Optimisation of β-cyclodextrin inclusion complexes with natural antimicrobial agents: thymol, carvacrol and linalool

Optimisation of β-Cyclodextrin Inclusion Complexes with Natural Antimicrobial Agents: Thymol, Carvacrol and Linalool

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Beta-cyclodextrin (β-CD) inclusion complexes with naturally derived antimicrobial (AM) agents: thymol, carvacrol, and linalool were prepared using a co-precipitation technique. Conditions including solvent composition, temperature, reaction time, and total solvent volume were investigated to optimise the inclusion efficiency (IE) and yield. Electrospray ionization mass spectrometry was used to confirm the formation of the thymol/β-CD complex and gas chromatography was used to quantify the amount AM agent that was encapsulated, absorbed onto the surface, or remaining in the filtered solvent. The systematic optimisation of the conditions improved both the yield of the complex and the IE of the AM agents compared to previously reported methods that have been applied to other agents. Using a 1:1 mole ratio of the AM agent to β-CD, the optimised parameters resulted in maximum yields of 87, 84 and 86% (w/w) for thymol, carvacrol and linalool respectively with IE's close to 100% (w/w) for each agent.

Keywords: antimicrobial; β-cyclodextrin; encapsulation; food packaging; inclusion complex

Introduction

Microencapsulation techniques are used in various industries to entrap a wide range of substances such as essential oils, oleoresins, aroma, and flavour mixtures (Balasubramani *et al.*, 2015, Michalska *et al.*, 2017). The encapsulation of these substances in an external matrix offers many advantages including: providing

stabilisation and protection against oxidation and thermal or light-induced decomposition (Wang *et al.*, 2014); reducing evaporation losses of volatile substances; converting liquid substances to solids to facilitate handling or separation (Reineccius, 1995, Desai and Hyun Jin, 2005); preventing discolouration; providing thickening; improving bioavailability (Xiao *et al.*, 2014, Lima *et al.*, 2016); and masking unpleasant aromas or flavours in food applications (Risch, 1995, Astray *et al.*, 2009). The most common encapsulation materials are the bio-derived cyclodextrins (CDs) such as α-, β- and γ-CDs, with 80–90% of all CDs primarily used in pharmaceutical and food-related applications (Astray *et al.*, 2009, Nieddu *et al.*, 2014, Pinho *et al.*, 2014). Given the high costs required to purify α- and γ-CDs, more than 97% of the CDs used are β-CDs.

The typical preparation of β-CD complexes involves the use of co-solvents such as ethanol, methanol, acetone, or dichloromethane to aid in the dissolution of non-polar active agents and/or to dissolve the β-CD (Szente and Szejtli, 1986, Bhandari *et al.*, 1998, Chen *et al.*, 2007, Nunes and Mercadante, 2007, López and Pascual-Villalobos, 2010, Lopez *et al.*, 2012, Gomes *et al.*, 2014, Mangolim *et al.*, 2014, Tang *et al.*, 2015). Although β-CD is not soluble in 100% ethanol (Coleman *et al.*, 1993), mixtures of ethanol with water are commonly used during β-CD complex preparation. However, many studies using ethanol report low inclusion efficiencies (IE's) and examples of relatively low or varied IE's in β-CD include: 31% for linalool (López and Pascual-Villalobos, 2010); 6% (Paramera et al., 2011) and 74% (Mangolim et al., 2014) for curcumin; 53% and 62% for red bell pepper extract prepared by magnetic stirring and ultrasonic mixing respectively (Gomes et al., 2014); and 35% for 2-nonanone (Abarca et al., 2016). The encapsulation of curcumin has also been studied using other co-solvents including methanol (Paramera et al., 2011) and acetone (Mangolim et al., 2014) resulting in maximum IEs of 17% and 14% respectively. Dichloromethane has

also been used to dissolve lycopene with a resulting IE of 50% (Nunes and Mercadante, 2007) and a mixture of dichloromethane/acetone has been used to dissolve astaxanthin with an IE of 49% (Chen et al., 2007). In each of these studies, there were no systematic investigations on the effect of optimising the level of co-solvent on the IE, the yield, or the quantification analysis.

The use of antimicrobial (AM) additives in food packaging films is a popular alternative to the direct addition of conventional preservatives to food products (Suppakul *et al.*, 2003, Muriel-Galet *et al.*, 2012, Makwana *et al.*, 2014, Sung *et al.*, 2014). Of the wide range of natural AM agents utilised in food packaging, essential oil extracts (EOEs) are prevalent due to their availability and, in many cases, broad-spectrum AM activity (Gutierrez *et al.*, 2008, Rodríguez *et al.*, 2014). Examples of EOEs include thymol, carvacrol and linalool, which are hydrophobic AM agents extracted from basil and oregano plants. These active components have shown significant promise in AM packaging applications (Kuorwel *et al.*, 2011), and thymol, for example, has been shown to exhibit strong antibacterial, AM and some antioxidant activity (Suhr and Nielsen, 2003, Mourtzinos *et al.*, 2008). Carvacrol and linalool have also been shown to possess antifungal, insecticidal, antioxidant, and antiparasitic properties (Lopez *et al.*, 2012, Ramos *et al.*, 2012).

The addition of the abovementioned and other AM agents directly into packaging films is limited however, primarily due to the volatile nature of EOEs which can result in unacceptable losses during the production of AM films (Suppakul *et al.*, 2011, Ramos *et al.*, 2012). Microencapsulation of these AM agents using β-CD offers a promising technique that can protect the AM agents during processing to significantly reduce these losses (Guarda *et al.*, 2011, Tao *et al.*, 2014). Several methods have been developed to prepare β-CD inclusion complexes including: co-precipitation, slurry,

paste, and dry mixing (Hedges, 1998, Wang *et al.*, 2011); kneading, freeze-drying and spray-drying (Marques, 2010); and neutralization (Choi *et al.*, 2001). The method of co-precipitation is one of the simplest techniques to prepare inclusion complexes, however, large-scale production is limited due to the size of the reaction tanks required and wastewater disposal considerations (Hedges, 1998). Although the encapsulation of thymol, carvacrol, and linalool using various methods have been previously reported (Ponce Cevallos *et al.*, 2010, Bonetti *et al.*, 2015, Guimarães *et al.*, 2015), these studies have not quantified the IE's of the processes.

Improving the IE, preventing rapid losses of core materials during storage, and minimizing the amount of un-encapsulated oil at the surface of wall materials are essential to producing high-quality inclusion complexes with maximum yields (Choi *et al.*, 2001, Jafari *et al.*, 2008). Volatile liquids, however, may also be susceptible to evaporation losses during the preparation of their inclusion complexes (Guarda *et al.*, 2011). It is therefore important to investigate factors influencing the efficacy of any technique in order to optimise the process of encapsulation (Serafini *et al.*, 2012). In view of the wide range of existing applications of inclusion complexes, the potential use of these to overcome the volatility problems associated with incorporating natural AM agents in food packaging materials, and the need for improved and efficient methods of complex preparation that deliver higher yields with greater IE, the present study was undertaken. The primary aims were to: (i) investigate the experimental parameters that affect the IE and the yield of β-CD inclusion complexes with the AM agents: thymol, carvacrol, and linalool, and (ii) hence produce the target complexes that are suitable for food packaging applications under a co-precipitation method that is more efficient than existing methods.

Materials and Methods

Materials

Ethanol (absolute) was purchased from Merck Australia and the following chemicals were purchased from Sigma-Aldrich Australia: isooctane (anhydrous, 99.8%), beta-cyclodextrin (β-CD, 98.5% purity, 1.5% (w/w) water content), the AM agents: thymol (99.5% purity), carvacrol (98% purity) and linalool (97% purity). Milli-Q water was used in all preparations.

Pre-Optimised Thymol/β-CD Complex Preparation

The pre-optimised preparation of a thymol/β-CD complex was performed according to a precipitation method reported previously (Szente and Szejtli, 1986, Bhandari *et al.*, 1998, Bhandari *et al.*, 1999) with minor changes to the drying method and the ratio of the components. A quantity of β-CD (3.8352 g, 3.33 mmol) was dissolved in 38 mL of a 50% (v/v) ethanol/water mixture at 55°C. Thymol (0.5000 g, 3.33 mmol) was dissolved in absolute ethanol and added with continuous stirring to the warm β-CD solution at a 10% (w/v) ratio and the solution was covered. After the addition of thymol, the heating was discontinued and the mixture was cooled to room temperature (RT) with stirring for 4 h. The solution was then further cooled to 4°C and maintained at this temperature for 24 h. The complex was collected by vacuum filtration and was dried for 5-9 days at room temperature.

Electrospray Ionization Mass Spectrometry Analysis

A mass of 0.1 g of thymol/β-CD complex was dissolved in 25 mL of water followed by the addition of 25 mL of ethanol with stirring. A Thermo Electron Corporation LCQ-DECA-XP-MAX electrospray ionization mass spectrometry (ES/MS) instrument was

used to record the mass spectrum by the direct diffusion method. The ESI probe voltage was 3 kV and samples were delivered at a flow rate of 30 μL min^{-1} *via* a syringe pump. The capillary temperature was maintained at 260°C and no sheath or desolvation gas was used.

Gas Chromatographic Analysis

Gas chromatographic analyses were performed using a Varian Star 3400-CX gas chromatograph (GC) equipped with a fused silica capillary column DB-5 (30 m × 0.32 mm i.d.; film thickness: 0.25 μm; J & W Scientific, USA). The GC was operated using the following conditions: injection volume: 1.0 μL; initial column temperature: 80°C; heating rate: 5°C min^{-1} up to 180°C; kept at this temperature for an additional 1 min; injector temperature, 250°C; split ratio: 1:60; FID detector temperature: 300°C; and carrier gas: nitrogen.

Determination of Yield, Inclusion Efficiency and Un-Complexed AM Agent

Yield of Complex

The yield of the complex (expressed as a mass percentage) was calculated as the ratio of the recovered mass of the dried complex to the theoretically expected mass for 100% conversion, based on the mass of the materials used (i.e. AM agent plus β-CD) in the synthesis.

Inclusion Efficiency

The total mass of AM agent associated with the β-CD complex comprises the encapsulated AM agent and any AM agent that may be adsorbed on the exterior surface of the β-CD host. The total mass of AM agent was determined by extraction in ethanol

that is a solvent capable of fully extracting the complexed AM agent from the host as well as any AM agent adsorbed to the surface of the host.

To determine the IE, a mass of *ca.* 40 mg of the complex was placed in a round-bottom flask and 25 mL of ethanol was added. The flask was sealed and its contents stirred for 150 min. A volume of 5 mL of the extract was filtered using a Phenex RC 0.45 µL syringe filter and an aliquot of the filtrate was directly analysed by GC under the conditions described previously. The content of AM agent in the extract was determined using a standard calibration curve and the results are expressed as an average of six extractions along with the corresponding standard deviation. The IE was calculated using the following equation:

$$\text{IE}\ (\%) = 100 \times (m_{\text{complex}} / m_{\text{theoretical}}) \qquad (1)$$

where m_{complex} is the mass of AM agent extracted from the inclusion complex and $m_{\text{theoretical}}$ is the theoretical mass of AM agent for the given mass of complex assuming a 1:1 guest/host ratio and complete complexation. The value of $m_{\text{theoretical}}$, assuming a 1:1 guest/host mole ratio, is determined using:

$$m_{\text{theoretical}} = m_{\text{complex}} \times \text{MW}_{\text{thymol}}/(\text{MW}_{\beta\text{-CD}} + \text{MW}_{\text{thymol}}) \qquad (2)$$

where $\text{MW}_{\text{thymol}}$ and $\text{MW}_{\beta\text{-CD}}$ are the molecular weights of thymol and β-CD respectively. The assumption of complete complexation excludes guest molecules being entrapped among adjacent β-CD monomers, dimers or polymers, and guest molecules being adsorbed on the surface (Loftsson *et al.*, 2002, Loftsson *et al.*, 2004).

To quantify the mass of AM agent that was adsorbed to the surface of the β-CD host, a mass of 0.1711 g of the complex was firstly mixed with 3 mL of isooctane in a sample vial. Isooctane is sufficiently non-polar so as not to dissolve the β-CD host and thereby extract the AM agent that is complexed. The vial was capped and thoroughly

agitated for 5 min using a vortex mixer set at high speed. The complex was then filtered under vacuum and an aliquot of the filtrate was directly analysed using GC under the conditions described previously. Extractions were performed in triplicate and the amount of adsorbed AM agent was calculated using a standard calibration curve. The amount of AM agent (AO), assumed to be present as an adsorbed oil, was calculated using the following equation:

$$\text{AO}\ (\%) = 100 \times (m_{\text{isooctane}} / m_{\text{theoretical}}) \quad (3)$$

where $m_{\text{isooctane}}$ is the mass of AM agent detected in the isooctane solvent and $m_{\text{theoretical}}$ is the theoretical mass of AM agent assuming a 1:1 guest/host ratio (see Equation (2)).

Quantification of Un-Complexed Thymol

In order to determine the amount of AM agent remaining in the filtrate following the initial synthesis step, a volume of 2 mL of filtrate was placed in a 15 mL vial. Isooctane (5 mL) was added and the vial capped and gently shaken to facilitate mixing before the organic phase was separated and placed in a volumetric flask. The filtrate was washed further with isooctane and the organic phase (total of 10 mL) collected. An aliquot of the collected organic phase was directly analysed using GC in accordance with the method described previously.

The amount of un-complexed agent (UA) was calculated as the ratio of recovered AM agent in the total volume of the filtrate to the amount of AM agent originally added to the reaction mixture:

$$\text{UA}\ (\%) = 100 \times (m_{\text{organic}} / m_{\text{added}}) \quad (4)$$

where m_{organic} is the mass of AM agent detected in the organic phase and m_{added} is the mass of AM agent originally added to the reaction mixture. The value of m_{added} varies from $m_{\text{theoretical}}$ in that m_{added} is the experimental mass of the agent added to the mixture.

The analysis was performed in triplicate and the amount of AM agent was calculated using a standard calibration curve.

Synthesis Optimisation Experiments

A series of optimisation experiments was performed in order to maximise the yield of the complex and IE of thymol. A summary of the experimental conditions is presented in Table 1.

Table 1. Experimental parameters of solvent composition, initial reaction temperature and total solvent volume and conditions used in optimizing the yield and inclusion efficiency of thymol in β-CD complexes (n=3).

Solvent composition: ethanol/water/ % (v/v)	Initial reaction temperature /°C	Total solvent volume/mL	Other experimental conditions
50/50 60/40 70/30 80/20 90/10 92.5/7.5 95/5 100/0	55	204	Mix reactants at 55°C, cool to RT with stirring over 4 h period, cool to 4°C and maintain at this temperature for 24 h
97.5/2.5	55 60 80	204	
99.5/0.5	55	204	Mix reactants at 55°C, maintain at 55°C with stirring for 4 h, cool to 4°C and maintain at this temperature for 24 h
99.5/0.5	55	204 122 102 60	Mix reactants at 55°C, cool to RT with stirring over 4 h period, cool to 4°C and maintain at this temperature for 24 h
100/0	55	60	Mix reactants at 55°C, maintain at 55°C with stirring for 4 h, cool to 26 ± 1°C with stirring over 4 h, cool to 4°C and maintain for 24 h

In these experiments, the effect of the concentration of ethanol co-solvent on the formation of the complex was investigated using a constant total solvent volume of 204 mL. The preparation of the complex was achieved by firstly dissolving β-CD (3.8352 g, 3.33 mmol) in water at 55°C. In each case, thymol (0.5000 g, 3.33 mmol) was dissolved in different volumes of ethanol and added to the β-CD solution. In this series of experiments, the final ethanol/water ratio was varied from 0-50% (v/v) and the remaining preparation conditions were as described previously.

To optimise the reaction conditions with respect to the heating and cooling regimes, the effect of using higher temperatures during the synthesis of the complex was also investigated with complexes prepared at 55, 60 and 80°C. In each of these experiments, the concentration of ethanol and the total volume of the reaction mixture were held constant at 2.5% (v/v) and 204 mL respectively. The remaining preparation conditions were as described above. In addition, the effect of maintaining the temperature at 55°C for 4 h under continuous stirring rather than removing the heat source once the components were mixed was explored. The remaining preparation conditions for these experiments were also as described previously.

The effect of the total volume of the ethanol/water solvent system was investigated by successively reducing the total solvent volume from 204 mL to 60 mL in separate experiments. The ethanol concentration was held constant at 0.5% (v/v) and all other conditions were the same as those described previously.

The effect of extending the time allowed for the reaction to be completed under continuous stirring on the yield of complex and the IE was also investigated. In these experiments (not summarized in Table 1), the stirring was continued for a further 48 h after the heating was discontinued and the temperature of the reaction mixture reached

26 ± 1°C. The ethanol concentration was held constant at 0.5% (v/v) with all other conditions being the same as those described above.

Synthesis of Complexes Under Optimised Conditions

Beta-cyclodextrin complexes encapsulating thymol as well as carvacrol and linalool were each prepared under the optimised conditions as determined from the previous experiments. In these cases, β-CD (3.8352 g, 3.33 mmol) was reacted with the AM agents: thymol, carvacrol, and linalool in a 1:1 mole ratio in each case. The β-CD was dissolved in 60 mL of water at 55°C to which the appropriate mass of AM agent was added and the reaction mixture vigorously stirred. The solutions were covered and maintained at 55°C for 4 h with continuous stirring after which the heating was discontinued and the mixture was continuously stirred for a further 4-5 h until the temperature decreased to 26 ± 1°C. The mixture was then cooled to 4°C, maintained at this temperature for 24 h and the precipitated complexes were recovered by vacuum filtration. The complexes were dried in a vacuum desiccator for 1 h after which the vacuum was removed. The complexes remained in the desiccator for a further 12 h to remove any residual water and until constant masses were attained. The yield, IE, absorbed AM agent on the surface of the β-CD, and amount of un-complexed agent were determined in accordance with the methods described above.

Data Analysis

All experiments were performed in triplicate with the exception of the yield which was performed in duplicate. Error bars and errors presented in the tables and text are based on the standard deviation from the mean.

Results and Discussion

Pre-Optimised Complex Yield and Inclusion Efficiency

The preparation of the thymol/β-CD complex in accordance with a previously reported precipitation method (Szente and Szejtli, 1986, Bhandari *et al.*, 1998, Bhandari *et al.*, 1999) resulted in a final product yield of 77.0%. In the current study, it was observed that under the same synthesis conditions, no precipitate formed after the addition of thymol at 55°C despite having used the minimum amount of solvent during the preparation. The precipitate started to form only at temperatures below 40°C and the precipitate that was eventually recovered was analysed using ES/MS revealing a low-intensity peak corresponding to the 1:1 mole ratio β-CD complex (*m*/*z* = 1284.2) as shown in Figure 1. Peaks corresponding to β-CD and free thymol were also detected in the spectrum. The 1:1 mole ratio observed for the thymol/β-CD in the current study is consistent with the work of Bethanis *et al.* (2013) who determined a 1:1 mole ratio of thymol/β-CD in the solid state using x-ray crystallography.

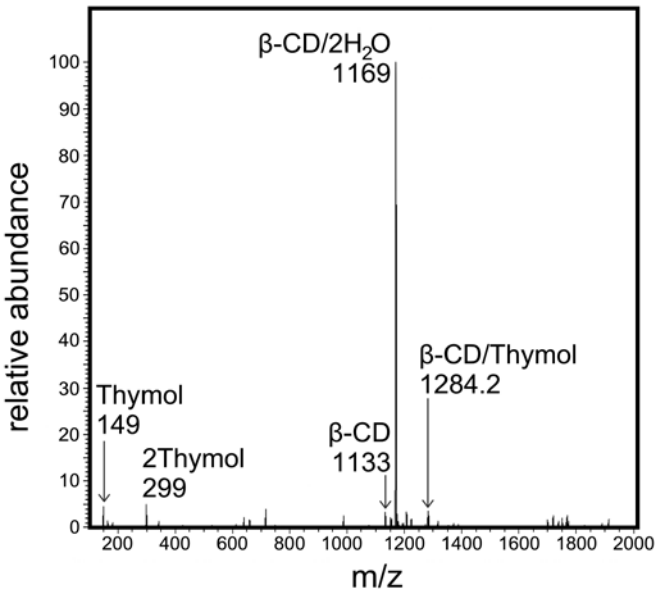

Figure 1. Example of an ES mass spectrum of thymol/β-CD complex prepared without ethanol confirming the presence of the inclusion complex at m/z = 1284.2.

Although the qualitative analysis using ES/MS indicates that thymol was complexed with β-CD, the results do not enable the efficiency of the process to be determined and so a quantitative analysis using GC was used to assess the IE. In this study the IE as defined by Gomes *et al.* (2014) was used as this provides the most accurate calculation of the percentage of the guest molecules that are complexed within the host cavity.

Other authors have defined and/or used terms such as "complexation efficiency" (Mangolim *et al.*, 2014), and "entrapment efficiency" (Hill *et al.*, 2013) to characterise the efficiency with which complexation has been achieved and it is important to note that these quantities are not directly comparable to each other or the "inclusion efficiency" as calculated in the current study. In this study, thymol was extracted from the thymol/β-CD complex using ethanol and the pre-optimised IE was 79.6 ± 0.5% (w/w) of the maximum that would be expected if each β-CD host molecule formed a complex with one molecule of the AM agent.

Optimised Preparation of Complex

Effect of Ethanol Co-Solvent

The effect of varying the concentration of the co-solvent ethanol on the yield and IE was investigated over the co-solvent range of 0-50% (v/v) ethanol/water. It was observed that for syntheses using concentrations of ethanol greater than 20% (v/v), no precipitate was formed unless the reaction mixture was cooled overnight at 4°C. Syntheses using lower amounts of ethanol resulted in a precipitate even at room temperature. As shown in Figure 2, the yield of complex decreased as the amount of ethanol was increased in the reaction mixture. This suggests that the ethanol content of the reaction mixture significantly influences the yield of complex. This is most likely

due to the complex being more soluble in the reaction mixture containing higher levels of ethanol; it therefore remains in solution and is not recovered, leading to lower isolated yields.

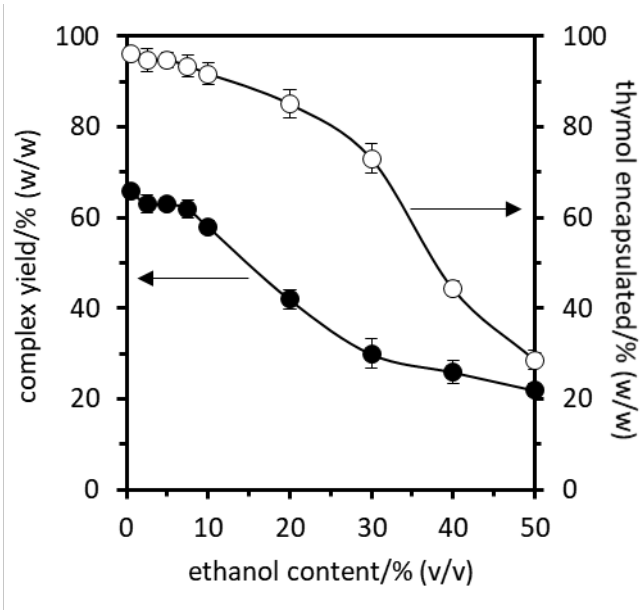

Figure 2. The effect of ethanol/water co-solvent composition on: (a) the yield of thymol/β-CD complex (filled circles) (n=2) and (b) the IE of thymol in the complex (open circles) (n=3).

Figure 2 also shows that the lower the amount of ethanol used in the synthesis, the higher the thymol/β-CD IE where the product prepared with 0.5% (v/v) ethanol has the greatest thymol IE of 96.2 ± 1.0% (w/w). These findings are consistent with the notion that ethanol is capable of disrupting the non-covalent bonding of thymol to the β-CD structure (Kalathenos and Russell, 2003). Hydrophobicity is the main driving force for guest/β-CD inclusion complexation and so the hydrophobic interactions necessary for encapsulation are stronger in polar aqueous environments compared with less polar solvent media such as ethanol/water mixtures. In the latter case, the solubilisation of thymol by ethanol is more favourable than the interaction of thymol with β-CD. In a similar study, the maximum complexation efficiency of curcumin in β-CD prepared by

co-precipitation in ethanol/water using a molar ratio of 1:2 was found to be 74% (Mangolim *et al.*, 2014). This low efficiency may be attributed to the combined use of ethanol and rotary evaporation to remove the co-solvent before cooling the mixture. However, it is important to note that in the latter study the authors calculated the efficiency based on the mass of the complexed curcumin to the total mass of curcumin added initially.

The filtrate from the syntheses in the present study was also analysed to investigate the amount of thymol that remained un-complexed with and unbound to the surface of the β-CD. Figure 3 shows plots of: (i) the percentage of the total thymol used in the synthesis that is adsorbed on or complexed with the β-CD and (ii) that which is un-complexed and remains in the filtrate, as a function of the percentage of ethanol in the solvent system that was used in the synthesis of the complex.

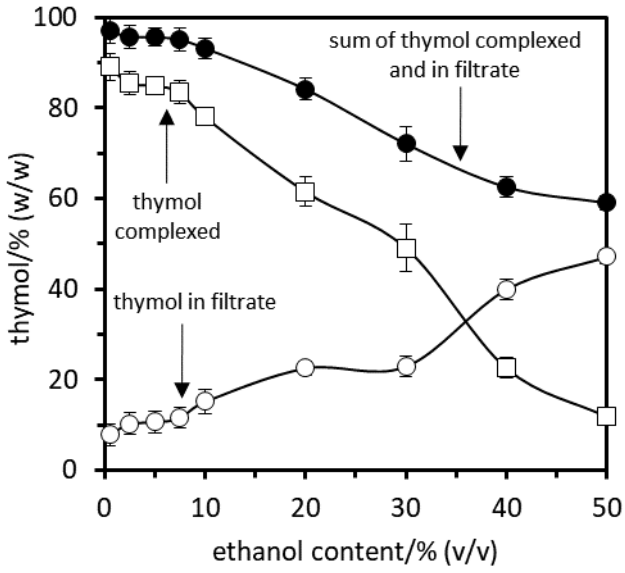

Figure 3. The effect of ethanol/water co-solvent composition on: (a) the percentage of the total thymol adsorbed/complexed with the β-CD (open squares), (b) percentage of un-complexed thymol in filtrate (open circles) and (c) the sum of (a) and (b) (filled circles) (n=3).

The percentage of thymol that was adsorbed/complexed (see Figure 3) was determined by quantitative GC analysis thymol in an ethanol extract of the prepared complex and the percentage of thymol in the filtrate was also obtained by GC analysis. Figure 3 also shows a mass balance plot in the form of the sum of the two other percentages. The results are consistent with the previous observation where reaction mixtures with lower ethanol content resulted in higher IE's of thymol in the β-CD host. Interestingly, the mass balance of the total thymol in the filtrate and that recovered from the β-CD complex suggests that some loss of thymol from the system occurred (see Figure 3). The loss also increased with increasing ethanol content in the synthesis solvent and this may be attributed to evaporation of thymol during the heating stage, or some of the thymol having remained in the aqueous phase in the form of the complex following the extraction process. In the case of the latter, the addition of isooctane was observed to result in the formation of a gel layer that may have trapped some of the thymol/β-CD complex. Some of the thymol adsorbed on the surface of the β-CD is also expected to have been lost by evaporation during the drying step (Bhandari *et al.*, 1998).

The thymol/β-CD complex was also prepared in the absence of ethanol to investigate the possibility of preparing the complex without using a co-solvent. It was found that without ethanol, the IE and yield were 99.8 ± 1.0% (w/w) and 87% (w/w) respectively. This suggests that in some cases the aqueous solubility of hydrophobic agents such as thymol may preclude the need for a co-solvent to aid dissolution (Chen *et al.*, 2007). Nonetheless, it is expected that this will not be the case in general and so it will usually be necessary to use small amounts of co-solvent to aid in the formation of the inclusion complex.

The amount of thymol absorbed on the surface of β-CD for samples prepared using only water as the solvent was determined by washing the complex with isooctane. Analysis of thymol in the washing solutions showed that only 0.26 ± 0.01% (w/w) thymol was adsorbed to the surface of the β-CD. This also confirms that isooctane did not extract the thymol from the β-CD complex and that isooctane is a good choice of solvent for washing the complex during the clean-up process.

Effects of Temperature, Reaction Time and Total Solvent Volume

The yield of the complex and IE of thymol at 55, 60 and 80°C using 2.5% (v/v) ethanol in the reaction mixture were also investigated. It is well known that the solubility limit of β-CD is up to 10 mM under ambient conditions (Marcolino *et al.*, 2011). It is expected that the thymol will be more soluble in aqueous solutions at the higher temperatures and this increased solubility may lead to greater IE's due to an increased concentration of thymol in solution (Mangolim *et al.*, 2014). The yields and IE's obtained at 60 and 80°C were found to be close to those obtained at 55°C which were 63% (w/w) and 94.7 ± 0.9% (w/w) respectively. This suggests that any difference in the solubility of the thymol at the increased temperatures does not affect the IE and therefore conducting the synthesis at higher reaction temperatures has no significant effect on the formation of the complex. Temperatures below 55°C were not explored as it was observed that β-CD is not completely soluble in the solvent system at these temperatures.

Under the pre-optimised conditions described above, the reaction mixture was stirred for 4-5 h after the heating was discontinued. To determine if an extended length of time may improve the yield and/or IE, a sample prepared using 0.5% (v/v) ethanol was reacted for a total of 48 h with continuous stirring. The extended time that allowed for the dissolution of thymol in the system resulted in no significant difference in the IE

or the yield. In addition, the total volume of the ethanol/water co-solvent was varied to assess its effect on the yield. Several complexes of β-CD with thymol were prepared using smaller total solvent volumes but maintaining the level of ethanol at 0.5% (v/v). Decreasing the total solvent volume from 204 mL to 60 mL improved the yield from 66±1 to 87±3% (w/w) and it was found that this increase was achieved without a compromise in the IE.

The minimum total volume of solvent that can be used is clearly dependent on the solubility of β-CD in the solvent system at the temperature used for the synthesis. Under the conditions used in the current study the minimum total volume was found to be 60 mL. Decreasing the solvent volume increases the amount of solute in the solution thereby producing more precipitate, resulting in a higher yield. Moreover, in the preparation of these complexes, as the total solvent volume is decreased keeping the amounts of thymol and β-CD the same, the concentrations of both thymol and β-CD will be subsequently increased, noting that the concentration of free thymol cannot be increased as much as that of β-CD due to its very low aqueous solubility. For a 1:1 inclusion complex of specific association constant *K*, the concentration of complex is given by the following:

$$[\text{complex}] = K[\text{free guest}][\beta\text{-CD}] \quad (5)$$

where [complex] is the concentration of the complex, [free guest] is the concentration of the free guest, and [β-CD] is the concentration of the free β-CD. Thus, it is expected that decreasing the total solvent volume, and thereby increasing the free guest and β-CD concentrations, will cause the dynamic equilibrium to be moved towards the direction of complexation resulting in a higher IE under these conditions.

Optimised Inclusion of Thymol, Carvacrol and Linalool

The optimum synthesis conditions for the inclusion of thymol in β-CD were applied to the inclusion of carvacrol and linalool in β-CD and a summary of the results is shown in Table 2. These parameters were calculated in accordance with the definitions given above in equations (1) to (3).

Table 2. Optimized yield (n=2) and inclusion efficiency as well as the amount of uncomplexed AM agent and amount of AM agent adsorbed in β-CD complexes with thymol, carvacrol and linalool (n=3).

AM Agent	Complex Yield/% (w/w)	Inclusion efficiency IE/% (w/w)	Amount of uncomplexed agent (UA)/ % (w/w)	Amount of adsorbed AM (AO)/ % (w/w)
Thymol	87 ± 3	99.8 ± 1.0	8.9 ± 1.8	0.27 ± 0.01
Carvacrol	84 ± 2	99.6 ± 0.9	8.4 ± 1.0	0.35 ± 0.09
Linalool	86 ± 2	99.3 ± 1.1	5.0 ± 0.3	0.30 ± 0.03

Similar to the case of thymol, both carvacrol and linalool were successfully complexed with β-CD at high yield using the optimised conditions with IE's close to 100% (w/w) suggesting this method is highly feasible for the inclusion of these agents. Guarda *et al.* (2011) reported the percentages of microencapsulated agents thymol and carvacrol in gum arabic *via* oil-in-water emulsions to be 98% and 91% respectively, which are slightly lower than the efficiencies achieved in the present study.

Evaluation of the AM agents adsorbed onto the surface of the β-CD indicated that surface adsorptions of carvacrol and linalool were very small as was observed in the case of thymol. Analysis of the filtrates showed the quantities of carvacrol and linalool remaining in solution after complexation were lower than that of thymol (see Table 2) which may be a result of the physical state of the agents with carvacrol and linalool being liquids at room temperature whereas thymol is a semi-crystalline solid.

In the present study, the maximum theoretical IE for thymol was found to be 13.23 g of thymol per 100 g β-CD. The maximum IE that was experimentally achieved was 13.20 g per 100 g of β-CD and was achieved without the use of ethanol as a co-solvent. The high IE of thymol, and indeed the high IE's of carvacrol and linalool, obtained in the present study may be the result of two important factors. Firstly, the use of water only as the solvent produced both higher yields and IE's. Secondly, maintaining stirring of the mixture at 55°C for 4 h may have contributed to improved IE's of the agents.

The IE's obtained for each of these complexes is close to 100% (w/w) indicating that in all cases studied a 1:1 mole ratio of guest/host exists. In the cases of the thymol/β-CD and linalool/β-CD complexes these results are confirmed by the respective x-ray crystallographic studies of Bethanis *et al.* (2013) who reported a 1:1 mole ratio for thymol/β-CD and Ceborska (2016) who reported a 2:2 molar ratio for the (-)-linalool/β-CD dimer. It is interesting to note, however, that the X-ray crystallographic study by Bethanis *et al.* (2013) suggests that a 1:2 carvacrol/β-CD ratio exists in the solid state where the complex was produced using a guest/host molar ratio of 1.5:1. Under the synthesis conditions used in the current work, if a complex was formed having a 1:2 mole ratio of guest/host then that would result in an observed IE of only 50% (w/w) so clearly a 1:2 complex was not obtained in the present study.

Conclusions

The natural AM agent thymol was successfully complexed with β-CD using the method of co-precipitation. The concentration of ethanol in the solution significantly influenced the complex formation with higher ethanol levels resulting in lower yields and lower IE's. The other optimum conditions comprised a reaction temperature of 55°C, a stirring time of 4-5 h after the complex had formed, and a reduced total solvent volume. Using

these optimised parameters, the average yields of the AM/β-CD complexes were 87, 84, and 86% (w/w) for thymol, carvacrol, and linalool respectively. For each complex, an IE that was close to 100% (w/w) was obtained suggesting that this optimised method is highly feasible for the complexation of these agents with β-CD for use in various applications.

Disclosure of Interest

The authors report no conflicts of interest.

References

Abarca, R.L., Rodríguez, F.J., Guarda, A., Galotto, M.J. & Bruna, J.E., 2016. Characterization of beta-cyclodextrin inclusion complexes containing an essential oil component. *Food Chemistry,* 196**,** 968-975.

Astray, G., Gonzalez-Barreiro, C., Mejuto, J.C., Rial-Otero, R. & Simal-Gándara, J., 2009. A review on the use of cyclodextrins in foods. *Food Hydrocolloids,* 23**,** 1631-1640.

Balasubramani, P., Palaniswamy, P.T., Visvanathan, R., Thirupathi, V., Subbarayan, A. & Prakash Maran, J., 2015. Microencapsulation of garlic oleoresin using maltodextrin as wall material by spray drying technology. *International Journal of Biological Macromolecules,* 72**,** 210-217.

Bethanis, K., Tzamalis, P., Tsorteki, F., Kokkinou, A., Christoforides, E. & Mentzafos, D., 2013. Structural study of the inclusion compounds of thymol, carvacrol and eugenol in β-cyclodextrin by X-ray crystallography. *Journal of Inclusion Phenomena and Macrocyclic Chemistry,* 77**,** 163-173.

Bhandari, B.R., D'arc, B.R. & Padukka, I., 1999. Encapsulation of Lemon Oil by Paste Method Using β-Cyclodextrin: Encapsulation Efficiency and Profile of Oil Volatiles. *Journal of Agricultural and Food Chemistry,* 47**,** 5194-5197.

Bhandari, B.R., D'arc, B.R. & Thi Bich, L.L., 1998. Lemon Oil to β-Cyclodextrin Ratio Effect on the Inclusion Efficiency of β-Cyclodextrin and the Retention of Oil Volatiles in the Complex. *Journal of Agricultural and Food Chemistry,* 46**,** 1494-1499.

Bonetti, P., De Moraes, F.F., Zanin, G.M. & De Cássia Bergamasco, R., 2015. Thermal behavior study and decomposition kinetics of linalool/β-cyclodextrin inclusion complex. *Polymer Bulletin***,** 13p.

Ceborska, M., 2016. Structural investigation of the β-cyclodextrin complexes with linalool and isopinocampheol – Influence of monoterpenes cyclicity on the host–guest stoichiometry. *Chemical Physics Letters,* 651**,** 192-197.

Chen, X., Chen, R., Guo, Z., Li, C. & Li, P., 2007. The preparation and stability of the inclusion complex of astaxanthin with β-cyclodextrin. *Food Chemistry,* 101**,** 1580-1584.

Choi, H.-G., Lee, B.-J., Han, J.-H., Lee, M.-K., Park, K.-M., Yong, C., Rhee, J.-D., Kim, Y.-B. & Kim, C.-K., 2001. Terfenadine–β-Cyclodextrin Inclusion

Complex with Antihistaminic Activity Enhancement. *Drug Development & Industrial Pharmacy,* 27**,** 857.

Coleman, A.W., Munoz, M., Chatjigakis, A.K. & Cardot, P., 1993. Classification of the solubility behaviour of β-cyclodextrin in aqueous–CO-solvent mixtures. *Journal of Physical Organic Chemistry,* 6**,** 651-659.

Desai, K.G.H. & Hyun Jin, P., 2005. Recent Developments in Microencapsulation of Food Ingredients. *Drying Technology,* 23**,** 1361-1394.

Gomes, L.M.M., Petito, N., Costa, V.G., Falcão, D.Q. & De Lima Araújo, K.G., 2014. Inclusion complexes of red bell pepper pigments with β-cyclodextrin: Preparation, characterisation and application as natural colorant in yogurt. *Food Chemistry,* 148**,** 428-436.

Guarda, A., Rubilar, J.F., Miltz, J. & Galotto, M.J., 2011. The antimicrobial activity of microencapsulated thymol and carvacrol. *International Journal of Food Microbiology,* 146**,** 144-150.

Guimarães, A.G., Oliveira, M.A., Alves, R.D.S., Menezes, P.D.P., Serafini, M.R., De Souza Araújo, A.A., Bezerra, D.P. & Quintans Júnior, L.J., 2015. Encapsulation of carvacrol, a monoterpene present in the essential oil of oregano, with β-cyclodextrin, improves the pharmacological response on cancer pain experimental protocols. *Chemico-Biological Interactions,* 227**,** 69-76.

Gutierrez, J., Barry-Ryan, C. & Bourke, P., 2008. The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *International Journal of Food Microbiology,* 124**,** 91-97.

Hedges, A.R., 1998. Industrial Applications of Cyclodextrins. *Chemical Reviews,* 98**,** 2035-2044.

Hill, L.E., Gomes, C. & Taylor, T.M., 2013. Characterization of beta-cyclodextrin inclusion complexes containing essential oils (trans-cinnamaldehyde, eugenol, cinnamon bark, and clove bud extracts) for antimicrobial delivery applications. *LWT - Food Science and Technology,* 51**,** 86-93.

Jafari, S.M., Assadpoor, E., Bhandari, B. & He, Y., 2008. Nano-particle encapsulation of fish oil by spray drying. *Food Research International,* 41**,** 172-183.

Kalathenos, P. & Russell, N.J., 2003. Ethanol as a food preservative. *In* N.J. Russell & G.W. Gould (eds.) *Food Preservatives.* New York: Kluwer Academic/Plenum Publishers 2nd ed.

Kuorwel, K.K., Cran, M.J., Sonneveld, K., Miltz, J. & Bigger, S.W., 2011. Essential Oils and Their Principal Constituents as Antimicrobial Agents for Synthetic Packaging Films. *Journal of Food Science,* 76**,** R164-R177.

Lima, P.S.S., Lucchese, A.M., Araújo-Filho, H.G., Menezes, P.P., Araújo, A.a.S., Quintans-Júnior, L.J. & Quintans, J.S.S., 2016. Inclusion of terpenes in cyclodextrins: Preparation, characterization and pharmacological approaches. *Carbohydrate Polymers,* 151**,** 965-987.

Loftsson, T., Magnúsdóttir, A., Másson, M. & Sigurjónsdóttir, J.F., 2002. Self-association and cyclodextrin solubilization of drugs. *Journal of Pharmaceutical Sciences,* 91**,** 2307-2316.

Loftsson, T., Másson, M. & Brewster, M.E., 2004. Self-association of cyclodextrins and cyclodextrin complexes. *Journal of Pharmaceutical Sciences,* 93**,** 1091-1099.

Lopez, M.D., Maudhuit, A., Pascual-Villalobos, M.J. & Poncelet, D., 2012. Development of formulations to improve the controlled-release of linalool to be applied as an insecticide. *Journal of Agricultural and Food Chemistry,* 60**,** 1187-1192.

López, M.D. & Pascual-Villalobos, M.J., 2010. Analysis of monoterpenoids in inclusion complexes with β-cyclodextrin and study on ratio effect in these microcapsules. *Julius-Kühn-Archiv***,** 705.

Makwana, S., Choudhary, R., Dogra, N., Kohli, P. & Haddock, J., 2014. Nanoencapsulation and immobilization of cinnamaldehyde for developing antimicrobial food packaging material. *LWT - Food Science and Technology,* 57**,** 470-476.

Mangolim, C.S., Moriwaki, C., Nogueira, A.C., Sato, F., Baesso, M.L., Neto, A.M. & Matioli, G., 2014. Curcumin–β-cyclodextrin inclusion complex: Stability, solubility, characterisation by FT-IR, FT-Raman, X-ray diffraction and photoacoustic spectroscopy, and food application. *Food Chemistry,* 153**,** 361-370.

Marcolino, V.A., Zanin, G.M., Durrant, L.R., Benassi, M.D.T. & Matioli, G., 2011. Interaction of curcumin and bixin with β-cyclodextrin: Complexation methods, stability, and applications in food. *Journal of Agricultural and Food Chemistry,* 59**,** 3348-3357.

Marques, H.M.C., 2010. A review on cyclodextrin encapsulation of essential oils and volatiles. *Flavour and Fragrance Journal,* 25**,** 313-326.

Michalska, P., Wojnicz, A., Ruiz-Nuño, A., Abril, S., Buendia, I. & León, R., 2017. Inclusion complex of ITH12674 with 2-hydroxypropyl-β-cyclodextrin: Preparation, physical characterization and pharmacological effect. *Carbohydrate Polymers,* 157**,** 94-104.

Mourtzinos, I., Kalogeropoulos, N., Konstantinou, K., Karathanos, V.T. & Papadakis, S.E., 2008. Encapsulation of nutraceutical monoterpenes in β-cyclodextrin and modified starch. *Journal of Food Science,* 73**,** S89-S94.

Muriel-Galet, V., López-Carballo, G., Gavara, R. & Hernández-Muñoz, P., 2012. Antimicrobial food packaging film based on the release of LAE from EVOH. *International Journal of Food Microbiology,* 157**,** 239-244.

Nieddu, M., Rassu, G., Boatto, G., Bosi, P., Trevisi, P., Giunchedi, P., Carta, A. & Gavini, E., 2014. Improvement of thymol properties by complexation with cyclodextrins: In vitro and in vivo studies. *Carbohydrate Polymers,* 102**,** 393-399.

Nunes, I.L. & Mercadante, A.Z., 2007. Encapsulation of lycopene using spray-drying and molecular inclusion processes. *Brazilian Archives of Biology and Technology,* 50**,** 893-900.

Paramera, E.I., Konteles, S.J. & Karathanos, V.T., 2011. Stability and release properties of curcumin encapsulated in Saccharomyces cerevisiae, β-cyclodextrin and modified starch. *Food Chemistry,* 125**,** 913-922.

Pinho, E., Grootveld, M., Soares, G. & Henriques, M., 2014. Cyclodextrins as encapsulation agents for plant bioactive compounds. *Carbohydrate Polymers,* 101**,** 121-135.

Ponce Cevallos, P.A., Buera, M.P. & Elizalde, B.E., 2010. Encapsulation of cinnamon and thyme essential oils components (cinnamaldehyde and thymol) in β-cyclodextrin: Effect of interactions with water on complex stability. *Journal of Food Engineering,* 99**,** 70-75.

Ramos, M., Jiménez, A., Peltzer, M. & Garrigós, M.C., 2012. Characterization and antimicrobial activity studies of polypropylene films with carvacrol and thymol for active packaging. *Journal of Food Engineering,* 109**,** 513-519.

Reineccius, G.A., 1995. Controlled Release Techniques in the Food Industry *In* S.J. Risch & G. Reineccius (eds.) *Encapsulation and Controlled Release of Food*

Ingredients ACS symposium series: 590. Washington, DC : American Chemical Society.

Risch, S.J., 1995. Encapsulation: Overview of Uses and Techniques *In* S.J. Risch & G. Reineccius (eds.) *Encapsulation and Controlled Release of Food Ingredients ACS symposium series: 590.* Washington, DC : American Chemical Society, 2-7.

Rodríguez, F.J., Torres, A., Peñaloza, Á., Sepúlveda, H., Galotto, M.J., Guarda, A. & Bruna, J., 2014. Development of an antimicrobial material based on a nanocomposite cellulose acetate film for active food packaging. *Food Additives & Contaminants. Part A: Chemistry, Analysis, Control, Exposure & Risk Assessment,* 31**,** 342-353.

Serafini, M.R., Menezes, P.P., Costa, L.P., Lima, C.M., Quintans, L.J., Jr., Cardoso, J.C., Matos, J.R., Soares-Sobrinho, J.L., Grangeiro, S., Jr., Nunes, P.S., Bonjadim, L.R. & Araújo, A.a.S., 2012. Interaction of p-cymene with β-cyclodextrin. *Journal of Thermal Analysis and Calorimetry,* 109**,** 951-955.

Suhr, K.I. & Nielsen, P.V., 2003. Antifungal activity of essential oils evaluated by two different application techniques against rye bread spoilage fungi. *Journal of Applied Microbiology,* 94**,** 665-674.

Sung, S.-Y., Sin, L.T., Tee, T.-T., Bee, S.-T. & Rahmat, A.R., 2014. Effects of Allium sativum essence oil as antimicrobial agent for food packaging plastic film. *Innovative Food Science & Emerging Technologies,* 26**,** 406-414.

Suppakul, P., Miltz, J., Sonneveld, K. & Bigger, S.W., 2003. *Active Packaging Technologies with an Emphasis on Antimicrobial Packaging and its Applications*.

Suppakul, P., Sonneveld, K., Bigger, S.W. & Miltz, J., 2011. Diffusion of linalool and methylchavicol from polyethylene-based antimicrobial packaging films. *LWT - Food Science and Technology,* 44**,** 1888-1893.

Szente, L. & Szejtli, J., 1986. Molecular Encapsulation of Natural and Synthetic Coffee Flavor with β-Cyclodextrin. *Journal of Food Science,* 51**,** 1024-1027.

Tang, P., Li, S., Wang, L., Yang, H., Yan, J. & Li, H., 2015. Inclusion complexes of chlorzoxazone with β- and hydroxypropyl-β-cyclodextrin: Characterization, dissolution, and cytotoxicity. *Carbohydrate Polymers,* 131**,** 297-305.

Tao, F., Hill, L.E., Peng, Y. & Gomes, C.L., 2014. Synthesis and characterization of β-cyclodextrin inclusion complexes of thymol and thyme oil for antimicrobial delivery applications. *LWT - Food Science and Technology,* 59**,** 247-255.

Wang, J., Cao, Y., Sun, B. & Wang, C., 2011. Physicochemical and release characterisation of garlic oil-β-cyclodextrin inclusion complexes. *Food Chemistry,* 127**,** 1680-1685.

Wang, X., Luo, Z. & Xiao, Z., 2014. Preparation, characterization, and thermal stability of β-cyclodextrin/soybean lecithin inclusion complex. *Carbohydrate Polymers,* 101**,** 1027-1032.

Xiao, C.-F., Li, K., Huang, R., He, G.-J., Zhang, J.-Q., Zhu, L., Yang, Q.-Y., Jiang, K.-M., Jin, Y. & Lin, J., 2014. Investigation of inclusion complex of Epothilone A with cyclodextrins. *Carbohydrate Polymers,* 102**,** 297-305.