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β-Cyclodextrins as Carriers of Monoterpenes into the Hemolymph of the Honey Bee (*Apis mellifera*) for Integrated Pest Management

BLAISE W. LEBLANC,*,† STEPHEN BOUÉ,‡ GLORIA DE-GRANDI HOFFMAN,† THOMAS DEEBY,† HOLLY MCCREADY,† AND KEVIN LOEFFELMANN§

Carl Hayden Bee Research Center, USDA ARS, 2000 East Allen Road, Tucson, Arizona 85719; Southern Regional Research Center, USDA ARS, New Orleans, Louisiana 70179; and Southwest Watershed Research, USDA ARS, 2000 East Allen Road, Tucson, Arizona 85719

The Varroa mite (*Varroa destructor*) is becoming ubiquitous worldwide and is a serious threat to honey bees. The cultivation of certain food crops are at risk. The most noted acaricides against Varroa mites are tau-fluvaninate and coumaphos, but the mites are showing resistance. Since these insecticides are used in the proximity of honey, it is desirable to use natural alternatives. Monoterpenoids such as thymol and carvacrol, that are constituents of oil of thyme and oil of origanum, show promise as acaricides against the Varroa mite (*Varroa destructor*), but the delivery of these compounds remains a challenge due to the low water solubility and uncontrolled release into the colony. β-cyclodextrin (β-CD) inclusion complexes of thymol, oil of origanum, and carvacrol were prepared on a preparative scale. Competitive binding was studied by fluorescence spectroscopy by using 6-*p*-toluidinylnaphthalene-2-sulfonate as a fluorescent probe. The complexes were characterized, and the competitive binding described by ^{1}H and ^{13}C NMR spectroscopy chemical shifts. The toxicity of β-CD and the prepared complexes in enriched sucrose syrup was studied by conducting caged honey bee (*Apis mellifera*) feeding trials. After the first and second weeks of feeding, hemolymph and gut tissue samples were acquired from the caged bee study. The levels of thymol and carvacrol were quantified by solid-phase microextraction gas chromatography mass spectroscopy, using an optimized procedure we developed. High (mM) levels of thymol and carvacrol were detected in bee tissues without any imposed toxicity to the bees, in an effort to deter Varroa mites from feeding on honey bee hemolymph.

KEYWORDS: Varroa; essential oils; phenolics; thymol; carvacrol; origanum; cyclodextrin; drug delivery

INTRODUCTION

The Varroa mite (*Varroa destructor*) is a widely distributed external parasite to honey bees that sustains itself by ingesting honey bees' hemolymph, thus robbing honey bees of energy and vitality, decreasing their adult lifespan, and thereby hampering effective crop pollination for specific crops (*1, 2*). It is considered the most serious threat to the beekeeping industry and has been attributed, in part, to the recent widespread Colony Collapse Disorder (CCD) as a disease vector (*3, 4*). In the United States, during the winter of 2006 to 2007, commercial beekeepers reported 38% of their colonies lost due to CCD in 12 of 13 states surveyed; they attributed the losses to starvation, Varroa mites, tracheal mites, small hive beetles, environmental reasons, and other unknown reasons (*4*). On the basis of this data, it was estimated that, in the United States, since 2005, the number of managed colonies has dropped from 4.5 to 2.4 million. Any methodology that could potentially mitigate the effects of Varroa, without harmful effects to honey bees, would increase their ability to resist undiagnosed aliments such as CCD and other Varroa transmitted diseases. Honey bee pollination is critical for cultivation of certain crops. For instance, successful cultivation of almonds and apples relies almost exclusively on honey bee pollination (*5*). The pollination effort for almond crops in California alone requires an estimated 1.2 million honey bee colonies with the monetary value, in the United States, estimated at 16 billion dollars (*6*).

Currently, the most effective acaricides to control Varroa are with the pyrethroid class of insecticide tau-fluvaliniate and with the organophosphate coumaphos; however, many others are used (*7, 8*). Late in the 1990s, Varroa was found to be resistant to tau-fluvalinate in Europe (*7*) and in the United States (*8*). Therefore, in 2001, the EPA reregistered the use of the widely used

* Author to whom correspondence should be sent. Voice: (520) 670-6481 ext. 124, FAX: (520) 670-6493, E-mail: blaise.leblanc@ars.usda.gov.
† Carl Hayden Bee Research Center.
‡ Southern Regional Research Center.
§ Southwest Watershed Research.

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organophosphate pesticide coumaphos for control of Varroa. Now, there is also documented resistance of Varroa against coumaphos (*9*). In addition, coumaphos and fluvalinate have been detected in wax from beehives (*10*). Beeswax makes an excellent absorbent and preservative for lipophilic organic compounds such as coumaphos, and there are recent reports of detectable levels of sulfonamides, tetracyclines, and many other veterinary drugs in honey in the United States and in Europe (*11*). Beeswax is another hive product that is used in the cosmetic industry that is devalued by pesticide residues (*10*). With the threat of such compounds propagating into the food chain for humans and compromising food and cosmetic sources, there is a need to use natural compounds such as terpenes and terpenoids and naturally ubiquitous compounds that are known to be effective as acaricides.

Terpenes, their oxygenated derivatives, terpenoids, and other aliphatic hydrocarbons comprise the components of several essential oils, which have the potential to control parasitic mites. Apilife Var is a commercially available, thymol based product for Varroa control that has been used in Italy for 15 years with some success and uses vermiculate to control the release of the thymol, eucalyptol, menthol, and camphor (*12*). Apiguard, which is produced in England, is a more recent thymol based product that uses poylacrylic acid gel to control the release of the thymol (*12*). The effective delivery of these products to colonies is a challenge due to low water solubility and the uncontrollable release with volatile organic solvents (*13*−*15*). β-cyclodextrin (β-CD) complexation has become an effective method for increasing the solubility of such compounds solubility in aqueous solutions and for providing a delivery shuttle to cross biological membranes (*16*−*18*). Since the interior of the cavity of β-CD is more hydrophobic than the exterior, monoterpenoids are ideal candidates to complex with β-CD. In the absence of the β-CD complexes, the monoterpenoid compounds simply oil out in aqueous sucrose solution. Apparently, the β-CD serves as a matrix modifier to homogenize the monoterpenoids. Therefore, stable β-CD complexes can be administered to bees in aqueous sucrose solutions.

We report here a novel approach to deliver monoterpene isomers such as thymol and carvacrol that are active acaricides, into the hemolymph of honey bees. The complex formation constants of guest molecules with β-CD have been monitored by circular dicroism, ultraviolet−visible, fluorescence, nuclear magnetic resonance spectroscopy, mass spectrometry, and other analytical methods (*17*). For our studies, we employed fluorescence spectroscopy as a comparative measure of guest inclusion into the β-CD cavity. The inclusion of 6-*p*-toluidinylnaphthalene-2-sulfonate (TNS) in β-CD forms a fluorescent complex in aqueous media (*18*). However, when the guest compound is introduced, TNS is displaced into the aqueous environment where it no longer retains its fluorescent property, and therefore, the stability of the inclusion complex can be compared to the decay of fluorescence (*16*−*18*). We also characterized the complexes with nuclear magnetic resonance spectroscopy (NMR). In this report, we describe the preparation of stable, host−guest complexes of β-CD with thymol, carvacrol, and oil of origanum. In addition, we report the toxicity of β-CD and the latter mentioned complexes to bees. The optimized detection method by gas chromatography mass spectrometry with solid-phase microextraction (SPME) for quantifying the guest compounds in the hemolymph (blood) and gut tissue of honey bees also is described.

MATERIALS AND METHODS

Honey Bees. Freshly emerged Italian honey bees (*Apis mellifera*) were used for the caged bee studies. Approximately 100 bees were placed into the cage for each caged bee trial (conducted in triplicate). Honey bees are not subject to the regulations that research animals such as primates and mammals are for laboratory research. Therefore, current laws do not require institutional animal care and use committee approval for honey bee research.

Materials and Equipment. Thymol, carvacrol, oil of origanum, and 6-*p*-toluidinylnaphthalene-2-sulfonate (TNS) were purchased from Sigma-Aldrich (St. Louis, MO), and the β-CD was purchased from TCI America (Portland, OR). The fluorometer used to record the spectra was a Biotek Synergy HT microplate reader (Winooski, VT), and the microplates were Costar clear bottom black side 96 well, product no. 3615. Melting point determinations are reported uncorrected and were recorded on a Mel Temp electronic melting point apparatus. Elemental analysis was performed by Atlantic Microlabs, Norcross, GA. ^{1}H and ^{13}C NMR spectra were recorded at 299.96 mHz and 74.99 mHz, respectively, on a Varian Unity 300 NMR spectrometer (Palo Alto, CA) at 0.200 M in DMSO-d_6 as solvent. Chemical shifts (σ) are referenced to trimethylsilane as an internal standard at 0.0 ppm. Peak multiplicities are reported as singlet (s), doublet (d), doublet of doublets (dd), triplet (t), heptet (h), and multiplet (m), and the coupling constants (J) are reported in hertz. Gas chromatography mass spectroscopy (GC/MS) was performed on a Varian 3800 series GC with a 2200 Saturn ion trap detector equipped with a SPME 1079 injection port. A Varian Factor Four 30 m × 0.25 mm ID chromatography column was used. The SPME fibers were purchased from Supelco, Bellefonte, PA.

Fluorescence Study of β-CD Inclusion Complexes and Stability. A modification of the method by Kondo, et al. (*19*) was employed. In a 2 mL Eppendorf tube, 700 μL of a solution of TNS (6.3 mM TNS in 10 mM PBS pH = 7.38) was combined with 500 μL of β-CD solution (1.00 mM in 10 mM PBS pH = 7.38). A 100 mM methanolic solution of the essential oil/guest compound (100 mM for single compound or 185 ppm for essential oil) was added, and the appropriate amount of methanol was added to maintain a constant volume. The tube was vortexed and allowed to incubate at room temperature for 20 min. If the solution contained precipitate, the solid was separated by centrifugation at 5000 RPM for 5 min, and the supernatant was transferred to a new tube. The supernatant (200 μL per well) was transferred to a clear bottom black side microplate (to prevent light spillage into adjacent wells). The samples were run in triplicate. The microplate was sealed with a Thermaseal transparent membrane. The fluorescence was recorded under the following conditions: 30 s (s) shake cycle, 30 s delay, 100 ms delay between plate movement, 1 ms delay between excitation and measurement, excitation 360 nm, emission 460 nm, bandwidth (emission) 20 nm, sensitivity = 60, excitation/emission read (bottom of plate).

Preparation of β-CD Complexes. To prepare the β-CD:thymol complex (T:β-CD), a modification of the method reported by Donze et al. was employed (*20*). β-CD (1.25 g, 1.01 mmol) was combined in a 150 mL Erlenmeyer flask with 50 mL of DI (18 M-Ohm) water. The solution was stirred with a magnetic stirring apparatus and was heated over a water bath until all solids were completely dissolved (∼50 °C). Thymol (0.758 g, 5.5 mM) was dissolved in absolute ethanol (80 mL) and dripped in at about 1 drop per 5 s, until all of the solution was added. The temperature was lowered if it approached 70 °C. For most of the reaction mixtures, cloudiness of solution and precipitation were evident and assumed indicative of β-CD encapsulation. The solutions were stirred an additional 60 min and cooled to room temperature, covered, and allowed to stand overnight. The following day the precipitate was suction filtered and allowed to air-dry. The yield was 1.11 g. Elemental analysis: %C,H,O (45.8, 6.81, 47.28). mp: 245.9−250.4 °C. ^{1}H NMR: δ 9.05 (s, 1H, Ar−OH), 6.94 (d, 1H, J = 7.6 Hz, Ar−H), 6.55 (m, 2H, J = 11.7 Hz, Ar−H), 5.72 (m, 7H, J = 12.7 Hz), 4.82 (d, 3.4H, J = 2.93 Hz), 4.56 (t, 3.4H, J = 5.86 Hz), 3.62 (m, 15H, J = 9.79 Hz), 3.38 (m, 14H, J = 11.47 Hz), 3.12 (h, 1H, J = 6.90 Hz, isopropyl−H), 2.15 (s, 3 H, methyl), 1.10 (d, 6H, J = 6.84 Hz, methyl). ^{13}C NMR: 154.17 (s), 135.33 (s), 131.21 (s), 125.68 (s), 119.69 (s), 115.57 (s), 101.99 (s), 81.60 (s), 72.47 (t), 59.98 (s), 26.05 (s), 22.67 (s), and 20.74 (s).

To prepare the β-CD:carvacrol complex (C:β-CD), exactly 30 g of β-cyclodextrin (26.4 mM) was combined in a 2 L round-bottom flask with 600 mL of water (DI, 18 mΩ). The suspension was warmed over

an oil bath with stirring. At about 60 °C, the suspension homogenized. Exactly 27.95 g of carvacrol (186 mM) was dissolved in 80 mL of ethanol and was dripped in over 6 h. The mixture was allowed to stir for 32 h at 60 °C; it was then removed from heat and allowed to stand overnight. The white solid was suction filtered, washed with cold water, and air-dried. The yield was 23.90 g. Elemental analysis: %C,H,O (46.89, 6.76, 46.31). mp: 240.2−241.5 °C. ^{1}H NMR: δ 9.04 (s, 1H, Ar−OH), 6.94 (dd, 1H, J = 7.57 Hz, Ar−H), 6.61 (s, 1H, Ar−H), 6.53 (dd, 1H, J = 7.5 Hz), 5.72 (m, 7H, J = 6.59 Hz), 4.82 (s, 3.3H), 4.46 (t, 3.3H, J = 5.13 Hz), 3.63 (m, 16H, J = 0.02 Hz), 3.39 (m, 18H, J = 0.03 Hz), 2.71 (h, 1H, J = 6.90), 2.04 (s, 3H), 1.12 (d, 6H, J = 6.84 Hz). ^{13}C NMR: 155.21 (s), 147.05 (s), 130.36(s), 121.01 (s), 116.71(s), 112.56(s), 102.00(s), 81.60 (s), 72.47 (t), 59.98 (s), 33.11 (s), 24.05 (s), and 15.65 (s).

To prepare the β-CD:origanum complex (O:β-CD), the procedure for T:β-CD was used. The yield was 1.08 g. Elemental analysis: %C,H,O (46.90, 6.94, 46.11). mp: 238.5−240.3 °C. ^{1}H NMR: δ 9.04(s, 1H, Ar−OH), 6.91 (dd, 1H, J = 7.58 Hz, Ar−H), 6.61 (s, 1H, Ar−H), 6.53 (dd, 2H, J = 7.42 Hz), 5.72 (m, 7H, J = 12.53 Hz), 4.82 (s,3H), 4.46 (t, 3H, J = 5.83 Hz), 3.63 (m, 16H, J = 6.75 Hz), 3.39 (m, 15H, J = 5.93 Hz), 2.71 (h, 1H, J = 6.90), 2.14 (s, 1H), 2.04 (s, 3H), 1.12 (d, 6H, J = 6.76 Hz). ^{13}C NMR: 155.21 (s), 147.04 (s), 130.36(s), 121.01 (s), 116.71(s), 112.56(s), 102.02(s), 81.62 (s), 72.48 (t), 59.98 (s), 33.11 (s), 24.05 (s), 22.66 (s), and 15.65 (s).

Sucrose Syrup Enriched Guest: β-CD Solutions for Bee Feeding. To prepare 0.01% (w/w) sucrose β-CD complex solution, 129 g of sucrose was added to 100 mL of tap water, and this was warmed on a water bath until the sucrose dissolved. The β-CD complex (0.0229 g) was added to the sugar solution, and the mixture was warmed and stirred with a magnetic stirring device until all solids dissolved. The 0.1 and 1.0% solutions were prepared by dissolving 0.229 and 2.29 g of the β-CD complexes, respectively. The solutions were stored at 55 °C until used.

Caged Bee Studies. The studies took place between November 2007 and February 2008. One hundred Italian honey bees (*Apis mellifera*) were placed in commercially manufactured, custom designed, plexiglass cages (6 in. high × 4 in. wide × 2.5 in. deep) with wire mesh on the sides to provide adequate ventilation and with removable, double mesh floors so that dead bees could be collected. The bees were provided with a sugar pollen mixture for protein and were fed the syrup ad libitum by gravity feeders. The caged bee studies were recorded in triplicate for each syrup treatment. The bees were housed in an environmental room maintained at 34 °C and 50% humidity to simulate bee hive conditions. Mortality counts were recorded at least three times a week, and dead bees were removed. Syrup mass was recorded twice during the first 12 days for each treatment to determine the average consumption.

Analysis of Volatiles by SPME. The method to detect volatiles from insects using SPME, including GC/MS parameters, was previously reported (*21*). After the first week of treatment, 5 live bees from the 0.1% T:β-CD, O:β-CD, and C:β-CD treatments were placed in a 50 mL Erlenmeyer flask. A screen cage was placed in the mouth of the flask to prevent the bees from physically contacting the fiber. A piece of aluminum foil was used to cover the flask. A SPME fiber (PDMS-DVB) was used to sample the volatiles for 10 min with a manual SPME device holder. The fiber was then placed into the injection port at 250 °C and desorbed for 10 min.

Extraction of Hemolymph. Hemolymph and gut were sampled after the first and second weeks of treatment. Three bees from each cage (9 bees total), of a specific dosage, were placed in a glass jar with a screen top. The bees were housed in a laboratory incubator before hemolymph extraction and were fed honey. The bees were placed in a freezer at 5 °C until motionless. The bees were then fastened to a filter paper with double sided tape with a harness across the dorsal portion until secure. A needle was used to puncture the thorax. A flame drawn Pasteur pipet was used to extract the hemolymph by capillary action. The hemolymph was pooled, maintained at 0 °C in a 2 mL Eppendorf tube in an ice bath and was centrifuged. Exactly 5 μL was added with a volumetric pipet to an Eppendorf tube containing 95 μL of sterile PBS buffered to pH 7.38. The contents were vortexed and then centrifuged. Exactly 75 μL of the 20× dilution (upper layer) was transferred to a clean Sarstedt vial, to separate away hemocytes, and the solution was stored at −70 °C until analyzed by SPME GC/MS. Gut samples were taken by dissecting the abdomen of the bee and removing the gut contents with a Pasteur pipet. The gut sample was treated as above for hemolymph.

Optimization of Detection and Determination of Thymol and Carvacrol in Bee Hemolymph and Gut Using SPME GC/MS. *Effects of Hydronium Ion.* To 1.8 mL GC vials was added 130 μL of water ranging from 7.38 to 0.80 pH units, that was adjusted with diluted HCl or NaOH solutions. To this was added 30 μL of 3.8 M NaCl solution, to normalize the ionic strength of the solutions, followed by 20 μL of carvacrol (37 μM) and benzophenone (20 μM). The vial was capped and vortexed, and the headspaced adsorbed for exactly 5 min with a PDMS-DVB fiber. The fiber was immediately placed in the injection port of a Varian 1079 modified for SPME, at 250 °C; the desorption time was 10 min. Oven conditions were 40 °C with a 2 min hold, then 20 °C/min to 250 °C, then a hold at 250 °C for 0.5 min. The run time was 13 min. The chromatographs were processed with Varian software version 6.9 and the ions 51, 77, 91, 105, 115, 135, 150, and 182 were used to process the data for peak area.

SPME Fiber Optimization. The SPME fibers were conditioned according to the instructions provided. To a 1.8 mL GC vial, 160 μL of a 3.8 M NaCl solution at pH 0.1 was mixed with 20 μL of ethanolic solutions of 20 μM benzophenone and 37 μM carvacrol and vortexed. The vial was immediately capped, and the representative SPME fibers (polyacrylate 85 μm (PA-85), PDMS-DVB 65 μm, PDMS, DVB/Carboxyn/PDMS cat. no. 57348-U, Carboxyn), previously conditioned according to the manufacturer's instructions, were used to absorb the headspace of the vials for 10 min. The mentioned GC conditions were used with the injection port increased to 280 °C to minimize carryover of benzophenone, and the mentioned ions were used to process the chromatograms for peak area.

Sample Preparation and Analysis. To a 1.8 mL GC vial was added 160 μL of a pH 1.07, 3.8 M NaCl solution. Methylbenzoate, 20 μL of a 10 μM ethanolic solution, was added as an internal standard followed by 20 μL of the bee hemolymph or bee gut PBS dilution. After every ninth sample, a carvacrol standard was analyzed followed by a blank. The samples were absorbed for exactly 5 min with a PA-85 fiber and desorbed for exactly 9 min. A standard calibration curve from 10 μM to 50 000 μM was used to convert peak area into μM. The ions 51, 77, 91, 105, 115, 135, 136, and 150 were used to process the chromatograms for peak area analysis. Calibration curves were generated by using carvacrol for the origanum O:β-CD and C:β-CD treated bees, and thymol was used for the T:β-CD bees, by plotting concentrations versus peak area. The detection limit was <10 μM and the percent recovery >82%. The following calibration curves were used to calculate tissue concentrations in millimolar.

$$\text{T:}\beta\text{-CD treated bees:}\quad [\text{thymol}]_{\text{mM}} = [20(\text{tissue dilution factor}) \times [(\text{peak area}) \times 0.0007 + 582]]_{\mu\text{M}} \times 10^{-3} \qquad R^2 = 0.995$$

$$\text{O:}\beta\text{-CD treated bees:}\quad [\text{carvacrol}]_{\text{mM}} = [20(\text{tissue dilution factor}) \times [(\text{peak area}) \times 0.0007 + 588.41]]_{\mu\text{M}} \times 10^{-3} \qquad R^2 = 0.993$$

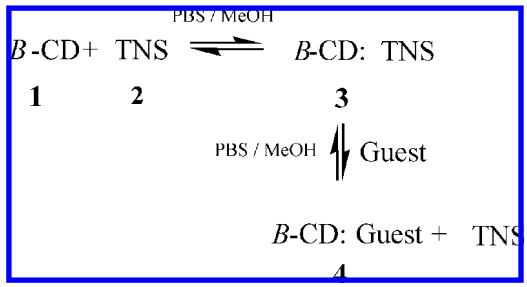
$$\text{C:}\beta\text{-CD treated bees:}\quad [\text{carvacrol}]_{\text{mM}} = [20(\text{tissue dilution factor}) \times [(\text{peak area}) \times 0.0008 + 679.12]]_{\mu\text{M}} \times 10^{-3} \qquad R^2 = 0.990$$

Statistics. Average consumption and mortality rates were compared among the treatment groups using the ANOVA technique with XLStat 2007.6 (Addinsoft). When ANOVA indicated differences among the means, a Dunnett (two sided) analysis of the differences was used for all comparisons. All data are reported as mean ± standard error.

RESULTS AND DISCUSSION

Chemists have analyzed CD inclusion by different means of spectroscopy. Some of the earlier reports in the literature

Scheme 1. Illustration of Fluorescence Decay Phenomena When Guest Molecules Displace the TNS Probe from the β-CD Cavity into Aqueous Media



reported on the complexation of fluorescent probes, namely 6-*p*-toluidinylnaphthanlene-2-sulfonate (TNS), with α-, β-, and γ-cyclodextrins, to form stoichiometric complexes (*18, 19, 21*). TNS was initially used as a fluorometric probe to analyze hydrophobic surfaces of biologically active molecules, such as proteins (*22*). Researchers found that aqueous solutions of TNS fluoresce strongly when α- or β-CD was added to the solution but that the fluorescence is quenched in the absence of CDs (*18, 19*). Kondo et al. concluded that the cavity of CDs are a relatively hydrophobic environment, and they exploited this phenomena to study TNS:CD complexes to study rate measurements for the hydrolysis of β-CD in sodium acetate buffer with amylases. The decay of fluorescence was unequivocally associated with the hydrolysis of β-CD in acidic sodium acetate buffer (*19*). There are speculations as to how the TNS was included, since it is a diaromatic system, and it was reported that at least two β-CD molecules can bind with one TNS to form a stable complex. Researchers have since studied this inclusion phenomena by nuclear magnetic resonance, ultraviolet, and fluorescence spectroscopies and have characterized the thermodynamic properties of this unusually stable complex (*18*).

Our initial investigations proceeded with the use of TNS and fluorescence for our studies of β-CD encapsulation. Since we used methanolic solutions of the guest compounds and there is a possibility that methanol could hydrogen bind to the pyranose ring hydroxyl groups of the β-CD and impede complexation with the guest molecule, we explored changing solvents. Fluorescence increased drastically when we changed solvents to acetonitrile. Fluorescence did not change though when the guest compound was added, so methanol was used as a solvent. Also, we found the results more reliable when excitation and emission were from the bottom of the microplate. This also allows for the plate to be sealed, so evaporation can be eliminated. The method we employed using TNS as a probe for the interior cavity of β-CD seems to be a sensitive method to judge guest molecules that are preferentially included into the β-CD cavity (**Scheme 1**). β-CD **1** forms a stable complex **3** with TNS **2**. Guest compounds are added in molar excess and can be compared under molar equivalent concentrations for competitive binding. The decrease in fluorescence with respect to concentration can be attributed to complexation **4** with β-CD (**Scheme 1**, **Figure 1**). For instance, an aliphatic ketone, such as 2-heptanone, that is also being investigated as an acaricide (*23*), does not complex well with β-CD according to our assay (**Figure 1**). Since there is less emission for carvacrol than for thymol at equivalent concentrations, it is included into the β-CD cavity slightly better. Menthol, the saturated analog of thymol, binds noticeably stronger than either of the latter, according to this assay. Oil of origanum contains carvacrol and myrcene as constituents, so an impure naturally produced material was not evaluated by this assay (*24*). Researchers have attempted to

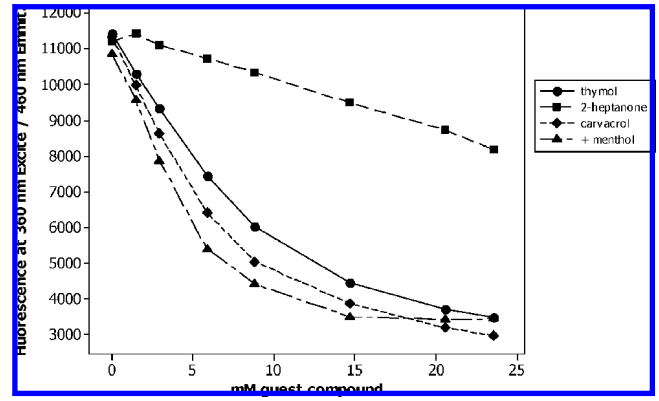


Figure 1. Fluorescence decay of TNS:βCD at 360 nm excitation and 460 nm emission, as a result of thymol, carvacrol, menthol, and 2-heptanone displacing TNS from β-CD into solution.

Table 1. ^{13}C NMR Shift Data for β-CD Complexes of Thymol and Cavacrol at 0.20 M in DMSO-d_6

carbon label T, C[a]	σ/ppm free T	σ/ppm complexed T:β-CD	σ/ppm complexed-free Δ = T: β-CD-T	σ/ppm free C	σ/ppm complexed C:β-CD	σ/ppm complexed-free Δ = C: β-CD-C
C1	154.64	154.17	−0.47	155.88	155.21	−0.67
C2	135.66	135.33	−0.33	147.92	147.05	−0.87
C3	131.62	131.21	−0.41	131.19	130.36	−0.83
C4	126.02	125.68	−0.34	121.91	121.01	−0.9
C5	120.05	119.69	−0.36	117.73	116.71	−1.02
C6	116.00	115.57	−0.43	113.49	112.56	−0.93
C7	26.49	26.05	−0.44	34.12	33.11	−1.01
C8	23.01	22.67	−0.34	24.62	24.05	−0.57
C9	21.09	20.74	−0.35	16.27	15.65	−0.62
C10	21.09	20.74	−0.35	16.27	15.65	−0.62

[a] Structures of carvacrol and thymol depicted below **Table 2**.

calculate association constants with guest molecules to determine binding affinity, but different methods produce contrary results for the same guest molecule, so the experimental conditions must be controlled precisely (*20, 25*−*27*).

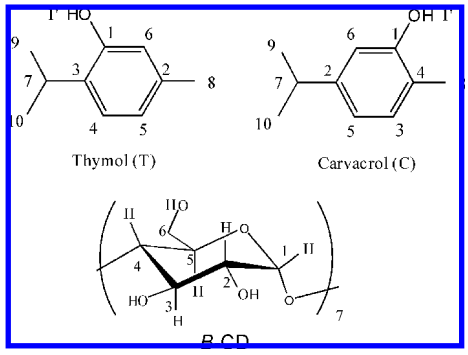
It is therefore desirable to have a second method to evaluate β-CD complexation reaction product outcomes. The proton and ^{13}C NMR chemical shift differences between the T:β-CD and C:β-CD complexes and the free compounds agree with the fluorescence data. As shown in **Table 1** for the ^{13}C NMR, the upfield chemical shift difference for carvacrol is greater than for thymol; therefore, carvacrol binds slightly better. It is interesting that, for caravcrol and thymol, the ortho and para carbons reveal the greatest change in chemical shift. The proton NMR data agrees with the ^{13}C data for the complexes, in terms of competitive binding. **Table 2** shows that the shift difference for the phenolic proton in carvacrol changes by 0.31, while for thymol the change is 0.01. Also, the ortho protons, label 6 in **Table 2**, for carvacrol and thymol have the greatest difference in shift for the complexed versus free compounds, with −0.06 ppm for the latter and −0.13 for the former. The ^{13}C chemical shift differences between the T:β-CD and C:β-CD subtracted from the chemical shifts of free β-CD reveal that it is inconclusive as to whether T or C binds better to β-CD. For instance, the chemical shifts were identical for all six carbons of the glucopyranose unit of β-CD, with the exception of the C-3 carbon, which differed by 0.01 ppm. The structure of the glucopyranose unit of β-CD is depicted in **Chart 1**. Other researchers reported similar ^{13}C NMR chemical shifts for β-CD complexed with the insecticides sulprofos and aldicarb (*28*),

Table 2. ^{1}H NMR Shift Data for β-CD Free and Complexed to Thymol and Carvacrol at 0.20 M in DMSO-d_6

proton	σ/ppm free	σ/ppm complexed	σ/ppm complexed-free	proton	σ/ppm free	σ/ppm complexed	σ/ppm complexed-free
label T	T	T:β-CD	Δ = T: β-CD-T	label C	C	C:β-CD	Δ = C: β-CD-C
1′	9.04 (s)	9.05 (s)	0.01	1′	8.73 (s)	9.04 (s)	0.31
4	6.95 (d)	6.94 (d)	−0.01	3	6.92 (d)	6.92 (d)	0.00
6	6.61 (s)	6.55 (s)	−0.06	6	6.74 (s)	6.61 (s)	−0.13
5	6.55(d)	6.54 (d)	−0.01	5	6.55 (d)	6.52 (d)	−0.03
7	3.16 (h)	3.12 (h)	−0.04	7	2.71 (h)	2.71 (h)	0.00
8	2.17 (s)	2.15 (s)	−0.02	8	2.17 (s)	2.04 (s)	−0.13
9, 10	1.14 (d)	1.10 (d)	−0.04	9, 10	1.16 (d)	1.12 (d)	−0.04

the fungicide tebuconazole (*29*), and for pyrazole-phenyl ether containing herbicides (*30*). The reasoning is that the glucopyranose ring is in a constant evolution of structural rearrangements, which leads to poor NMR structural resolution (*26, 27*). Theoretical calculations support the experimental observations of CD ring distortion (*31*).

Chart 1



For the preparation of the β-CD complexes, we followed a procedure based on a method reported by Donze, which involves dripping in an ethanolic solution of excess molar equivalents of the guest compound into a aqueous solution of β-CD (*20*). The ^{1}H NMR and ^{13}C NMR spectra of the O:β-CD and C:β-CD are nearly identical, so it is obvious that β-CD is encapsulating carvacrol from the origanum oil and offering a means of separation. From the ^{1}H NMR spectra integral of the aromatic protons on the number 4 and 3 carbons of T and C and the average of the integrals on the protons attached to the alcohol 2 or 3 carbons on the glucose of β-CD (**Chart 1**), it is possible to determine the ratios of β-CD to guest for the T:β-CD, O:β-CD, and C:β-CD complexes as 1:3.48, 1:3.71, and 1:3.37, respectively. Theoretical elemental analysis for these ratios for percent C, H, and N for the mentioned ratios would be 45.78, 6.36, 47.86 for percent C, 45.5, 6.69, 47.80 for percent H, and 45.66, 6.35, and 47.99 for percent N. The NMR data reveals that the O:β-CD complex has the highest amount of uncomplexed β-CD. The C:β-CD complex has the lowest ratio of β-CD to guest, so this data agrees with the fluorescence and the ^{13}C NMR data. Other investigators have reported on and characterized β-CD complexes with fraction ratios of guest molecules (*29*). A reason for the oil of origanum complex having a higher ratio is that carvacrol is a major component of oil of origanum, but there are other terpene components that might compete for binding and there is of course less carvacrol per unit mass than with the pure compound.

Since bees will eat sugar syrup, we first investigated dissolving the complexes in high fructose corn syrup and water. Sucrose, glucose, and fructose aqueous solutions provide an ideal (like) environment for oligosaccharides such as β-CD that are composed of dextrose. However, we used sucrose because it enabled us to more precisely formulate the mixtures. We found that the 0.01 and 0.1% (w/w) β-CD complexes remained in solution but the 1.0% complex treatments crystallized in the feeders so the consumption of syrup for the 1.0% (w/w/) solution was less than the latter mentioned formulations (**Figure 2A**). The T:, O:, and C:β-CD complexes were less soluble in the sucrose solution than β-CD itself. This was expected as it is known that β-CD complexes generally have a lower solubility in water than β-CD (*28*). Also, thymol at 0.01% (w/w) crystallized out of a 50% sucrose solution and carvacrol, at the same concentration, oiled out. However, the 0.01% T: and C:β-CD complexes in 50% (w/w) sucrose solutions remained dissolved. In either case, fermentation did not occur with the sucrose solution standing covered at room temperature for prolonged periods (weeks). Sucrose solutions typically ferment after two weeks standing at room temperature. The antifungal and antibacterial properties of phenolics and monterpenes have been reported by other researchers (*32, 33*).

During the caged bee experiments, syrup consumption was measured from the start of the feeding trial through day 12 and the average rates of consumption per cage are reported (**Figure 2A**). There is toxicity associated with β-CD consumption for bees. For instance, **Figures 2B**, **2C**, and **3** show that the 1.0% β-CD is substantially more toxic than the control or the 0.01 and 0.1 β-CD formulations. Our initial toxicity trials were with 5.0% (w/w) β-CD formulations and the toxicity was markedly obvious because within the first two weeks all of the bees were dead. The 0.01 and 0.1% (w/w) concentrations, however, were not found to be significantly more toxic than the sucrose control (**Figures 3**, **2B**, and **2C**). We expected the bees to experience toxicity to β-CD, as complex sugars, and especially pectin are highly toxic to bees. For instance, Barker reported 82% mortality in a caged bee study of bees fed on a 4% pectin formulation (*34*). It is interesting that trisaccharides such as raffinose and stachiose were found to be substantially toxic (*34*).

The toxicity of β-CD and the complexes is demonstrated with dose mortality (**Figures 3**, **2B**, and **2C**). A low rate of consumption of the 1.0% syrups can be attributed to crystallization in the feeding bottle therefore impeding the flow of the solution. Also, the 1% formulation had a much stronger aroma, so the bees may have been repelled. After 25 days, the toxicity became much more apparent, with 1.31 ± 2.27, 6.52 ± 4.41, and 8.93 ± 3.1% survival for the 1.0% T:β-CD, O:β-CD, and C:β-CD complexes, respectively (**Figure 2C**). β-CD is a torus shaped, non-natural, enzyme-produced, cyclic oligosaccharide polymer, with seven maltodextrin units (*35*), and bees may not be capable of digesting them. In addition, they may include or chelate to molecules or metal ions that are biologically important to bees. The C:β-CD 1.0% treatments were less toxic than the O:β-CD or the T:β-CD for both 12 and 25 days (**Figures 2**B and **C**). The C:β-CD formulations provided for higher consumption with less bee mortality (**Figures 2A** and **2C**). After weeks 1 and 2, the hemolymph and gut contents from three live bees were analyzed to determine the amount of volatile organic compounds present.

SPME combined with GC/MS, is a highly sensitive technique for detecting organic compounds in aqueous matrices and has been reported to quantify picogram per milliliter levels of benzene and substituted aromatics in municipal drinking water (*36*). Organic compounds with a carboxylic acid moiety can be selectively extracted by adjusting the pH of the aqueous solution

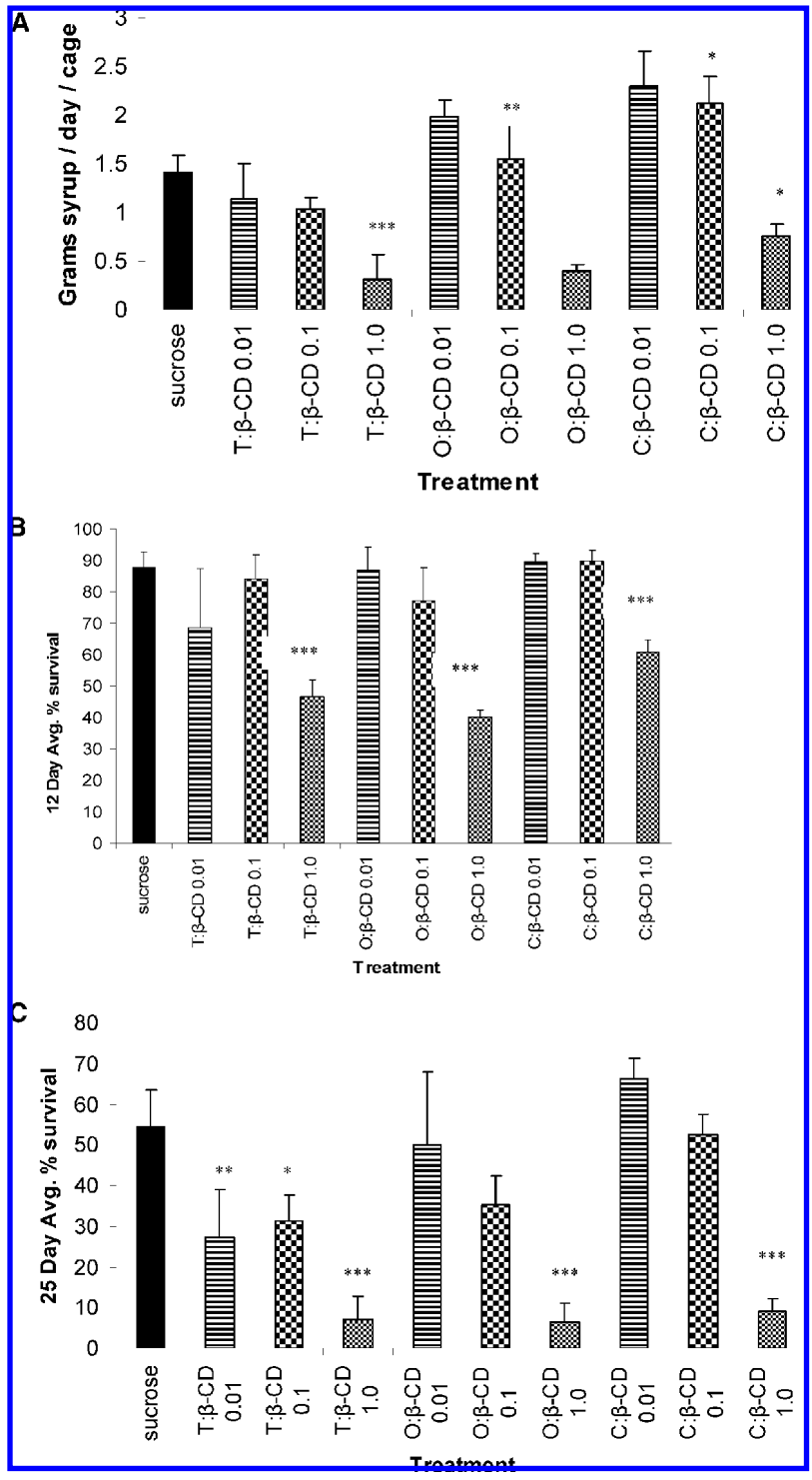


Figure 2. (**A**) Consumption data for T:, O:, and C:β-CD complexes at 0.01, 0.1, and 1.0% expressed in grams of syrup per cage per day. (**B**) Average percent survival at 12 days for bees fed β-CD syrups at 0.01, 0.1, and 1.0%. (**C**) Average survival at 25 days for bees fed β-CD syrups at 0.01, 0.1, and 1.0%. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ indicate significant differences compared with sucrose control.

(*36*). The technique has been used to selectively adsorb target nitrol and amine substituted phenolic analytes based on pK_a values. It was reported that the polyacryate (PA) SPME fiber was the most pH selective for these analytes compared to other commercially available fibers (*37*). Some of the important factors that can be utilized for optimizing detection limits that were optimized for our technique are adsorption time, pH of solution, salinity, fiber immersion depth, and sample agitation (*38, 39*).

After the first week of treatment with the 0.1% formulations, sampling was started. We detected thymol and carvacrol volatiles emanating from five live bees by a SPME GC/MS technique previously reported for detecting pheromones (*21*). The potential to deter Varroa is, therefore, very encouraging. To optimize the detection limits for the analysis of carvacrol and thymol in the tissue samples, we first determined the optimum pH of the buffer (**Figure 4**). We used a PDMS-DVB to monitor solutions ranging from 0.08 to 7.38 pH units. Even though the PA fiber was more sensitive than the PDMS-DVB fiber, the signal response is attributed to the analytes in the vapor phase, so the analyte signal is realized with other fibers (*37*). Increased salinity raises the vapor pressure of organic analytes, especially in aqueous media (*39*). There is a noticeable increase in the analyte and internal standard signal with

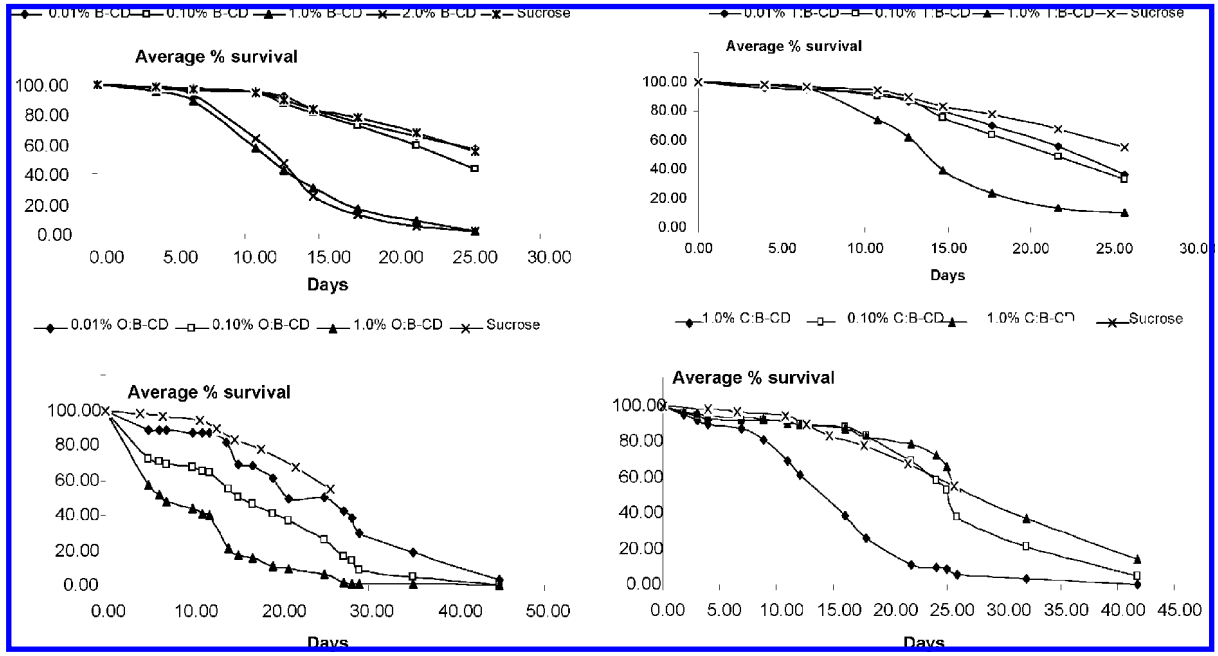


Figure 3. Mortality data for β-CD, T:β-CD, O:β-CD, C:β-CD, and sucrose syrup.

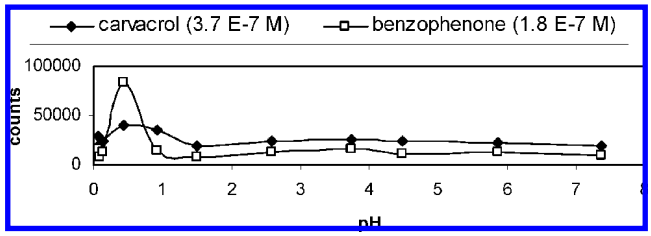


Figure 4. Effects of hydronium ion on analyte and internal standard signal.

solutions having a pH of less than 1 (**Figure 4**). We noticed that artifact, siloxane peaks from the fiber occur with the lower pH solutions. These artifacts have been observed by others and are attributed to the polymer coating on the fiber degrading under acidic conditions (*39, 40*). For the buffer, we selected a saturated NaCl solution with a pH of 1.07. The Carboxyn fiber showed drastically lower sensitivity to the carvacrol signal and the benzophenone internal standard than the other fibers tested, while the PA fiber was clearly the most sensitive (**Figure 5**). In an effort to reduce adsorption time, we plotted peak area versus time in minutes for the PA and PDMS-DVD fibers and fit the data using linear regression analysis. The equation for the PA fiber was $-1570.9 + 3458.8 \times$ time ($R^2 = 0.981$) and $2927.5 + 2087.5 \times$ time ($R^2 = 0.997$) for the PDMS-DVB fiber. We found that there was carry over with benzophenone, with the PA fiber, even after increasing the injection port temperature to 300 °C. Carryover with benzophenone derivatives has been observed by other investigators when using PA fibers (*37*). To overcome the carryover effect, the absorption time was decreased to less than 4 min (*38*). We used methylbenzoate as an internal standard, so that the sensitivity of the analytes was not compromised by decreasing the absorption time. The PA fiber has a polyacrylate coating and therefore is theoretically more sensitive to polar organics, but the PA fiber can form π bonds with π orbitals of aromatics, so this may account for the greater sensitivity of the PA fiber, for the aromatic analytes, over the PDMS-DVB fiber (*40*). This may also account for the carryover effect of benzophenone with the PA fiber.

During the analysis of gut samples, the internal standard had decreased due to competition from other compounds adsorbing to the fiber. When analyzing liquid samples for headspace using SPME, there is equilibrium between the liquid and vapor state and the vapor state with the SPME fiber. Chemicals such as an internal standard and an analyte should therefore be as close as possible to one another, chemically, if the internal standard signal is to be relied on for analyte signal correction. This is because different compounds have a different partition coefficient between the liquid and gas sample (*38, 40*). Sample adsorption time was directly proportional to the signal for carvacrol, thymol, and the internal standards we used.

We found the T:β-CD treated bees had lower levels of thymol in the hemolymph after week 1 compared to the other hemolymph samples for most treatments but had much lower levels of the guest compound in the gut tissue (**Table 3**). One would expect that in the second week, groups would have higher levels of the analytes, but we found that the first week had predominantly higher levels of carvacol or thymol. One possible explanation is that, when bees are first introduced into the cage, such as during the first week, they ingest high amounts of honey or sugar syrup, if it is available to them. Beekeepers feed sugar syrup to bees to promote honeybee brood and brood foundation (*41*). The O:β-CD and C:β-CD treated bees had levels of carvacrol greater than 100 mM in the gut tissue of the bees. It is encouraging that these high levels were not toxic to the bees. This might be because thymol and carvacrol are naturally occurring monterpenoids. Bees are likely resistant to the toxic effects of many monterpenoids and polyphenolic compounds because they utilize propolis, which is a rich source of the latter, that bees collect from tree saps and plant resins (*42*). The hemolymph levels for the O:β-CD and C:β-CD are comparable. But, considering that the concentrations in **Table 3** are expressed in millimolar, they are considered high for a living organism.

Cyclodextrins have recently been reported to complex with lemon oil components (*43*), and since CD complexation is a method of controlling the volatility of semivolatile compounds, there are applications to the cosmetics and fragrance industry (*44*). Most applications of cyclodextrins for agricultural uses reported in the literature are β-CD complexes with insecticides (*28*), fungicides (*29*), and herbicides (*30*), so that effective concentrations can be decreased, and therefore, less chemicals can be perpetually discharged into the environment. β-CD is the most common and

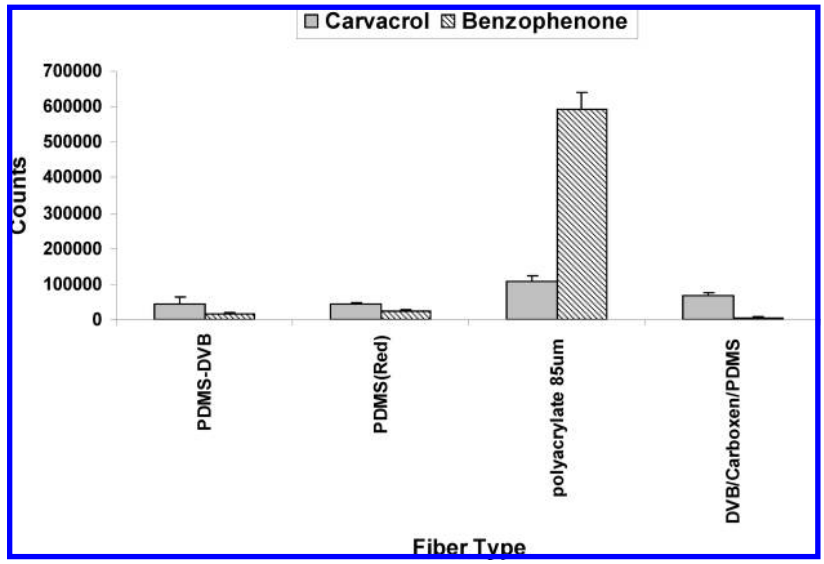


Figure 5. Comparison of the sensitivity of SPME fibers.

Table 3. Thymol and Carvacrol Detected with SPME-GC/MS at Millimolar Concentrations in Bee Hemolymph and Gut Tissue from Bees Fed β-CD, T:β-CD, O:β-CD, and C:β-CD at Indicated Concentrations

treatment	tissue	week	conc 0.01%	conc 0.1%	conc 1.0%
T:β-CD	hemolymph	1	0.11 ± 0.008	0.15 ± 0.008	1.16 ± 0.004
T:β-CD	hemolymph	2	0.10 ± 0.005	0.20 ± 0.037	0.10 ± 0.006
T:β-CD	gut	1	12.4 ± 0.114	13.20 ± 0.981	23.0 ± 1.61
T:β-CD	gut	2	4.2 ± 1.17	14.3 ± 0.781	52.2 ± 2.20
O:β-CD	hemolymph	1	0.187 ± 0.150	0.251 ± 0.105	0.178 ± 0.076
O:β-CD	hemolymph	2	0.200 ± 0.002	0.143 ± 0.001	0.978 ± 0.104
O:β-CD	gut	1	0.552 ± 0.275	4.89 ± 0.955	104. ± 14.3
O:β-CD	gut	2	0.855 ± 0.174	11.7 ± 0.083	51.2 ± 5.18
C:β-CD	hemolymph	1	0.259 ± 0.108	0.357 ± 0.090	1.26 ± 0.014
C:β-CD	hemolymph	2	0.058 ± 0.014	0.526 ± 0.107	0.505 ± 0.036
C:β-CD	gut	1	1.48 ± 0.262	39.5 ± 0.769	145 ± 11.7
C:β-CD	gut	2	0.741 ± 0.191	16.31 ± 2.46	137 ± 6.92

least expensive of the cyclodextrins (*28*). Essential oils and their components are receiving more attention due to their antibacterial and antimicrobial properties as well (*32, 33*). Therefore, the potential to apply hydrophobic compounds for many other fields of research can be realized with CD complexation (*44*).

We found that β-CD can be used to create stable sucrose solutions with thymol and carvacrol β-CD complexes. The solutions at 0.01, 0.1, and 1.0% (v/v) of these complexes are readily taken up by bees, and the levels of these analytes are detectable in the millimolar level in the gut and hemolymph, without introducing toxicity with the 0.01 and 0.1% (w/w/) formulations. Since Varroa mites feed on bee hemolymph and there are reports of the use of essential oils to control Varroa, we believe that this will offer alternatives to coumaphos, fluvalinate acaricides, and possibly the oxalic acid and formic acid, which are also used by beekeepers. One explanation for the lack of mortality with the high levels of thymol and carvacrol we detected is that honey bees utilize propolis in their hives for sealing up cracks and as a type of adhesive. As with propolis, ethanolic tinctures, which investigators also have shown to have miticidal activity against Varroa mites, without harmful effects to bees, the monoterpenoids that we investigated in this research are tolerated at high levels (*45*). It is our hypothesis that low levels of the essential oils in the bees' hemolymph will deter the mites from feeding and thereby reduce mite reproduction. The combination of the terpenoids as guests and cyclodextrin carriers has the potential to evolve into a delivery system that could effectively control parasitic mites in honey bee colonies and potentially other parasites and pathogens of honey bees as well.

Hopefully, the work presented here will stimulate researchers to investigate other types of delivery systems and naturally occurring compounds for honey bee health. Future work will involve testing these formulations on Langstroth hives infested with Varroa mites to see if the thymol and carvacrol are detected in bee larvae to deter the mites from feeding on the hemolymph.

ABBREVIATIONS USED

EPA, Environmental Protection Agency; β-CD, β-cyclodextrin; mM, millimolar; μM, micromolar; M, mega (10^6); mL, milliliter; SPME, solid-phase microextraction; T:β-CD, β-cyclodextrin−thymol complex; O:β-CD, β-cyclodextrin−origanum complex; C:β-CD, β-cyclodextrin−carvacrol complex; DI, deionized; TNS, 6-*p*-toluidinylnaphthalene-2-sulfonate.

SAFETY

Appropriate safety precautions were observed where necessary.

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