ZEIN ENCAPSULATION OF AMPHIPHILIC COMPOUNDS

BY

CHIN-PING SU

THESIS

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Master’s Committee:

Professor Keith Cadwallader, Chair
Research Professor Graciela Padua, Director of Research
Assistant Professor Youngsoo Lee
Associate Professor Hao Feng
Functional compounds including flavors, essential oils, antioxidants or nutraceuticals are widely added into food systems to enhance sensory properties or for health purposes. However, these compounds might not be stable in food systems during processing, storage and food preparation. Encapsulation has been proposed as a practical approach to stabilize these compounds and control their release. Zein is an amphiphilic protein originally obtained from corn. Its film-forming and coating ability make it potentially useful as a wall material for encapsulation. Amphiphilic compounds have been recognized to form well defined microspheres by evaporation induced self-assembly (EISA) in binary solvents, which is useful in encapsulation. The goals of this research were to improve our understanding of the development of zein encapsulation structures. The main objective was to investigate the effect of the hydrophobicity of core materials as measured by contact angle on the formation and structure of zein encapsulates obtained by evaporation induced self-assembly of ethanol-water systems. A second objective was to apply the knowledge learned in the above objective to propose a strategy to capture and encapsulate flavors generated in frying oils. Results showed that citral was effectively encapsulated with zein. Zein encapsulation morphology was related to the droplet formation ability of core materials in ethanol-water systems. Amphiphilic compounds were believed to form stable droplets in ethanol-water, which favored zein encapsulation. Hydrophobic compounds showed phase separation in ethanol-water which led to the formation of films rather than closed structures. A model system consisting of mixtures of a flavor and a hydrophobic carrier was used to study the effect of carriers on encapsulation ability of zein by the self-assembly process. The presence of the carrier negatively affected encapsulation effectiveness. Citral was recovered from the flavor-carrier mixture by extraction and phase separation at sub-freezing temperatures. Recovered citral was effectively encapsulated by zein. Fried chips flavors were also recovered from frying oil and encapsulated
in zein by ethanol extraction and phase separation at low temperatures. Sensory ranking test was used to confirm the presence of recovered flavors after zein encapsulation.
To my family, Sui-Peng Su, Hui-Mei Lin and Yu-Wen Su...

…for their encouragement, support, trust and love.
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CHAPTER I

INTRODUCTION

Flavor compounds are routinely added to foods to enhance their sensorial quality. However, flavors might escape or degrade under food processing and storage conditions. For example, (E,Z)-2,6-nonadienal (NDEA) and (E)-2-nonenal (NEA), present in cucumber flavor, degrade into their corresponding acids by oxidation or enzymatic reactions within a few hours of storage (Cho and Buescher 2011). Other food compounds and additives including phenol, anthocyanin, Vitamin C and Vitamin A are also lost through oxidation during processing and storage of foods (Whited, Hammond et al. 2002; Burdurlu, Koca et al. 2006; Patras, Brunton et al. 2011).

Encapsulation has been proposed as an effective method to maintain the quality and stability of these compounds in food products (Lakkis 2007; Huang, Given et al. 2009). For example, fish oil was encapsulated in cyclodextrins and whey protein to prevent lipid oxidation (Na, Kim et al. 2011). Orange oil was encapsulated in lactose and caseinate to prevent degradation by exposure to moisture and light (Edris and Bergnstahl 2001). Folic acid was encapsulated by ethyl cellulose to inhibit deterioration in acidic conditions (Prasertmanakit, Praphairaksit et al. 2009). Furthermore, encapsulation allows liquid compounds to be incorporated into dry wall materials, turning them into powders to facilitate their handling.

Zein, a protein original from corn, is recognized for its coating ability. It is potentially useful as an encapsulation material to protect sensitive flavors and bioactive compounds. For example, zein was used to encapsulate flax oil (Quispe-Condori, Saldana et al. 2011), curcumin (Gomez-Estaca, Balaguer et al. 2012) and cranberry procyanidins (Zou, Li et al. 2011) by spray drying, freeze drying and coacervation methods. Zein has an amphiphilic molecule due to its relatively high content of non-polar amino acids. It is capable of self-assembly owed to its structural morphology and amphiphilic character. Microspheres and lamellar films formed by evaporation induced self-assembly of zein in ethanol-water were observed by Wang, Yin et al. (2008) and Wang and Padua (2010). Evaporation induced self-assembly (EISA) is a method first described for the encapsulation of oil containing gold particles in a surfactant dispersed in
aqueous phase (Brinker, Lu et al. 1999). Wall and core materials are dispersed in a binary solvent such as ethanol-water. Upon evaporation of the light solvent, wall materials self-assemble, due to changes in solvent polarity, to form various structures including micro- and nano-sized spheres, sponges and films. The type of structures formed depends on the materials involved and processing conditions. Self-assembly allows for better control of encapsulate size, morphology, and functional properties.

The effectiveness of zein encapsulation is believed to depend on the formation of core micron size droplets in a solvent medium where zein can also be dispersed. Both events are only possible when the hydrophilic/hydrophobic balance is properly tuned and the ratio of core to shell materials is optimized. The goals of this research are to improve the understanding of structure development of zein encapsulates. The specific objectives were to:

1. Investigate the effect of the hydrophobicity of core materials as measured by contact angle on the formation and structure of zein encapsulates.

2. Apply the knowledge learned in the above objective to propose a strategy to capture and encapsulate flavors generated in frying oils.
CHAPTER II
LITERATURE REVIEW

2.1 Encapsulation

2.1.1 Definition
Encapsulation has been applied for several decades in food, pharmaceutical and cosmetic industry. Encapsulation can be defined as a process that incorporates discontinuous solid particles, liquid droplets, or gaseous bubbles within another continuous film forming materials and forms a closed system in solid, liquid capsules or gas cells. Depending on capsule size of final products, encapsulation is categorized to nano-encapsulation, microencapsulation and macroencapsulation (King 1995; Desai and Park 2005; Marcuzzo, Sensidani et al. 2010).

2.1.2 Purposes
The main purposes of encapsulation are (Barbosa-Cánovas, Ortega-Rivas et al. 2005; Desai and Park 2005; Poncelet 2006; Lakkis 2007):

*Protection:*  
To enhance stability of core materials such as flavors. Flavor compounds play an important role in food system by offering the overall sensations. They are also a key factor to determine human consumption choice. In general, flavor compounds are volatile and liable to oxidation and degradation during handling and storage under different atmospheric conditions or by microbial growth (Reineccius and Heath 2006). Ruiz Perez-Cacho and Rouseff (2008) studied the flavor variation and degradation in orange juice during the thermal processing and storage. It was found that thermal processing will cause the degradation of desirable flavor compound such as citral and lead to off-flavor compounds 4-vinylguaiacol, p-cymene and carvone resulting from Millard, Strecker reactions and acid catalyzed hydration. Lubbers, Decourcelle et al. (2004) investigated flavor release during the storage of fat-free strawberry yogurts. They found that flavors such as methyl 2-methyl butanoate, ethyl hexanoate, and hexyl acetate were lost in half during the storage after 28 days. Moreover, the flavor release can be affected by handling process, composition of protein and polysaccharide in food system.
Encapsulation technique could help on preventing deterioration of flavor quality. For example, Patel, Hu et al. (2010) encapsulated curcumin by zein in order to improve the stability under pH variation and irradiation conditions. They found that the stability of curcumin under pH variation from pH 1 to pH 9 and ultraviolet was improved. Orange oil was encapsulated by lactose and caseinate in order to prevent the deterioration caused by heat, humidity, light, and oxygen (Edris and Bergnstahl 2001). Other than flavor compounds, polyunsaturated fatty acids and vitamins were also encapsulated for the purpose of protecting them from external effects including light, temperature, air, moisture and microorganisms. For example, alpha-tocopherol was encapsulated by zein/chitosan complex in order to against degradation in acidic gastric environment and improves the quality (Luo, Zhang et al. 2011). Fish oil was encapsulated by cyclodextrin and whey protein to inhibit lipid oxidation (Na, Kim et al. 2011). McClements (2009) coated fruits and vegetables by chitosan/eugenol laminated film to protect from microbial growth and enhance the quality.

Physical modification:
Encapsulation could turn liquid flavors into powders. Besides, it could also improve rheology, compression, miscibility, dispersible properties or density in order to improve the utilizations. For example, Wang, Yan et al. (2010) encapsulated water-insoluble drug, thiocoraline, by mesoporous silica for the purpose of improving the water solubility.

Controlled release:
Delay release (e.g. flavor release while heating of foods), sustainable release (e.g. encapsulating flavor or sweetness for chewing gum) and control the release at certain time (drug release while reaching certain organs). For example, cinnamon oil and garlic oil were encapsulated by β-cyclodextrin in order to extend their release time and shelf life (Ayala-Zavala, Soto-Valdez et al. 2008). Folic acid was encapsulated by ethyl cellulose to prevent destruction from acidic stomach condition and allows the release until reaching intestinal tract (Prasertmanakit, Praphairaksit et al. 2009). Hoad, Rayment et al. (2011) encapsulated sunflower oil in alginate to demonstrate the potentiality to reduce bioavailability of lipid in GI tract and control lipid absorptions by slow release.

Mask:
Block undesirable tastes, odors in foods or drugs. (e.g., micronutrients, probiotics) For example, antioxidant extraction from *Myrtus communis* and antitumor extraction from olive leaf were encapsulated in liposomes and β-Cyclodextrin, respectively for masking their bitter taste in food system (Mourtzinos, Salta et al. 2007; Gortzi, Lalas et al. 2008).

### 2.2 Wall materials

Wall materials serve to enclose core materials within its structure and protect the core materials from external environment conditions. They generally have the characteristics of: film-forming, rheological properties at high concentration, being dispersible in solvent, ability to form a stable emulsion with core materials, having bland taste, being non-reactive with core materials and solvents during the process and storage, and low costs (Shahidi and Han 1993; Barbosa-Cánovas, Ortega-Rivas et al. 2005; Zuidam and Heinrich 2010). In food industry, wall materials have to be selected to meet GRAS requirement and be soluble in GRAS solvent for human consumption (Shahidi and Han 1993). Widely utilized wall materials in food industries as described by Shahidi and Han (1993) & Lakkis (2007) are summarized in Table 2.1. They can be categorized to waxes and lipids, proteins, carbohydrate, celluloses, gums and food grade polymers. Wall materials can be selected for encapsulation based on characteristics of core materials, solvent, equipment and desirable behaviors of final products such as release mechanism, compositions, particle sizes, cost and physical form. For example, lactose and caseinate were utilized to encapsulate orange oil to protect it from deterioration caused by temperature, moisture or light (Edris and Bergnsthall 2001). Jayalalitha, Balasundaram et al. (2012) utilized whey protein to encapsulate iron in order to prevent the iron derived lipid oxidation in iron fortified probiotic yogurt. Vernon-Carter and Welti-Chanes (2011) reported that mesquite gum can serve as a good wall material to encapsulate anthocyanins and helped on stabilizing its color and preventing from degradation. Zheng, Ding et al. (2011) found that encapsulation of antioxidant bayberry polyphenol by ethyl cellulose showed better stability and antioxidant capacity.

#### 2.2.1 Zein

In terms of good film forming and barrier properties, zein is widely utilized as a wall material for encapsulation of flavors, essential oils and bioactive compounds. It provides the
functionalities including increasing stability and control release of core materials. For example, α-tocopherol and essential oils, oregano oil and red thyme oil, were encapsulated by zein and zein/chitosan complex, respectively, for the purposes of control release and protection from decomposition in acidic gastric environment (Parris, Cooke et al. 2005; Luo, Zhang et al. 2011). Antimicrobial compounds, lysozyme and nisin, were encapsulated by zein in order to sustainable release and improve antimicrobial efficiency in food products (Zhong, Jin et al. 2009; Xiao, Davidson et al. 2011). Zein was also utilized to encapsulate antioxidant compound, cranberry procyanidins, and essential fatty acid, ω-3 fatty acid in flax oil, for control release and improve stability in delivery systems (Quispe-Condori, Saldana et al. 2011; Zou, Li et al. 2011).

Amino acid composition:
Zein is a protein derived from corn endosperm. Based on the solubility in ethanol-water, zein can be categorized into four types: α, β, γ and δ. α-zein is dominant type in total zein from corn and which contains more than 70%, followed by γ-zein (10–20%), β-zein (1–5%), and δ-zein (1–5%) (Wilson 1991). In the industry, α-zein and γ-zein are two types mainly utilized. Based on molecular weight, zein can be further separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Thompson and Larkins (1989) reported that the bands shown on SDS-PAGE were 19 and 22 kDa for α-zein, 14 kDa for β-zein, 16 and 27 kDa for γ-zein and 10 kDa for δ-zein.

The amino acids composition of zein was reported by Holding and Larkins (2009) as shown in Table 2.2 that α-zein contains a high proportion of glutamine, leucine and alanine, γ-zein contains high portion of proline and δ-zein is rich in methionine. Nonthanum, Lee et al. (2012) reported the ratio between polar and non-polar amino acids in zein. α-zein is around 42.6% to 57.4%. β-zein is around 50% to 50%. γ-zein at 16 kDa is around 52% to 48%. γ-zein at 27 kDa is around 48% to 52%. δ-zein is around 33.3% to 66.7%.
Molecular structure:
α-zein is abundant in total zein. The dimensional structure model of α-zein was proposed by Matsushima, Danno et al. (1997) that α-zein performed like as a $13 \times 1.2 \times 3$ nm$^3$ prism which was constructed by 10 α-helices plus one N-terminal domain aligned side by side in antiparallel orders. The whole structure is stabilized by the hydrogen bonds between the α-helices and connected by a glutamine-rich bridge on the top or the bottom of the structure. Lai, Geil et al. (1999) reported the amphiphilic property of zein that zein has hydrophobic surface located on the side of the stack which is formed by the outer surface of the helices and hydrophilic surface on the top and the bottom of the glutamine-rich area.

Properties:
The characteristics of zein mainly account for its composition and structure morphology as described above. Because of the sequence of amino acids and high composition of hydrophobic amino acids in zein, it is insoluble in water but can be dispersed in 50-90% ethanol-water mixture (Shukla and Cheryan 2001). Zein is an amphiphilic protein because of containing both hydrophobic and hydrophilic amino acids. Wang, Wang et al. (2004) found that zein had ability to attach with both hydrophilic and hydrophobic surfaces by the different surfaces. Because of the amphiphilicity of zein, it is able to self-assembly and give rise to several well-defined structures include micelles, vesicles, lamellar films, fibers and tubes constructed by van der Waals, hydrophobic attractions, hydrogen bonds or electrostatic interactions (Löwik and van Hest 2004; Grason 2006; McClements 2009). Different zein structures after evaporation-induced self-assembly in ethanol-water (EISA) were investigated by Wang, Yin et al. (2008). It was observed that zein could form spheres, sponge and lamellae structures depending on hydrophilic–lipophilic balance (HLB) conditions in system. The morphology tended to transform from spheres, spongy to smooth films by additions of hydrophobic, hydrophilic and amphiphilic compounds, respectively. Wang and Padua (2010) investigated the effect of zein/solvent ratio and ethanol/water volume ratio on zein structural behavior after EISA in 30-95% ethanol-water with zein concentration range from 0.25 to 150 mg zein/ml in ethanol-water. They constructed a phase diagram that described the structural changes from microspheres to bicontinuous and lamellar film with increased zein.
concentration. As reviewed by Shukla and Cheryan (2001) and Lawton (2002), zein is able to form tough, glossy, hydrophobic and greaseproof coatings that are resistant to microbial attack with excellent flexibility and compressibility. However, these characteristics subject to change with humidity. Wang and Padua (2004) proposed a method to produce zein film with oleic acid as plasticizer to improve water vapor barrier property. They concluded that plasticizer plays an important role on determining water absorption characteristic of zein. Besides, several studies have shown sphere-forming ability of zein which offers zein a possibility to use as a good wall material for encapsulation (Wang, Yin et al. 2008; Padua and Wang 2009; Nonthanum, Lee et al. 2012; Wang and Padua 2012).

In another respect, Wang, Fujimoto et al. (1991) studied antioxidant property of zein by mixed zein with methyl linoleate and stored at 60°C in dark. They found that zein has antioxidant property to prevent oxidation of methyl linoleate under high water activity conditions by binding methyl linoleate. Nonthanum, Lee et al. (2012) studied the effect of different types of zein on rheology characteristic and it was found that the presence of γ-zein helped on providing better gelation and networking property than α-zein only.

2.3 Encapsulation methods
Methods that are utilized to encapsulate are presented as followed:

*Spray drying:*

Spray drying is the most widely utilized technique for producing encapsulated products. The main processing procedures are simplified as followed (Zuidam and Heinrich 2010):

1. Dispersion of wall material in an aqueous phase
2. Dispersion of core material into above wall material aqueous phase
3. Homogenization and emulsion of the mixture
4. Atomization of the mixture and spraying into a hot chamber
5. Dehydration and forming of encapsulated particles

The final products perform as dry powders. Based on the polarity or volatility of core compounds, particle size and retention ability could be varied. Generally, the less volatile
compounds, of larger molecular weight, the larger particle size product will generate. The more hydrophobic compounds tend to obtain better retention upon spray-drying (Zuidam and Heinrich 2010).

Advantages of spray drying are low cost, availability of equipment, small particle size and good protection. However, limitation includes not suitable for heat sensitive compounds, shorter shelf life and limited number of shell materials (Brannonpeppas 1993; Barbosa-Cánovas, Ortega-Rivas et al. 2005; Desai and Park 2005).

*Coacervation:*

Coacervation is also called phase separation methods. It involved three different immiscible phases in the system. They are liquid manufacturing vehicle phase, core material phase and coating material phase. The main principle is that the coating material phase will well surround core material phase within the vehicle phase to form the liquid capsules. Lastly, the liquid capsules will be solidified and separated from vehicle phase and forming dry products (Shahidi and Han 1993). The whole process includes three main steps (Barbosa-Cánovas, Ortega-Rivas et al. 2005):

1. Formation of the three immiscible phases while mixing under controlled conditions.
2. Deposition of the coating material around the core material and forming liquid capsules.
3. Shrinkage and solidification of the liquid capsules to form the solid microcapsules.

Some advantages of coacervation methods are good core and shell life and the high loading rate. Nevertheless, shortcomings include limited food grade wall materials, large particle size (20µm-2mm) and the materials are required to be immiscible with each other (Brannonpeppas 1993).

*Freeze drying:*

Freezing drying is also called lyophilization. It is a good method to encapsulate natural and heat sensitive compounds especially for the volatile compounds. Since the whole process is under low temperature, the product usually contains relatively high compound loading (Barbosa-Cánovas, Ortega-Rivas et al. 2005). The main process steps include (Desai and Park
2005):
1. Mixing of core material in a coating material solution
2. Freeze-drying of the mixture

In the freeze-drying process, the product is first frozen and following by the steps of sublimation of ice and removal of water vapor under vacuum or very low pressure state (Barbosa-Cánovas, Ortega-Rivas et al. 2005).

The advantages of freeze drying include simple and mild process, good protections of heat sensitive compounds and high loading rate. However, the long processing time and high cost are still the big concerns (Shahidi and Han 1993).

**Evaporation induced self-assembly (EISA):**

Evaporation induced self-assembly can be described as that wall materials, dispersed in a binary solvent, start to aggregate during solvent evaporation. The materials that could perform EISA are normally amphiphilic. Upon solvent evaporation, polarity in the system becomes more hydrophilic that triggers self-assembly of amphiphilic materials by hydrophobic attraction. Amphiphilic materials could form spherical micelles that incorporate hydrophobic compounds within its structure by attaching them with their hydrophobic parts and maintain hydrophilic parts in contact with water phase. After water evaporation, a dry powder encapsulated particles could be produced. Other than hydrophobic attraction, the forces including hydrogen bonding, Van der Waals forces and electrostatic forces also involve in stabilizing structure after EISA (Brinker, Lu et al. 1999; Brinker 2004). However, the microstructures obtaining through EISA are various from spheres to film depending on the concentration of solutes, the types of solvent, and volumetric ratio of solvent (Govor, Parisi et al. 2009; Wang and Padua 2010). The main processes of EISA include:
1. Dispersion of wall and core materials in a binary solvent
2. Performing self-assembly upon solvent evaporation.
3. Water evaporation and produce dry powder product
2.4 Morphology of encapsulated products
Various structures of encapsulated products can be produced based on interaction between wall materials and core materials and the processing methods as shown in Figure 2.1. As reviewed by Barbosa-Cánovas, Ortega-Rivas et al. (2005), such structures include: single core with regular or irregular shapes, multiple cores, matrix and multi-walled structure. Single core structure is produced by mechanism that core material is well surrounded by wall material layer or membrane. Matrix structure does not necessarily perform a clear boundary between core and wall materials. Instead of that, core materials are locked into wall material molecule which provides similar effect as a continuous capsule wall. Multiple cores structure can be produced when several core material particles are incorporated into a same particle surrounding by wall material. In some cases, core materials could also be coated by several layers of wall materials for storage or handling purposes (Edris and Bergnstahl 2001).

**SEM (Scanning electron microscope):**
The overview of SEM application was introduced by Wang and Petrova (2012). Scanning electron microscope (SEM) was widely utilized to investigate and analysis surface morphology of micro and nano-particles in food science area. Image formation principle of SEM is similar to standard microscope that light reflect by sample objectives and detect by human eyes. However, light source of SEM is replaced by short wavelengths of electrons. It is focused by electromagnetic lenses and reflection is detected by instrument. Higher resolution of image could be performed by utilizing electrons as a light source comparing to visible light because of its short wavelengths. During investigation, electron beam will strike and penetrate sample surface in certain depth depending on energy of beam and compositions of sample. Sample atoms will be energized upon penetrations. Several signals are generated rely on depth of penetration. These signals include Auger electron, Secondary electrons (SEs), Backscattered electrons (BSEs), Characteristic X-rays and Continuum X-ray. Normally, the signals come from near-surface region such as SEs is collected to form an image with better resolution in order to investigate the surface morphology. The other signals such as X-ray and Auger electron spectra might be recorded for the purpose of determining sample composition. Before SEM examination, non-conductive sample is gold coated to improve conduction and electrons penetration. In food industries, SEM has been applied on characterizing
microstructure of food materials such as zein and encapsulation particles. For instance, the morphology of ethyl cellulose encapsulated bayberry polyphenol and zein encapsulated Vitamin D particles were characterized by SEM in Zheng, Ding et al. (2011) and Luo, Teng et al. (2012)’s research. However, limitation of SEM includes sample damage caused by collision of electron beam and bad image performance by low conductive materials.

**TEM (Transmission electron microscope):**

TEM supplies good mapping and structural analysis functions for specimen with thin layers. Compare to SEM, the electron beam in TEM can transit through specimen with or without changing direction and losing the energy. During examination, electron beam will focus and scan on interested area of sample to produce diffracted and scattered electrons. These electrons will be selected and collected by the detector behind specimen for image formation. TEM has been utilized to determine locations of target core materials within encapsulation particle. For example, Qi, Sun et al. (2012) utilized TEM to investigate internal structure of chitosan encapsulated heparin capsules. Based on the TEM images showed in the research, the encapsulated heparin and the wall material, chitosan, presented a good core and shell structure image which provided evidence of encapsulation. However, limitation of TEM includes first, sample damage by high energy electron beams and second, difficulty to discern overlap sample, third, limited study area that small study area might not be fully representative to whole sample and lastly, high vacuum environment might not truly reflect sample’s original morphology (Lei 2012).

### 2.5 Release mechanisms

Several releasing mechanisms have been developed in order to properly release encapsulated core materials at specific time at certain rate or location. Releasing mechanisms could be controlled by pH, temperature, enzymes and moisture content determined by characteristics of wall materials (Desai and Park 2005; Madene, Jacquot et al. 2006). These release mechanisms include:

**Fracturation:**

Core materials can be released by breaking down of wall materials. Release can be achieved
by external forces including pressure, shearing force and ultrasonic or by adjusting temperature or pH. An example in food system is to release encapsulated sweeteners in chewing gum by mastication. Some other methods such as: increasing temperature to melt wax or oil base wall materials, increasing moisture content to break water absorbed wall materials, adjusting pH to breaking pH sensitive wall materials and dissolving wall materials by its solvent (Brannonpeppas 1993; Shahidi and Han 1993; Madene, Jacquot et al. 2006).

**Diffusion:**
Some of wall materials can serve as a semi-permeable membrane for conducting diffusion. Diffusion process is determined by permeability and solubility of core materials in wall materials system. It is noticeable that diffusion mechanism would not be achieved if core material is not soluble in wall material. The driving force of diffusion include concentration gradient or vapor pressure of volatile substance between each side of capsule (Shahidi and Han 1993; Madene, Jacquot et al. 2006). Main diffusion release procedures include, first, diffusion of core material to wall material, second, partition of core material in wall material following by the release of core materials from surface of wall materials to exterior environment (Madene, Jacquot et al. 2006).

**Degradation:**
Release of core materials by degradation can be accomplished by certain biodegradable wall materials and enzymes. Degradation is performed by slow erosion with homogeneous and heterogeneous types. Heterogeneous erosion occurs in capsule with a thin layer, whereas homogenous erosion presents when a capsule is under a homogeneous environment with a constant releasing rate throughout polymer matrix (Pothakamury and Barbosa-Cánovas 1995). For example, lipid base wall materials could be degraded by lipase (Yazawa 1974). Zein encapsulated essential oil particles were found having a fast release while presence of microbial enzyme (Parris, Cooke et al. 2005).
2.6 Tables and figures

Table 2.1 Wall materials for encapsulation used in industry*

<table>
<thead>
<tr>
<th>Types</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipids</td>
<td>Beeswax, candelilla and carnauba waxes, paraffin, tristearic acid,</td>
</tr>
<tr>
<td></td>
<td>diglycerides, mono glycerides, wax emulsions, glycerol distearate</td>
</tr>
<tr>
<td></td>
<td>and natural or modified fats</td>
</tr>
<tr>
<td>Proteins</td>
<td>Gelatins, soy proteins, whey proteins, zein, casein, albumin,</td>
</tr>
<tr>
<td></td>
<td>hemoglobin, peptides and gluten</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>Starches, maltodextrins, sucrose, glucose, lactose, dextran, corn</td>
</tr>
<tr>
<td></td>
<td>syrup and sucrose cyclodextrins</td>
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<tr>
<td>Cellulose</td>
<td>Carboxy methylcellulose, methylcellulose, ethylcellulose, nitrocellulose,</td>
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<td>acetylcellulose, cellulose acetate-phthalate and cellulose acetate-</td>
</tr>
<tr>
<td></td>
<td>butylate-phthalate</td>
</tr>
<tr>
<td>Gums</td>
<td>Gum acacia, agar, sodium alginate and carrageenan</td>
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<tr>
<td>Food grade polymers</td>
<td>Polypropylene, polyvinylacetate, polystyrene and polybutadiene</td>
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</table>

* Adapted from Shahidi and Han (1993) and Lakkis (2007).
Table 2.2 Amino acid composition of the predominant zein storage proteins (in mol %)*

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<tr>
<th></th>
<th>19-kDa α-zein</th>
<th>22-kDa α-zein</th>
<th>27-kDa γ-zein</th>
<th>16-kDa γ-zein</th>
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<td>9.3</td>
<td>8.5</td>
<td>13.3</td>
</tr>
<tr>
<td>Tyr</td>
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</tr>
<tr>
<td>Phe</td>
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<td>3.3</td>
<td>1.0</td>
<td>4.3</td>
<td>4.0</td>
</tr>
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<td>0.0</td>
<td>0.0</td>
<td>0.7</td>
</tr>
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<td>1.8</td>
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<td>0.0</td>
<td>0.6</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Holding and Larkins (2009).

Figure 2.1 Structures of microcapsule
CHAPTER III

EFFECT OF CORE HYDROPHOBICITY ON ZEIN ENCAPSULATION EFFECTIVENESS

3.1 Introduction
Core-shell encapsulation of liquid flavors in zein by evaporation induced self-assembly (EISA) depends initially on the formation of flavor droplets in the solvent system. Droplet forming ability of flavor compounds in ethanol-water relies on its hydrophobic characteristics.

Contact angle is the tangent angle between a liquid droplet and a solid surface (Figure 3.1). It depends on the surface properties of both liquid and solid surface (Kwok and Neumann 1999). It is used as an index to characterize surface properties of relevant samples. For example, if oil and water droplets are placed on a hydrophilic surface, the contact angle of oil will be larger than that of water, indicating a hydrophobic behavior.

Zein has been used to encapsulate essential oils, bioactive compounds and flavor oils by spray drying, freeze drying and coacervation methods, as described in Chapter 2. In this work, zein was utilized to encapsulate flavor compounds by evaporation induced self-assembly (EISA) in ethanol-water. EISA is an efficient structure forming method to produce well defined microstructures including spheres and films. As described by Brinker (2004), an amphiphilic compound may undergo evaporation induced self-assembly (EISA) in binary solvents driven by changes in polarity during solvent evaporation. The effect of experimental conditions on structure development of zein after EISA was investigated by Wang and Padua (2010). They observed the formation of microspheres, bicontinuous, and lamellar films. Wang and Padua (2010) constructed a phase diagram that described structural changes with increased zein concentration. The formation of microspheres was proposed for encapsulation purposes.

Flavors are often dispersed in hydrophobic carriers to protect them from degradation and facilitate their handling. However, the surface properties of carriers may influence the effectiveness of flavor encapsulation by zein. Neobee 1053 is a medium chain triglyceride
widely utilized as a carrier in the flavor industry. Choi, Decker et al. (2009) reported the use of Neobee 1053 to protect citral from degradation in acid environments.

The objective of this experiment was to investigate the relation between surface properties of flavor compounds, as evaluated by contact angle, with the microstructure of zein encapsulated flavors. A second objective was to investigate the effect of carrier properties on effectiveness of flavor encapsulation by zein.

3.2 Materials and methods

3.2.1 Materials
Triacetin, benzaldehyde, trans-2 hexenal, citral, cis-3 hexen-1-ol were purchased from Sigma Aldrich® (St. Louis, MO, U.S). Carriers, Neobee M5 and Neobee 1053 were from Stepan Company (Maywood, New Jersey). Glass microscope slides (TruView 0110B 76 × 26 mm 1.0 mm thickness) were obtained from Tru Scientific, LLC (Bellingham, WA, U.S). Zein (Amazein®) was obtained from Prairie Gold Inc. (Bloomington, IL). Ethanol (USP) was from Decon Labs, Inc. (King of Prussia, PA).

3.2.2 Contact angle measurement
The contact angle of benzaldehyde, trans-2 hexenal, citral and cis-3 hexen-1-ol, triacetin, Neobee M5, Neobee 1053, and water was measured on a hydrophilic glass microscope slide with a goniometer (CAM 200 goniometer KSV Instruments, Inc., Monroe, CT) equipped with a FireWire camera (resolution: 512 x 480 pixels). Before the measurement, glass microscope slides were etched with a reactive ion etcher (RIE, March Jupiter III) with gas (O₂, Ar and ArH₂) flow for 1 minute to produce hydrophilic surfaces on glass slides. Droplets of water, benzaldehyde, trans-2 hexenal, citral and cis-3 hexen-1-ol, and carriers, triacetin, Neobee M5 and Neobee 1053, were placed on the microscope slides. Images of droplets making contact with the slides were recorded with the camera starting when droplets touched the surface at the rate of 1 image/ second for 10 seconds. Contact angle was determined from the images by measuring the tangent angle between the droplet and the solid surface. Instrument software was used to fit the Young-Laplace equation to the shape of the drop. Measurements were run
in triplicate. Statistical analysis of the differences between each sample was performed by ANOVA with mean separation at $\alpha=0.05$.

### 3.2.3 Encapsulation
Zein encapsulated samples were prepared by evaporation induced self-assembly (EISA) of zein dispersions in ethanol-water. Zein (10 mg) was dispersed in 10 ml of 70 or 90% ethanol followed by sonication with ultrasonic processor (Model: GEX 750, Geneq Inc. Quebec, Canada) with ¼ inch tip (CV33 with) for 2 minutes at 40% amplitude. Flavor/carrier dispersions were prepared by the same method using 10 ml of 70 or 90% ethanol with 10µl of flavors or carriers. Dispersions were then combined after sonication and placed on aluminum dishes (vol. 42 ml, Fisher Scientific, Pittsburgh, PA) to carry out evaporation induced self-assembly. EISA was carried out in a chamber with constant air flow at room temperature (25°C) for 24 hours to fully evaporate the solvent. The final products were collected as dry powders.

To observe the effects of carrier on flavor-zein structure, Neobee 1053 and citral were selected as model system for carrier and flavor. Neobee 1053 (2.5, 6.6, 15 and 40 µl) was added with 10 µl citral to prepare samples of Neobee 1053 to citral volume ratio of 1:4, 2:3, 3:2 and 4:1. Mixtures were continuously stirred for 2 hours at room temperature with a magnetic stirrer. The contact angle of Citral/Neobee 1053 samples were then measured by the method as described above. After that, Citral/Neobee 1053 samples were dispersed in 10 ml of 90% ethanol followed by sonication as described above. Zein dispersions were prepared as above using 10 mg zein in 90% ethanol. Zein and flavor-carrier dispersions were combined after sonication and placed on aluminum dishes for EISA to obtain dry powders.

### 3.2.4 Morphology characterization
*Scanning electron microscope (SEM):*
Zein encapsulated flavor or carrier samples were sputter coated with gold (300 Å) using Emitech K575 sputter coater (Ashford, UK) for 20 seconds to improve electrical conductivity of sample surfaces, allowing image collection and reducing thermal damage. The microstructure of the coated sample was examined and imaged by a JEOL 6060LV General
Purpose SEM (Peabody, MA, U.S) and Hitachi s-4700 high resolution SEM (Schaumburg, IL, U.S) with accelerating voltage between 15-20 kV and 8-12 mm working distances.

Transmission electronic microscope (TEM):
Samples of zein encapsulated flavor or carrier were prepared by placing a carbon-coated copper micro-grid at the bottom of the aluminum dish during EISA. After the process, the dry powder sample was attached to the sample grid. The sample grid was then put on a sample holder and inserted into a JEOL 2100 Cryo TEM (Tokyo, Japan) for examination. The experiment was conducted at 200 kV and TEM images were taken with a video rate camera for real time imaging and a slow scan CCD camera for final images.

3.3 Results and discussion

3.3.1 Contact angle

Contact angle measurement:
The surface properties of flavors and carriers were considered critical to determine the formation of core and shell encapsulation structures. Contact angle was considered a good parameter to describe surface properties of core compounds. Figure 3.2 shows droplets of water, trans-2 hexenal, benzaldehyde, citral, cis-3 hexen-1-ol, triacetin, Neobee M5 and Neobee 1053 in contact with the hydrophilic surface of glass. Contact angle values are shown in Table 3.1. Hydrophilic compounds had lower contact angle values. Contrarily, hydrophobic compounds had higher contact angle values. That is, hydrophobicity increased as follows: water, trans-2 hexenal, benzaldehyde, citral, cis-3 hexen-1-ol, triacetin, Neobee M5 and Neobee 1053.

Microstructure morphology by SEM:
Detection of spherical structures was relevant in this work because sphere structures were considered to have a higher potential for flavor/carrier encapsulation than films. It was believed that compounds with intermediate interfacial tension in solvent will form stable droplets after sonication thus leading to sphere structure by EISA. When interfacial tension between core and solvent was high, droplets formed by sonication tended towards aggregation and phase separation, thus preventing formation of spherical structures with zein.
The morphology of flavors/carriers encapsulated with zein in 70% ethanol was observed by SEM as shown in Figures 3.3 to 3.9. Contact angle was measured for trans-2 hexenal (14.3°) and benzaldehyde (15.1°). Figures 3.3 and 3.4 show spheres of 800-1,600 nm in diameter. The microstructure of encapsulated citral (17.3°) and cis-3 hexen-1-ol (26.5°) (Figures 3.5 and 3.6) shows spheres of 750-2,500 nm in diameter, fusing together. The microstructure of encapsulated triacetin (31.8°) (Figure 3.7) shows a spongy structure. The microstructure of encapsulated Neobee M5 (33.5°) and Neobee 1053 (39°) (Figures 3.8 and 3.9) shows film structures. It was observed that microstructure of zein encapsulated compounds transformed from spheres, fused spheres, sponge to films with increasing contact angle. This trend was attributed to increased interfacial tension between flavors/carriers and solvent which led to phase separation in 70% ethanol.

The morphology of flavor/carriers encapsulated with zein in 90% ethanol is shown in Figures 3.10 to 3.16. The microstructure of encapsulated trans-2 hexenal (14.3°), benzaldehyde (15.1°), citral (17.3°) and cis-3 hexen-1-ol (26.5°) (Figures 3.10, 3.11, 3.12 and 3.13) shows spheres of 800-3,600 nm in diameter. The microstructure of encapsulated triacetin (31.8°) (Figure 3.14) was a sponge. The microstructure of encapsulated Neobee M5 (33.5°) and Neobee 1053 (39.0°) (Figures 3.15 and 3.16) shows film-like structures with embedded spheres. Microstructure of zein encapsulated compounds changed from spheres, to sponge and films with increasing contact angle. This trend was believed to result from decreased interfacial tension leading to core and solvent miscibility in 90% ethanol.

Differences in morphology were observed with respect to ethanol content and contact angle values. Amphiphilic compounds with contact angle values around 15° (trans-2 hexenal and benzaldehyde) easily formed droplets in both 70 and 90% ethanol leading to sphere formation. Amphiphilic compounds with contact angle values between 17 and 27° (citral and cis-3 hexen-1-ol) also formed droplets. However, droplets were unstable in 70% ethanol and tended to coalesce leading the formation of a structure of connected or fused spheres due to comparably higher interfacial tension. Droplets were more stable in 90% ethanol, a relatively hydrophobic solvent, and generated a structure of packed individual spheres due to decreased interfacial tension in more hydrophobic conditions. More hydrophobic compounds with contact angle larger than 30° (Triacetin, Neobee M5 and Neobee 1053) had low interfacial
tension which decreased droplet forming ability in 70 and 90% ethanol, thus generated sponge structures (triacetin) and films (Neobee M5 and Neobee 1053) in 70% ethanol and fused spheres (triacetin) and films with embedded spheres (Neobee M5 and Neobee 1053) in 90% ethanol. Contact angle evaluated compounds ability to form droplets. Therefore, amphiphilic compounds were more likely to form droplets than highly hydrophobic compounds in ethanol-water. It is believed that interfacial tension of more hydrophobic compounds in more hydrophilic environment (70% ethanol) is increased thus leading to unstable droplets or phase separation. Therefore, the compounds with contact angle larger than 15° (citral, cis-3 hexen-1-ol, triacetin, Neobee M5 and 1053) may not easily form stable droplets in 70% ethanol. In 90% ethanol, it provided more hydrophobic environment that decreased interfacial tension of more hydrophobic compounds in solvent thus produce more sphere-like structures.

The octanol-water partition coefficient (Log P) is used to identify hydrophobic/ hydrophilic character of compounds. It is given by the concentration ratio of a compound in the octanol phase to that in the water phase at equilibrium. Compounds with higher Log P are more hydrophobic and compounds with lower Log P are more hydrophilic (Sangster 1997). Log P values of trans-2 hexenal, benzaldehyde, citral, cis-3 hexen-1-ol, triacetin, Neobee M5 and Neobee 1053 are presented in Table 3.2. Log P value may be correlated to contact angle values which reflect the surface behavior of compounds. Thus, it may be related to microstructure of zein encapsulated products. Compounds, including trans-2 hexenal, benzaldehyde, citral and cis-3 hexen-1-ol, with Log P ranging from 1.5 to 3.3, formed sphere structures. Compounds, including Neobee M5 and Neobee 1053, with Log P estimated at higher than 3.3 (Popplewell, Brain et al. 2008) formed films. Triacetin, had a low log P indicating a hydrophilic character but a high contact angle value on hydrophilic surface, suggesting a high cohesiveness and a good droplet forming ability. Comparing Log P value to contact angle value, contact angle value provides better indication for zein encapsulation microstructure.

**Microstructure morphology by TEM:**

Core and shell structure of sphere forming samples formed by 90% ethanol were characterized by TEM. Citral was found to have core and shell structure as shown in Figure 3.17. Zein, has higher density, was shown in a darker shade that formed outer membrane of sphere. Citral, has
a lower density, was shown in a light shade that formed core within sphere. Both single-core and multiple-core spheres were observed zein encapsulated citral as shown in Figure 3.17A and Figure 3.17B, respectively. The average particle size is around 2000 nm with a core around 1200nm in diameter for single-core sphere. The particle size is around 2300 nm with core size around 800nm in diameter for multiple-cores sphere. Therefore, it is believed that citral was encapsulated with zein. However, the encapsulation of other sphere-forming flavors (trans-2 hexenal, benzaldehyde and cis-3 hexen-1-ol) cannot be confirmed because they all showed solid sphere by TEM as shown in Figure 3.18, 3.19 and 3.20, respectively. In the future, in order to encapsulate trans-2 hexenal, benzaldehyde and cis-3 hexen-1-ol, the mass ratio of zein to flavors and ethanol content in the solvent may be modified.

3.3.2 Effect of carrier on microstructure of encapsulated citral

Contact angle values and microstructure observations of zein encapsulated citral/Neobee 1053 mixtures after EISA in 90% ethanol are shown in Table 3.3 and Figure 3.21. Contact angle increased with the content of Neobee 1053 in the mixtures. Citral/Neobee1053 with volume ratio of 4:1 (Figure 3.21 D, contact angle: 16.6°) formed sphere structures while Citral/Neobee1053 at 1:4 (Figure 3.21A, contact angle: 34.9°) showed continuous film-like structures. At intermediate Citral/Neobee1053 ratio, 3:2 (Figure 3.21 C, contact angle: 22.5°) and 2:3 (Figure 3.21 B, contact angle: 24.4°), structure changed from fused spheres to film-like but with embedded spheres. The internal structure of the only sphere forming sample, citral/ Neobee 1053:: 4:1, was examined by TEM (Figure 3.22). The image shows a solid sphere which was not useful to confirm citral encapsulation.

Based on previous results, the presence of Neobee 1053 affected citral encapsulation with zein. It led to film-like structures and interfered with the formation of core and shell. Zein encapsulated microstructures were transformed from film-like to distinct spheres with decreasing amount of Neobee 1053 in the mixtures. As discussed above, the droplet forming ability of hydrophobic compounds such as Neobee 1053 in 90% ethanol might be low due to their decreased interfacial tension in the solvent. The droplet forming ability of citral in ethanol-water may have decreased with the presence of Neobee 1053 resulting from the decreased interfacial tension of mixture droplets in 90% ethanol thus interfering with zein encapsulation.
3.4 Conclusions
The droplet forming ability of core compounds is determined by its interfacial tension in ethanol-water. Droplet formation controls microstructure in zein encapsulation. Amphiphilic compounds with contact angle between 14° and 30° on hydrophilic surfaces were considered to have intermediate interfacial tension leading to stable droplet forming ability in 90% ethanol. EISA of zein and such amphiphiles resulted in the formation of spheres. With decreased hydrophobicity in solvent from 90 to 70% ethanol, the amphiphilic compounds with comparably higher hydrophobicity (contact angle between 17° and 27°) transformed from spheres to connected and fused spheres because of increased interfacial tension that lowers droplet stability. More hydrophobic compounds did not form droplets in 70 or 90% ethanol. They formed fused spheres, spongy structures and films due to low interfacial tension. Citral was encapsulated with zein in 90% ethanol. However, the droplet forming ability of citral in 90% ethanol was decreased with added hydrophobic compounds (Neobee 1053), which interfered with encapsulation. The next chapter will focus on the separation of citral from Neobee 1053 to minimize the negative effects of Neobee 1053 on citral-zein encapsulation.
3.5 Tables and figures

Figure 3.1 Image of water droplet forming on zein film surface, \( \theta = 65^\circ \).

Figure 3.2 Images of A) water, B) trans-2 hexenal, C) benzaldehyde, D) citral, E) cis-3 hexen-1-ol, F) triacetin, G) Neobee M5 (>98%) and H) Neobee 1053 (100%) droplets in contact with a hydrophilic surface.
Table 3.1 Contact angle of flavors/carriers on hydrophilic surfaces and morphology of encapsulation microstructures.

<table>
<thead>
<tr>
<th>Flavors/Carriers</th>
<th>Contact Angle *</th>
<th>Microstructure morphology</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>70% ethanol</td>
</tr>
<tr>
<td>Water</td>
<td>5.9±0.6 E</td>
<td>-</td>
</tr>
<tr>
<td>Trans-2-hexenal</td>
<td>14.3±0.7 13</td>
<td>Spheres</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>15.1±1.1 13</td>
<td>Spheres</td>
</tr>
<tr>
<td>Citral</td>
<td>17.3±0.2 17</td>
<td>Fused spheres</td>
</tr>
<tr>
<td>Cis-3 hexen-1-ol</td>
<td>26.5±2.3 C</td>
<td>Fused spheres</td>
</tr>
<tr>
<td>Triacetin</td>
<td>31.8±3.8 4</td>
<td>Spongy</td>
</tr>
<tr>
<td>Neobee M5 (&gt;98%)</td>
<td>33.5±1.7 4</td>
<td>Films</td>
</tr>
<tr>
<td>Neobee 1053 (100%)</td>
<td>39.0±4.4 A</td>
<td>Films</td>
</tr>
</tbody>
</table>

*Superscripts indicate significance at α=0.05.

Table 3.2. Log P and contact angle values of selected flavors/carriers.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Log P</th>
<th>Contact angle values</th>
<th>Microstructure morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trans-2-hexenal</td>
<td>1.7 1</td>
<td>14.3±0.7</td>
<td>Spheres</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>1.5 2</td>
<td>15.1±1.1</td>
<td>Spheres</td>
</tr>
<tr>
<td>Citral</td>
<td>3.3 3</td>
<td>17.3±0.2</td>
<td>Spheres</td>
</tr>
<tr>
<td>Cis-3 hexen-1-ol</td>
<td>1.6 4</td>
<td>26.5±2.3</td>
<td>Spheres</td>
</tr>
<tr>
<td>Neobee M5 (&gt;98%)</td>
<td>&gt;3.3 5</td>
<td>33.5±1.7</td>
<td>Films</td>
</tr>
<tr>
<td>Neobee 1053</td>
<td>&gt;3.3 5</td>
<td>39.0±4.4</td>
<td>Films</td>
</tr>
<tr>
<td>Triacetin</td>
<td>0.3 6</td>
<td>31.8±3.8</td>
<td>Sponges</td>
</tr>
</tbody>
</table>

1 Seuvre, Philippe et al. (2006), 2 Material safety data sheet (benzaldehyde purum, ≥98.0% (GC), Sigma-Aldrich), 3 Benigni, Andreoli et al. (1996), 4 Relkin, Fabre et al. (2004), 5 Popplewell, Brain et al. (2008), 6 Ivanova, Lindman et al. (2000)
Table 3.3 Contact angle of citral/ Neobee 1053 mixtures on hydrophilic surfaces and morphology of encapsulation microstructure.

<table>
<thead>
<tr>
<th>Citral/1053</th>
<th>Contact Angle *</th>
<th>Microstructure</th>
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<tbody>
<tr>
<td>1/4</td>
<td>34.9±1.3</td>
<td>Films</td>
</tr>
<tr>
<td>2/3</td>
<td>24.4±1.8</td>
<td>Films w/embedded spheres</td>
</tr>
<tr>
<td>3/2</td>
<td>22.5±0.5</td>
<td>Fused spheres</td>
</tr>
<tr>
<td>4/1</td>
<td>16.6±0.1</td>
<td>Spheres</td>
</tr>
<tr>
<td>5/0</td>
<td>17.3±0.2</td>
<td>Spheres</td>
</tr>
</tbody>
</table>

*Superscripts indicate significance at α=0.05.
Figure 3.3 SEM images showing morphology of zein encapsulated trans-2 hexenal after EISA in 70% ethanol.

Figure 3.4 SEM images showing morphology of zein encapsulated benzaldehyde after EISA in 70% ethanol.

Figure 3.5 SEM images showing morphology of zein encapsulated citral after EISA in 70% ethanol.
Figure 3.6 SEM images showing morphology of zein encapsulated cis-3-hexen-1-ol after EISA in 70% ethanol.

Figure 3.7 SEM images showing morphology of zein encapsulated triacetin after EISA in 70% ethanol.

Figure 3.8 SEM images showing morphology of zein encapsulated Neobee M5 after EISA in 70% ethanol.
Figure 3.9 SEM images showing morphology of zein encapsulated Neobee 1053 after EISA in 70% ethanol.

Figure 3.10 SEM images showing morphology of zein encapsulated trans-2 hexenal after EISA in 90% ethanol.

Figure 3.11 SEM images showing morphology of zein encapsulated benzaldehyde after EISA in 90% ethanol.
Figure 3.12 SEM images showing morphology of zein encapsulated citral after EISA in 90% ethanol.

Figure 3.13 SEM images showing morphology of zein encapsulated cis-3-hexen-1-ol after EISA in 90% ethanol.

Figure 3.14 SEM images showing morphology of zein encapsulated triacetin after EISA in 90% ethanol.
Figure 3.15 SEM images showing morphology of zein encapsulated Neobee M5 after EISA in 90% ethanol.

Figure 3.16 SEM images showing morphology of zein encapsulated Neobee 1053 after EISA in 90% ethanol.
Figure 3.17 TEM images showing internal structure of zein encapsulated citral after EISA in 90% ethanol. A) zein encapsulated citral with single core and B) zein encapsulated citral with multiple cores.

Figure 3.18 TEM images showing internal structure of zein encapsulated cis-3-hexen-1-ol after EISA in 90% ethanol.
Figure 3.19 TEM images showing internal structure of zein encapsulated trans-2 hexenal after EISA in 90% ethanol.

Figure 3.20 TEM images showing internal structure of zein encapsulated cis-3-hexen-1-ol after EISA in 90% ethanol.
Figure 3.21 SEM images showing morphology of zein encapsulated citral/Neobee 1053 mixture after EISA in 90% ethanol. A) citra/1053 at the ratio of 1:4, B) citra/1053 at the ratio of 2:3, C) citra/1053 at the ratio of 3:2 and D) citra/1053 at the ratio of 4:1.

Figure 3.22 TEM images showing internal structure of zein encapsulated citral/Neobee 1053 mixture at the ratio of 4:1 with zein after EISA in 90% ethanol.
CHAPTER IV

FLAVOR RECOVERY TO MINIMIZE EFFECT OF CARRIER ON FLAVOR ENCAPSULATION BY ZEIN

4.1 Introduction

Citral is one of the main volatile components from citrus oil or lemon grass oil which provides natural lime note and which is widely utilized as an index to evaluate the quality of citrus or lemon grass oil. Citral is a mono-terpene aldehyde naturally present as two geometrical isomers, neral (cis-citral) and geranial (trans-citral) (Shaw 1979; Bhat, Nagasampagi et al. 2005). Several studies have confirmed that citral is unstable and readily decomposes in the presence of oxygen and light, such degradation occurs more rapidly at low pH. Under these unfavorable conditions, citral will undergo a series of cyclization and oxidation to produce off-odor compounds including p-cresol, p-methylacetophenone, and p-cymene, which generate phenolic, bitter almond-like and gasoline-like odors (Schieberle and Grosch 1988; Ueno, Masuda et al. 2004; Choi, Decker et al. 2010).

To stabilize citral, Choi et al (2009 &2010) proposed the addition of surfactants, such as Tween 80, polyglycerol polyricinoleate (PGPR), medium chain triglycerides (MCT, Neobee 1053) and triacetin, to prevent citral degradation. They proposed that surfactants would surround and encapsulate citral droplets. Yang, Tian et al. (2011) reported that antioxidants improved the stability of citral in acidic aqueous solutions.

Results from Chapter 3, indicate that pure flavor compounds can be encapsulated by zein. However, in industry, flavors are often mixed with carriers, which may interfere with encapsulation. In this chapter, a model flavor-carrier system consisting of citral and Neobee 1053 will be used to develop a strategy for effective flavor encapsulation from flavor-carrier mixtures. Neobee 1053, a medium-chain triglyceride, contains caprylic and capric acids at a ratio of 56 to 44. It is miscible with citral and was proposed as an emulsifier to protect citral from chemical degradation by Choi, Decker et al. (2009). The proposed strategy consists of
separating citral from Neobee mixtures by extraction with ethanol-water, followed by encapsulation with zein.

Ethanol is a good solvent for a number of chemicals and it is widely utilized to extract flavors, nutrients, essential oils and antibacterial components from several natural substrates. Ethanol was used to extract vanilla flavors from vanilla beans (Cicchetti and Chaintreau 2009; Cicchetti and Chaintreau 2009). Sulong (2006) reported the extraction of essential oils from jasmine flowers with ethanol-water. Different combinations of ethanol-water have been utilized as extraction solvents to recover nutraceuticals, including α-tocopherol, saponins and naringin from okra seed, ginseng and citrus peel, respectively (Kwon, Belanger et al. 2003; Andras, Simándi et al. 2005; Khan, Abert-Vian et al. 2010). Ethanol is also useful to extract citral from lime essential oil and lime peel (Chienthavorn and Insuan 2004; Dupuy, Athes et al. 2011).

4.2 Materials and methods

4.2.1 Materials

Citral (natural, ≥95%, FCC) was obtained from Sigma Aldrich® (St. Louis, MO, U.S). Neobee 1053 was from Stepan Company (Maywood, New Jersey). Ethanol (USP) was purchased from Decon Labs, Inc. (King of Prussia, PA). Diethyl ether (Anhydrous) was received from Fisher Scientific (Fair Lawn, New Jersey). Zein (Amazein®) was obtained from Prairie Gold Inc. (Bloomington, IL).

4.2.2 Sample preparation

Preparation of model System:

A mixture of citral (100 µl, equivalent to 88 mg) and Neobee 1053 MCT (900 µl, equivalent to 854.1 mg) was prepared by continuous stirring for 2 hours at room temperature with a magnetic stirrer.
Citral recovery:
Citral was recovered from the model system by liquid–liquid extraction with 60%, 70% and 80% ethanol-water. Ethanol-water (10 ml) was added to the citral-Neobee 1053 mix (1 ml) and continuously stirred for 2 hours to form a cloudy dispersion. Dispersions were stored at three different temperatures, 25, 5 and -15°C for 48 hours to allow for phase separation. For the samples stored at 25 and 5°C, Neobee 1053 showed a phase separation from ethanol-water phase. The upper clear ethanol phase was carefully separated from the bottom MCT phase for further citral quantification. In the samples stored at -15°C, the bottom MCT phase became frozen. The upper clear ethanol phase was separated by filtration at -15°C for further citral quantification. Three replicates were run for each temperature at each ethanol-water solvent.

4.2.3 Citral quantification
Gas chromatography-Flame Ionization Detector (GC-FID):
A GC-FID system equipped with 15 m × 0.32 mm (id) × 0.5 µm film thickness capillary column (RTX-5, Bellefonte, PA) and hot split injector was used in this experiment. The helium gas flow rate was kept constant with a split ratio at 10:1 and 1.5 ml/min with injector maintained at 250°C. The oven temperature program was set for initial 130°C to final 180°C at a rate of 10°C/min with final holding time for 2 minutes. Citral was detected by FID at 250°C.

Standard curve:
An external standard was used to determine citral concentration in the ethanol phase. To construct an external standard correlation curve, 11.2 mg citral were placed in a 10 ml volumetric flask and diluted with diethyl ether to 10 ml to form a standard citral solution of 1120 ppm. The standard was further diluted with diethyl ether to form series solutions with citral concentration of 560 ppm, 280 ppm and 140 ppm. 1 µl of each diluted sample was injected into GC-FID. The resulting peak area was measured in every case. Citral is a mixture of geranial and neral, therefore it showed two peaks in the GC-FID spectrogram (Figure 4.1). The calculated peak area represented the summation of those two peaks (geranial and neral) as calculated by the program Chem Station (Rev. B. 01.03 [204], Agilent Technologies, 2001-2005). Three replicates were used to calculate average peak area. Citral concentration was plotted against average peak area and the curve was fit by linear regression (MS-Office).
**Citral quantification and recovery efficiency:**

Ethanol-water extracts were diluted with diethyl ether (1 ml extract and 9 ml diethyl ether). 1 µl of ether diluted extract was injected into GC-FID at the same conditions as described in the previous section to obtain the peak area. Samples were injected three times. The citral content was calculated from the standard curve. The recovery efficiency was obtained by the equation below. Data was statistically analyzed by ANOVA (SAS, SAS Institute Inc., Cary, NC) with mean separation at 95% confidence.

\[
\text{Recovery Efficiency}(\%) = \frac{\text{Citral content in ethanol extract (ppm)}}{\text{Citral content in model system (ppm)}} \times 100\%
\]

**4.2.4 Encapsulation of citral recovered from citral/Neobee 1053 mixtures**

**Sample preparation:**

Zein (10 mg) was dispersed in 10 ml 90% ethanol-water followed by sonication, as described in Chapter 3. Citral extracts (2 ml) from each 60, 70 and 80% ethanol were diluted with 12 to 17 ml of 95%, 93% and 91% ethanol, respectively as shown in Table 4.1 to standardize citral content of samples to 1 µl citral/ml 90% ethanol. Citral extracts were sonicated and combined with above zein dispersion. Combined liquid mixtures were placed under a hood to allow for EISA and produce flavor encapsulated dry powders.

**Sample morphology:**

The morphology of encapsulated samples was examined by SEM and TEM. Sample preparation methods and equipment were described in Chapter 3.

**4.3 Results and discussion**

**4.3.1 Citral quantification and recovery efficiency**

**Standard curve:**

The standard curve for citral concentration is shown in Figure 4.2. It shows a liner relation between citral concentration and GC-FID peak area. The equation representing the best fit is \( y = 4.2309x - 52.811 \) with \( R^2 \) of 0.9933, where \( y \) stands for citral concentration (ppm) and \( x \) stands for cumulative peak area.

*Citral recovery efficiency from citral/Neobee 1053 mixture:*
Citral was extracted from the citral/Neobee 1053 mixture with 60%, 70% and 80% ethanol in water at room temperature and the ethanol extracts were phase separated at 25°C, 5°C and -15°C. Citral was effectively extracted at all three ethanol content levels. Table 4.2 summarizes the citral recovery efficiency of 60% 70% and 80% ethanol phase separated at 25°C, 5°C and -15°C. The effect of ethanol content on recovery efficiency was significant at 5 and 25°C, with lower efficiency at 60% ethanol (68% vs. 82-83%). No significant differences due to ethanol content were found at -15°C. The effect of temperature was significant at all ethanol levels. Significantly higher recovery efficiency was observed at -15°C for 60% ethanol (91 vs. 68%), 70% ethanol (96 vs. 78-82%) and 80% ethanol (94 vs. 83-84%). No significant differences due to temperature were observed at 25 and 5°C.

The improvement of citral extraction efficiency at -15°C was attributed to the crystallization of Neobee 1053 which has a melting point of -5°C (Stepan Company, NEOBEE® Brochure, 2008). Crystallization of octadecane was reported to increase the partition of citral into aqueous phases from due to the formation of pure lipid crystals (Mei, Choi et al. 2009). Compounds dispersed in a lipid phase are expelled during lipid crystallization as reported by Ghosh, Peterson et al. (2006) and Müller, Mäder et al. (2000). In the lab, citral remained liquid at -15°C, but Neobee 1053 was crystallized. It was suggested that crystallization of Neobee 1053 improved the partition of citral into the ethanol-water phase. Also, crystallization of Neobee 1053 helped in the separation of solid MCT from the liquid ethanol phase thus minimizing its negative effects on sphere formations.

4.3.2 Morphology of zein encapsulated citral

SEM:
The morphology of recovered citral encapsulated by zein was examined by SEM. Images are shown in Figures 4.3 to 4.11. The effect of ethanol content when phase separation was performed at 25°C is shown in Figures 4.3, 4.4 and 4.5. All samples showed a film-like structure, which was related to the presence of hydrophobic material (see Chapter 3). At low ethanol content (60%), spheres of 700 to 1,250 nm in diameter were present, which suggested a lower content of hydrophobic material was extracted with 60% ethanol. When ethanol phase separation was performed at 5°C, the structure corresponding to 60 and 70% ethanol
extraction, Figures 4.6, 4.7, show the formation of spheres of 500 to 1200 nm in diameter. The structure corresponding to 80% ethanol extraction, Figure 4.8, showed a film-like structure suggesting more triglycerides were extracted with 80% ethanol. At -15°C, samples for all three ethanol content levels showed sphere formation with 200 to 1300 nm in diameter, Figures 4.9, 4.10 and 4.11, suggesting a good separation of triglyceride.

The structure of encapsulated recovered citral showed an increased formation of spheres as ethanol content of the solvent decreased from 80% to 60% and separation temperature decreased from 25 to -15°C. A lower content of triglycerides in the recovered citral is believed due to lower hydrophobicity of 60% ethanol that decreased solubility of triglycerides. Therefore, it is presumed that there was more amount of Neobee 1053 in 80% than 70% than 60% ethanol. Moreover, a lower separation temperature led to a better separation of the lipid phase from ethanol phase. At -15°C, crystallization of Neobee 1053 further facilitated its separation thus minimizing its effect on citral-zein encapsulation.

**TEM:**
TEM was utilized to probe the internal structure of sphere-forming samples. Figures 4.12, 4.13 and 4.14 show encapsulated structures when citral was extracted with 60% ethanol and separated at 25, 5 and -15°C, respectively. Figures 4.15 and 4.16 show encapsulation structures extracted with 70 and 80% ethanol and separated at -15°C. Core and shell structures were observed in all samples. The sample extracted with 60% ethanol and separated at -15°C showed the highest number of core-shell structures suggesting higher encapsulation efficiency. However, further research is needed to investigate citral encapsulation efficiency.

**4.4 Conclusions**
Citral was recovered from a model citral-carrier (Neobee 1053) mixture by extracting it with 60, 70, 80% ethanol-water and separating the extract from the carrier phase at 25, 5 and -15°C. The highest recovery efficiency was observed at -15°C due to crystallization of triglycerides. The morphology of zein encapsulated citral was characterized by SEM. Ethanol content in the extraction solvent and separation temperature affected the formation of encapsulation spheres.
Decreasing ethanol content and decreasing separation temperature promoted the formation of spheres due to better separation of triglycerides from ethanol phase. The core-shell structure of spheres was characterized by TEM. Core and shell structures were observed on all sphere forming samples. The citral-zein structure formed by 60% ethanol extract separated at -15°C showed the highest number of core and shell structures. Based on these results, ethanol-water is a useful solvent to extract flavor components from oil phases. Extract separation is facilitated at low temperature, below the freezing point of the lipid phase.

4.5 Tables and figures

Figure 4.1 Typical citral GC-FID spectrogram: 1120 ppm.
Figure 4.2 GC-FID citral standard curve.

Table 4.1 Standardization of citral/ethanol ratio before zein encapsulation.

<table>
<thead>
<tr>
<th>Separation temperature (°C)</th>
<th>Ethanol content of extraction solvent *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60%</td>
</tr>
<tr>
<td>25</td>
<td>12 ml 95% ethanol</td>
</tr>
<tr>
<td>5</td>
<td>12 ml 95% ethanol</td>
</tr>
<tr>
<td>-15</td>
<td>16 ml 95% ethanol</td>
</tr>
</tbody>
</table>
Table 4.2 Effects of ethanol content of extraction solvent and separation temperature on citral recovery efficiency (%).

<table>
<thead>
<tr>
<th>Separation temperature (°C)</th>
<th>Ethanol content of extraction solvent *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60%</td>
</tr>
<tr>
<td>25</td>
<td>68.0±3.9% (^{B,B})</td>
</tr>
<tr>
<td>5</td>
<td>68.6±5.1% (^{B,B})</td>
</tr>
<tr>
<td>-15</td>
<td>91.3±4.7% (^{A,A})</td>
</tr>
</tbody>
</table>

* First superscript indicates significance (α=0.05) of ethanol content at the same separation temperature.

Second superscript indicates significance (α=0.05) of separation temperature at the same ethanol content.
Figure 4.3 SEM images showing morphology of zein encapsulated citral extracted with 60% ethanol and separated at 25°C.

Figure 4.4 SEM images showing morphology of zein encapsulated citral extracted with 70% ethanol and separated at 25°C.

Figure 4.5 SEM images showing morphology of zein encapsulated citral extracted with 80% ethanol and separated at 25°C.
Figure 4.6 SEM images showing morphology of zein encapsulated citral extracted with 60% ethanol and separated at 5°C.

Figure 4.7 SEM images showing morphology of zein encapsulated citral extracted with 70% ethanol and separated at 5°C.

Figure 4.8 SEM images showing morphology of zein encapsulated citral extracted with 80% ethanol and separated at 5°C.
Figure 4.9 SEM images showing morphology of zein encapsulated citral extracted with 60% ethanol and separated at -15°C.

Figure 4.10 SEM images showing morphology of zein encapsulated citral extracted with 70% ethanol and separated at -15°C.

Figure 4.11 SEM images showing morphology of zein encapsulated citral extracted with 80% ethanol and separated at -15°C.
Figure 4.12 TEM images showing internal structure of zein encapsulated citral extracted with 60% ethanol and separated at 25°C.

Figure 4.13 TEM images showing internal structure of zein encapsulated citral extracted with 60% ethanol and separated at 5°C.
Figure 4.14 TEM images showing internal structure of zein encapsulated citral extracted with 60% ethanol and separated at -15°C.

Figure 4.15 TEM images showing internal structure of zein encapsulated citral extracted with 70% ethanol and separated at -15°C.
Figure 4.16 TEM images showing internal structure of zein encapsulated citral extracted with 80% ethanol and separated at -15°C.
CHAPTER V

RECOVERY AND ENCAPSULATION OF FRIED CHIPS FLAVOR

5.1 Introduction
Frying is a method of preparing food. It is widely utilized in food industries because it is a quick cooking process which offers crispy texture, specific flavors and desirable colors for fried foods (Saguy and Dana 2003). However, the main concerns of consuming fried foods are their high oil and high energy intake. Several studies have declared the connection of high energy and high oil diet with obesity and cardiovascular diseases (Lin, Thomas et al. 2000; Gielen and Hambrecht 2004; Guallar-Castillón, Rodríguez-Artalejo et al. 2007). The two major mechanisms which cause high oil content retaining in fried foods were proposed by Saguy and Dana (2003). They are: first, replacement of evaporated water by oil during frying process and, second, oil absorption upon cooling. Potato chip products are considered high oil content snacks, generally having 34.6% oil (USDA ARS, 2000; Saguy and Dana 2003).

Rodin, Mancuso et al. (1991) studied the relation between food craving and body mass in women groups. It was found that higher ratios of “more over-weight women” prefer chips and popcorn compare to “less over-weight women”.

To overcome the health issues of consuming fried products include potato chips, Miller, Castellanos et al. (1998) studied on the effects of utilizing olestra as oil alternative to produce fat-free potato chips to decrease overall fat and energy intakes. On the other hands, the industries have been utilized different heating process on producing potato chips such as baked chips in the favor of lowering oil content in the products. Nevertheless, in terms of taste, the unique flavor notes produced during frying process are still difficult to be generated during baking.

Based on the results from Chapter 4, ethanol is considered a good solvent to extract flavors from an oil phase. Zein was also found to be a good material to encapsulate flavor compounds in ethanol-water by EISA. In this work, a strategy based on Chapter 4 is proposed to recover
fried potato chips flavor from fried potato chips oil following by separation of oil phase at freezing temperature and encapsulation with zein.

The objective of this study was to investigate the feasibility of recovering flavors from fried potato chips oil and encapsulating them with zein. Successful completion of this study may offer the snack industry a strategy to improve the flavor of baked chips, which offer consumers a low fat product but lags on flavor behind fried chips. The presence of fried flavors in the encapsulated product was investigated by sensory tests. R-index was selected to detect added fried flavor to baked chips. It is a simple method to analyze flavor intensity, sensitivity, preference and similarity (Meilgaard, Civille et al. 2007; Lawless and Heymann 2010).

5.2 Materials and methods

5.2.1 Materials
Potatoes, vegetable oil (Crisco, Orrville, OH) and potato chips (Frito Lay’s Classic, Frito Lay’s Baked, Original Flavor, Plano, TX) were purchased from a local supermarket. Zein (Amazein®) was obtained from Prairie Gold Inc. (Bloomington, IL). Ethanol (USP) was purchased from Decon Labs, Inc. (King of Prussia, PA).

5.2.2 Methods
Preparation of fried potato chips flavor:
Frying process:
Potatoes (2.4 Kg) were washed, removed the skins and thin sliced with a slicer to a consistent thickness of 1 mm. Vegetable oil (9 L) was heated in a fryer (15 lb. countertop fryer, Vollrath, Sheboygan, WI) to 175°C. The potato slices were placed into the fryer and processed with the fryer switched off for 3 minutes. The frying oil was collected after frying 8 batches of potato slices with 300 g potato slices per batch and stored in freezer maintained at -15°C until used.

Extraction process:
Ethanol-water at 60% and 70% (50 ml) was mixed with 50 ml of oil from the frying process and continuously stirred for 4 hours at room temperature (25°C). The oil-ethanol mixture was
then moved to a freezer maintained at -15°C and kept for 48 hours to allow for phase separation. After 48 hours, the oil formed a solid phase and precipitated at the bottom and the ethanol extract was separated from the solid oil by filtration at -15°C.

**Encapsulation:**
Series of increasing amounts of fried flavor extracts (1, 3, 5, 7 and 10 ml) from 60 and 70% ethanol were used for zein encapsulation. Extracts were mixed with 10 ml zein dispersions in 90% ethanol (1 mg zein /ml). The ensuing encapsulation process was described in Chapter 3.

**Morphology of encapsulated flavor:**
Scanning electron microscopy was utilized to examine the morphology of encapsulated samples. The same equipment and sample preparation methods were described in Chapter 3.

**Sensory evaluation:**
**Pre-screening test:**
Pre-screening test was conducted by a small group of four panelists to determine the intensity of potato chips oil flavor on baked potato chips sample. The samples were prepared by adding baked potato chips with zein encapsulated potato chips oil flavor from 60% or 70% ethanol extracts. The ratio of encapsulated flavor to baked chips was 5mg of chips flavor powder to 1g baked chips. The pre-screen test determined that the baked chips sample made with 10 ml of 70% ethanol-water extracted chips oil flavor gave the most intense flavor perception. Therefore, the potato chips oil flavor made by 10 ml 70% ethanol water extract was chosen to conduct the sensory test by R-index ranking.

**R-index ranking test:**
Applying R-index into ranking test increases precision by reducing response bias and generating quantitative value to describe similarity between samples (O'Mahony 1992; Lee and Van Hout 2009).
Fried potato chips (Lay’s classic potato chips) and baked chips (Lay’s Baked chips) were utilized as substrates. Every panelist received 5 samples, a “Control” sample (Fried potato chips, Frito Lay’s Classic) is labeled as “C” and the 4 test samples: baked chips (Frito Lay’s Baked, Original Flavor), baked chips plus zein protein, baked chips with zein encapsulated
potato chips oil flavor and control sample again as noise. Test samples were coded with a 3-digit random number. All samples were crushed to improve the distribution of additives in the samples. The sample of baked chip with potato chips oil flavor extract was prepared by adding 0.5 g of potato chips oil flavor extracts into 100 g of crushed baked chips. The sample of baked chip with zein was prepared by adding 0.5 g of zein into 100 g of crushed baked chips. Samples (3 g) were placed in sample cups with lids for the sensory test.

The sensory experiment was approved as IRB Protocol number 12584 of the University of Illinois. The R-index ranking test was conducted by a panel of 25 individuals (10 males and 15 females, with an age range between 23 and 49) recruited from the Champaign-Urbana area. Every panelist received a total of 5 samples as described above. Tests were conducted under incandescent light at room temperature (25°C) and 30% RH. Panelists were asked to taste the control sample first then proceed with the test samples from left to right as shown by the serving order. Panelists were required to have a rest period about 20 seconds between each sample and clean their palate between samples with provided distilled water and plain crackers. During testing, panelists were asked to compare the overall taste of test samples with the control sample C and order them by using the template provided with the samples on how similar the tested samples were to control sample C, giving a score of 1 to the most similar and 4 to the least similar. After the panelists were satisfied with the final ranking, the 3-digits sample code on the sample was written on the space provided on the score sheet.

Statistical analysis:
The R-index value based on John Brown computations against control expressed in percentage (O'Mahony 1992) was calculated for all sensory data. The R-index computation for each sample involved counting the times that the tested sample preceded the noise control sample and dividing it by the total number of panelists. R-index was expressed in percent basis. R-Index values ranging from 100% through 50% indicate the degree of similarity (the higher the R-Index, the more similar to the control reference than the noise reference) and the R-Index ranging from 50% through 0% indicates the degree of difference (the lower the R-Index, the more different the test product from the reference) (Lee and Van Hout 2009). To detect a significant difference on the overall taste between test samples and the control, Table I in BI and O'Mahony (1995)’s research was used. For R-index values in the 0 - 50% range,
values were deducted from 50% before comparing them to the tabular critical value. Significant differences were determined when the deducted R-index was larger than a critical value according to sample size and confidence interval. Moreover, Friedman analysis was conducted to understand the differences among test samples. T value and LSD\textsubscript{rank} were calculated based on Equation 1 and Equation 2, respectively, as shown below, to determine significant differences between each group (Meilgaard, Civille et al. 2007). At least one sample is significantly different if T is higher than the table critical $\chi^2$-square based on sample size at $\alpha=0.05$ (Meilgaard, Civille et al. 2007). Any two groups whose rank sums differ by more than the LSD\textsubscript{rank} are determined significantly different (Meilgaard, Civille et al. 2007).

\[
T = \left(\frac{12}{bt(t+1)}\sum_{j=1}^{t} X_j^2\right) - 3b(t+1) \quad \text{(Equation 1)}
\]

Where $b$ is the number of panelists, $t$ is the number of the tested samples. $X_j$ is the rank sum from all the panelists corresponding to sample $j$.

\[
LSD_{\text{rank}} = Z_{\alpha/2} \sqrt{bt(t+1)/6} \quad \text{(Equation 2)}
\]

Where $b$ is the number of panelists, $t$ is the number of the tested samples. $Z$ value was obtained from Table 5.2 in Ott and Longnecker (2008) at $\alpha=0.05$.

5.3 Results and discussion

5.3.1 Morphology characterization by SEM

Fried potato chips flavors were extracted with 60% and 70% ethanol and the extracts were phase separated at -15°C. The microstructure of zein encapsulated flavor extracts after EISA was characterized by SEM. Figures 5.1 to 5.5 show the morphology of encapsulates prepared with 1, 3, 5, 7, 10 ml of 60% ethanol-water extracts, respectively. The microstructure in these samples showed the formation of spheres 1400 to 5200 nm in diameter. Figures 5.6 to 5.10 show the morphology of encapsulates prepared with 1, 3, 5, 7, 10 ml of 70% ethanol-water extracts, respectively. The microstructure in these samples showed the formation of spheres, 600 nm to 12 µm. It was found that the overall particle size of samples prepared with 70%
ethanol extracts was larger than particles obtained with 60% ethanol. Particle size also increased as the amount of 70% flavor extract used for encapsulation.

5.3.2 Sensory test
The sensory evaluation panel was asked to report their consumption of potato chips products. Their answers are reported in Table 5.1.

John Brown R-index computations:
The R-index value was calculated based on John Brown computations and significant differences between tested samples and control sample were determined by the method described by BI and O'Mahony (1995). Results are shown on Table 5.2. The R-index value was 16% for baked chips sample, 20% for baked chips with zein and 16% for baked chips with zein encapsulated fried flavors. To determine significance, 50% was deducted from the above values, yielding 34%, 30% and 34%, respectively. Deducted values are higher in every case than the critical value of 18.57% at α=0.05 and n=25. Therefore, it was concluded that all three samples were significantly different from the control fried chips sample.

Friedman analysis:
Friedman analysis was utilized to determine differences on overall taste among the four samples. T value, calculated by Equation 1, was -34.1 which was lower than the critical χ-value of 0.352 at α=0.05 for 3 degrees of freedom. Therefore, it was concluded that at least one of the samples was significantly different. LSD_{rank} = 17.9 was calculated by Equation 2 and used for sample comparisons. As shown in Table 5.3, the rank sum for each sample was 38, 59, 71, and 82 for fried potato chips, baked potato chips, baked potato chips with zein and baked potato chips with zein encapsulated flavor, correspondingly. It was found that the overall taste of baked potato chips with or without additives was significantly different from fried potato chips. Among the baked potato chips samples, there was no significant difference between baked potato chips with or without zein. However, a significant difference was found between baked potato chips and baked potato chips with zein encapsulated flavor. Based on these results, it was inferred that zein effectively encapsulated flavor compounds which had been extracted with ethanol-water from frying potato chips oil. However, flavor compounds extracted by ethanol-water may not represent all flavor compounds in fried commercial potato chips or those may be present in low amounts.
In the future, in order to enhance the flavor profile of fried potato chips oil extract, different solvents may be used to extract a wider range of flavor compounds from fried potato chips oil. Moreover, the flavor profile of baked potato chips could be identified and compared to that of fried potato chips in order to determine major differences in flavor. The missing flavors in baked potato chips could be added to enhance sensory properties.

5.4 Conclusions
The sphere morphology of zein - flavor structures suggested an effective flavor encapsulation process. SEM samples prepared with 60% and 70% ethanol extracts showed well-formed spheres. Particle size increased with the ethanol content of extraction solvent and with the proportion of 70% extract in the sample. Zein encapsulated potato chips flavor was detected by a sensory panel when added to commercial baked potato chips. Baked chips with added zein encapsulated flavor were significantly different on overall taste from commercial baked potato chips based on their rank sum value, suggesting that the flavor compounds in frying oil could be recovered by ethanol-water and effectively encapsulated by zein. However, baked chips with added zein encapsulated flavor were also found different in overall taste from commercial fried potato chips. Further research is needed to determine if extracted flavor compounds represent the full array of flavor compounds in fried chips.
5.5 Tables and figures

Table 5.1 Potato chips consumption by panelists.

<table>
<thead>
<tr>
<th>Consumption Rate</th>
<th>Fried Potato Chips</th>
<th>Baked Potato Chips</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2 times per week</td>
<td>16%</td>
<td>12%</td>
</tr>
<tr>
<td>1 time per two weeks</td>
<td>28%</td>
<td>4%</td>
</tr>
<tr>
<td>1 time per month</td>
<td>40%</td>
<td>24%</td>
</tr>
<tr>
<td>Rarely consume</td>
<td>16%</td>
<td>56%</td>
</tr>
<tr>
<td>Never consume</td>
<td>0%</td>
<td>4%</td>
</tr>
</tbody>
</table>

Table 5.2 John Brown R-index values of test samples.

<table>
<thead>
<tr>
<th>Sample group</th>
<th>R-index JB(^#) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baked chips</td>
<td>16.0(^*)</td>
</tr>
<tr>
<td>Baked chips + zein</td>
<td>20.0(^*)</td>
</tr>
<tr>
<td>Baked chips + encapsulated flavor</td>
<td>16.0(^*)</td>
</tr>
</tbody>
</table>

\(^\#\)R-index is calculated based on John Brown computations (O'Mahony 1992) against control (fried chips) (n=25)
* Significantly different from control at \(\alpha=0.05\).

Table 5.3 Rank sum value of overall taste of potato chips samples based on Friedman analysis.

<table>
<thead>
<tr>
<th>Sample group</th>
<th>Rank sum*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fried potato chips</td>
<td>38(^A)</td>
</tr>
<tr>
<td>Baked potato chips</td>
<td>59(^B)</td>
</tr>
<tr>
<td>Baked potato chips + Zein</td>
<td>71(^BC)</td>
</tr>
<tr>
<td>Baked potato chips + Zein encapsulated flavor</td>
<td>82(^C)</td>
</tr>
</tbody>
</table>

* Different superscripts indicate significant differences at \(\alpha=0.05\), \(\text{LSD}_{\text{rank}}=17.9\).
Figure 5.1 SEM images of zein encapsulated fried flavor using 1 ml of 60% ethanol flavor extract.

Figure 5.2 SEM images of zein encapsulated fried flavor using 3 ml of 60% ethanol flavor extract.

Figure 5.3 SEM images of zein encapsulated fried flavor using 5 ml of 60% ethanol flavor extract.
Figure 5.4 SEM images of zein encapsulated fried flavor using 7 ml of 60% ethanol flavor extract.

Figure 5.5 SEM images of zein encapsulated fried flavor using 10 ml of 60% ethanol flavor extract.
Figure 5.6 SEM images of zein encapsulated fried flavor using 1 ml of 70% ethanol flavor extract.

Figure 5.7 SEM images of zein encapsulated fried flavor using 3 ml of 70% ethanol flavor extract.

Figure 5.8 SEM images of zein encapsulated fried flavor using 5 ml of 70% ethanol flavor extract.
Figure 5.9 SEM images of zein encapsulated fried flavor using 7 ml of 70% ethanol flavor extract.

Figure 5.10 SEM images of zein encapsulated fried flavor using 10 ml of 70% ethanol flavor extract.
REFERENCES


Encapsulation Technologies for Active Food Ingredients and Food Processing." 127-160.


Meilgaard, M., G. V. Civille, et al. (2007). Sensory evaluation techniques. CRC.


### Table A.1 Peak areas of standard citral sample on the spectrogram obtained by GC-FID.

<table>
<thead>
<tr>
<th>Citral concentration in Diethyl Ether (ppm)</th>
<th>Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rep. 1</td>
</tr>
<tr>
<td>140</td>
<td>39.35</td>
</tr>
<tr>
<td>280</td>
<td>79.12</td>
</tr>
<tr>
<td>560</td>
<td>154.23</td>
</tr>
<tr>
<td>1120</td>
<td>271.45</td>
</tr>
</tbody>
</table>
Table A.2 Quantification of citral in ethanol-water extraction with 60, 70 and 80% ethanol from MCT phase and separation at 25°C.

<table>
<thead>
<tr>
<th>Ethanol-Water Concentration (%)</th>
<th>60%</th>
<th>70%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Replication</strong></td>
<td>Rep1</td>
<td>Rep2</td>
<td>Rep3</td>
</tr>
<tr>
<td>Citral (mg)</td>
<td>92.2</td>
<td>91</td>
<td>93.7</td>
</tr>
<tr>
<td>1053 (mg)</td>
<td>854.6</td>
<td>867.4</td>
<td>854.3</td>
</tr>
<tr>
<td>1053 (ml)</td>
<td>0.90</td>
<td>0.91</td>
<td>0.90</td>
</tr>
<tr>
<td>Peak Area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>164.0</td>
<td>148.8</td>
<td>170.1</td>
<td></td>
</tr>
<tr>
<td>165.6</td>
<td>146.8</td>
<td>172.3</td>
<td></td>
</tr>
<tr>
<td>162.4</td>
<td>151.6</td>
<td>166.9</td>
<td></td>
</tr>
<tr>
<td>Peak area average</td>
<td>164.0</td>
<td>149.1</td>
<td>169.7</td>
</tr>
<tr>
<td>Citral conc. in 10 ml ethanol water (ppm)</td>
<td>6411.8</td>
<td>5778.6</td>
<td>6653.0</td>
</tr>
<tr>
<td>Original citral concentration (ppm)</td>
<td>9220.0</td>
<td>9100.0</td>
<td>9370.0</td>
</tr>
<tr>
<td>Extraction efficiency (%)</td>
<td>69.5</td>
<td>63.5</td>
<td>71.0</td>
</tr>
<tr>
<td>Ave. Extraction Efficiency (%)</td>
<td>68.0</td>
<td>82.5</td>
<td>82.3</td>
</tr>
</tbody>
</table>
Appendix A. Continuation

Table A.3 Quantification of citral in ethanol-water extraction with 60, 70 and 80% ethanol from MCT phase and separation at 5°C.

<table>
<thead>
<tr>
<th>Ethanol-Water Concentration (%)</th>
<th>60%</th>
<th>70%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>Rep1</td>
<td>Rep2</td>
<td>Rep3</td>
</tr>
<tr>
<td>Citral (mg)</td>
<td>91.3</td>
<td>92.3</td>
<td>90.1</td>
</tr>
<tr>
<td>1053 (mg)</td>
<td>863.7</td>
<td>858.6</td>
<td>875.0</td>
</tr>
<tr>
<td>1053 (ml)</td>
<td>0.91</td>
<td>0.90</td>
<td>0.92</td>
</tr>
<tr>
<td>Peak Area</td>
<td>170.1</td>
<td>157.23</td>
<td>151.4</td>
</tr>
<tr>
<td></td>
<td>171.4</td>
<td>146.15</td>
<td>157.7</td>
</tr>
<tr>
<td></td>
<td>174.5</td>
<td>151.35</td>
<td>162.7</td>
</tr>
<tr>
<td>Peak area average</td>
<td>171.9</td>
<td>151.58</td>
<td>157.3</td>
</tr>
<tr>
<td>Citral conc. in 10 ml ethanol water (ppm)</td>
<td>6748.1</td>
<td>5884.9</td>
<td>6125.6</td>
</tr>
<tr>
<td>Original citral concentration (ppm)</td>
<td>9130.0</td>
<td>9230.0</td>
<td>9010.0</td>
</tr>
<tr>
<td>Extraction efficiency (%)</td>
<td>73.9</td>
<td>63.8</td>
<td>68.0</td>
</tr>
<tr>
<td>Ave. Extraction Efficiency (%)</td>
<td>68.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table A.4 Quantification of citral in ethanol-water extraction with 60, 70 and 80% ethanol from MCT phase and separation at -15°C.

<table>
<thead>
<tr>
<th>Ethanol-Water Concentration (%)</th>
<th>60%</th>
<th>70%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>Rep1</td>
<td>Rep2</td>
<td>Rep3</td>
</tr>
<tr>
<td>Citral (mg)</td>
<td>90.8</td>
<td>90.8</td>
<td>95.1</td>
</tr>
<tr>
<td>1053 (mg)</td>
<td>868.8</td>
<td>867.8</td>
<td>860.2</td>
</tr>
<tr>
<td>1053 (ml)</td>
<td>0.92</td>
<td>0.91</td>
<td>0.91</td>
</tr>
<tr>
<td>Peak Area</td>
<td>200.1</td>
<td>221.7</td>
<td>212.9</td>
</tr>
<tr>
<td>Peak area average</td>
<td>201.6</td>
<td>217.1</td>
<td>215.5</td>
</tr>
<tr>
<td>Citral conc. in 10 ml ethanol water (ppm)</td>
<td>7989.1</td>
<td>8783.2</td>
<td>8477.6</td>
</tr>
<tr>
<td>Original citral concentration (ppm)</td>
<td>9080.0</td>
<td>9080.0</td>
<td>9510.0</td>
</tr>
<tr>
<td>Extraction efficiency (%)</td>
<td>88.0</td>
<td>96.3</td>
<td>89.1</td>
</tr>
<tr>
<td>Ave. Extraction Efficiency (%)</td>
<td><strong>91.3</strong></td>
<td><strong>96.6</strong></td>
<td><strong>94.1</strong></td>
</tr>
</tbody>
</table>
APPENDIX B. ADDITIONAL SEM IMAGES

Figure B.1 SEM images showing morphology of zein encapsulated trans-2 hexenal after EISA in 70% ethanol.

Figure B.2 SEM images showing morphology of zein encapsulated benzaldehyde after EISA in 70% ethanol.

Figure B.3 SEM images showing morphology of zein encapsulated citral after EISA in 70% ethanol.
Appendix B. Continuation

Figure B.4 SEM images showing morphology of zein encapsulated cis-3-hexen-1-ol after EISA in 70% ethanol.

Figure B.5 SEM images showing morphology of zein encapsulated triacetin after EISA in 70% ethanol.

Figure B.6 SEM images showing morphology of zein encapsulated Neobee M5 after EISA in 70% ethanol.
Appendix B. Continuation

Figure B.7 SEM images showing morphology of zein encapsulated trans-2 hexenal after EISA in 90% ethanol.

Figure B.8 SEM images showing morphology of zein encapsulated citral after EISA in 90% ethanol.

Figure B.9 SEM images showing morphology of zein encapsulated cis-3-hexen-1-ol after EISA in 90% ethanol.
Appendix B. Continuation

Figure B.10 SEM images showing morphology of zein encapsulated triacetin after EISA in 90% ethanol.

Figure B.11 SEM images showing morphology of zein encapsulated Neobee M5 after EISA in 90% ethanol.

Figure B.12 SEM images showing morphology of zein encapsulated Neobee 1053 after EISA in 90% ethanol.
Figure B.13 SEM images showing morphology of zein encapsulated citral/Neobee 1053 mixture at ratio 1:4 after EISA in 90% ethanol.

Figure B.14 SEM images showing morphology of zein encapsulated citral/Neobee 1053 mixture at ratio 2:3 after EISA in 90% ethanol.

Figure B.15 SEM images showing morphology of zein encapsulated citral/Neobee 1053 mixture at ratio 3:2 after EISA in 90% ethanol.
Appendix B. Continuation

Figure B.16 SEM images showing morphology of zein encapsulated citral/Neobee 1053 mixture at ratio 4:1 after EISA in 90% ethanol.

Figure B.17 SEM images showing morphology of zein encapsulated citral extracted with 60% ethanol and separated at 25°C.

Figure B.18 SEM images showing morphology of zein encapsulated citral extracted with 70% ethanol and separated at 25°C.
Appendix B. Continuation

Figure B.19 SEM images showing morphology of zein encapsulated citral extracted with 80% ethanol and separated at 25°C.

Figure B.20 SEM images showing morphology of zein encapsulated citral extracted with 60% ethanol and separated at 5°C.

Figure B.21 SEM images showing morphology of zein encapsulated citral extracted with 60% ethanol and separated at -15°C.
Appendix B. Continuation

Figure B.22 SEM images showing morphology of zein encapsulated citral extracted with 70% ethanol and separated at -15°C.

Figure B.23 SEM images showing morphology of zein encapsulated citral extracted with 80% ethanol and separated at -15°C.

Figure B.24 SEM images of zein encapsulated fried flavor using 1 ml of 60% ethanol flavor extract.
Appendix B. Continuation

Figure B.25 SEM images of zein encapsulated fried flavor using 3 ml of 60% ethanol flavor extract.

Figure B.26 SEM images of zein encapsulated fried flavor using 5 ml of 60% ethanol flavor extract.

Figure B.27 SEM images of zein encapsulated fried flavor using 7 ml of 60% ethanol flavor extract.
Appendix B. Continuation

Figure B.28 SEM images of zein encapsulated fried flavor using 10 ml of 60% ethanol flavor extract.

Figure B.29 SEM images of zein encapsulated fried flavor using 1 ml of 70% ethanol flavor extract.

Figure B.30 SEM images of zein encapsulated fried flavor using 3 ml of 70% ethanol flavor extract.
Appendix B. Continuation

Figure B.31 SEM images of zein encapsulated fried flavor using 5 ml of 70\% ethanol flavor extract.

Figure B.32 SEM images of zein encapsulated fried flavor using 7 ml of 70\% ethanol flavor extract.
APPENDIX C ADDITIONAL TEM IMAGES

Figure C.1 TEM images showing internal structure of zein encapsulated citral extracted with 60% ethanol and separated at 5°C.

Figure C.2 TEM images showing internal structure of zein encapsulated citral extracted with 60% ethanol and separated at -15°C.
Appendix C. Continuation

Figure C.3 TEM images showing internal structure of zein encapsulated citral extracted with 70% ethanol and separated at -15°C.
APPENDIX D. ADDITIONAL SPECTROGRAMS

Figure D.1 Citral GC-FID spectrogram: 140 ppm

<table>
<thead>
<tr>
<th>Replications</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>39.35</td>
</tr>
<tr>
<td>2</td>
<td>37.28</td>
</tr>
<tr>
<td>3</td>
<td>39.05</td>
</tr>
</tbody>
</table>

Figure D.2 Citral GC-FID spectrogram: 280 ppm

<table>
<thead>
<tr>
<th>Replications</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>79.12</td>
</tr>
<tr>
<td>2</td>
<td>81.42</td>
</tr>
<tr>
<td>3</td>
<td>80.94</td>
</tr>
</tbody>
</table>
Appendix D. Continuation

Figure D.3 Citral GC-FID spectrogram: 560 ppm

Figure D.4 Citral GC-FID spectrogram: 1120 ppm
Appendix D. Continuation

Figure D.5 Spectrogram: citral extracted with 60% ethanol and separated at 25°C
Appendix D. Continuation

Figure D.6 Spectrogram: citral extracted with 70% ethanol and separated at 25°C

<table>
<thead>
<tr>
<th>Repeat 1</th>
<th>Replications</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>187.27</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>204.26</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>195.56</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Repeat 2</th>
<th>Replications</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>180.99</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>181.98</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>181.69</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Repeat 3</th>
<th>Replications</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>181.75</td>
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<tr>
<td></td>
<td>2</td>
<td>185.59</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>187.93</td>
</tr>
</tbody>
</table>
Figure D.7 Spectrogram: citral extracted with 80% ethanol and separated at 25°C
Appendix D. Continuation

Figure D.8 Spectrogram: citral extracted with 60% ethanol and separated at 5°C
Appendix D. Continuation

Figure D.9 Spectrogram: citral extracted with 70% ethanol and separated at 5°C
Figure D.10 Spectrogram: citral extracted with 80% ethanol and separated at 5°C
Appendix D. Continuation

Figure D.11 Spectrogram: citral extracted with 60\% ethanol and separated at - 15°C
Appendix D. Continuation

Figure D.12 Spectrogram: citral extracted with 70% ethanol and separated at -15°C
Appendix D. Continuation

Figure D.13 Spectrogram: citral extracted with 80% ethanol and separated at -15°C
APPENDIX E. SENSORY SCORE CARD

Description of Test:

In this test, you are asked to compare the overall flavor sensation of multiple test samples with the control sample. You will be asked to rank them from most similar to least similar to the control. Please rate each sample in the proper order. After completion of the entire set, you may go back and re-evaluate samples, and change the order if you wish.

Please read the instructions below, and make sure that they are completely clear to you. If you have any question, please feel free to ask the experimenter. Thank you.

Instructions:

1. You have received 5 samples, a “Control” sample labeled C and 4 test samples each labeled with a 3-digit number.
2. Taste the control sample and try to use the first impression (initial taste) to evaluate the sample. Proceed with the test samples from left to right as shown by serving order below. Please rinse your mouth with water and wait about 10 seconds between each sample.
3. Compare the test samples with C, and order them using the template in front of you on how similar or different they are from C, with 1 = most similar to 4 = least similar.
4. After you are satisfied with your final ranking, please write sample codes of the test samples in the space provided below.
5. If samples appear the same, please make a “best guess”.

<table>
<thead>
<tr>
<th>Serving order</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rank</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Most similar</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Least similar</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample code</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure E.1 Score card for R-index ranking test
Appendix E. Continuation

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**Additional Questions**

The questions below are to allow the researchers to better understand the group participating in this sensory test. No individually identifiable information will be published.

1. What is your gender?  
   
2. How old are you?  
   
3. How do you describe yourself? Please mark the one option that best describes you:
   
   ___ American Indian or Alaska Native
   ___ Asian or Asian American
   ___ Black or African American
   ___ Hawaiian or Other Pacific Islander
   ___ Hispanic or Latino
   ___ Non-Hispanic White

4. How often do you consume these potato chips? Please mark the category that best describes your averaged beverage consumption:

   **4.1 Frying chips**
   ___ more than 5 times/week
   ___ 3-5 times/week
   ___ 1-2 times/week
   ___ 1 time/every two weeks
   ___ 1 time/month
   ___ rarely
   ___ never

   **4.2 Baked Chips**
   ___ more than 5 times/week
   ___ 3-5 times/week
   ___ 1-2 times/week
   ___ 1 time/every two weeks
   ___ 1 time/month
   ___ rarely
   ___ never

---

Figure E.2 Question sheet