The influence of selected food ingredients on the release of aroma compounds from \( \beta \)-cyclodextrin in aqueous solutions

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Abstract

Flavor encapsulation has been widely used to protect flavors against degradation during processing and storage. Among the encapsulation methods, molecular inclusion by cyclodextrins (CyDs) is one of the simplest encapsulation systems. However, little information is available on the competitive binding between the volatiles and a food ingredient in an inclusion complex system of a CyD so far. One question that needs to be answered is whether if volatiles from a β-CyD:volatile inclusion complex will be released in a food system as a result of replacement by a food ingredient. To obtain good flavor retention and desirable sensory attributes, it is important to understand the competitive binding between a flavor compound and a food ingredient for CyD. This study provided insight into the competitive binding between volatiles and selected commonly used food ingredients.

In this study, the release of volatiles (three esters – ethyl acetate, ethyl butyrate, and ethyl heptanoate), was measured using Gas Chromatography (GC). Food ingredients from different groups were selected - carbohydrates, emulsifiers, and proteins. Additionally, the effect of pH on volatile release from the β-CyD cavity was also evaluated.
The molecular inclusion of model esters in β-CyD had a significant effect on their release into water and consequently the headspace, and the influence increased with increasing carbon chain length. The headspace concentrations decreased by 20, 70, and 80% in the presence of β-CyD in the order of ethyl acetate, ethyl butyrate, and ethyl heptanoate (with all statistical significance level set at 5%).

Upon the addition of casein and soy protein isolate (SPI), the headspace concentration of ethyl heptanoate was reduced by 47 and 16% compared to a simple CyD, water, and aroma compound system, respectively. The observed reduction upon the addition of CyD can be attributed to the high hydrophobicity of the ester. In addition to its interaction with the CyD cavity, it can also interact with the proteins via hydrophobic interactions.

The release of ethyl butyrate increased by 87% with the addition of lecithin compared to reference 2 (the volatiles included in β-CyD). The increased release of ethyl butyrate is attributed to freeing of the ester from the inclusion complex into the headspace as a result of the replacement of it by lecithin. Ethyl acetate is hydrophilic and has a relatively high solubility in water, which upon the addition of a hydrophobic substance such as a protein, had little or no influence on its partition - a large portion of ethyl acetate had already partitioned into the aqueous phase. For ethyl heptanoate, because it is the most hydrophobic of the
three esters, it had a high affinity for any hydrophobic components added to the system, for example the CyD cavity, a protein, or a hydrophobic fatty acid part of lecithin. For the same volatile, the type of the emulsifier does not affect its release significantly. However, similar to what happened to the protein samples, the release of the volatiles decreases significantly with the increasing carbon chain length upon the addition of the emulsifiers.
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1. Review of Literature

1.1 Introduction and Objectives

Molecular inclusion of volatiles using cyclodextrins (CyDs) has been reported by many researchers (Ciobanu et al. 2013, Reineccius et al. 2002, Madene and Jacquot et al. 2006, Kant et al. 2004, Del Valle, et al. 2004). CyD inclusion complexes of volatiles are used to protect and stabilize volatiles against degradation from exposure to light, elevated heat, and oxygen during processing and storage (Del Valle, 2004, Astray et al. 2010). However, there is little information available in the literature on the competitive binding between volatiles and a food ingredient with the CyD. When the CyD:flavor inclusion complex is incorporated in a food, a hydrophobic ingredient such as protein or emulsifier can interact with the CyD and replace the volatiles. This phenomenon would result in freeing the volatiles into the product or product headspace thereby influencing flavor intensity and possibly flavor balance. Therefore, it is important to understand the competitive binding between a volatile and a food ingredient.

In this study, some commonly used food ingredients were selected including whey protein isolate (WPI), casein, soy protein isolate (SPI), OSA-modified starch, lecithin, Tween 20, and sucrose for the investigation of competitive binding.
Furthermore, food exists in various pH conditions. The interactions between a volatile and a food ingredient (e.g. a protein) may be influenced by pH. Therefore, it was also of interest to study the effect of pH on the competitive binding.

Another relevant factor regarding the competitive binding and flavor release behavior is the order of addition. Would volatile release behavior differ if a different order of ingredient addition was used? For instance, would volatile headspace concentration be different if a food ingredient was included in the CyD cavity in the first place instead of the volatile? So far, little information has been known on the effect of addition order. Therefore, it is of our interest to evaluate the influence of the order.

Therefore, the objectives of this study were as below:

a. Investigate the competitive binding between volatiles and selected food ingredients with β-CyD and its effect on volatile release

b. Evaluate the effect of pH on volatile release in a β-CyD:volatile:food ingredient systems.

c. Assess the effect of addition order on volatile release from β-CyD inclusion complexes.
1.2 Flavor:food ingredient interactions

Flavor is one of the key factors controlling consumer acceptability of a food product (Hansen and Booker, 1996; Plug and Haring, 1994; Bower and Whitten, 2000). Aroma compounds are volatiles that can reach the nasal cavity (olfactory epithelium) and be perceived. If an aroma compound binds/interacts with a food ingredient, its volatility reduces and it will not be freed into the gas phase or be perceived. The intensity of the perceived aroma is directly related to the amount that is retained by the food matrix. A food matrix is a very complex system containing many ingredients such as carbohydrates, proteins, stabilizers, and thickening agents, etc. They all can interact with flavor compounds and change the flavor profile (e.g. reduce intensity, lose desirable flavor compound, and potentially generate an off-flavor compound). Sometimes, even a small change in the flavor profile (e.g. a change to a characteristic aroma compound) can become unacceptable to the consumers. For example, one of the major proteins in whey – β-Lactoglobulin (β-Lg) interacts and binds with many flavor compounds and renders less flavors available to be released or perceived (Hansen and Booker, 1996; Guichard, 2002). Besides interacting chemically, food stabilizers and thickeners can also reduce flavor retention by providing resistance to mass transfer due to their high viscosity.
Consumer awareness of health risks of high fat food products calls for a formulation change to reduce fat. The biggest challenge of reducing fat is related to the resulting unbalanced flavor profile and reduced flavor quality (Plug and Haring, 1994). Because most flavors are hydrophobic and fat is a good solvent for them, removing fat increases the partition of the flavors between the aqueous phase and the gas phase (Le Thanh et al. 1998). Consequently, the release into the headspace is unpleasantly high even if the same flavor concentration is used. Additionally, some flavors are products of lipid oxidation (Frankel 1980). Thus, this reformulation would not make the same rich and full profile because some flavors will be lost inevitably. Among all the fat replacers, protein is one of the most popular. When fat reduction is accompanied by increased protein concentration, decreased flavor intensity can be expected in most cases, because proteins can bind with many flavor compounds in general. Understanding the interactions between protein and volatiles provides insight into obtaining a desirable flavor profile and sensory attributes. Other food ingredients such as starch, food stabilizers and thickeners also interact with flavors through various interactions (e.g. hydrogen bonding, hydrophobic interactions, and physical entrapment). Therefore, it is of utmost importance to understand flavor interactions with food ingredients to design and formulate a successful product.
1.3 Flavor encapsulation

In addition to interactions with food ingredients, flavor is subject to chemical deterioration during processing and storage. When exposed to light, oxygen, and elevated temperature, flavor degradation can take place. To reduce flavor loss, encapsulation techniques have been widely used to protect and improve their stability. Technologies have been developed to encapsulate flavor including spray drying, extrusion, freeze-drying, coacervation, and molecular inclusion. Among these techniques, molecular inclusion using cyclodextrins (CyDs) has advantages such as simple preparation, relatively low cost, and good encapsulation (Ciobanu et al. 2013, Madene and Jacquot et al. 2006).

1.4 Cyclodextrins (CyDs)

The CyDs are a family of cyclic oligosaccharides with α-1, 4 linked glucopyranose units as the building block (Ciobanu et al. 2013). The family contains α-, β-, and γ-CyDs that consist of 6, 7, and 8 D-glucose units, respectively (Reineccius et al. 2002). These cyclic glucose-based oligosaccharides have a relatively unique molecular structure that resembles a bucket in shape. In aqueous solutions, the glucose molecules orient in such a manner that the interior is hydrophobic and the exterior is hydrophilic. Typically, only one guest molecule can fit into the cavity. Among the three cyclodextrins, β-
CyD is generally the most efficacious of the three common cyclodextrins and is the lowest-priced (Del Valle et al., 2004).

In this study, β-CyD is used to include flavor compounds. The structure and dimensions of the CyDs are shown in Figure 1. The size of the cavity is increased in the order of α-, β-, to γ-CyD. The height of the cavity is the same across the three CyDs. Because the volatiles are typically small molecules, they can form the inclusion complexes with the CyDs and this has been applied to encapsulate flavor. In most cases, a guest molecule forms the inclusion complex with a CyD on 1:1 molar ratio due to the size of the CyD cavity (Kant et al 2004).

![Figure 1. Dimensions of α-, β-, and γ-CyD. Reproduced from Ciobanu et al. 2013.](image)

When a guest molecule is included in the CyD cavity, an equilibrium between the inclusion complex and the free guest molecule is established in the solution
(Reineccius et al. 2002). Factors such as the properties of the CyD and the flavor compounds, temperature, moisture content, and matrix composition have an influence on the equilibrium constant (Reineccius et al. 2002). Shiga et al. studied the release rate of d-limonene and ethyl n-hexanoate encapsulated by a blend of β-CyD and gum acacia. It was found that the release rate of the volatiles depends on the type of the flavor and the composition of the encapsulation system. Additionally, the molecular size, geometry, and the hydrophobicity of the flavor compound also affects its ability to form the inclusion complex with the CyDs (Kant et al. 2004).

1.4.1 Applications of the CyDs

CyDs have been applied to improve the stability of flavor compounds against light, oxygen, and heat-induced degradations (Del Valle, 2004), reduce or mask off-flavors, remove bitterness (Rouseff, 1990), and control flavor release in food and beverage. In addition to encapsulating flavors, it is also used to stabilize vitamins, essential oils, and fragrances (Astray et al. 2010).

CyDs have a wide range of applications in the food, drugs, and cosmetic products (Marques 2010; Astray et al. 2009; Goubet et al. 1998; Del Valle, 2004; Davis and Brewster, 2004; Challa et al. 2005). It is of most interest to use CyDs to encapsulate flavors, essential oils, fatty acids, carotenoids, and vitamins for
stabilization purpose. In addition, it is applied to reduce off-flavors and dissolve flavors with poor solubility in water (Fenyvesi et al. 2015). Moreover, elimination of bitterness and other unpleasant odors in food and drugs is also achieved by CyD inclusion (Szejtli and Szente, 2005). Due to its many functionalities and properties, CyDs are in high demand globally. Some examples of the applications of the CyDs are given in the following sections.

It was used to encapsulate and stabilize pure cinnamaldehyde and benzaldehyde against oxidation (Szente and Szejtli 1988). The dried CyD inclusion complex was found to improve the stability of flavors and essential oils under the stress of heat (Furuta et al. 1996). β-CyD was used to encapsulate benzaldehyde, citral, L-menthol, and vanillin for processed foods. The retentions of encapsulated flavors were significantly higher than those in a non-encapsulated liquid formulation. In particular, an 86-fold increased retention of lemon flavor was achieved upon β-CyD treatment compared to one without β-CyD treatment (Reineccius and Reineccius. 2004). The addition of β-CyD of 15 mM was also found to increase the color intensity of pear juice and avoid reducing flavor retention (Andreu-Sevilla et al. 2011).

One great advantage of the CyDs over the conventional encapsulation materials is its ability to dissolve low water solubility flavorings. Attributed to the good solubility of the CyDs in water, it promotes the dissolution of the flavor in water
after they form the inclusion complex with the CyDs. One example is the improved solubility of volatile antimicrobials in cinnamon leaf and garlic oils, upon inclusion complex formation. The more soluble antimicrobials can function more effectively, and consequently inhibition of deleterious microorganisms was found after CyD treatment (Ayala-Zavala et al. 2008). In addition to flavors, CyDs are employed to stabilize oxidation sensitive PUFAs as dietary nutritional supplements. It has been applied both to protect the PUFAs from degradation as well as to mask the fishy rancid smell (Cao et al. 2011, Gorska et al. 2011).

Another important application of CyDs are to mask or remove bitterness in foods. Unpleasant tastes, such as naringin in citrus juice or chlorogenic acid in coffee, can be eliminated completely or reduced treatment with a CyD (Szejtli and Szente, 2005). β-CyD reduced the bitter taste of grapefruit significantly with the addition of 1.3% CyD (Shaw and Wilson, 1983). The addition of 0.3% of β-CyD resulted in a significant reduction of the bitter taste in grapefruit or mandarin juices. Another example of CyD reducing bitterness is the application of β-CyD to whey protein hydrolysate (WPH). The bitterness of maltodextrin/β-CyD encapsulated (spray dried) WPH was significantly reduced compared to the original state (Yang et al. 2012). Bitterness from plant extracts, such as bergamot and curcuma, was also blocked or reduced by CyDs (Lu and Li, 2005; Kishi et al. 2012). Moreover, its effectiveness in removing bitterness in drugs has also been well documented. The sensory threshold of bitterness is typically very low and
generally tolerable at ppm levels. Its application in this area has been reviewed extensively (Szejtli and Szente, 2005).

Another interesting and very useful application was using β-CyD to remove cholesterol. Cholesterol can be included by β-CyD and then becomes insoluble in water. β-CyD has been applied to remove cholesterol in eggs and other dairy products effectively (Awad et al. 1997; Roderbourg et al. 1990). It was reported that 92.2% to 95.3% of cholesterol was removed by adding 0.5 to 1.5% of β-CyD when mixed for only 10 min in homogenized milk (Lee et al. 1999).

1.4.2 Approval status of CyDs in food

Cyclodextrins have been approved for generally recognized as safe (GRAS) status in the US including as a flavor encapsulation material in human food (Szente and Szejtli, 2004). A summary of the legal status in various countries and regions is given in Table 1 below.
Table 1. Approval status of use in food of the CyDs. Reproduced from Marques 2010.

<table>
<thead>
<tr>
<th></th>
<th>α-CyD</th>
<th>β-CyD</th>
<th>γ-CyD</th>
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<td>USA</td>
<td>GRAS(^b) (Jan. 2004)</td>
<td>GRAS(^c) (Oct. 2001)</td>
<td>GRAS(^b) (Sept. 2000)</td>
</tr>
<tr>
<td>Canada</td>
<td>Filed for novel food status (July 2006)</td>
<td>GRAS(^b) (October 2000)</td>
<td>GRAS(^c) (Oct. 2001)</td>
</tr>
<tr>
<td>EU</td>
<td>Novel food approved (2008)</td>
<td>Carrier for food additives (&lt;g/1Kg)</td>
<td>Novel food Filed (Jan. 2010)</td>
</tr>
<tr>
<td>Japan</td>
<td>Natural product</td>
<td>Natural product</td>
<td>Natural product</td>
</tr>
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</table>

GRAS\(^b\) in a wide range of intended use in food; GRAS\(^c\) as a flavor protectant.

1.4.3 Competitive binding of the CyDs

Flavorings are often complex mixtures of many flavor compounds. The flavor compounds vary in their physicochemical properties such as size (molecular volume), geometry, and hydrophobicity, etc. As a result, their binding affinities to β-CyD are different and consequently, their retentions in the CyDs vary. The retentions of the two esters ethyl hexanoate and ethyl propionate by β-CyD were compared to understand the relationship between volatile hydrophobicity and their retention in CyD inclusion complex (Goubet et al. 2001).
Figure 2. Retention of esters after freeze-drying of cyclodextrins initially saturated with 2 mol of ethyl propionate per mol of β-CyD, in which increasing amounts of ethyl hexanoate have been added. Reproduced from Goubet et al. 2001.
As shown in Figures 2 and 3, when a β-CyD and 75% water system was saturated with 2 mol of ethyl propionate per mol of β-CyD in the first place, the retention of ethyl hexanoate was increased greatly corresponding to decreased retention of ethyl propionate upon the addition of ethyl hexanoate. As the addition order of the two flavor compounds reversed, however, the retention of ethyl propionate was slightly increased. This result can be explained by the hydrophobicity and vapor pressure of the two compounds. The log P values of ethyl hexanoate and ethyl propionate are 2.83 and 1.24, respectively. With higher hydrophobicity, ethyl hexanoate has an 8.3 times higher affinity constant than...
ethyl propionate with Ka of 318 M⁻¹ and 38 M⁻¹, respectively (Goubet et al. 2001). Therefore, ethyl hexanoate binds with CyD stronger than ethyl propionate and thus showed higher retention. Also, the saturated vapor pressure of ethyl propionate was much higher than that of ethyl hexanoate, with 5374 Pa and 238 Pa at 25 °C, respectively. During freeze-drying, ethyl propionate is more easily vaporized than ethyl hexanoate.

Besides the two esters, the competitive bindings of two acids as well as the alcohols were also investigated in the same study. However, when the same experiment for the alcohols was repeated for the acids (hexanoic acid and 2-methylbutyric acid), the less hydrophobic 2-methylbutyric acid was retained more by the CyD. The authors hypothesized that the spatial conformation and size of the two acids influenced their retentions. Comparing to the linear hexanoic acid, 2-methylbutyric acid has a larger molecular volume and more contact with the cavity. Additionally, the authors speculated that the small linear hexanoic acid interacts with the cavity mainly through hydrophobic interactions while the large 2-methylbutyric acid, the stronger dispersion forces resulted in its’ better retention.

In addition to the competition of binding between the flavor compounds themselves (i.e. esters, acids, and alcohols, etc.), another important aspect is the competition of binding sites between the flavors and the food ingredients. Food
contains many ingredients such as protein, carbohydrate, lipids, and emulsifiers, etc. Many food ingredients are hydrophobic or to some extent, hydrophobic. They can compete for the hydrophobic CyD cavity with the flavor compounds. If an ingredient has a stronger affinity for the CyD cavity, the flavor compound(s) would be replaced or have no chance to be included inside of the CyD cavity. Flavor compounds can end up being released into the headspace, which increases their headspace concentration. So far, no publication has reported the competitive binding between the flavor compounds and a food ingredient. Understanding this mechanism and behavior can help: 1, to predict the flavor retention by the complex; 2, to design an encapsulation system to obtain a balanced flavor profile and sensory attribute of a target food product application.

1.5 Review of selected food ingredients

1.5.1 Carbohydrate:flavor interactions

Carbohydrates are the most common ingredients in food. As mentioned earlier, the cyclodextrins are one type of carbohydrates - an oligosaccharide. Based on the molecular weight of a carbohydrate, they are classified into three classes: simple sugars (mono - and disaccharides), oligosaccharides, and polysaccharides.
1.5.1.1 Simple sugars

The role of simple sugars on influencing flavor retention is mainly through their interaction with water molecules. Hydroxyl groups of simple sugars interact with water molecules through hydrogen bonding. This is also the reason why small molecular weight carbohydrates have high solubility in water. Interactions between simple sugars and water in the aqueous solution can increase the concentration of the volatiles in the headspace - this is the so-called “salting out” effect. This phenomenon is attributed to less free water available to interact and retain the volatiles in the aqueous solution as water interacts with the sugar molecules. However, this effect only takes place when the concentration of sugar is very high (over 60%), which is not common in a food system. In addition, this effect is also found to be flavor compound dependent. For some flavor compounds, the influence is very little. Limonene, for example, was found not affected upon the addition of simple sugars (Hansson et al. 2001). It was found that the release of the flavor compounds such as isopentyl acetate, ethyl hexanoate, L-menthone, cis-3-hexenyl acetate, and linalool was increased as sucrose and invert sugar were added in the aqueous solution at concentrations over 60% (Hansson et al. 2001).

1.5.1.2 Starch
Starch is the energy storage and the second most abundant biopolymer in nature, after cellulose (Ghasemlou et al. 2015). Its building block is the D-glucopyranose unit. Each starch variety differs in the number and proportion of glucose linkage types. Starch consists of two major components – amylose and amylopectin. Amylose is a linear polymer with the glucose unit bonded through the α-1, 4 linkages. This linear structure of amylose forms the backbone of starch. In comparison, amylopectin consists of short chains and is highly branched. A schematic description of the starch polymer is shown in Figure 4 below.

![Figure 4. Structure of long chain amylose and highly branched short chain amylopectin (Sheldrake, 2009).](image)

The amylopectin fraction grows out of the linear amylose backbone via the branching point at the C 6 position of the glucose (α-1, 6 linkages). Figure 5 shows the molecular structure of the amylose and amylopectin polymers and the α-1, 4 and the α-1, 6 linkages.
While starches can vary greatly in the proportions of amylose and amylopectin, starch generally contains about 65-85% of amylopectin. Amylopectin is considered as the major component of the structure of starch granules (Hoover 2001). The remainder of the starch (20 to 30%) consists of amylose. As noted, the proportion of these two starch structures varies with the botanical source of the starch. In the waxy starches, the amylose content is nearly zero (Shi et al. 1998).

1.5.1.2.1 Starch:Flavor interactions
Aroma compounds can interact with the amylose fraction of starch. Amylose often exists in helical structures. The helixes orient in a way that is similar to the CyD. The hydroxyl groups are on the outside of the coil with the hydrophobic regions inside (Plug and Haring, 1994). This allows the amylose to form inclusion complexes with flavor compounds through hydrophobic interactions (Rutschmann and Solms, 1990). Similar to an inclusion complex with the cyclodextrins, the size and geometry of a guest or flavor compounds must fit the amylose helix to be included. It was found that inclusion complexes were formed with several flavor compounds – decanal, menthone, 1- naphthol in binary or ternary model systems (Rutschmann and Solms, 1990).

Besides binding with starch through hydrophobic interactions, flavor compounds can also be retained by starch through physical sorption and entrapment. Compared to the hydrophobic interactions, this type of adsorption and entrapment is non-specific. Native starch powders contain porosity on the surface of the granules and aroma compounds can be retained by physical sorption in theses pores (Boutboul et al. 2000). In a glassy system where starch is used to encapsulate flavors, the mobility of the flavors is very limited due to the high viscosity of the system and consequently, flavor diffusion through the matrix is greatly reduced (Escher et al. 2000).
1.5.1.3 Modified Starch

The use of native starch is limited due to its cohesive, soft, and rubbery structure and texture upon heating (Abbas et al. 2010). It also lacks other functional properties such as emulsification ability, which limits its use as an emulsifier and encapsulation agent (Chiu and Solarek 2009). For these reasons, starch has been modified to obtain certain desirable characteristics such as good solubility in water and emulsification ability. Starch can be chemically modified by reacting a hydroxyl group of the glucose residues with a hydrophobic substitute i.e., octenyl succinic anhydride (OSA) through a covalent bond (by esterification). Other modification, e.g. acetylation, has also been used but the OSA modification is one of the most important and commonly used one in the food industry. One of the advantages of the Octenyl Succinic Anhydride Modified Starch (OSA-MS) is that it is colorless and tasteless in solution (Hategekimana et al. 2014). The chemical structure of the OSA-modified starch is given in Figure 6. The OSA-modification can take place at C2, C3, and/or C6 on the glucose residue (Figure 6). The reaction mechanism of starch modification by the OSA reagent is shown in Figure 7.

Besides increased solubility, the added hydrophobic character of the OSA-modified starch provides a variety of properties such as emulsification ability, film forming, and gel formation properties (Sweedman et al. 2013). The added
hydrophobic feature of the OSA-MS has offered several important applications particularly in stabilizing o/w emulsions, encapsulation, and coating in the food, cosmetic, and pharmaceutical industries (Jiang et al. 2016).

![Chemical structure of OSA-modified starch and the possible modification sites. Reproduced from Wang et al. 2010.](image1)

**Figure 6.** Chemical structure of OSA-modified starch and the possible modification sites. Reproduced from Wang et al. 2010.

![Reaction mechanism and structure of OSA-Modified Starch.](image2)

**Figure 7.** Reaction mechanism and structure of OSA-Modified Starch. Reproduced from Sweedman et al. 2013.
As shown in Figure 6, the OSA-MS contains a glucose residue and an OSA residue. The hydrophilic glucose residues can bind with water or the aqueous phase while the octenyl residues bind with the hydrophobic flavor oil phase. Thus, the OSA-modified starch is used as an emulsifier. In addition to its application as an encapsulation wall material, it has also been used in food products (e.g. salad dressing, food emulsion, etc.), fragrances, and adhesives, etc. (Liu et al. 2008). When used in food, the degree of substitution is not allowed to exceed the maximum of 3 (w/w) % by the FDA (Liu et al. 2008).

1.5.1.3.1 Effect of modified starch on flavor release

OSA-modified starch is one of the most popular encapsulation materials in the flavor industry. Due to its high molecular weight, it forms a much thicker film than the small molecular weight surfactants. As a result, it creates a good barrier to keep the flavor oil droplets from coalescence. Flavorings do not diffuse easily through the emulsion and thus, slows down phase separation and achieves controlled release.

Modified starch stabilizes food emulsions not only serving as an emulsifier but also by increasing the viscosity of the system. The increased viscosity of the emulsion system provides barrier and resistance to mass transfer, and therefore, traps, slows down, and reduces the transfer and release of the flavors.
1.5.2 Emulsifiers: Flavor interactions

1.5.2.1 Lecithin

Lecithin is one of the few natural emulsifiers that has been granted GRAS status (generally recognized as safe) by the FDA. “Lecithin”, by definition, is a mixture of several phospholipids, a natural constituent of animals and plants (Xu et al. 2011). Since the primary source of lecithin is egg yolk, it serves for non-vegan applications, but not economically feasible for industrial applications (Xu et al. 2011). Lecithin is also found in plant and vegetable such as soybean, rapeseed, and sunflower. Because phospholipids are an integral part of the cell membrane, lecithin is abundant in animals, plants, and vegetables. Besides egg yolk, soybean oil seeds are another abundant source of lecithin and in fact, commercial lecithin is mostly manufactured from soybeans instead of egg yolk due to the lower cost (Gladkowski et al. 2012; Xu et al. 2011). Soy lecithin has an advantage over egg lecithin since it is suitable for vegan applications (Cui and Decker 2016). Lecithin is the most popular natural emulsifier with an estimated market of 150,000 to 170,000 tons around the world (Cui and Decker 2016). It is widely used in food products such as cookies, chocolates, milk replacers, and low-fat spreads (Van Nieuwenhuyzen et al. 2008).

Lecithin is a small molecule with a molecular weight around 729 Da (Pan et al. 2013). Soy lecithin contains about 65-75% of phospholipids (PLs), 34%...
triglycerides, and smaller amounts of carbohydrates, pigments, sterols, and sterol glycosides (Dickinson, 1993). The phospholipid composition varies with its origin (Gladkowski et al. 2012). Also, the type (carbon chain length and degree of saturation) and composition of the fatty acids in lecithin varies with source (Cui and Decker 2016). The general structure of lecithin is shown in Figure 8. PLs have a glycerol backbone with its third carbon atom attached to a phosphate group (Xu et al. 2011). The PLs have hydrophilic heads as well as hydrophobic long chain fatty acid tails. Therefore, it can adsorb to the w/o interfaces and thereby reduce surface tension.
Figure 8. Molecular structure of the phospholipids. Reproduced from Xu et al. 2011.

1.5.2.2 Tween 20

Tween 20 or Polysorbate 20 is a non-ionic surfactant that forms micelles at 0.08% w/v in water at ambient temperature (Lei et al. 2013). It is a small molecule with a molecular weight around 1227 Da (Scheffler et al. 2009). It is quite hydrophilic among the nonionic emulsifiers. The structure of Tween 20 is
shown in Figure 9 below. The emulsification ability of Tween 20 comes from its structure – it has hydrophilic sorbitan polyoxyethylene bonded or esterified to a laurate hydrophobic tail. Tween 20 has better solubility in water than the other Tween emulsifiers. The Tween emulsifiers have broad applications – they are widely used in the food, cosmetic, and pharmaceutical industry (Zhang et al. 2013; Mandal et al. 2015).

![Structure of Tween 20](image)

**Figure 9.** Structure of Tween 20 (with x+y+z = 20). Reproduced from Ruiz et al. 2003.

Although the Tweens and lecithins are widely used emulsifiers, there is very little information reported on their interaction with flavor compounds. One study reported that the partition coefficients of various flavor compounds were affected by the concentration and type of the surfactants evaluated (van Ruth et al. 2002). Therefore, it is of interest to evaluate the effect of different type of emulsifier/surfactant (e.g. lecithin and Tween 20) on flavor retention.
1.5.3 Protein:Flavor interactions

Protein is one of the most important and widely present ingredients in food. Protein itself has little flavor, but the presence of protein can significantly change flavor profile by various interacting with the flavor. The interactions can result in reducing or losing certain sensory attribute or generating off-flavors (O’Neill, 1996).

The physicochemical properties and functionalities of the most commonly used proteins in food systems including whey protein, casein, and soy protein are reviewed in the following section. A discussion of the interactions between proteins and flavor compounds will follow.

1.5.3.1 Whey Protein Isolate (WPI)

Normal bovine milk proteins can be classified into two types of proteins – casein 80% w/w and whey protein 20% w/w. Whey is defined as “the liquid substance obtained by separating the coagulum from milk, cream, or skim milk in cheese-making”, based on the definition from the United States Code of Federal Regulations (21 CFR 184.1979). Whey protein is obtained as a by-product from cheese manufacture from cow’s milk. It is separated from the other components in milk after casein is precipitated out or removed during the cheese-making
process. Historically, whey was thought of as a waste product and disposed of as such. Today, the value of whey, particularly the protein, is well recognized. Four major, unique protein fractions make up whey protein: β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA), and immunoglobulin (Gangurde et al. 2011). Whey proteins contain other minor proteins including lactoferrin, lactollin, glycoproteins, lactoperoxidase, and transferrin (Killara and Panyam, 2003). The whey proteins are more soluble than casein and their isoelectric point is around pH 5. Besides solubility, whey protein is also to be of higher quality i.e. more bioavailable than casein. It takes less time to digest whey protein than casein. For this reason, whey protein has also been considered as a better choice than casein for those who have interest in building body mass (Gangurde et al. 2011). It is also used in infant formula, weight loss products, sports drinks, and nutrition bars (Killara and Vaghela 2004). For these reasons, whey proteins are of great importance and have been widely used in food. This is also one of the reasons why the whey proteins were selected for this study.

In addition to whey protein being considered of high quality and nutritional value, it also has many desirable functional properties. It has surface-active properties, film-forming properties, and good solubility in water. Therefore, it has been used as a wall material for flavor encapsulation. When combined with small molecular weight carbohydrates, it shows very good barrier properties (Young et al. 1993). Several studies have investigated the performance of protein carriers for
encapsulation (Sheu and Rosenberg, 1995; Rosenberg and Sheu, 1996; Noshad et al. 2015; Rosenberg 1997; Rosenberg and Young 1993). Protein carriers – whey protein isolate, soy protein isolate, and sodium caseinate have been studied to encapsulate limonene. Their encapsulation efficiencies were compared with OSA-MS and gum acacia (GA). All the protein carriers retained significantly more limonene than that of OSA-MS and GA (Charve and Reineccius 2009). However, the protein carriers also have certain drawbacks, which is the reason why they have not been more commonly used as encapsulation materials. These issues include allergens, religious issues, undesirable reactions or flavor degradation (e.g. the Maillard reaction).

Whey protein isolate (WPI) is the whey protein ingredient with highest protein content containing ≥ 90% protein (Bansal and Bhandari. 2016). The other well-known concentrated form of whey proteins is whey protein concentrate (WPC), which contains a lesser proportion of protein than WPI: 35-85% of protein (Ennis et al. 2000). This difference in the proportion of protein is the main difference between the WPI and the WPC (Bansal and Bhandari 2016). Besides proteins, WPI powders also contain moisture 4-6%, lactose 0.2-2.0%, fat 0.2-1.5%, and ash 0.3-4.5% (Morr and Ha, 1993; Foegeding et al., 2011).
1.5.3.1.1 Interactions between whey proteins and flavor compounds

Proteins interact with flavor compounds through various interactions (covalent, hydrophobic interactions, hydrogen bonding, etc). These interactions would likely result in changes in flavor profiles, flavor release, and sensory attributes. Even the addition of milk proteins at low concentration can significantly change a given flavor profile causing imbalanced flavor profile (Kühn et al. 2006). The binding between a flavor compound and a protein can be reversible or irreversible (Kühn et al. 2006). The type of the interactions is related to the structure of the protein as well as the flavor compound itself. Other factors can be involved including pH, temperature, and concentration, etc. The structure and binding sites of the major component of whey protein, β-Lg, will be reviewed in the following section.

1.5.3.2 Structure and binding sites of β-lactoglobulin (β-Lg)

β-Lg is one of most extensively studied among all the proteins in the food system. It contains two β-sheets, a hydrophobic pocket, and a flanking three-turn α-helix (Papiz et al. 1985). The structure is shown in Figure 10.
The hydrophobic pocket (noted as “CALYX” with the hydrophobic ligand of closed circle atoms) is the primary binding site for flavor and the groove near the outer surface is the secondary binding site. The binding between a protein and a flavor compounds in most cases is reversible (Lubbers et al. 1998). However, in some cases, the binding can be through covalent bonds which are not reversible. For example, aldehydes bind with amino and sulfhydryl groups covalently (Motttram et al. 1996).

The reversible interactions include hydrophobic interactions, ionic interactions, and hydrogen bonding. The kind of interactions/bonding between a flavor
compound and milk protein depends on the characteristics of both the protein and the flavor compound. A more detailed discussion and aldehyde:protein reactions will be provided later. Many researchers have investigated the interactions between flavor compounds and milk proteins. For example, it was reported that the odor intensities of the methyl ketones and eugenol were decreased significantly upon the addition of only 1% of β-Lg in water (Andriot et al. 2000). Alkanes, ketones, aldehydes, and lactones were also found to react with β-Lg (Guichard 2002).

1.5.3.3 Structure and properties of α-lactalbumin (α-La)

α-La contains six subunits of 122 amino acids. It is a unique protein because it binds with cations such as Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, and Na$^+$. In addition to binding cations, it was also found to bind with carbonyls (Franzen and Kinsella 1974; Jasinski and Kilara 1985). α-La consists of two domains – a large α-helix and a small β-sheet. These two domains are connected by a loop that is the calcium-binding site. This protein has a strong ability to bind with calcium, and it is used as study material on the physiological importance regarding protein calcium binding (Permyakov and Berliner, 2000). Moreover, the ability of the protein to bind calcium helps stabilize the protein from irreversible thermal
denaturation (Hiraoka and Sugai, 1984; Liu et al. 2011).

1.5.3.4 Effect of pH on Protein:Flavor interactions

pH is known to have a substantial influence on protein conformation. A change in protein conformation may have an influence on flavor:protein binding. Additionally, changes in pH affect the charges on the protein and consequently, affect the interactions. When the pH of a food matrix is at the isoelectric point of a given protein, the protein aggregates and precipitates. For example, casein is isolated by isoelectric precipitation from milk during cheese-making. Isoelectric precipitation will also change the conformation of the protein and thus its interaction with ingredients such as a flavor compounds. The effect of pH on β-Lg has been reported by several studies (Weel et al. 2003; van Ruth and Villeneuve, 2002; Benjamin et al. 2012). So far, the underlying mechanism of the effect of pH on the interactions between flavors and protein has not been elucidated.

The effect of pH on flavor binding and retention has also been investigated on soy protein. When soy protein denatured at pH of 3, the structure of the soy protein changed significantly and resulted in reduced retention of hexyl acetate (Semenova et al. 2002).
1.5.3.5 Influence of protein denaturation on flavor:protein interactions

The denaturation by heat had a different effect on interactions between volatiles and protein (e.g. casein and β-Lg). When sodium caseinate undergoes heat denaturation, it shows good stability (solubility). Studies on interactions with vanillin found there was no difference in vanillin intensity between native and denatured casein. This observation was attributed to the structure of the protein, that is, casein has little secondary or tertiary structure (Fox and Mulvihill 1982). β-Lg, in comparison, showed increased binding capacity after denaturation by heat. When a native β-Lg was denatured at 70°C for 30 min, binding of benzaldehyde with β-Lg increased from 38 to 63% (Hansen and Booker 1996). This was a result of more accessibility and increased number of binding sites on the protein owning to the unfolding of the protein structure upon heat treatment. Additionally, it was also postulated that the binding of flavor compounds was decreased in the later stage of protein denaturation owning to the aggregation of the protein molecules.

Additionally, the whey proteins have globular conformation and readily denature at 72°C upon heating. As the whey proteins are denatured, they adopt a more random structure with the exposure of the sulfhydryl and hydrophobic groups. As a result, interactions between the polypeptide chains are enhanced, which leads to protein aggregation (Ennis et al. 2000).
1.5.3.6 Casein

As mentioned earlier, casein is the major protein in milk. It is a mixture of different proteins: $\alpha_{S1}$ (44%), $\alpha_{S2}$ (11%), $\beta$ (32%), and $\kappa$ (11%). However, unlike whey proteins, casein does not have secondary or tertiary structure but instead, its structure is more flexible and disordered in aqueous solutions. In milk, casein exits as “micelles”. A casein micelle is a cluster of many protein molecules. The diameter of the micelle can range from 50 to 600 nm with the mean diameter around 150 nm. An image of a casein micelle is shown in Figure 11 below.

Figure 11. Electron micrograph of an individual casein micelle. Scale bar = 200nm. Reproduced from Dalgleish et al. 2004.
The structure and network of a casein micelle are held together by calcium phosphate. Calcium phosphate interacts and crosslinks with serine-phosphate and some glutamate residues in the casein proteins and forms the structure of the micelle (O'Regan et al. 2009).

In addition to structural differences, caseins and whey proteins also have very different amino acid profiles. Casein contains more aromatic amino acids such as methionine, arginine, and histidine than whey proteins. Therefore, hydrophobic interactions between casein proteins and flavor compounds are more dominant than with whey proteins. Also, whey proteins contain more cysteine and lysine residues than casein proteins, and these amino acid residues can bind with flavor compounds (e.g. an aldehyde) covalently (Hansen and Heinis, 1992).

1.5.3.7 Soy Protein Isolate (SPI)

Soybeans are a rich source of high quality protein. Soybeans also contain nutraceuticals/phytochemicals such as isoflavones that are claimed to have health benefits (Messina 1999). Due to its plant source origin, soy protein has the advantage of being relatively inexpensive, more biodegradable, and renewable compared to dairy proteins (Wang et al. 2015; Tang and Li, 2013). The health benefits of soy-based foods have been researched in great deal. The claimed beneficial effects include lowering of plasma cholesterol as well as reducing
chances of diabetes, obesity, and cancer (Friedman and Brandon. 2001). Soy protein is nutritionally not an ideal protein since it is deficient in methionine. Whey protein and casein protein are of higher nutritional quality than the soy protein. Another disadvantage of soy protein is the presence of endogenous inhibitors of digestive enzymes that contribute to the poor digestibility upon the consumption of raw soybean meal (Friedman and Brandon. 2001).

Despite there being some health benefits to soy proteins, they have not enjoyed the same demand as whey proteins and have been relatively low in consumer acceptance. A major problem that has limited the use of soy protein and other soy products in the western market is its characteristic flavor. It’s flavor is often described as “metallic, bitter, grassy, and painty” (Kinney 2003).

Soy protein has its advantages and disadvantages as discussed: some argue that it is a better encapsulation material than whey proteins due to reasons such as its plant origin, low cost, biodegradability, and renewability (Tang and Li, 2013).

Soy protein consists of two major globular proteins: the hexameric glycinin (Mwt 3 to 3.8×10^5) and the trimeric β-conglycinin (Mwt 1.8 to 2×10^5). These two proteins account for around 40% and 30% of the total soy proteins, respectively (Tromelin et al. 2006). Similar to whey proteins, soy proteins also have many
desirable properties and functionalities including surface activity, film-forming capacity, and good solubility. Therefore, it has also been used as a wall material for encapsulation (Charve and Reineccius 2009). Besides flavors, bioactive compounds such as vitamins have also been successfully encapsulated by SPI (Teng et al. 2013; Nesterenko et al. 2014; Nesterenko et al. 2012). SPI in combination with MS was reported to be an effective wall material for β-carotene (Deng et al. 2014). Additionally, the oxidative stability of SPI encapsulated orange oil was better than sodium caseinate and gum acacia (Kim et al. 1996).

The binding between volatiles and soy protein has also been reported (O'Keefe et al. 1991a, O'Keefe et al. 1991b, Damodaran and Kinsella, 1981b). It was found that binding was mainly through hydrophobic interactions. Investigations on the binding between carbonyls and soy protein showed that with each increase in methylene group in the hydrocarbon chain of the carbonyl, the binding constant with soy protein increased 3 orders of magnitude (Damodaran and Kinsella, 1981a).

Interactions between aldehydes and soy protein has also been reported. It was found that the retention of aldehydes corresponded with chain length or hydrophobicity. For example, the retention (the percentage of retained flavor /initial added flavor) of octanal, nonanal, and decanal was 83-85%, 90-93%, and 94-97%, respectively (Gremli 1974). Additionally, like whey proteins, some of the
hydrophobic binding sites of soy protein are also hidden inside of a globular structure when in its native state. When hydrophobic binding sites become available upon denaturation (e.g. by heat or pH treatment), the binding affinity increases. The binding constant of 2-nonanone with native soy and denatured soy (heat at 90 °C for 1 h) increased from 930 M\(^{-1}\) to 1240 M\(^{-1}\), respectively (Damodaran and Kinsella, 1981a).

Understanding the interactions between volatiles and soy protein is of importance in obtaining desirable sensory attributes. When a flavorist creates a flavor or a food product developer formulates a food product, it is necessary to have a knowledge of what kind of reactions/interactions might occur between a flavor compound and a given protein. Based on the understanding of these mechanisms, one can determine if excess amount of a certain flavor compound is needed to adjust it to a desirable intensity when certain binding is expected. Therefore, the objectives of this study were as below:

a. Investigate the competitive binding between volatiles and selected food ingredients with β-CyD and its effect on volatile release
b. Evaluate the effect of pH on volatile release in a β-CyD:volatile:food ingredient systems.
c. Assess the effect of addition order on volatile release from β-CyD inclusion complexes.

2. Materials and Methods

2.1 Materials

Cyclodextrins (pharmaceutical grade, 98% purity) were purchased from Wacker Biochemical Corp. (Munich, Germany). Ethyl acetate, ethyl butyrate, and ethyl heptanoate were purchased from Sigma Aldrich Chemical Co. (Milwaukee, WI, U.S.A); Modified starch (OSA modified) was provided by Cargill Food & Pharma Specialties North-America (Cedar Rapids, IA). Whey protein isolate (BiPro) was purchased from Agropur (Le Sueur, MN). Lecithin was purchased from Spectrum Chemical Mfg. Corp. (New Brunswick, NJ). Casein was bought from Fisher Scientific (Fair Lawn, NJ). Sucrose was purchased from ICN Biomedicals Inc. (Costa Mesa, CA), and soy protein isolate (SPI, Pro Fam 781) from ADM (Decatur, IL). Tween 20 was purchased from Croda International PLC. (Edison, NJ).

2.2 Methods

2.2.1 Preparation of External Standard
A stock solution (600 ml) was prepared by stirring 1% (wt/wt) β-CyD in distilled water for 20 min using a stir bar at ambient temperature. The three model flavor compounds, ethyl acetate, ethyl butyrate, and ethyl heptanoate, were then added to this stock solution (at equimolar ratios) and mixed for 20 min using a stir bar. The total amount of the three esters added was calculated based on a 1:1 molar ratio of esters to β-CyD. The concentration of the β-CyD and the three esters in the 600 ml stock solution are calculated as below.

Weight of β-CyD: 600 g of water × 1% = 6 g

Mole of β-CyD: \[
\frac{6g}{1135g/mM} = 5.286 \text{ mM}
\]

A molar ratio of 1:1 of the guest:host is used in this study to form the inclusion complex.

Mole of each of the three of the esters = 1/3 of the Mole of the β-CyD

Mole of each of the three of the esters = 5.286 mM × 1/3 = 1.762 mM

2.2.2 Sample preparation before GC analysis

After preparing the stock solution, 10 ml of the stock solution was pipetted
into a 20 ml headspace sampling vial. The vial was sealed using a Teflon-faced septum lined aluminum crimp cap. Triplicates were prepared for each sample. The samples were equilibrated for 24 h before the GC analysis.

2.2.3 Preparation of the references

Reference 1 - esters and water:

The esters were added into distilled water (without CyD added) and mixed for 20 min using a stir bar. The concentration of the esters in water was the same as calculated in the last section. After mixing the esters in water, 10 ml of the solution was transferred into a headspace sampling vial (20 ml) sealed with Teflon-faced septa lined aluminum crimp caps and allowed to equilibrate for 24 h before GC analysis. Three replicates (10 ml sample) were prepared for GC analysis.

The first reference was prepared to compare with Reference 2 and other sample groups with food ingredient added. All the references and samples were prepared at ambient temperature.

Reference 2 - esters, β-CyD, and water
The second reference was prepared to evaluate the effect of β-CyD on flavor release from an aqueous model flavor system. Its other purpose was to allow comparison with the release behavior of the volatiles from the samples containing other ingredients. In this system, samples were prepared by mixing β-CyD and esters together without the food ingredients added. First, a stock solution of 1% (wt/wt) β-CyD in 600 ml water was prepared. The β-CyD (6g) was dissolved in water and mixed for 20 min using a stir bar. Secondly, the flavor compounds were added and mixed for another 20 min to allow the inclusion complex formation to take place.

The equal molar ratio of the β-CyD to the total of the three esters was used (as same as calculated above). Ten ml sample was then transferred from this stock solution into a 20 ml headspace sampling vial sealed with Teflon-faced septa lined aluminum crimp caps and allowed to equilibrate for 24 h before GC analysis. Three replicates (10 ml of each sample) were prepared for GC analysis.

2.2.4 Sample preparation method for the evaluation of the effect of pH on volatile release in the presence of a selected food ingredient

A stock solution of 1% β-CyD in distilled water was prepared by stirring for 30 min using a stir bar. The flavor compounds were then added to form the inclusion complex with the β-CyD with an equal molar ratio using the preparation
conditions same as described above. The food ingredient was added last into the β-CyD:flavor inclusion complex solution and stirred for 30 min using a stir bar. The food ingredient was added individually to each solution to study its effect on the release of the volatile from the β-CyD cavity. The concentrations of each of the ingredient was 5 wt % WPI, SPI, and casein, 1 wt % Tween 20 and lecithin, 5 wt % sucrose, and 5 wt % modified starch. Sodium hydroxide or hydrochloric acid of 0.1 M was used to adjust the pH. Three pH conditions were used for each sample – pH 4, 6, and 8. All samples were prepared at ambient temperature and in triplicate.

A 10 ml aliquot of each solution was transferred into 20 ml headspace sampling vials sealed with Teflon-faced septa lined aluminum crimp caps and then equilibrated at ambient temperature for 24 h before analysis. Three replicates (10 ml sample) were prepared for GC analysis. Sodium hydroxide or hydrochloric acid (0.1 M) was used to adjust the pH.

To verify that the 24 h equilibration time was adequate for equilibration, the samples were also equilibrated for 36 and 48 h. No significant difference in headspace concentration of the flavors over time was found upon the GC analysis.
2.2.5 Sample preparation method for the evaluation of the effect of the addition order on volatile release

This study was aimed to investigate the effect of the order of addition of the β-CyD and the food ingredient on the release of the flavor compounds.

2.2.5.1 Addition Order 1

The purpose of this order of addition was to allow the flavor compounds to form the inclusion complex with the CyD first. Then the food ingredient was added to the complex to evaluate the effect of the food ingredient on flavor release – that is, to determine if the ingredient would replace the flavor in the CyD or not. If the ingredient added replaced the flavor compounds included, the flavor compounds would be freed to release into the headspace and consequently, increase their headspace concentration. In this experimental design, the binding between the esters and the ingredient (e.g. WPI) is negated.

The sample preparation procedure is as follows: first, the flavor-β-CyD reference solution was prepared as described above (reference 2). Then, one of the food ingredients (such as WPI, OSA-MS, or lecithin) was added and mixed for 30 min using a stir bar. Each sample contained the three flavor compounds, 5 wt % β-CyD and an individual ingredient with concentrations of 5 wt % WPI, SPI, and
casein, 1 wt % Tween 20 and lecithin, 5 wt % sucrose, and 5 wt % modified starch. All samples were prepared at ambient temperature.

2.2.5.2 Addition Order 2

A solution of CyD (1%) was first prepared. One of the food ingredients was added to the β-CyD solution and then mixed another 20 min. Finally, the three esters were added and mixed for 30 min. This order of addition allows the CyD to form a complex with the food ingredient first. To investigate the ability of the flavors to replace the ingredient from the CyD cavity, the flavor compounds were added later. If the ingredient is replaced, the headspace concentrations of the flavor compounds would be decreased proportionally.

2.2.6 Gas Chromatography (GC) Analysis – GC conditions

An Agilent 5890 gas chromatograph equipped with an Agilent 7694 Headspace auto - sampler was used to analyze flavor release into the sample headspace. A flame ionization detector (250 ºC) and an HP-5 column (30 m, 0.25 mm I.D., 0.5 μm film thickness) were used (J&W Scientific, Inc., Rancho Cordova, CA). A headspace injection (1 ml) with a split ratio of 1:20 was made. Helium was used as the carrier gas. The gas flow rate through the column was 1
ml/min. The oven temperature program was: initial temperature 50°C, initial hold 2 min, heating rate 10°C/min, final temperature 120°C, final hold 5 min.

2.2.7 Data Analysis

Analysis of variance (ANOVA) was conducted with the R.3.3.2 statistical software package. In the addition order study, the two orders of addition and food ingredients were studied as the source of variation on their effects on flavor interactions. The three pHs served as the source of variation to study their influence on flavor interactions. The significance level was set at 5%. Two-way ANOVA was used with R.

3. Results and Discussion

3.1 The effect of β-CyD on flavor release

To investigate the effect of a selected food ingredient on the release of the volatiles included in the β-CyD inclusion complexes, the headspace concentration of a volatile in samples with an ingredient added was compared with that of reference 1 (esters in water) and 2 (esters and β-CyD inclusion complex solution).
Figure 12. The effect of β-CyD on volatile release from aqueous solution. The headspace concentrations of reference 2 (volatile: β-CyD inclusion complex) were standardized by the reference 1 (esters in water) - assume the headspace concentrations of reference 1 were 100%.

As shown in Figure 12, the headspace concentrations of all three esters were decreased significantly in the CyD+Esters control compared to Esters+water control – the esters in water control without CyD added. Specifically, the headspace concentrations of ethyl acetate, ethyl butyrate, and ethyl heptanoate decreased 20, 70, and 80% in the presence of β-CyD. The reduction of the volatile release is related to the carbon chain length, i.e. the longer the carbon
chain length, the less of the volatile is released to the headspace. This result is consistent with findings in literature. It was reported that the percentages static equilibrium headspace intensity of the ethyl esters decrease from 99, 68, 45, to 9 in the order of methyl acetate, ethyl butyrate, ethyl hexanoate, and ethyl octanoate relative to the aqueous control (Kant et al. 2004). The same trend was found for the alcohols. The standardized headspace concentration decreased from 129 to 117 from ethanol to butanol. With the ketones in the order of heptanone, octanone and decanone, the standardized headspace concentration decreased from 56 to 9.

The decreased release with increasing carbon chain length is attributed to the hydrophobicity of the guest volatile compound. The longer the carbon chain, the more hydrophobic the volatile is and therefore, the more it is retained by the β-CyD cavity due to higher affinity toward the CyD cavity. Consequently, less is freed or released into the headspace. The physicochemical properties of the esters used in this study are given in Table 2.
Table 2. Physicochemical properties of the selected esters.

<table>
<thead>
<tr>
<th>Aroma compound</th>
<th>Molecular weight (g/mol)</th>
<th>Partition Coefficient (log P)(^b)</th>
<th>Solubility in water 25°C (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate</td>
<td>88</td>
<td>0.73</td>
<td>80</td>
</tr>
<tr>
<td>Ethyl butyrate</td>
<td>116</td>
<td>1.85</td>
<td>4.9</td>
</tr>
<tr>
<td>Ethyl heptanoate</td>
<td>158</td>
<td>3.32</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Saturated vapor pressures were estimated using the Gomez Thodos model (26). \(^b\)log P is a hydrophobic constant, calculated using Rekker’s method.

Log P is defined as the logarithm of the oil:water partition coefficient in an octanol:water system (Comer and Tam, 2001). It indicates the ability of a flavor compound to partition into an organic phase (octanol). A higher log P value would mean a stronger tendency to partition into an organic phase than an aqueous phase, and therefore greater hydrophobicity. As given in Table 2, with the highest log P of 3.32, ethyl heptanoate is the most hydrophobic ester. The volatile hydrophobicity decreases as the carbon chain length decreases. The correspondence found between the hydrophobicity with the carbon chain length of the esters is consistent with findings of others (Madene, et al. 2006; Reineccius, et al. 2002; Goubet, et al. 2001; Del Valle, 2004; Kant, et al. 2004).
3.2 Effect of selected food ingredients on volatile release from β-CyD inclusion complexes

3.2.1 Proteins

The addition of WPI to Reference 1 (aqueous solution of CyD + volatiles) resulted in an increased headspace concentration of ethyl acetate by 7%, and ethyl butyrate by 103%. We assume that WPI addition had a lesser effect on the release of ethyl acetate than ethyl butyrate since there is little ethyl acetate...
included in the β-CyD to be released (as shown in the high headspace concentration from Reference 2). The increases in headspace concentration observed for these two esters are due to the net effect of the WPI replacing the esters in the CyD cavity (increase headspace concentration) and any binding that would then occur between the esters and the WPI (decrease headspace concentration). The effect of adding WPI on ethyl heptanoate headspace concentration was minor (ca. +5%). It is unknown if the WPI could not replace the ethyl heptanoate in the CyD or if it was replaced but then bound by the WPI. Because of the relatively high hydrophobicity of ethyl heptanoate, upon the addition of the WPI, ethyl heptanoate can not only bind with the CyD cavity but also the proteins through hydrophobic interactions.

While similar trends were found for the addition of other proteins (casein and SPI), subtle differences were noted. For casein and SPI, the release of ethyl heptanoate decreased by 47 and 16%, respectively. The reduced release of ethyl heptanoate (compared to that of reference 2) from the casein and SPI samples may be explained by the interactions between the volatiles and the proteins in the aqueous phase as explained earlier. All three proteins can bind nonpolar compounds through hydrophobic interactions as discussed in the review of literature.
As shown in Figure 13, the headspace concentrations of the volatiles decreased with increasing carbon chain length – headspace concentration was the highest for ethyl acetate and the lowest for ethyl heptanoate. This result suggests that the binding affinity of the volatiles (with both proteins and the CyD) increased as their hydrophobicity increases. The concept that protein:aroma compound binding increases with hydrophobicity of the aroma compound has also been demonstrated for protein: aliphatic ketones (O’Neill and Kinsella, 1987, See Table 3).


<table>
<thead>
<tr>
<th>Ligand</th>
<th>K, M⁻¹</th>
<th>ΔG, kcal/mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Heptanone</td>
<td>150</td>
<td>-2.98</td>
</tr>
<tr>
<td>2-Octanone</td>
<td>480</td>
<td>-3.66</td>
</tr>
<tr>
<td>2-Nonanone</td>
<td>2440</td>
<td>-4.62</td>
</tr>
</tbody>
</table>

As shown in Table 3 above, the binding constant and the free energy of binding were compared across the 2-alkanones with increasing carbon chain length. The free energy of binding 2-alkanones to β-Lg decreased with increasing carbon chain length. Among the three 2-alkanones, the free energy needed to bind with β-Lg was the lowest for 2-nonanone. Also, the association constants increased with increasing carbon chain length, which was consistent with the free energy
data. These findings suggested again that the binding was hydrophobic interaction in nature.

In another study, the binding affinity of volatiles to whey protein and casein was compared (Hansen and Booker, 1996). The authors first mixed model flavor compounds - citral, d-limonene, vanillin, and benzaldehyde, with ice cream mix. Then, they fractionated the ice cream mix into fat, casein, and whey. The relative flavor concentrations in each of these fractions were quantified to determine the level of flavor:protein binding. It was found that for vanillin, about 50% was recovered from the whey protein fraction whereas 37.1% was recovered from the casein fraction. In addition to the analytical data, sensory tests were also conducted by a trained sensory panel.

It was found that, in general, flavor intensity was lower in the WPC fraction than casein fraction. The intensity of benzaldehyde (relative to the 0.168 mM benzaldehyde reference) dropped from 0.45 to 0.25 as the WPC concentration increased from 0.5 to 1% whereas no significant difference in benzaldehyde intensity was found for the CAS sample. For d-limonene, its intensity (relative to 0.389 mM d-limonene reference) dropped from 0.41 to 0.27 as the WPC concentration increased from 0 to 0.5%. The data for intensity decrease in the presence of CAS was not given, however, the authors noted that the decrease was the most marked for the WPC fraction. In general, it was concluded that as
Pasteurization took place, the \( \beta \)-Lg of the whey protein fraction bonded the flavors to a greater degree than casein. The \( \beta \)-Lg was more sensitive to heat than casein, and upon heat treatment, \( \beta \)-Lg unfolded and exposed its hydrophobic binding sites, which resulted in increased binding and reduced flavor intensity.

The nature of the interactions between WPI and aroma compounds has been found to be mainly through hydrophobic interactions (Guichard and Langourieux et al, 2000). Binding sites were determined to be in the hydrophobic pocket of the \( \beta \)-Lg fraction (Guichard and Langourieux et al, 2000). For a variety of hydrophobic compounds, such as alkanes, sodium dodecyl sulfate, N-methyl-2-anilino-6-napthalenesulfonic acid, and retinol, the hydrophobic pocket was found to be the only binding site for this protein.

3.2.2 Emulsifiers
Figure 14. The effect of the emulsifiers – Modified starch, Lecithin, and Tween 20, on volatile release from the β-CyD inclusion complex.

Similar to proteins, the greatest effect of emulsifier addition on ester release from CyD complexes was observed for ethyl butyrate (no significant effect on ethyl acetate or ethyl heptanoate, Fig 14). For example, the headspace concentration of ethyl butyrate increased by 87% compared to reference 2 upon the addition of lecithin. The reasons for our observations are likely different for emulsifiers vs proteins. As explained earlier, the amount of free esters in the protein systems was the net result of binding either with the CyD cavity or the protein. In the case of emulsifiers, there would be no equivalent binding of esters...
to the emulsifiers: esters freed from the CyD cavity due to preferential binding of
the emulsifier would be in the “solution”. However, the solution would have a very
different polarity when it contained emulsifier than when it is pure water. It would
be much more non-polar meaning that it would be a better solvent for
hydrophobic molecules (e.g. ethyl heptanoate). This would reduce compound
volatility (sensory contribution) even though not being bounded.

One can also recognize that the amount of emulsifier added to the system can
influence aroma compound volatility. Once the concentration of an emulsifier
exceeds its critical micelle concentration (CMC), the emulsifier or surfactant self-
aggregates and forms micelles. The hydrophobic regions of the emulsifier orient
toward the core whereas the hydrophilic heads orient toward the aqueous phase.
Once the micelles are formed, volatiles can be accumulated and trapped. This
would further reduce the sensory impact of aroma molecules that have a high
solubility in the non-polar phase. The concentration of Tween 20 as well as
lecithin used in this study (1%) exceeded their CMC of 0.0694 mM and 10^{-7} mM,
respectively (Held, 2014, Tanford, 1980).

There was no significant difference in the volatile release across the three
eumsifiers for the same volatile. For example, no significant difference was
found between the addition of, OSA-MS, lecithin, or Tween 20 on the release of
ethyl acetate. However, there were significant differences in the headspace
concentrations across the three esters upon the addition of the same emulsifier. For example, for the MS sample, the releases of the three esters were significantly different from each other. This can be explained by the differences in the hydrophobicity of the volatiles as discussed previously.

3.2.3 Carbohydrates

3.2.3.1 Sucrose
Figure 15. The effect of sucrose on volatile release from the β-CyD inclusion complex.

Figure 15 shows a similar effect of adding sucrose to the CyD+esters: as was observed for the proteins and the emulsifiers. Again, the largest change took place for the release of ethyl butyrate. The addition of sucrose increased the headspace concentration of ethyl butyrate by 90%. Similar to the other model ingredients – ethyl butyrate increased to the largest extent whereas lesser changes were observed for ethyl acetate and ethyl heptanoate.
Previous work has shown that a “salting out” may occur with esters in aqueous solution but only when sucrose concentrations get in the 20 to 60 w% range (Roberts et al. 1996). When adding sucrose at high concentration, sucrose interacts with water and leaves less water available to interact and solubilize the volatiles, which results in freeing the volatiles into the headspace. The addition of high concentration of sucrose would be expected to increase the release of hydrophilic flavor compounds into the sample headspace and a reduced release of hydrophobic compounds. This takes place because the solution is made more hydrophobic as sucrose interacts with water and renders less “free water” available to solubilize the polar flavor compounds. On the other hand, the increased hydrophobicity of the solution now can better solubilize non-polar flavor compounds. However, because the sucrose concentration was only 5 wt % in this study, it was unlikely to be a result of the salting out effect. Thus, we are at a loss to explain why the addition of sucrose to the CyD system resulted in greater aroma release into the sample headspace.

3.3 Effect of pH on volatile release from β-CyD inclusion complexes

3.3.1 Effect of pH on the release of ethyl acetate in the protein samples
Figure 16. Effect of pH on the release of ethyl acetate from the β-CyD inclusion complex in the protein samples. The three letter groups: 1. x and y; 2. a, b, and c; 3. the asterisk *, denote the significant differences between the pHs in each of WPI, casein, and SPI, respectively. Different letters or asterisk symbols indicate significant difference at 5% significant level.

Figure 16 shows the effect of three pH conditions on volatile release from protein:CyD systems. One will note that pH changes have very similar effects on volatile release from both casein and WPI, i.e. the pattern of the release as well
as the changes in the headspace concentrations. The headspace concentration of ethyl acetate decreased as the pH increased from 4, 6 to 8 in casein and from 6 to 8 in WPI. It has been previously reported that as the system approaches the isoelectric point of whey protein (in the range of 4 to 6), the solubility of the whey proteins (WPC and WPI) do not decrease and the whey proteins remain soluble in the solution (de Carvalho-Silva et al. 2013). In consistent with their findings, the release of ethyl acetate from the WPI samples did not show a significant change from pH 4, 6, to 8 in our study. In other words, it is possible that the changes in pH did not significantly affect the solubility, conformation or the binding of the protein with ethyl acetate.

The release of ethyl acetate from the SPI samples did not show any significant change across the pH range. However, the headspace concentration of the casein samples decreased significantly from pH 6 to 8. The significant reduction in the headspace concentration of ethyl acetate might be attributed to an increased binding of the ester to casein. One possibility can be that when the pH of the solution is above the isoelectric point, which is 4.6 for casein, casein becomes negatively charged. As the pH continued to increase, the negatively charged casein repulsed each other by electrostatic repulsion and resulted in the slowing down of the aggregation (Liu and Guo, 2008; Madadlou et al. 2009). As a result, more casein remained in solution instead of precipitating out.
Consequently, there was more casein available to bind with the volatile compound and reduce its release.

3.3.2 Effect of pH on the release of ethyl butyrate across the protein samples

Figure 17. Effect of pH on the release of ethyl butyrate in the protein samples.

The three letter groups: 1. a, b, and c; 2. x and y; 3. the asterisk *, denote the significant differences between the proteins at the pH of 4, 6, and 8, respectively.
Different letters or asterisk symbols indicate significant difference at 5% significant level.

Figure 17 shows that the release of ethyl butyrate is significantly higher from casein than SPI sample at pHs 4 and 6. It is possible that the binding affinity of the ester to SPI what higher than that to casein at both of pH 4 and 6. Additionally, in the casein sample, the release of ethyl butyrate significantly decreased from 4 to 8. This may be attributed to the higher solubility of casein at pH 8 than 4. The increased solubility of casein in the solution allowed it to bind more ethyl butyrate and resulted in the reduced release into the headspace. As discussed previously, casein becomes negatively charged above pH 4.6, and the negatively charged casein repulses each other and keeps them from aggregation. Therefore, the solubility of casein increases as the pH increases from 4 to 8 and resulting in greater binding of casein with ethyl butyrate. It is also possible that the structure of the protein changed upon the pH change, thereby some hydrophobic region(s) of the protein (i.e. an amino acid side chain or a hydrophobic segment of the protein) were exposed. The hydrophobic region interacted with the CyD cavity and resulted in flavor release.

3.3.3 Effect of pH on the release of ethyl heptanoate across the protein samples
Figure 18. Effect of pH on the release of ethyl heptanoate in the protein samples.

The letters denote the significant difference between the headspace concentration of ethyl acetate, with different letters indicate significant difference at the significant level of 5%.

As shown in Figure 18, the headspace concentration of ethyl heptanoate was significantly higher at pH 4 than 6 from the WPI sample. This observation can be possibly explained by the protein conformation change upon pH change.
The isoelectric point of the WPI was found to be 5.2. The maximum turbidity and protein aggregation were also observed at this pH, together with the increased solubility of the protein both above and below its PI (Ju and Kilara, 1998). More importantly, they observed aggregation at pH 6.2 using the dynamic light scattering (DLS) method. The presence of the aggregations was further confirmed by the turbidity method. Additionally, it was found that the aggregations were gradually reduced as the pH was reduced from 6 to 4. The increased hydrophobic interactions between the proteins contributed to the formation of the aggregates. When the pH was reduced toward the pH of 5.2, the electrostatic repulsion was reduced. Also, this change in the pH can induce changes in the conformational structure of the protein – more hydrophobic groups or bindings sites were exposed to interact with each other or with another hydrophobic substance, in this case, ethyl heptanoate. This could be a possible explanation of the significant reduction in the headspace concentration of ethyl heptanoate at pH 6.

At pH 6, the release of ethyl heptanoate from SPI was significantly higher than that from casein. The isoelectric point of SPI is 4.5 – very close to that of casein (Hefnawy and Ramadan, 2011). The difference in the release of ethyl heptanoate could be possibly a result of the difference in hydrophobicity of the two proteins - SPI was less hydrophobic than casein and therefore will bind less of the ester.
Another possibility is that the hydrophobic amino acid side of SPI interacted with the CyD and results in the replacement of ethyl heptanoate and its release.

3.4 Effect of order of addition on volatile release

Two orders of adding esters, β-CyD and food ingredient were compared to evaluate their effects on flavor interactions. The first addition order was prepared by mixing the esters with β-CyD first to form the flavor:β-CyD inclusion complex and then one of the food ingredients was added. In the second order of addition, one of the food ingredients was mixed with β-CyD first and then the esters were added.

For the SPI system, higher headspace concentrations of ethyl acetate and ethyl butyrate resulted from order 1 than order 2 by 18 and 23%, respectively. One possible explanation can be the difference in the hydrophobicity of the solutions resulting from the two orders of addition. When SPI was added last into the solution in order 1, the solution could have been made more hydrophobic as compared to the addition order 2. When SPI was added first to mix with the CyD, the hydrophobic region of SPI (e.g. a hydrophobic side chain) could have already been included inside of the CyD cavity. As a result, the sample made by order 2 can be less hydrophobic and therefore, can better solubilize ethyl acetate, which resulted in the reduced release comparing to addition order 1. Another possible
explanation is that the system had not reached equilibration yet, this was also the reason why significant differences in release behaviors were observed. Because if equilibration had been reached, the most hydrophobic one (whether it is a hydrophobic region of a protein or a hydrocarbon chain of an emulsifier) would be included in the CyD cavity eventually - owning to their higher binding affinity and larger binding constant.

Additionally, because the lower hydrophobicity of ethyl acetate and ethyl butyrate, the binding affinities to the CyD cavity or SPI were lower than that of ethyl heptanoate. Therefore, it was easier for the smaller two esters to be partitioned into the headspace. However, for a more hydrophobic volatile compound – ethyl heptanoate, attributed to its high hydrophobicity, its bindings with both the CyD cavity and SPI were stronger. As a result, the addition order of these two (CyD and SPI) would not have affected its release or headspace concentration. This was probably the reason why no significant difference in the headspace concentration of ethyl heptanoate was found.

Additionally, higher headspace concentration of ethyl heptanoate from order 1 was also observed in the OSA-MS samples. The octenyl succinic anhydride fraction of the OSA-MS is hydrophobic and is expected to compete with ethyl heptanoate for the CyD cavity. This was probably the reason why the increased headspace concentration of ethyl heptanoate in the sample of the addition order
was observed comparing to the order 2. Another important possibility was the same as noted earlier, which was related to binding kinetics and system equilibration. It is possible that the system had not reached equilibrium, if given enough time, there would not be a significant difference.

4. Conclusions

The interactions we would expect between our esters and the food components studied were mainly through hydrophobic interactions (no charge effects or covalent reactions are anticipated). Headspace concentrations of our model compounds in pure water generally increased with their increasing polarity and volatility. In the presence of CyDs, flavor compounds with high hydrophobicity and low volatility would have higher affinity to the CyD cavity and thus, might be reduced in release. One can also expect an impact on the sensed or perceived sensory characteristics of a certain flavor profile because of the difference in the level of release.

In real food systems, food ingredients may compete with volatiles for forming inclusion complexes with β-CyD to various degrees depending on the volatile hydrophobicity and general fit into the CyD cavity. This competition for the CyD cavity would potentially release the more hydrophobic volatiles (those in the CyD complex). This difference in the release behavior, again, can result in the amount
of volatiles being sensed and consequently perceived as imbalanced flavor profile. An additional consideration is that a food ingredient or part of a food ingredient (e.g. hydrophobic portion of an emulsifier) may be included in a CyD and as a result, possibly lose its functionality (e.g. an emulsifier).

It is important to note that the competitive binding and release behavior were observed within the time frame of our experiments. The most hydrophobic substance with the highest binding affinity to the CyD cavity would be included if enough time is given for the system to reach equilibration. Furthermore, it was found that pH influenced the protein:CyD:flavor system to a greater degree compared to the emulsifier:CyD:flavor system. This may be attributed to a change in protein structure as a result of pH change. The hydrophobic region of the protein may have been exposed upon pH induced denaturation, which allowed it to bind more with the CyD or a hydrophobic volatile. As a result, reduced volatile release was observed. Also, it is possible that pH affected protein solubility as a result of electrostatic repulsion. Better solubilized protein can bind more volatiles and thus decrease their release.

The findings from this study can contribute to the prediction of volatile release behavior in a food system. Understanding the competitive binding between food ingredients and volatiles to CyD can help formulate and develop a food product with desirable flavor profile and sensory attributes.
5. Comprehensive Bibliography


Lei, M., Hewitt, D., Cornell, C., Skidmore, K., Kao, Y. H., Sugahara, J., ... & Ji, J. (2013). The effects of membrane filters used in biopharmaceutical processes on
the concentration and composition of polysorbate 20. Biotechnology progress, 29(6), 1503-1511.


compounds under dynamic conditions. Journal of Agricultural and Food Chemistry, 44(5), 1321-1326.


