

# Encapsulation of Nutraceutical Monoterpenes in $\beta$ -Cyclodextrin and Modified Starch

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**ABSTRACT:** The encapsulation of thymol and geraniol in  $\beta$ -cyclodextrin ( $\beta$ -CD) and modified starch (MS) by spray- and freeze-drying was studied. The formation of thymol/ $\beta$ -CD and geraniol/ $\beta$ -CD inclusion complexes was confirmed by differential scanning calorimetry (DSC). Oxidative DSC revealed that the monoterpenes enclosed in the  $\beta$ -CD cavity were protected against oxidation, remaining intact in temperatures at which free monoterpenes were oxidized. Phase solubility studies showed that the inclusion complexes of thymol and geraniol with  $\beta$ -CD are more soluble in water than the free molecules themselves. Furthermore, in order to evaluate the fraction of monoterpenes that can be released from their complexes with MS in aqueous media, a series of release experiments were conducted.

**Keywords:** differential scanning calorimetry, modified starch, monoterpenes, phase solubility,  $\beta$ -cyclodextrin

## Introduction

Encapsulation has been extensively used during the last decades in the cosmetics and drug industry. Furthermore, it has found many applications in the food industry as a flavor carrier and as a treatment to impart some degree of protection against evaporation, reaction, or migration of food substances (Zeller and others 1999; Gouin 2004). Encapsulation provides the necessary protection of active compounds against oxidation, while—by increasing their solubility—it allows them to be used in the formulation of several fortified and functional foods (Schrooyen and others 2001). The simplest means of encapsulation is to emulsify the ingredient of interest in a solution containing the so-called “wall” material followed by freeze- or spray-drying (Desorby and others 1997).

Cyclodextrins (CDs) are chemically and physically stable molecules, formed by the enzymatic modification of starch (Hedges and others 1995). They have the ability to form inclusion complexes with a wide variety of organic compounds, which enter entirely or partly into the relatively hydrophobic cavity of CDs. The size of CD cavity allows the selective complexation of guest molecules (Szejtli 1998). Encapsulation with CDs may drastically modify the physical, chemical, and biological properties of the encapsulated molecules (Polyakov and others 2004) by increasing the dissolution rate, membrane permeability, and bioavailability of low-solubility nutraceuticals. The CDs act as flavor carriers, while furthermore they protect against oxidation, light-induced decomposition, and heat-induced changes. Moreover, CDs can improve the shelf life of food products and mask or reduce undesired smell or taste (Szente and Szejtli 2004). Probably the most important consequence of encapsulation in CDs is the increase of water solubility of various sparingly soluble compounds.

Carbohydrates such as hydrolyzed starches, emulsifying starches, and gums are the most common carrier materials

(Reineccius 1988, 1989). These hydrophilic materials have little affinity for hydrophobic oils. Modifying them with *n*-octenyl succinic anhydride can alter their hydrophilic nature. These hydrophobic octenyl side chains also impart emulsifying capability to the starches (Qi and Xu 1999). Carriers based on these new modified starches (MS) and their blends with other components have been successfully optimized for flavor encapsulation with spray drying (Buffo and Reineccius 2000).

Thymol is a terpenoid found in the essential oils of thyme (*Thymus vulgaris*) oregano (*Origanum vulgare*) and related species and has the characteristic smell of thyme. Thyme essential oil and its ingredients have been shown to exhibit strong antibacterial and antimicrobial activity. Components of thyme, mainly thymol and carvacrol, were also suggested to exhibit some antioxidant activity (Aydin and others 2005). Dietary supplementation with thyme oil tended to maintain higher polyunsaturated fatty acids (PUFA) levels in cell membranes of various rat tissues, as a result of its antioxidant activity (Youdim and Deams, 1999). Thymol and thymol hemisynthetic derivatives have promising antileishmanial potential and could be considered as new leading structures in the search for novel antileishmanial drugs (Robledo and others 2004).

Geraniol, an acyclic monoterpene alcohol (Carnesecchi and others 2002; Lijima and others 2004), is a natural component of plant essential oils. Geraniol shows *in vitro* and *in vivo* antitumor activity against various cancer cell lines. Geraniol sensitizes Caco-2 cells to an anticancer drug (Carnesecchi and others 2002). Burke and others (1997) found that geraniol, farnesol, and perillyl alcohol suppress pancreatic tumor growth. Palmarosa oil, which is composed of 65% geraniol and 20% geranyl acetate, shows antimicrobial action on *Saccharomyces cerevisiae* (Prashar and others 2003). Ji and others (2002) proved that geraniol prevents acute allograft rejection by inhibiting lymphocyte proliferation, the inhibition being concentration dependent.

Differential scanning calorimetry (DSC) is among the methods used to confirm the formation of a complex in the solid state. The disappearance of thermal events of guest molecules when they are examined as CD complexes is generally considered as a proof of real inclusion (Williams and others 1998; Pralhad and Rajendrakumar 2004). Furthermore, DSC may be used under oxidative

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conditions to study the oxidation of a molecule (Karathanos and others 2007).

In this work, encapsulated forms of thymol and geraniol in  $\beta$ -CD and MS were prepared in solid state. The verification of encapsulation was accomplished by DSC under either inert or oxidative conditions. The solubility of monoterpenes in the presence of  $\beta$ -CD was examined by a phase solubility study. Furthermore, the encapsulation efficiency of  $\beta$ -CD and MS was determined and the release of encapsulated monoterpenes from MS was studied.

## Materials and Methods

### Reagents and chemicals

Thymol was obtained from Riedel-de Haen (Seelze, Germany), geraniol was obtained from MP Biomedicals (Illkirch, France);  $\beta$ -CD was purchased from Aldrich Chemie GmbH (Steinheim, Germany). Tween 80 was obtained from Merck (Steinheim). MS (sodium octenyl succinate modified starch) was obtained from Cargill Inc. (Cedar Rapids, Iowa, U.S.A.). The schematic structure of octenyl succinate MS is given in Figure 1. Hexane of analytical grade was purchased from Aldrich.

### Preparation of the samples

**Preparation of inclusion complexes.** The inclusion complexes of thymol and geraniol in  $\beta$ -CD were prepared by dispersing thymol (49 mg) or geraniol (50 mg) in 20 mL aqueous solution of  $\beta$ -CD (16 mM) in 1:1 molar ratio of terpene to  $\beta$ -CD and mixed in a laboratory stirrer for 24 h at room temperature. In order to remove any insoluble monoterpenes, the suspensions were filtered through a 0.45- $\mu$ m PVDF filter (Chromafil P-45/25, Macherey-Nagel, Duren, Germany). The filtrates were frozen at  $-40$  °C for 24 h and then lyophilized in a Telstar Cryodos (Terrassa, Spain) freeze dryer. The powders thus obtained were stored in gas-tight bottles at  $-40$  °C until further handling.

**Preparation of the physical mixtures.** Physical mixtures consisting of thymol or geraniol with  $\beta$ -CD of the composition as the freeze-dried complexes were also prepared, by admixing monoterpenes and  $\beta$ -CD in a mortar with a pestle for 5 min to obtain a homogeneous blend. The blends were also stored in gas-tight bottles at  $-40$  °C until further analysis.

**Preparation of encapsulated monoterpenes in modified starch.** Emulsions of the monoterpenes studied were prepared by dissolving MS (20 g) in deionized water (50 mL), followed by the addition of thymol or geraniol (1 g), while mixing with the aid of a mechanical stirrer and employing Tween 80 (50 mg) as emulsifier. The resulting crude emulsions were homogenized for 1 min at 9500 rpm with an Ultra Turrax T25 homogenizer (IKA Instruments,

Staufen, Germany) and were subsequently turned to powder by a benchtop spray dryer (model SD-04, Lab-Plant Ltd., Huddersfield, U.K.). Ambient air was introduced into the dryer via a blower delivering around 500 g of air/min (0.39 m<sup>3</sup>/min at STP). The air was heated to a temperature of 130 °C. A peristaltic pump delivered the feed solution to a 2-fluid stainless-steel atomizer with a liquid jet of inside diameter 0.5 mm. The flow rate of compressed air to the atomizer was set at the maximum possible value. The drying air flowed cocurrently with the spray through the main chamber of the dryer and then through the cyclone. The feed solution flow rate was controlled through the speed of the peristaltic pump, and it was usually set at the maximum possible value permitting successful drying for the given drying air inlet temperature. The maximum evaporative capacity of the dryer is 20.8 mL of water/min at 250 °C drying air inlet temperature. In this work the feed flow rate was set at 10 mL/min and the outlet air temperature was around 85 °C. Spray-dried powder samples were collected in the flask at the base of the cyclone and were stored in hermetically sealed containers at  $-40$  °C until analyzed.

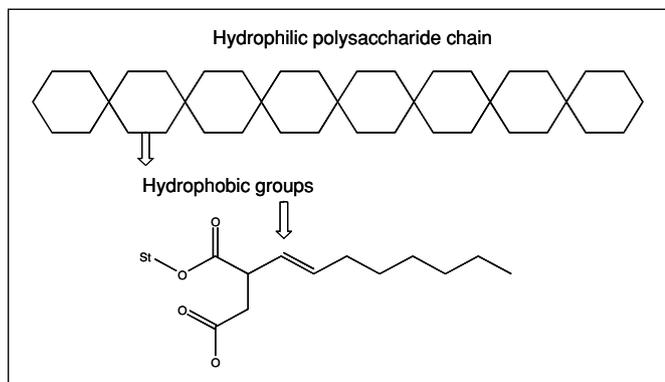
**Determination of surface-adsorbed monoterpenes.** The amount of surface-adsorbed monoterpenes was determined by extracting 2.6 mg of the geraniol or thymol complexes with MS or  $\beta$ -CD with 3 mL hexane by shaking for 30 min at ambient temperature. The suspensions were filtered and aliquots (0.5 mL) of the filtrates were sealed in GC vials and directly analyzed by capillary GC-MS (Soottitawat and others 2005).

**GC-MS analysis of monoterpenes.** An Agilent (Wallborn, Germany) HP series GC 6890N gas chromatograph equipped with a HP-5973 MS detector (EI, 70eV), split-splitless injector, and an HP 7683 autosampler were used for the determinations. Aliquots of standards or sample solutions (1  $\mu$ L) were injected into the gas chromatograph at a split ratio 1:20. Separation was achieved on a 30 m, 0.25-mm internal diameter, HP-5 MS capillary column, coated with a 250  $\mu$ m thick film of 5% phenyl-95% methyl polysiloxane. Helium was used as a carrier gas at a flow rate of 1 mL/min. The injector and transfer line temperature were set at 280 and 300 °C, respectively. The oven temperature program was initial temperature 80 °C for 2 min, 80 to 140 °C at 5 °C/min, where it was held for 6 min. Under these conditions geraniol and thymol were eluted in 11.98 and 13.17 min, respectively.

MSD Chemstation (Agilent Technologies Inc.) software was used for quantification of the monoterpenes. Calculations were carried out by means of reference curves constructed by analyzing a series of 7 standard mixtures of geraniol and thymol in hexane with concentrations ranging from 5 to 75  $\mu$ g/mL and 3 to 50  $\mu$ g/mL, respectively. The linearity of the mass selective detector was found satisfactory for the aforementioned range of concentrations with correlation coefficients equal to 0.996 and 0.995 for geraniol and thymol, respectively.

**Study of thymol/ $\beta$ -CD complex formation by DSC.** A Perkin Elmer differential scanning calorimeter (Perkin Elmer, DSC-6, Boston, Mass., U.S.A.) was employed. Indium was used for enthalpy calibration. Samples were weighed with an accuracy of  $\pm 0.01$  mg, placed in 40- $\mu$ L closed aluminum pans, and scans were conducted under nitrogen between 40 and 210 °C at a 10 °C/min rate. DSC curves of 4 types of samples were obtained: (a) pure  $\beta$ -CD, (b) pure thymol, (c) the physical mixture of (a) and (b), and (d) the inclusion complex of (a) and (b). Determinations were duplicated.

**Oxidation study of thymol, geraniol, and their encapsulated forms by DSC.** DSC was furthermore employed to study the thermo-oxidation stability of the samples. Samples of pure terpenes as well as their complexes with  $\beta$ -CD and their



**Figure 1 – Schematic structure of octenyl succinate modified starch.**

encapsulated forms in MS containing around 2 mg of terpenes were placed in 40- $\mu$ L aluminum pans having a hole in their lid. The specimens were heated in pure oxygen atmosphere from room temperature to 120 °C at 90 °C/min, remained at 120 °C for 1 min, to ensure a uniform temperature distribution within the sample, and then heated up to 420 °C at 10 °C/min.

**Phase solubility studies.** Phase solubility studies were carried out as described by Higuchi and Connors (1965). Briefly, an excess amount of thymol or geraniol was mixed with an aqueous solution of  $\beta$ -CD of increasing concentrations (0 to 0.015 M). A laboratory shaker was used and the samples were equilibrated for 2 d at 25, 35, and 45 °C at gas-tight bottles. Then the samples were filtered with a syringe through 0.45- $\mu$ m pore diameter PVDF filters, and the amount of thymol and geraniol in solution was determined spectrophotometrically by measuring the absorbance at 280.2 and 221.8 nm, respectively. Reference curves were constructed from the absorbance measurements of 5 different standard monoterpenes' concentrations (0 to 1.2 mg). The experiments were carried out in triplicate at each temperature.

The stability constants,  $K_C$ , of the reaction between monoterpenes and  $\beta$ -CD toward a complex formation were calculated from the straight-line portion of the phase solubility diagram according to the Higuchi-Connors equation (Eq. 1):

$$K_C = \frac{\text{slope}}{\text{intercept} \cdot (1 - \text{slope})} \quad (1)$$

**Release experiments and retention determination of monoterpenes in modified starch.** Release experiments of monoterpenes were carried out by dissolving into 3 mL of H<sub>2</sub>O 2.6 mg of either geraniol or thymol encapsulated in MS. After stirring at room temperature for predetermined time periods (0 to 20 h), released monoterpenes were extracted with hexane. Aliquots (0.5 mL) of the hexane solutions obtained from the release experiments of encapsulated samples, were sealed in GC vials and directly analyzed by capillary GC-MS. The release experiments were conducted in separate tubes, each one containing the same amount of complex and solvent (water) and kept at the same temperature, altering only the release time, after which the sample

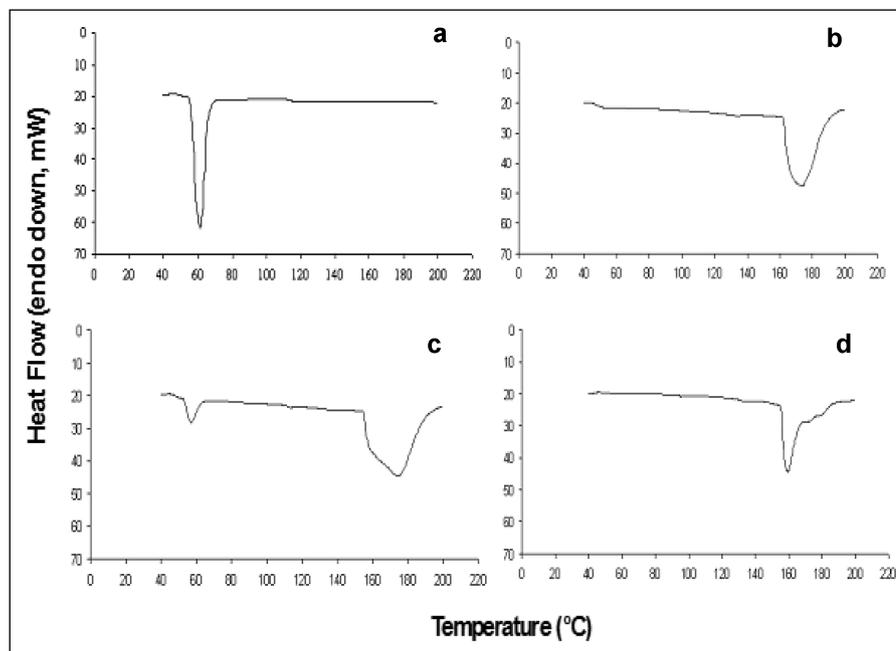
was extracted with hexane. The terpenes in the organic phase were measured by GC-MS as described above.

## Results and Discussion

The inclusion complexes' formation was confirmed by DSC in an inert atmosphere. This is the method of preference for  $\beta$ -CD complexes (Williams and others 1998). Figure 2 depicts the DSC results for the different types of samples: (a) pure thymol, (b) pure  $\beta$ -CD, (c) physical mixture of thymol with  $\beta$ -CD, and (d) the respective thymol/ $\beta$ -CD inclusion complex. Pure thymol DSC scan exhibited an endothermic peak at 61.6 °C (Figure 2a), which corresponds to thymol's melting point. The DSC thermogram of  $\beta$ -CD showed an endothermic peak at 170 °C (Figure 2b), possibly due to elimination of water. The DSC scan of the physical mixture was similar to those of pure thymol and  $\beta$ -CD, exhibiting the respective endothermic peaks (Figure 2c), thus offering an indication that no inclusion occurred by simple mixing. As it can be seen in Figure 2b and 2d, the thermograms of  $\beta$ -CD and the inclusion complex did not show any sharp endothermic peak in the temperature range of thymol's melting point. The elimination of the endothermic peak at 61.6 °C—assigned to pure thymol—(Figure 2a and 2d) indicates that an inclusion complex rather than a simple physical mixture has been formed between thymol and  $\beta$ -CD.

Since pure geraniol is liquid at room temperature, no DSC thermographs can be obtained for this substance in order to prove the formation of its inclusion complex. The inclusion of geraniol can be confirmed using DSC indirectly by comparing the thermal stability of free geraniol compared with geraniol encapsulated in  $\beta$ -CD molecules. The expected higher thermal stability of encapsulated geraniol may be considered as an indirect proof of inclusion.

In Figure 3, the DSC oxidation curves of (a) pure geraniol, (b) the inclusion complex of geraniol with  $\beta$ -CD, and (c) geraniol encapsulated in MS are presented. The exothermic peak observed at 174 °C for pure geraniol is related to geraniol's oxidation. This peak was not present in the DSC scan of the geraniol/ $\beta$ -CD complex, indicating that geraniol is protected from oxidation, being inside the  $\beta$ -CD cavity, and offering an indirect proof of geraniol's inclusion. Similar results were obtained by DSC of the geraniol/MS sample under oxidative conditions at the same temperatures, (Figure 3),



**Figure 2—DSC thermograms of (a) thymol, (b)  $\beta$ -CD, (c) their physical mixture, and (d) inclusion complex.**

indicating that geraniol is also more stable when it is encapsulated in MS. The exothermic peaks emerging after 322 °C for geraniol/ $\beta$ -CD complex and after 276 °C for geraniol/MS complex are possibly due to the decomposition of geraniol and  $\beta$ -CD due to the decomposition of MS and  $\beta$ -CD.

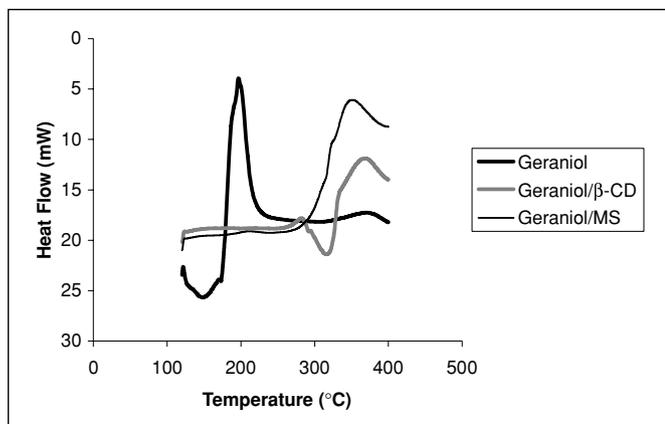
The same approach was followed for thymol by comparing the oxidation stability of the encapsulated and free molecule, and the respective oxidation curves are presented in Figure 4. The DSC thermograph of pure thymol under oxidation conditions reveals that thymol is oxidized at 166 °C as an exothermic process begins at this temperature. The exothermic curves emerging after 322 °C for thymol/ $\beta$ -CD complex and after 276 °C for thymol/MS complex in the respective thermographs are possibly due to the decomposition of  $\beta$ -CD and MS.

### Calculation of encapsulation efficiency

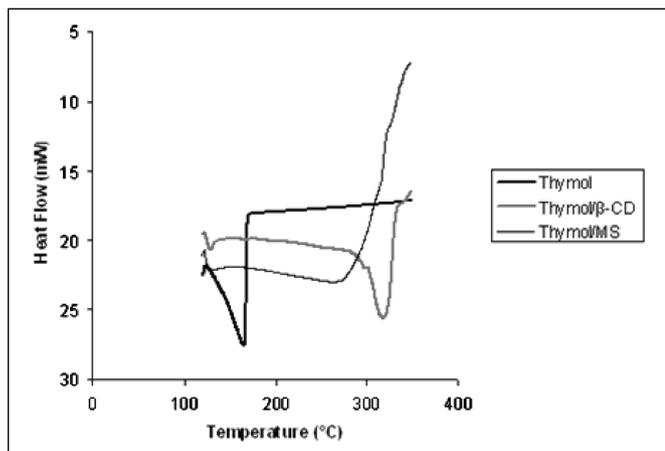
The encapsulation efficiency (EE%) was calculated from the following Eq. (2):

$$EE(\%) = \frac{\text{actual amount of encapsulated monoterpenes}}{\text{theoretical amount of encapsulated monoterpenes}} \times 100 \quad (2)$$

where the actual amount of encapsulated monoterpenes is the difference between the total monoterpenes used for encapsulation (= theoretical amount of encapsulated monoterpenes) and the surface-adsorbed monoterpenes.



**Figure 3** – DSC thermograms of pure geraniol, complex of geraniol/ $\beta$ -CD, and geraniol/modified starch under oxidative conditions.



**Figure 4** – DSC thermograms of pure thymol, complex of thymol/ $\beta$ -CD, and thymol/modified starch under oxidative conditions.

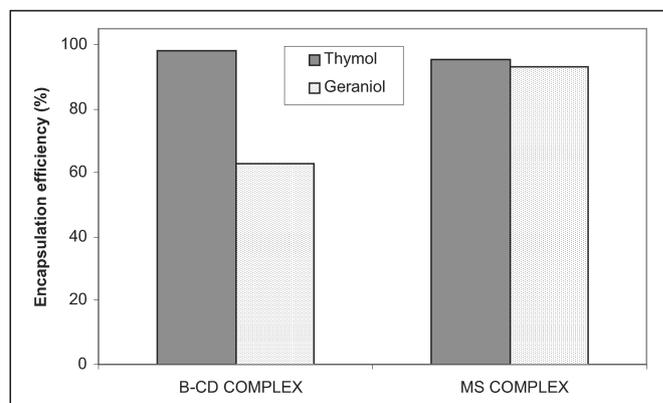
The encapsulation efficiencies of thymol and geraniol in  $\beta$ -CD and MS are depicted in Figure 5. As can be seen, MS encapsulated both molecules very effectively, probably as the result of reduced emulsion size obtained by the combined use of MS and Tween 80, which improves the stability of the inclusion complexes. On the other hand,  $\beta$ -CD encapsulated thymol almost quantitatively, and geraniol to a lesser degree. This difference may be assigned to  $\beta$ -CD, which offers the optimum cavity environment for encapsulating phenyl-ringed molecules like thymol (Clarot and others 2000).

### Phase solubility study

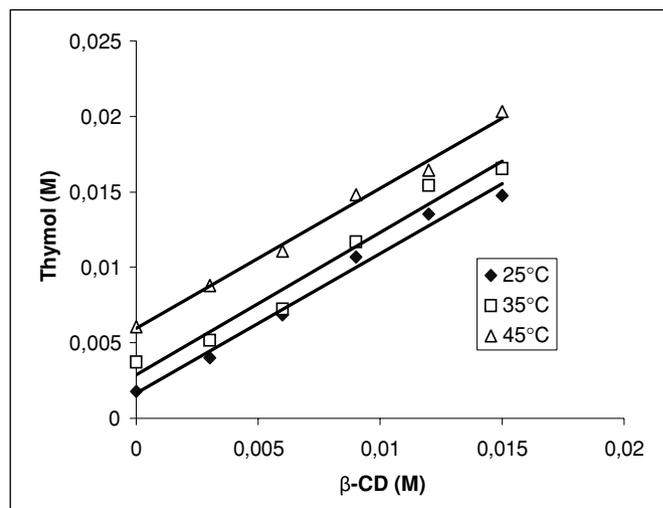
Phase solubility studies were carried out at 3 temperatures in order to calculate the stability constants,  $K_C$ , and the thermodynamic parameters for the complex formation. The phase solubility diagrams of thymol and geraniol with  $\beta$ -CD at the selected temperatures (25, 35, 45 °C) are presented in Figure 6 and 7, respectively. The plots show linear trends in all the temperatures studied. Diagrams exhibiting linear relationship are considered as AL-type, according to Higuchi and Connors (1965).

The stability constants,  $K_C$ , of the monoterpene/ $\beta$ -CD complexes at the selected temperatures (25, 35, 45 °C) were calculated by Eq. (1) and are presented in Table 1.

From the data provided in Table 1 it is obvious that the water solubility of both thymol and geraniol increases with increasing temperature (intercept of lines), and the same was true for the total



**Figure 5** – Encapsulation efficiency of thymol and geraniol in  $\beta$ -CD and MS.



**Figure 6** – Phase solubility study of thymol with  $\beta$ -CD in water at 25, 35, and 45 °C.

**Table 1 – Phase solubility study parameters and stability constants,  $K_C$ , of thymol/ $\beta$ -CD and geraniol/ $\beta$ -CD complexes at different temperatures (25, 35, 45 °C).**

Temperature (°C)	Intercept		Slope		$K_C$ (per M)	
	Thymol	Geraniol	Thymol	Geraniol	Thymol	Geraniol
25	0.0017	0.0042	0.9265	0.9629	7415	6179
35	0.0028	0.0055	0.9449	0.9668	5913	5294
45	0.0059	0.0072	0.9296	0.9772	2238	2227

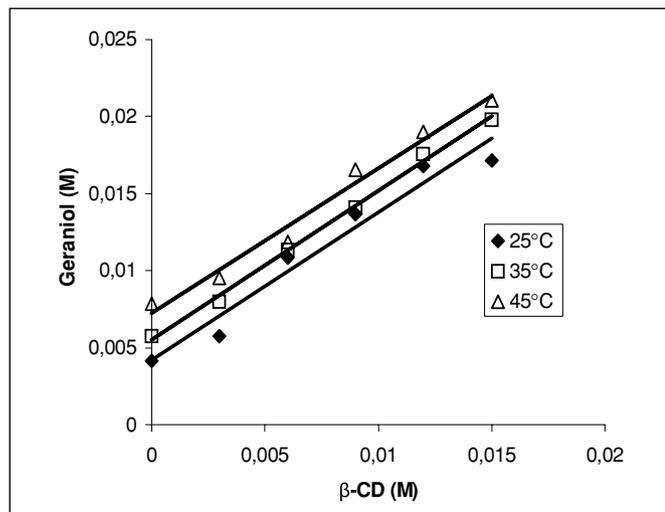
concentrations of these compounds in aqueous media containing  $\beta$ -CD. The decrement of  $K_C$  constants with increasing temperature is expected for an exothermic process. A similar effect of temperature on the stability constants has been reported for vanillin (Karathanos and others 2007).

The phase solubility data furthermore permit the derivatization of the values of various thermodynamic parameters involved in the complex formation. The integrated form of the Van't Hoff equation (Eq. 3) relates  $K_C$  to the absolute temperature  $T$ :

$$\ln K_C = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (3)$$

where  $R$  is the universal gas constant and  $\Delta H$  and  $\Delta S$  are the enthalpy and entropy changes, respectively. From the experimentally determined data of  $K_C$  compared with  $T$ , the values of  $\Delta H$  and  $\Delta S$  can be estimated (Tommasini and others 2004). According to Eq. (3), a plot of  $K_C$  compared with the inverse of the absolute temperature ( $1/T$ ) should give a straight line, and that was the case for the complexes of thymol/ $\beta$ -CD and geraniol/ $\beta$ -CD. The thermodynamic parameters calculated from the straight lines are presented in Table 2.

The negative  $\Delta H$  values indicate that the interaction processes between thymol and  $\beta$ -CD or geraniol and  $\beta$ -CD are exothermic. The magnitude of the enthalpy changes ( $\Delta H$ ) provides an indication of low-energy interactions such as hydrophobic interactions due to the displacement of water molecules from the cavity of the  $\beta$ -CD, increase of van der Waals intermolecular interactions, and hydrogen bonds formation. The negative values of  $\Delta S$  in these processes can be explained considering that the complex formation causes a decrease in translational and rotational degrees of freedom of the complexed molecules as compared with the free ones, leading to a more ordered system. The Gibbs free energy change for the interactions that take place during the inclusion process can be

**Figure 7 – Phase solubility study of geraniol with  $\beta$ -CD in water at 25, 35, and 45 °C.**

calculated from the following Eq. (4):

$$\Delta G_{25} = \Delta H - T \cdot \Delta S \quad (4)$$

The calculated Gibbs free energies at 298 K were found to be negative (Table 2), indicating that the inclusions studied are spontaneous processes.

### Release experiments

Release experiments were conducted by determining the amounts of monoterpenes released from the spray- or freeze-dried powders in water. In Figure 8, the release curves of thymol and geraniol from MS as a function of time are presented. As can be seen, shortly after dispersion in water, most of geraniol is released in solution (approximately 80% release in 5 min), while thymol's release is less prolonged (26%). After 10 h, the release of geraniol is almost quantitative (95%), while thymol still appears to show stronger affinity for MS, as only 30% is released.

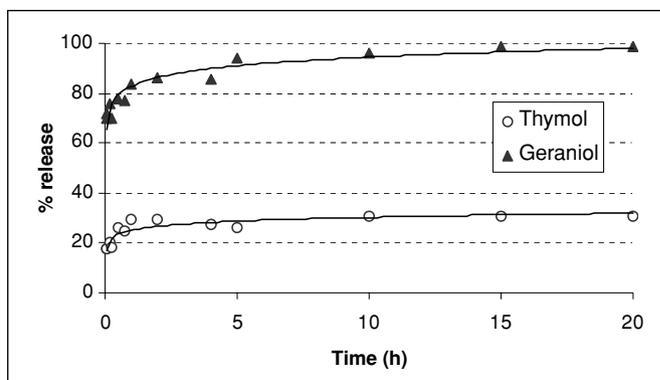
The delayed release of monoterpenes from MS could be attributed to the increased retention caused by the small droplet size emulsion, produced by the combination of MS with a 2nd surfactant, as reported by Soottitantawat and others (2005), who studied the encapsulation of l-menthol in MS.

### Conclusions

Encapsulation of thymol and geraniol in  $\beta$ -CD and MS increased the water solubility of the active compounds. Moreover, oxidative DSC studies revealed that thymol and geraniol were protected from oxidation when complexed with  $\beta$ -CD and MS. Release experiments showed that MS encapsulated geraniol is quantitatively released in water, while a significant fraction (70%) of thymol remains

**Table 2 – Values of thermodynamic parameters for complex formation of thymol and geraniol with  $\beta$ -CD.**

Thermodynamic parameter	Thymol values	Geraniol values
$\Delta H$ (kJ/mol)	-46.854	-39.883
$\Delta S$ (J/mol/K)	-82.0	-60
$\Delta G_{25}$ (kJ/mol)	-22.391	-22.003

**Figure 8 – Time related release of MS encapsulated thymol and geraniol in water.**

encapsulated in MS. It is concluded that the terpene complexes with  $\beta$ -CD and MS can be used as additives to the foods, to which they are normally added as flavors, with the advantage of higher stability.

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