ENCAPSULATION OF ORANGE POMACE POLYPHENOLS IN ZEIN/PECTIN NANOPARTICLES

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THESIS: 

ENCAPSULATION OF ORANGE POMACE POLYPHENOLS IN ZEIN/PECTIN NANOPARTICLES

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ABSTRACT

Orange juice industries produce byproducts, known as orange pomace, that contains the oranges’ discards like their pulp, membrane tissue, and peels. Orange pomace contains polyphenols that exhibit beneficial effects on health like antioxidant, antimicrobial, and anti-inflammatory properties. Although these polyphenols show great potential as nutraceutical additives for functional foods and beverages, they are difficult to utilize within the food industry due to their poor water solubility and bioaccessibility. The polyphenol in focus for this research was aglycone hesperetin (HT), the bio-derivative of hesperidin. This project aimed to develop a protein nanoparticle colloidal delivery system using zein and pectin that could effectively encapsulate hesperetin to improve its water dispersibility, stability, antioxidant activity, and in vitro bioaccessibility. Hesperetin-loaded zein (HZ) nanoparticles were formed at pH=4.0 with optimal final hesperetin concentration at 500 µg/mL (particle diameter (PD) = 179.23 ± 5.0 nm, PDI = 0.146 ± 0.02, zeta-potential = 23.23 ± 0.8 mV, and encapsulation efficiency = 92.78 ± 1.0 %). The addition of optimal final pectin concentration (0.5 mg/mL) onto HZ nanoparticles yielded a PD of 356.83 ± 10.7 nm, PDI = 0.119 ± 0.03, zeta-potential = -22.56 ± 0.4 mV, and encapsulation efficiency = 93.97 ± 0.2 %. The interaction between hesperetin and zein in the fluorescence spectrometer yielded a binding constant of 4.94 x 10^5 M^-1 with the # of binding sites at 1.2126. Fourier-Transform Infrared (FTIR) confirmed that the interactions between hesperetin and zein were hydrogen bonds and hydrophobic interactions with electrostatic attraction between pectin and zein. Hesperetin-loaded zein/pectin (HZP) nanoparticles were proven stable against different environments (pH 2.0-8.0, 90°C heating, and 50 mM NaCl exposure)
compared to HZ nanoparticles, which destabilized at pH 5.0-6.0, 90°C, and 25 mM NaCl. HZP nanoparticles have shown ~3x more antioxidant activity (0.09 ± 0.008 µg/mL ascorbic acid equivalent (AAE) per 1 µg/mL hesperetin) compared to hesperetin dispersed in water (0.03 ± 0.02 µg/mL AAE per 1 µg/mL hesperetin). Both HZ and HZP nanoparticles have exhibited ~5.5x more bioaccessibility (64.32 ± 0.5 % and 64.55 ± 0.4 %, respectively) compared to hesperetin dispersed in water (11.49 ± 2.1 %). These results show promise in the use of a protein nanoparticle colloidal delivery system using zein and pectin to improve the incorporation of hesperetin, and possibly other polyphenols from orange pomace, into a variety of foods and beverages as nutraceutical additives and functional ingredients. This process will bring greater value to the byproducts produced within the orange juice industry as opposed to being commonly used for animal feed and pectin extraction.
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1. INTRODUCTION

The orange juicing industry produces byproducts, known as pomace, that contains discards of oranges like their pulp, peels, membrane tissues, and seeds. Orange pomace accounts for 55-60% of the citrus weight and the orange juicing industry produces 1 million tons of byproduct each year (Castro et al., 2020; Fernández-Fernández, 2020). In addition, orange pomace is commonly discarded into landfills, but can also be utilized as animal feed and land fertilizer within the industry (Ignatia et al., 2022). While compounds like pectin are commonly extracted from orange pomace, polyphenols are not as commonly extracted (Rafiq et al., 2018).

Polyphenols from orange pomace, like hesperidin, are commonly found in oranges like Citrus sinensis. These polyphenols exhibit beneficial effects on health, such as antioxidant, anti-inflammatory, antimicrobial, and anti-mutagenic activity (Pyrzynska., 2022; Gabriele et al., 2017). The polyphenols within the wastes of citrus fruits have beneficial properties that can be utilized as nutraceuticals in foods and beverages, on which the global herbal supplements market is expected to make $120 billion in 2028 (Vantage Market Research, 2022). Hesperitin, the bioderived aglycone of hesperidin, has enhanced radical scavenging properties as antioxidants and have more affinity to bind to proteins due to the lack of glycosides (Alam et al., 2014; Cho, 2006; Vallejo et al., 2016).

While these polyphenols have great therapeutic values, the industry is unable to use them directly due to their poor bioavailability, poor chemical stability, and low water solubility, limiting incorporation into aqueous based foods that leads to low bioaccessibility (Pyrzynska, 2022; Jabbari et al., 2017; Lucas-Abellán et al., 2019). Additionally, the poor water dispersibility of these polyphenols leads to crystallization or
sedimentation within food products that can showcase poor visual appeal (Caballero et al., 2022). A potential way to tackle this issue is by encapsulating these orange pomace polyphenols into a protein nanoparticle colloidal delivery system.

The protein that will be used is zein, a prolamin storage protein found in maize that can encapsulate the polyphenols due to the abundant amount of non-polar amino acid side chains (Tortorella et al., 2021; Gianazza et al., 1977). By encapsulating hesperetin in zein/pectin nanoparticles for a nanoparticle delivery system, hesperetin’s water solubility could significantly increase and lead to an improved bioaccessibility with incorporation into functional foods and beverages as nutraceutical additives. Hesperetin will be covered in zein protein through hydrogen-bonding and hydrophobic interactions. Zein protein is used to protect sensitive flavors and bioactive compounds from digestive enzymes (Jafari, 2019; Wang et al., 2018; Hu & McClements, 2016). This process helps in improving the polyphenols’ bioaccessibility during In vitro digestion, which is a measurement of the concentration of bioactive and lipophilic compounds that are incorporated into mixed micelles, composed of bile salts, at the end of a simulated intestinal phase with similar physiological conditions (Hur et al., 2011; McClements & Li, 2010). Lastly, zein protein is desirable as nanocarriers for their reduced toxicity in the environment and for humans for an eco-friendlier carrier system (Oliveira et al., 2018). Specifically, when a stock solution of zein in aqueous alcohol is diluted with water, zein becomes insoluble in water and precipitates to form colloidal particles as nanocarriers without other added solvents (Patel & Velikox, 2014). This also allows zein to be an eco-friendlier carrier within humans as opposed to other delivery systems like silver nanoparticles where nano silver can produce toxic effects in major organs (Zhang & Wang et al., 2022).
There has been limited research on orange pomace polyphenol encapsulation and stability. Additionally, there have been studies demonstrating how zein nanoparticles used to encapsulate polyphenols such as resveratrol or curcumin improve their water dispersibility, chemical stability, and bioaccessibility (Davidov-Pardo et al., 2015; Hu et al., 2015). Even though there are studies of the encapsulation of orange pomace polyphenols in emulsions (Ting et al., 2013), liposomes (Mohanty et al., 2020), cyclodextrins (Tommasini et al., 2005), and encapsulation of the orange pomace polyphenol tangeretin in zein nanoparticles (Chen et al., 2014), to the best of the author’s knowledge, there has not been a study on encapsulation of hesperetin with zein nanoparticles specifically. There are other studies of nanoencapsulation on hesperetin like hydroxypropyl-beta-cyclodextrin inclusion complex (Wang et al., 2023), propylene glycol liposomes with lecithin (Vaz et al., 2021), and pea protein with high-methoxyl pectin electrolyte complex (Caballero et al., 2022). However, zein nanoparticles are simpler to fabricate with anti-solvent precipitation compared to using a homogenizer for propylene glycol liposomes with lecithin (Vaz et al., 2021) and a crystallization tank for hydroxypropyl-beta-cyclodextrin inclusion complex (Wang et al., 2023). In addition, higher hydrophobicity proteins like zein allow a longer and more controlled release of the bioactive compound throughout the gastrointestinal tract compared to hydrophilic pea protein (Caballero et al., 2022; Kianfar, 2021; Hong et al., 2020; Saha et al., 2016).

The goals of this research are to assess the formation of a nanoparticle colloidal system formed from zein protein, to determine and characterize the concentration of hesperetin that could be bound with zein nanoparticles, and to assess the impact of
encapsulation on hesperetin’s dispersibility, antioxidant activity and In vitro bioaccessibility.
2. LITERATURE REVIEW

2.1. Oranges

Oranges are a type of citrus fruit that comes from the species Citrus sinensis and the Rutaceae family, which originated in China (Petrotos et al., 2021; Wu et al., 2018). An estimation of 48.8 million tons of oranges will globally be produced this year (USDA, 2022). The California Department of Food and Agriculture also forecasts 70.0 million cartons (1.3 million tons) of navel oranges will be produced this year (NASS/USDA, 2021). Although oranges are sold as a whole fruit, they are ranked as the most consumed fruit juice in the United States, as shown in figure 1, (USDA, 2021). Valencia oranges and navelina oranges are the most often used oranges for global orange juice production (USDA, 2022).

![Figure 1: Food availability of various fruit and different types of consumption of those fruits in the U.S. (USDA, 2021).](image-url)
2.2. Orange Juice Industry

From the 48.8 million tons of oranges globally produced, USDA reports have shown that 1.7 million tons of orange juice were produced globally in 2022 and 3.6 million tons of oranges were used to produce the 1.7 million tons of orange juice (USDA, 2022). From the 3.6 million tons of oranges, the remaining difference (1.9 million tons) is classified as orange wastes, which is similar to ~54% of the total global oranges classified as orange peels as reported by Li et al. (2023). Even though global orange juice production decreased in 2019/2020 due to Huanglongbing (HLB), or citrus greening, in Mexico, California, and Florida, global production is rising again in 2022 (USDA, 2022). The U.S. produced 3.6 million tons of oranges and is responsible for 215,000 tons of orange juice production from 2021/2022 (USDA, 2022). California produced 49,000 tons of oranges in 2021/2022 (USDA/NASS, 2022).

The pulp, seeds, and peels of citrus make up about 50% of the total weight of the fruit and are the composition of industrial waste during commercial juice production (Fernández-Fernández, 2021). Over a thousand tons of citrus pomace are produced annually at an industry level and burden the industry with its disposal (Berk, 2016). Within orange processing facilities, there is a possibility of clogging waterways during industrial processes and pollution in residual water (Oluseun Adejumo & Adebiyi, 2021; Cypriano et al., 2018). Disposal of the wastes from oranges requires more energy, mainly to decrease their water content and can be very costly to the industry (Cypriano et al., 2018). Specifically, the waste from oranges contains high proportions of water that are difficult to retain for long-term storage and must be treated immediately to prevent putrefaction (Li & Putra et al., 2023). One common example of waste management
within the industry is through combustion for thermal energy but can release high levels of air pollutants into the environment like sulfur dioxide, carbon monoxide, and nitrogen oxides (Russo et al., 2021; Siles et al., 2016). Another example of waste management is through anaerobic digestion where the digested waste produces both biogas for plant energy requirements and fertilizers for orange crops (Ortiz et al., 2020). Although this method is more environmentally friendly than combustion, anaerobic digestion incurs very high costs with large amounts of water consumed during the process (Ortiz et al., 2020).

2.3. Orange Processing Byproducts (Orange Pomace)

The byproducts of oranges are also known as orange pomace. Orange pomace is a solid waste byproduct from the orange juice industry that accounts for approximately 50% of the citrus weight that is processed into juicing (Petrotos et al., 2021). Specifically, pomace consists of the albedo, the white inner layer of the orange, and the flavedo, the surface of the orange peel (Rafiq et al., 2018). The image of the composition of orange pomace from an orange is shown in Figure 2. The processing byproducts from oranges have some utilization like cattle feed, but they produce little to no profit for the company in comparison to the amount of input energy required (Widmer et al., 2010). In addition to cattle feed, pectin can be extracted from oranges. Pectin is present in the cell walls of plants and is often used as a thickening agent and gelling agent for foods and is a source of soluble dietary fiber (Videocoq et al., 2011; Fissore et al., 2012). However, pectin can also be extracted from other agriculture products like other citrus, apple pulp, or sugar beet solids, so oranges are not often the only target for pectin (Putnik et al., 2017). To
process these orange byproducts, however, they must be worked quickly as they are susceptible to pests and microbial spoilage due to oranges’ high moisture and sugar content (Berk, 2016). Although pectin extraction and cattle feed are primarily the orange processing byproducts, polyphenols from orange pomace are an underutilized processing byproduct component (Rafiq et al., 2018).

![Figure 2: Components of orange pomace from a peeled orange (Tomita et al., 2019).](image)

**2.4. Orange Pomace Polyphenols**

Polyphenols are phenolic compounds that are naturally abundant in fruits like oranges that help in the defense against aggression by pathogens and ultraviolet radiation (Iglesias-Carres et al., 2019; Pandey et al., 2009). Consumption of plants high in plant polyphenols can help protect against developing diseases like cancer and diabetes (Pandey et al., 2009). The polyphenols predominant in orange pomace are known as flavonoids. Flavonoids have a 15-carbon skeleton that consists of a benzo-γ-pyrone moiety with an attached benzene ring (Russo et al., 2021). The general chemical structure of a flavonoid is shown in Figure 3. These flavonoids are categorized into either flavanols, flavones, flavanones, flavonols, isoflavones, or anthocyanidins, and their
subgroups are dependent on their methylation and glycosylation (Hu, Liu, et al., 2017). The different classifications, structures, and properties of flavonoids are shown in Table 1.

Orange pomace is a valuable raw material to produce high-value products, especially for substantial quantities of flavonoids and polyphenols (Petrotos et al., 2021). In a comparison of phenolic compounds between juice and pomace of oranges, it was shown that the phenolic content of the pomace was 3- to 4-fold higher than in the juices (Multari et al. 2020). The polyphenols predominant in orange pomace are known as flavonoids. Common flavonoids and their classifications, structures, functional properties, and limitations are shown in Table 1.

Figure 3: Flavonoids and their different classifications based on their methylation and glycosylation (Russo et al., 2021).
<table>
<thead>
<tr>
<th>Compound + Structure</th>
<th>Family + Classification</th>
<th>Properties</th>
<th>Challenges</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hesperidin</td>
<td>Flavanone Glycoside</td>
<td>Antioxidant Antidepressant Antiviral Antimicrobial Antidiabetic</td>
<td>Low water solubility Sedimentation</td>
<td>Kuntić et al., 2014 Zhu et al., 2020 Ding et al., 2018 Panasiak et al., 1989 Visnagri et al., 2014,</td>
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<tr>
<td>Naringin</td>
<td>Flavanone Glycoside</td>
<td>Reduce cholesterol levels Antioxidant Activity</td>
<td>Low water solubility Sedimentation Bitterness</td>
<td>Baskaran et al., 2015 Luo et al., 2012 Mahmoud et al., 2012,</td>
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<tr>
<td>Nobiletin</td>
<td>Flavone Polymethoxylated</td>
<td>Anti-diabetes Anti-inflammatory Antimicrobial</td>
<td>Low water solubility Sedimentation</td>
<td>Kumar et al., 2018 Lai et al., 2008 Yao et al., 2012,</td>
</tr>
</tbody>
</table>

Table 1: Functional properties and structures of common polyphenols derived from orange peels.
Table 1 (cont’d): Functional properties and structures of common polyphenols derived from orange peels.

<table>
<thead>
<tr>
<th>Polyphenol</th>
<th>Functional Properties</th>
<th>Molecular Structure</th>
<th>Source(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tangeretin</td>
<td>Polymethoxylated</td>
<td><img src="image" alt="Tangeretin Structure" /></td>
<td>Boye et al., 2021, Caballero et al., 2021</td>
</tr>
<tr>
<td>Flavone</td>
<td>Antioxidant, Anti-proliferative (inhibit cell growth)</td>
<td><img src="image" alt="Flavone Structure" /></td>
<td></td>
</tr>
<tr>
<td>Neohesperidin</td>
<td>Glycoside, Creation of dihydrochalones (sweetness)</td>
<td><img src="image" alt="Neohesperidin Structure" /></td>
<td>Frydman et al., 2005, Caballero et al., 2021</td>
</tr>
<tr>
<td>Hesperetin</td>
<td>Aglycone, Antioxidant, Anti-inflammatory, Anticarcinogenic</td>
<td><img src="image" alt="Hesperetin Structure" /></td>
<td>Parhiz et al., 2015, Iranshahi et al., 2015</td>
</tr>
<tr>
<td>Naringenin</td>
<td>Aglycone, Antioxidant, Anti-inflammatory, Antibacterial</td>
<td><img src="image" alt="Naringenin Structure" /></td>
<td>Smruthi et al., 2022, Salehi et al., 2019</td>
</tr>
</tbody>
</table>
Within the peels of oranges, hesperidin and naringin are often the most abundant flavonoids (Pereira et al., 2017). From Pereira et al. (2017), the concentrations of these polyphenols in orange peels are ~37 mg g\(^{-1}\) and 6.7 mg g\(^{-1}\) for the wet basis of hesperidin and naringin, respectively. Even though there are small quantities of polyphenols for extraction, the utilization of these polyphenols will add more value to the orange pomace (Petrotos et al., 2021).

### 2.4.1. Incorporating orange pomace polyphenols into the functional food and beverage industry

Incorporating orange pomace polyphenols will be a great addition to the functional food and beverage industry. In addition to sourcing the wastes from orange pomace, there is a demand for new nutraceuticals and “all-natural” additives. There is an expected $120 billion profit in the global herbal supplements and around 56% of U.S. adults are seeking functional foods (Hartman Group, 2021; Vantage Market Research, 2022).

### 2.5. Hesperetin

Bioaccessibility and solubility of high molecular weight phenol compounds like hesperidin are lower compared to its aglycone counterpart hesperetin due to its sugar residue of a glycoside known as glycone (Grgic et al., 2018). The lack of glycone attached to the flavonoids allows hesperetin to strongly enhance their bioaccessibility and bioavailability (Furtado et al., 2012; Slámová et al., 2018; Thilakarathna et al., 2013).
Hesperetin also has enhanced radical scavenging properties as antioxidants due to the lack of glycosides (Alam et al., 2014; Cho, 2006; Vallejo et al., 2016). The process of deglycosylation, selective trimming of the sugar residue of a glycoside using a glycosidase enzyme, is used in the production of the aglycone hesperetin from their glycone counterpart hesperidin (Slámová et al., 2018). The glycone of hesperidin has also shown poor binding to proteins compared to its aglycone hesperetin (Shpigelman et al., 2014). The hydrophilic sugar moiety within glycones interferes with the hydrophobic binding sites of proteins (Pastukhov et al., 2007). Thus, the aglycone hesperetin was selected as the polyphenol for this project. The deglycosylation of hesperidin to hesperetin is shown in Figure 4.

![Figure 4: Schematic of biotransformation of hesperidin to hesperetin (Weiz et al., 2019).](image)

In Figure 4, when hesperidin is biotransformed into hesperetin, it undergoes enzymatic hydrolysis where α-rhamnosyl-β-glucosidases (αRβG) I and II along with water, to lyse the 7-O-linked flavonoid rutinosides and result in hesperetin and rutinose (Weiz et al., 2019).
2.6 Delivery Systems for Orange Pomace Polyphenols

A lot of polyphenols present within orange pomace have low water solubility that limits their incorporation into aqueous-based foods and poor bioaccessibility under gastrointestinal conditions (Smruthi et al., 2022; Tsirigotis-Maniecka et al., 2017). In addition to their low water solubility, the poor water dispersibility of polyphenols could also lead to crystallization and visible sedimentation that makes food products visually unappealing (Caballero et al., 2021). A potential way to address these issues is by using nanotechnology-based delivery systems. This method can incorporate polyphenols into foods and beverages by encapsulating chemically unstable and hydrophobic bioactive compounds. There are various delivery systems including inclusion complexes, liposomes, biopolymer nanoparticles, hydrogels, and more.

2.6.1. Liposomes

Liposomes are part of a delivery system that uses spherical vesicles that are comprised of one or more phospholipid bilayers commonly made out of lecithin, which can be derived from a variety of sources like egg yolk, rice beans, and soy (Pasarin et al., 2023). This is produced in the laboratory by combining the bioactive compound and emulsifier in an organic solvent like ethanol, vortexing, evaporating the organic solvent to leave a thin film, and then redispersing the film in water (Šturm & Ulrih, 2021). Liposomes are capable of encapsulating polyphenols but would not be ideal to use within the food industry due to their low stability from their fragile phospholipid membranes and the low number of polyphenols successfully being encapsulated along with their
phospholipids prone to lipid oxidation during storage (Pasarin et al., 2023, Šturm & Ulrih, 2021).

2.6.2. Emulsions

Oil-in-water (O/W) emulsions have oil droplets dispersed in a continuous aqueous phase with emulsifiers that stabilize the thermodynamically unstable system in the oil-water interface (Jaiswal et al., 2014). Components require oil, emulsifiers, and aqueous phases for emulsions and some methods can involve ultrasonic emulsification, high-pressure homogenization, microfluidization, and/or phase inversion temperature (Jaiswal et al., 2014). An example of a type of emulsion is nanoemulsions. Nanoemulsions have oil droplets that are lower than 200 nm, which further improves the emulsion’s kinetic stability and the droplets’ resistance to gravitational separation and aggregation (Caballero et al. 2021; Singh et al. 2017; McClements, 2011). O/W emulsions could be used as nanocarriers for polyphenols but requires the use of high-cost equipment like homogenizer and ultrasonication for decreasing the droplet size along with the need for high concentrations of surfactants when the nanoemulsions are influenced by temperature and pH (Sabjan et al., 2020).

2.6.3. Solid Lipid Nanoparticles

Solid lipid nanoparticles are lipid-based colloidal delivery systems that have aqueous dispersion of small lipid particles that are fully solidified (Fathi et al., 2012). Generating solid lipid nanoparticles have each lipid particle covered in an emulsifier, like nanoemulsions, and can either be created through hot homogenization or cold
homogenization (Duan et al., 2020). Hot homogenization is when polyphenols can be encapsulated into a melted lipid phase and emulsified during hot temperatures while cold homogenization is when polyphenols can be encapsulated into a melted lipid phase and then cooled into lower temperatures and homogenized in cold water (Caballero, 2021). This delivery system would not be ideal, as encapsulated compounds could be expelled from the particles if the lipid phase crystallizes or promote aggregation (Subroto et al., 2023).

2.6.4. Molecular Inclusion Complexes

Molecular inclusion complexes have a host-guest relationship where the host molecule like β-cyclodextrin encapsulates the guest molecules in its hydrophobic cavity and stabilizes the polar part by the polar rims (Saha et al., 2016). In the case of creating the host polysaccharide, they are formed by enzymatic degradation of starch by cyclodextrin-transferases (Wei et al., 2019). Although cyclodextrins could potentially be used as nanocarriers for aglycone polyphenols, they are expensive ingredients that reduce commercial feasibility, and derivatives of cyclodextrins are unable to be utilized in a clean label or natural products as final products (Tiwari et al., 2010).

2.6.5. Hydrogels

Hydrogels are made from synthetic, semi-synthetic, or natural polymers that are covalently or physically crosslinked to form gels that are comprised of highly hydrated mesh networks (Nairon et al., 2020). This method of delivery system uses polymerization and crosslinking in the creation of hydrogels for encapsulation (Onaciu et al., 2019).
These gels could have low encapsulation of polyphenols at a nano size and increasing hydrogels to a macro size will increase encapsulation of polyphenols but at the cost of negatively impacting the texture and stability of food products (Caballero et al., 2021).

2.6.6. Biopolymer Nanoparticles

Biopolymer nanoparticles are nanoparticle colloidal delivery systems that are formed by the agglomeration of proteins and/or polysaccharides, which are induced by nucleation and crystal growth mechanisms (Vodyashkin et al., 2022; Qian et al., 2021). These biopolymer nanoparticles are promising for the delivery of polyphenols from orange pomace as this method requires general hydrophobicity rather than specific molecular structures (Caballero et al., 2021, Nitta & Numata, 2013). Table 2 lists the different delivery systems used for the encapsulation of various polyphenols derived from orange peels.

Table 2: Overview of different delivery systems for polyphenols found within orange pomace.

<table>
<thead>
<tr>
<th>Polyphenols from Orange Peels</th>
<th>Delivery System</th>
<th>Overall Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hesperidin</td>
<td>Beta-Cyclodextrin inclusion complex</td>
<td>~10% radical inhibition increase</td>
<td>Tommasini et al., 2005</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>Sodium alginate-sodium carboxymethyl cellulose macroparticle hydrogel beads</td>
<td>Stable in simulated intestinal conditions.</td>
<td>Tsirigotis-Maniecka et al., 2017</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>Solid lipid nanoparticles (precirol, span 80, and Poloxamer 188)</td>
<td>Physically stable after 6 month storage in 25 ± 1 C</td>
<td>Shahraki et al., 2023</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>Lipid-polymer hybrid nanoparticles with lecithin, chitosan, and poly vinyl alcohol</td>
<td>~92.8% entrapment efficiency, ~80% drug release, increased radical inhibition</td>
<td>Jangde et al., 2022</td>
</tr>
<tr>
<td>Hesperetin</td>
<td>Pea protein-high methoxyl pectin electrolyte complex</td>
<td>Significant improvement of water dispersibility, bioaccessibility, and antioxidant activity</td>
<td>Caballero et al., 2022</td>
</tr>
<tr>
<td>---------------</td>
<td>----------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Hesperetin</td>
<td>Proposomal gel (propylene glycol liposomes with lecithin)</td>
<td>Enhanced In vitro drug release to ~95% and effective radical inhibition</td>
<td>Vaz et al., 2021</td>
</tr>
<tr>
<td>Hesperetin</td>
<td>Hydroxypropyl-Beta-Cyclodextrin inclusion complex</td>
<td>Improved antitumor effect on non-small cell lung cancer</td>
<td>Wang et al., 2023</td>
</tr>
<tr>
<td>Hesperetin</td>
<td>Beta-Cyclodextrin inclusion complex</td>
<td>~25% radical inhibition increase</td>
<td>Tommasini et al., 2005</td>
</tr>
<tr>
<td>Naringin</td>
<td>Biopolymer nanoparticles from Beta-lactoglobulin-amylose-linoleic acid</td>
<td>~78% encapsulation efficiency, stable in simulated gastric fluid, prolonged release SIF</td>
<td>Feng et al., 2017</td>
</tr>
<tr>
<td>Naringin</td>
<td>DPPC/Chol/DSPE-020CN Liposomes</td>
<td>Significant inhibition of proinflammatory markers</td>
<td>Mohanty et al., 2020</td>
</tr>
<tr>
<td>Naringenin</td>
<td>Lactoferrin inclusion complex</td>
<td>Reduced overall bitterness</td>
<td>Nunes et al., 2020</td>
</tr>
<tr>
<td>Naringenin</td>
<td>O/W microemulsion with triacetin, CRH40, and PEG400</td>
<td>Significantly improved drug absorption by 1.95 fold and prolonged release</td>
<td>Ma et al., 2022</td>
</tr>
<tr>
<td>Naringenin</td>
<td>Polylacticco-glycolic acid</td>
<td>Significant decrease of degenerative neurons from histopathology in paraquat-induced rats</td>
<td>Dashputre et al., 2023</td>
</tr>
<tr>
<td>Naringenin</td>
<td>Whey Protein Isolate inclusion complex</td>
<td>~50% increase in antioxidant activity, 73%</td>
<td>Yin et al., 2020</td>
</tr>
<tr>
<td>Polyphenol</td>
<td>Delivery System</td>
<td>Encapsulation Efficiency</td>
<td>Bioaccessibility/Properties</td>
</tr>
<tr>
<td>---------------</td>
<td>--------------------------------------------------------</td>
<td>--------------------------</td>
<td>---------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Nobiletin</td>
<td>Gelled (WPC/HPMC) O/W emulsions</td>
<td>73% In vitro bioaccessibility</td>
<td></td>
</tr>
<tr>
<td>Tangeretin</td>
<td>Zein/β-lactoglobulin protein nanoparticles</td>
<td>Improved stability of particles at low salt concentrations and pH levels</td>
<td></td>
</tr>
<tr>
<td>Tangeretin</td>
<td>Viscoelastic O/W emulsions with lecithin stabilizer</td>
<td>Crystals locked in gel</td>
<td></td>
</tr>
<tr>
<td>Neohesperidin</td>
<td>Pectin-chitosan conjugated nanoliposome</td>
<td>~64.25% encapsulation efficiency, ~75% stable in gastrointestinal conditions, increased antioxidant activity</td>
<td></td>
</tr>
</tbody>
</table>

The different nanotechnology-based delivery systems showed to be an effective method to increase the potency of these polyphenols from orange pomace, blocking their undesirable traits, and protecting from the different environmental conditions within the gastrointestinal tract. This includes an increase in radical inhibition as antioxidants, reducing their bitterness, and stability through the body for an improved bioaccessibility. Compared to liposomes, emulsions, nanoemulsions, solid lipid nanoparticles, and hydrogel, have the advantage of using food-grade biopolymers to fabricate delivery systems that can be incorporated into a wide range of commercial products and are biodegradable, natural, and label friendly (Hu et al., 2015).

Desirable nanoparticles would be measured through their particle diameter, zeta potential, and polydispersity index. The small particle diameter, high positive or negative charges, and low polydispersity index are desirable traits for an effective nanoparticle colloidal delivery system (Kashanian et al., 2011). Particle diameter can significantly
influence the in vivo absorption and distribution between the particles and cells (Li et al., 2017). Zeta potential is a measurement of how likely the particles will be electrostatically stable under certain conditions and would ideally want it higher than absolute 30 mV (Kashanian et al., 2011). The polydispersity index represents information about the homogeneity of particle size distribution and ideally should have a value less than 0.3 with a range from 0.0 for perfectly uniform particle sizes to 1.0 with a very diverse multiple particle size population (Pathak & Nagarsenker, 2019; Danaei et al., 2018). Encapsulation efficiency is described as the amount of compound that has successfully been encapsulated (Li et al., 2017). A higher encapsulation efficiency is more desired as it would signify a high amount of loading efficiency of the compound which will lead to a less likely occurrence of the challenges of these compounds like crystallization and bitterness (Caballero et al., 2022).

2.6.7. Why Protein Nanoparticles Are Selected?

The focus for the delivery system of this project will be protein nanoparticles because this type of delivery system is actively used as a tool within the functional foods and pharmaceutical fields due to their biodegradability and low toxicity (Hong et al., 2020; Jacob et al., 2018). Specifically, the proteins used for nanoparticles are of biological origin, so they are advantageous with their non-toxicity and non-immunogenicity (Hong et al., 2020). Protein nanoparticles can improve the stability of polyphenols from enzymatic degradation, which can be used in a variety of targeted therapies like lung and cancer therapy (Hong et al., 2020). There were also previous studies that have shown success in using protein nanoparticles for encapsulation of
polyphenols from orange pomace (Nallamuthu et al., 2021; Chen et al, 2014). In Nallamuthu et al.’s study, the protein nanoparticles involved naringin-loaded zein/casein nanoparticles and they studied the effects of naringin’s anti-adipogenic activity, the ability to prevent the conversion of pre-adipocytes to adipocytes that can effectively manage obesity (2021). Their main results showed that nano-forms of naringin had significantly higher efficiency on anti-adipogenesis compared to free-form naringin (Nallamuthu et al., 2021). They concluded that this delivery system of protein nanoparticles are a promising carrier of naringin for anti-adipogenesis (Nallamuthu et al., 2021). Chen et al.’s study involved tangeretin-loaded zein nanoparticles with β-lactoglobulin and tested their stability at different environmental conditions that could be incorporated into a variety of commercial products (2014). The particles showed stability to aggregation in low salt concentrations (<50 mM), pH levels higher than 5.5, and at temperatures between 30-60 °C (Chen et al., 2014). Their overall results showed successful encapsulation of tangeretin that protected tangeretin from the aqueous environment and would be able to be incorporated into aqueous-based food products (Chen et al., 2014).

2.7. Antisolvent Precipitation to Create Nanoparticles

One proven method for the successful creation of protein nanoparticles is called antisolvent precipitation. This method primarily involves the solute of interest, a solvent for dissolving the solute, and an anti-solvent that does not dissolve the solute. Antisolvent precipitation is a common method used by dissolving the solute with the solvent and then mixing in the anti-solvent to induce supersaturation on the solute, create nucleation, and
ultimately generate nanoparticles. (Joye and McClements, 2013). Figure 5 shows a visual representation of the nanoparticle formation through antisolvent precipitation. Different conditions and specificity of materials such as solvent/anti-solvent ratio, speed of the mixing rate, concentration of the solute, and mixing sequence, are required for the generation of nanoparticles (Li et al., 2017). A study by Li et al. showed the different consequences of changing these parameters when making coumarin-loaded zein/sodium caseinate nanoparticles (2017). High solvent/antisolvent ratio can result in large particle diameter and a high polydispersity index and decreased mixing speed led to increased particle size (Li et al., 2017). Increasing the solute concentration led to larger particle diameter with broader size distributions while mixing sequences can influence faster or slower nucleation which can possibly lead to larger particles for faster nucleation (Li et al., 2017).

![Figure 5](image-url): Visual representation of antisolvent precipitation to generate nanoparticles (Joye & McClements, 2013 with special permission from ScienceDirect Journals).

2.8. Zein Protein

Zein is a prolamin storage protein found as a main byproduct in the corn-processing industry (Gianazza et al., 1977). Zein has an abundant number of amino acids
with non-polar side chains, thus it is only soluble in aqueous alcohol solutions (Jafari, 2019). The plentiful amount of non-polar amino acid side chains allows the hydrophobic compounds like the polyphenols from orange pomace to be encapsulated (Tortorella et al., 2021). The amino acid profile of zein protein showing the amount of nonpolar and polar amino acids is shown in Table 3. Zein protein bodies consist of α-zein (most abundant at 80%), β-zein, γ-zein, and δ-zein (Kim et al., 2002).

**Table 3:** Amino acid profile and composition of zein (Zhang et al., 2017).

<table>
<thead>
<tr>
<th>Amino Acid Class</th>
<th>Amino Acid</th>
<th>ng·mg⁻¹</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonpolar</td>
<td>Glycine</td>
<td>6,514.847</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Valine</td>
<td>17,211.984</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>Isoleucine</td>
<td>17,782.280</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Phenylalanine</td>
<td>39,886.276</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td>57,419.553</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>Proline</td>
<td>56,749.686</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>118,507.485</td>
<td>19.8</td>
</tr>
<tr>
<td>Sulfur Groups</td>
<td>Methionine</td>
<td>6,565.298</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Cysteine</td>
<td>7,857.047</td>
<td>1.3</td>
</tr>
<tr>
<td>Alcohol Groups</td>
<td>Threonine</td>
<td>15,271.866</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Serine</td>
<td>31,716.602</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>Tyrosine</td>
<td>29,833.976</td>
<td>5.0</td>
</tr>
<tr>
<td>Acidic</td>
<td>Glutamic Acid</td>
<td>150,275.829</td>
<td>25.1</td>
</tr>
<tr>
<td></td>
<td>Aspartic Acid</td>
<td>31,076.188</td>
<td>5.2</td>
</tr>
<tr>
<td>Basic</td>
<td>Histidine</td>
<td>5,043.198</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>428.899</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Arginine</td>
<td>7,262.791</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Zein nanoparticles are resistant to digestive enzymes which leads to a more controlled release of functional molecules (Patel and Velikov, 2014). Zein protein is also used for the protection of sensitive bioactive compounds from environmental stresses and digestive enzymes and is biodegradable (Jafari, 2019; Wang et al., 2018; Hu &
McClements, 2016). The zein protein is also low cost and is identified as generally safe by the U.S. Food and Drug Administration (Weissmueller et al., 2016). These properties from zein protein are desirable for their reduced toxicity in the environment and to humans for an eco-friendlier carrier system (Oliveira et al., 2018). Zein as the protein for nanoparticle colloidal delivery systems has shown to be an effective encapsulant for hydrophobic compounds, as shown in Table 4. This includes increasing digestive stability (Cheng et al., 2019), antioxidant activity (Wang et al., 2018), and an overall improvement on the stability of nanoparticles (Chen et al., 2014; Patel et al., 2012). However, the overall stability of zein nanoparticles is not possible without some sort of coating. This is due to zein’s overall hydrophobicity from its abundant amount of non-polar amino acids that only allows itself to be soluble in alcohol and fosters flocculation in aqueous environments (Smruthi et al., 2022).

**Table 4:** Summary of different zein nanoparticle colloidal delivery systems using the antisolvent precipitation method.

<table>
<thead>
<tr>
<th>Encapsulated Compound</th>
<th>Coating Used on Zein Nanoparticles</th>
<th>Particle Results</th>
<th>Overall Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apigenin</td>
<td>Leciithin</td>
<td>PD: 189.3 ± 2.2 nm ZP: 24.7 ± 1.6 mV PDI: 0.43 ± 0.02</td>
<td>Stability against sunlight irradiation</td>
<td>Poureini et al., 2021</td>
</tr>
<tr>
<td>Beta-Carotene</td>
<td>Carboxymethyl Chitosan + Tea Polyphenols</td>
<td>PD: 70.41 ± 0.67 nm ZP: -45.1 ± 0.19 mV PDI: 0.195 ± 0.05</td>
<td>~92% encapsulation efficiency ~40% increase in antioxidant activity</td>
<td>Wang et al., 2018</td>
</tr>
<tr>
<td>Coumarin</td>
<td>Sodium Caseinate</td>
<td>PD: 168 ± 1.60 nm</td>
<td>Prolonged drug release</td>
<td>Li et al., 2017</td>
</tr>
<tr>
<td>Compound</td>
<td>Coating</td>
<td>PD</td>
<td>ZP</td>
<td>PDI</td>
</tr>
<tr>
<td>-------------------</td>
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<td>---------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Cannabidiol</td>
<td>Whey Protein</td>
<td>PD:140 – 160 nm ZP: ~(-40 mV) PDI: 0.18</td>
<td>Improved oral bioavailability</td>
<td>Wang et al., 2022</td>
</tr>
<tr>
<td>Lutein</td>
<td>N/A</td>
<td>PD:72.1 ± 25.3 nm ZP: N/A PDI: N/A EE: 61.39% ± 6.6455</td>
<td>~58% increase digestive stability</td>
<td>Cheng et al., 2019</td>
</tr>
<tr>
<td>N/A</td>
<td>Tween 80</td>
<td>PD:~80 nm ZP: ~15 mV PDI: 0.13</td>
<td>Stable particles at low pH 2.5-4.0 and pH 6.5-8.0</td>
<td>Hu &amp; McClemments, 2014</td>
</tr>
<tr>
<td>Quercetin</td>
<td>Sodium Caseinate</td>
<td>PD:132.2 nm ZP: -40.6 mV PDI: 0.1</td>
<td>Enhanced stability to alkaline pH and UV irradiation</td>
<td>Patel et al., 2012</td>
</tr>
<tr>
<td>Tangeretin</td>
<td>Beta-lactoglobulin</td>
<td>PD:249 ± 4 nm ZP: ~(-15 mV) PDI: N/A</td>
<td>Improved stability of particles</td>
<td>Chen et al., 2014</td>
</tr>
<tr>
<td>Taxifolin</td>
<td>Sodium Caseinate</td>
<td>PD:168.74 ± 0.35 nm ZP: -57.67 ± 0.25 mV PDI: 0.13 ± 0.02 EE: 85.83 ± 0.89 %</td>
<td>Enhanced bioavailability within rats</td>
<td>Li et al., 2023</td>
</tr>
</tbody>
</table>

*Abbreviations: PD = Particle Diameter, ZP = Zeta Potential, PDI = Polydispersity Index, EE = Encapsulation Efficiency

### 2.9. Pectin as a Coating for Protein Nanoparticles

Pectin is an anionic polysaccharide that consists of galacturonic acid and methylated galacturonic acid (Chan et al., 2017). Commercial pectin can have different
classifications depending on its degree of methylation and can either be low methoxy pectin with <50% degree of methylation or high methoxy pectin with >50% degree of methylation (Chan et al., 2017). Pectin is used within the food industry as a gelling agent, thickening agent, or stabilizer and can also be utilized as a coating for biopolymer nanoparticles (Chan et al., 2017; Huang et al., 2017). For this project, pectin has proven to help further improve the stability of nanoparticles by strong electrostatic and steric repulsion that helps prevent aggregation of particles (Table 5). The addition of coatings further helps biopolymer nanoparticles in becoming more reliable as a vehicle for bioactive delivery within the food and pharmaceutical industries (Huang et al., 2017). Moreover, the source of pectin can also be from orange pomace, which further adds more value to the waste of oranges (Fernández-Fernández, 2020).

Table 5: Summary of stability of nanoparticles with pectin coatings.

<table>
<thead>
<tr>
<th>Encapsulated Compound</th>
<th>Nanoparticle Composition</th>
<th>Overall Nanoparticle Stability</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hesperetin</td>
<td>Pea protein-high methoxyl pectin electrostatic complex</td>
<td>Reduced particle aggregation with higher pectin concentration</td>
<td>Caballero et al., 2022</td>
</tr>
<tr>
<td>Curcumin</td>
<td>Iron oxide nanoparticles with pectin coating</td>
<td>ZP w/out Pectin: -4.55mV ZP w/ pectin: -35.9mV More stable particles with pectin coating</td>
<td>Ganguly et al., 2017</td>
</tr>
<tr>
<td>Curcumin</td>
<td>Zein/caseinate nanoparticles with pectin coating</td>
<td>Significantly improved stability under simulated gastrointestinal tract</td>
<td>Chang et al., 2017</td>
</tr>
<tr>
<td>Quecertin</td>
<td>Hordein nanoparticles with pectin coating</td>
<td>Hordein w/out pectin: 56.5 ± 0.2 %</td>
<td>Zhang et al., 2023</td>
</tr>
</tbody>
</table>
2.10. Bioaccessibility Through *In Vitro* Digestion

Bioaccessibility is defined as the compounds that are released from food and into the digestive tract and the number of compounds that are potentially available for absorption or bioavailable (Quirós-Sauceda et al., 2014). Followed by bioaccessibility is bioavailability, which is the ingested compound that reaches the blood circulation and goes to specific sites where the compounds can exert its functions (Bohn et al., 2017). Measurement of the bioaccessibility from phenolic compounds is most common through the processes of simulated digestion from In vitro gastrointestinal tract (Carbonell-Capella et al., 2014). *In vitro* conditions for digestion, studies are preferred since they are very useful in predicting outcomes of *in vivo* digestion and *in vivo* studies are not always financially and ethically possible using human/animal subjects (Bohn et al., 2017). In addition, the measurement of *in vitro* bioaccessibility well reflects results for in vivo bioavailability while measuring bioavailability first can be difficult in determining bioaccessibility. *In vitro* bioaccessibility is a measurement of the concentration of bioactive and lipophilic compounds that are incorporated into mixed micelles, composed of bile salts, at the end of a simulated intestinal phase with similar physiological conditions (Hur et al., 2011; McClements & Li, 2010). For *in vitro* digestions, samples are exposed to physiological conditions that match the human gastrointestinal tract. *In vitro* digestion goes through the mouth, stomach, small intestines, and then the colon and a general schematic are shown in figure 6 (McClements & Li, 2010). These physiological conditions primarily include the addition of simulated gastric fluid, simulated intestinal
fluids, bile salts, and digestive enzymes like pepsin and pancreatin at different In vitro digestion stages (Davidov-Pardo et al., 2015). With the measurement of bioaccessibility through *in vitro* digestion from hesperetin that is encapsulated in zein, this should measure the polyphenols’ stability in simulated gastric and intestinal phases.

**Figure 6:** General schematic diagram of the conditions present within different regions of the human gastrointestinal tract (McClements & Li, 2010; with special permission from Royal Society of Chemistry).
3. MAIN GOAL AND SPECIFIC OBJECTIVES

Main Goal: This research aimed to assess the impact of encapsulation in protein nanoparticle colloidal delivery systems made from zein protein and pectin on hesperetin’s dispersibility, antioxidant activity, and in vitro bioaccessibility.

Specific Objective 1: To determine the effects of hesperetin addition on zein nanoparticle characteristics.

The first objective aimed to optimize the concentrations of hesperetin for the formation of zein protein nanoparticles. The dependent and independent variables for this objective are shown in table 6.

Table 6: Independent and dependent variables used for specific objective #1.

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Dependent Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final hesperetin concentration (0, 166, 333, 500, 666, and 833 ug/mL)</td>
<td>Particle diameter</td>
</tr>
<tr>
<td></td>
<td>Zeta-potential</td>
</tr>
<tr>
<td></td>
<td>Polydispersity index</td>
</tr>
<tr>
<td></td>
<td>Encapsulation efficiency</td>
</tr>
</tbody>
</table>

H₀₁: There will be no significant difference in the particle diameter, zeta-potential, or encapsulation efficiency of hesperetin-loaded zein nanoparticles at different final hesperetin concentrations.

H₁: There will be a significant difference in the particle diameter, zeta-potential, or encapsulation efficiency of hesperetin-loaded zein nanoparticles at different final hesperetin concentrations.
Specific Objective 2: To determine the effects of pectin coating on the characteristics of hesperetin loaded nanoparticles.

The second objective aimed to assess the effect of a pectin coating onto zein nanoparticles for an optimal pectin concentration. The dependent and independent variables for this objective are shown in table 7.

**Table 7: Independent and dependent variables for specific objective #2**

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Dependent Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final pectin concentrations (0, 0.5, 1, 1.5, 2, and 2.5 mg/mL)</td>
<td>Particle diameter</td>
</tr>
<tr>
<td></td>
<td>Polydispersity index</td>
</tr>
<tr>
<td></td>
<td>Zeta-potential</td>
</tr>
</tbody>
</table>

$H_02$: There will be no significant difference in the particle diameter or zeta-potential of pectin coated zein nanoparticles at different final pectin concentrations.

$H_{A2}$: There will be a significant difference in the particle diameter or zeta-potential of pectin coated zein nanoparticles at different final pectin concentrations.
Specific Objective 3: To characterize the chemical interactions of hesperetin, zein, and pectin within nanoparticles.

The third objective aimed to characterize the interactions between hesperetin, zein, and pectin. There is no statistical hypothesis for Table 8. The dependent and independent variables are shown in tables 9 for the statistical hypothesis in this objective.

Table 8: Samples and results obtained with fluorescence spectrometer and Fourier-Transform Infrared (FTIR) for specific objective #3.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Results Obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final hesperetin concentrations (0, 5, 10, 20, 40, 60, 80, and 100 µM) in 25 µM zein</td>
<td># of binding sites</td>
</tr>
<tr>
<td>Hesperetin</td>
<td>Binding Constant</td>
</tr>
<tr>
<td>Pectin</td>
<td>FTIR Peak Shifts</td>
</tr>
<tr>
<td>Zein nanoparticles</td>
<td></td>
</tr>
<tr>
<td>Zein/pectin nanoparticles</td>
<td></td>
</tr>
<tr>
<td>Hesperetin-loaded zein nanoparticles</td>
<td></td>
</tr>
<tr>
<td>Hesperetin-loaded zein/pectin nanoparticles</td>
<td></td>
</tr>
</tbody>
</table>

Table 9: Independent and dependent variables used for specific objective #3.

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Dependent Variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Encapsulated and non-encapsulated hesperetin with zein nanoparticles</td>
<td>Free Radical Scavenging %</td>
</tr>
<tr>
<td>Coated and noncoated hesperetin-loaded zein nanoparticles with high-methoxyl pectin</td>
<td></td>
</tr>
</tbody>
</table>

H_03: There will be no significant difference in the free radical scavenging % of hesperetin in either both or one of dependent variables; encapsulated or non-encapsulated hesperetin with zein nanoparticles, or coated/noncoated nanoparticles with high-methoxyl pectin.
HA3: There will be significant difference in the free radical scavenging % of hesperetin in either both or one of dependent variables; encapsulated or non-encapsulated hesperetin with zein nanoparticles or coated/noncoated nanoparticles with high-methoxyl pectin.

Specific Objective 4: To compare the stability of HZ and HZP nanoparticles at different environments.

The fourth objective aimed to assess the stability of HZ and HZP nanoparticles at different common environmental conditions found within foods. The dependent and independent variables for this objective are shown in Table 10.

Table 10: Independent and dependent variables used for specific objective #4.

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Dependent Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Storage in 4C (0, 7, 14, 21, and 28 days)</td>
<td>Particle diameter</td>
</tr>
<tr>
<td>pH (2, 3, 4, 5, 6, 7, and 8)</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>Temperature (40, 50, 60, 70, 80, and 90°C)</td>
<td>Zeta-potential</td>
</tr>
<tr>
<td>NaCl Concentration (0, 25, 50, 75, 100, and 125 mM)</td>
<td></td>
</tr>
<tr>
<td>Coated and noncoated hesperetin-loaded zein nanoparticles with high-methoxyl pectin</td>
<td></td>
</tr>
</tbody>
</table>

H04: There will be no significant difference in particle diameter or zeta-potential of coated/noncoated hesperetin-loaded zein nanoparticles with high-methoxyl pectin at different storage days, pH, temperature, or NaCl concentrations.

H04: There will be significant difference in particle diameter or zeta-potential of coated/noncoated hesperetin-loaded zein nanoparticles with high-methoxyl pectin at different storage days, pH, temperature, or NaCl concentrations.
Specific Objective 5: To compare the bioaccessibility of hesperetin encapsulated in zein nanoparticles with and without pectin coating.

The aim of the fifth objective was to measure the bioaccessibility of free and encapsulated hesperetin that goes through in vitro digestions, which consists of gastric and intestinal phases. The concentration of hesperetin solubilized in intestinal fluid at the end of the intestinal phase was used as a measure of bioaccessibility. The independent and dependent variables for this objective are shown on table 11.

Table 11: Independent and dependent variables used for specific objective #5.

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Dependent Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Encapsulated and nonencapsulated hesperetin with zein nanoparticles</td>
<td>Particle diameter distribution and zeta potential after each In vitro gastrointestinal stage</td>
</tr>
<tr>
<td>Coated and noncoated nanoparticles with high-methoxyl pectin</td>
<td>Concentration of hesperetin in micelle phase</td>
</tr>
</tbody>
</table>

H₀₅: There will be no significant difference(s) in particle diameter or zeta-potential after each in vitro gastrointestinal state or hesperetin concentration in micelle phase in either both or one of dependent variables; encapsulated or non-encapsulated hesperetin with zein nanoparticles, or coated/noncoated nanoparticles with high methoxyl pectin.

H₅: There will be significant difference(s) in particle diameter or zeta-potential after each in vitro gastrointestinal state or hesperetin concentration in micelle phase in either both or one of dependent variables; encapsulated or non-encapsulated hesperetin with zein nanoparticles, or coated/noncoated nanoparticles with high methoxyl pectin.
4. MATERIALS, ACRONYMS, AND METHODS

4.1. Materials

Materials that were used included powdered hesperetin (98% purity) (Frontier Scientific, Logan, UT), powdered zein (Kobayashi Perfumery Co. Ltd., Japan), powdered pectin from citrus peel (galacturonic acid ≥ 74%) (Sigma Aldrich, St. Louis, MO), pepsin (from porcine gastric mucosa, ≥250 units/mg solid) (Sigma Aldrich, St. Louis, MO), pancreatin (USP) (MP Biomedicals, LLC, Solon, OH), ox bile extract (MP Biomedicals, LLC, Solon OH), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (>97% purity) (TCI, Portland, OR), crystalline L-Ascorbic acid (Sigma Aldrich, St. Louis, MO), ethanol (200 proof pure) (Koptec, King of Prussia, Pennsylvania) potassium phosphate monobasic anhydrous (VWR, Solon, OH), hydrochloric acid (Fischer Scientific, Fair Lawn, NJ), ultrapure water (resistivity, 18.4 MΩ cm), and Acetonitrile (gradient HPLC grade).

4.2. Acronyms

Table 12: Notable acronyms used throughout sections 4, 5, and 6.

<table>
<thead>
<tr>
<th>Full Word</th>
<th>Acronym</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle diameter</td>
<td>(PD)</td>
</tr>
<tr>
<td>Polydispersity index</td>
<td>(PDI)</td>
</tr>
<tr>
<td>Hesperetin-loaded zein</td>
<td>(HZ)</td>
</tr>
<tr>
<td>Hesperetin-loaded zein/pectin</td>
<td>(HZP)</td>
</tr>
<tr>
<td>High-Performance Liquid Chromatography</td>
<td>(HPLC)</td>
</tr>
<tr>
<td>Fourier-Transform Infrared</td>
<td>(FTIR)</td>
</tr>
<tr>
<td>Ascorbic Acid Equivalent</td>
<td>(AAE)</td>
</tr>
<tr>
<td>2,2-diphenyl-1-picrylhydrazyl</td>
<td>(DPPH)</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>(NaCl)</td>
</tr>
<tr>
<td>Gastrointestinal Dilution Factor</td>
<td>(GI)</td>
</tr>
<tr>
<td>Dilution Factor</td>
<td>(DF)</td>
</tr>
</tbody>
</table>
4.3. Methods

4.3.1. Zein Nanoparticle Formation

Zein nanoparticle formulations were fabricated through anti-solvent precipitation from the Smruthi et al. (2022) method with some modifications. A 3% zein solution (w/v) was made by dissolving 30 grams of zein in an 85% ethanol solution (v/v) and stirred for 1 hour at 500 rpm. This solution was then filtered and 4 mL zein solution was added to 20 mL of 5mM citrate-phosphate buffer at pH 4.0 for 2.5 minutes at 500 rpm. The solution was then put into a Buchi Rotavapor R-300 rotary evaporator (Buchi, New Castle, DE, USA) or Fisherbrand Isotemp Model 282A vacuum oven (Fisherbrand, Waltham, MA, USA) to evaporate the ethanol and then compensate for the lost volume with the 5mM citrate phosphate buffer at pH 4.0. As for loading hesperetin in zein nanoparticles, hesperetin was added to the filtered zein solution (solvent phase) before the mixture with the antisolvent phase for 1 hour at 500 rpm with final hesperetin concentrations at 0, 166, 333, 500, 666, and 833 µg/mL. Characterization of nanoparticles was performed to select the preferred polyphenol concentration. The nanoparticles that yielded the highest encapsulation efficiency (procedure 4.2.4) with the least number of changes in the samples’ particle diameter, zeta potential, and polydispersity index (procedure 4.2.2) were chosen for the optimal hesperetin concentration.

4.3.2. Characterization of Nanoparticles

A Zetasizer Nano ZS 90 (Malvern Panalytical Inc., Westborough, MA, USA) was used to determine the nanoparticles’ diameter size and polydispersity index through the
dynamic light scattering method. The Zetasizer Nano ZS 90 was also used to measure the particles’ zeta potential through the particle electrophoresis method. The samples were diluted in 5mM citrate phosphate buffer at pH 4.0 to a dilution factor of 10 for decreasing back light scattering. The Smoluchowsky model was used to measure the particles’ electrophoretic mobility. The refractive index of zein particles dispersed in water was set at 1.49 (De Boer et al., 2018).

4.3.3. Hesperetin Quantification

Table 13: HPLC gradient flow profile of A and B for hesperetin quantification.

<table>
<thead>
<tr>
<th>Time</th>
<th>100% Acetonitrile (A)</th>
<th>0.1% Acetic Acid (v/v) (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20 %</td>
<td>80 %</td>
</tr>
<tr>
<td>15</td>
<td>40 %</td>
<td>60 %</td>
</tr>
<tr>
<td>20</td>
<td>40 %</td>
<td>60 %</td>
</tr>
<tr>
<td>22</td>
<td>80%</td>
<td>20 %</td>
</tr>
<tr>
<td>30</td>
<td>80 %</td>
<td>20 %</td>
</tr>
<tr>
<td>35</td>
<td>20 %</td>
<td>80 %</td>
</tr>
</tbody>
</table>

The concentration of hesperetin was determined using an Agilent 1100 Series High-Performance Liquid Chromatography (Agilent, Santa Clara, CA, USA) with a UV detector and an autosampler. This method was based on Zheng & Zhang (2019) and Zhang et al. (2013) with some modifications. The column was an Agilent Poroshell 120 C18 column (100mm length x 4.6 mm diameter, 2.7 µm particle size). The parameters for HPLC run included a flow rate of 1 mL/min, UV detector at 280 nm, injection volume of 50 uL, a mobile phase of 100% Acetonitrile (v/v) as A and 0.1% acetic acid solution (v/v) with Milli-Q Ultrapure water for B. The flow composition was set to a gradient flow as shown in table 13 for a total of 30 minutes. A calibration curve of hesperetin was made with 85% ethanol (v/v) at ranges of concentrations between 0.1-20 µg/mL. Samples
were filtered with 0.2 uM filters before HPLC runs. The chromatograms were saved for recording the peaks’ retention times and peak areas in the creation of a calibration curve for hesperetin.

### 4.3.4. Encapsulation Efficiency of Hesperetin

The encapsulation efficiency of hesperetin within nanoparticles was based on Cuevas-Bernardino et al.’s (2018) method with some modifications. Two mL of the nanoparticle samples were transferred to an Amicon Ultra-2 Centrifugal Filter Unit with 10 kDa molecular weight cut off (Millipore Sigma, Burlington, MA, USA) and centrifuged with an Allegra 21R centrifuge (Beckman Coulter Inc., Palo Alto, CA, USA) at 4,000 rpm for 2 hours. This method was used to separate the unencapsulated hesperetin (filtrate) from the encapsulated polyphenol (retained in the filter). The nanoparticles containing the hesperetin (total polyphenol) and filtrate (free polyphenol) were dissolved in 85% ethanol (v/v) in a dilution factor of 50, which was then filtered with 0.2 uM filter before running through HPLC following the procedure described in section 4.2.3. The encapsulation efficiency of the polyphenol was determined based on the equation below:

\[
Encapsulation\ Efficiency\ (%) = (1 - (H_f/H_t)) \times 100\%
\]

From this equation, \(H_f\) is the concentration of free hesperetin (ug/mL) and \(H_t\) is the concentration of the total hesperetin (ug/mL).

### 4.3.5. Coating of Nanoparticles with Pectin

A pectin stock solution (10 mg/mL) was made by dissolving it in deionized water with magnetic stirring for 1 hour and then stored at 4 °C until ready to use. The stock
solutions were then diluted (0, 1, 2, 3, 4, and 5 mg/mL pectin) with deionized water before the coating of zein nanoparticles. Zein nanoparticles and the diluted pectin solutions were mixed in a 1:1 ratio (final concentrations of pectin at 0, 0.5, 1, 1.5, 2, and 2.5 mg/mL) with constant stirring at 500 rpm for 20 minutes and at pH 4.0 (Smruthi et al., 2022). The characterization of the pectin-coated nanoparticles followed the protocol from section 4.2.2. The nanoparticles were then stored at 4 °C refrigeration for future use. Minimum pectin concentration was selected based on zein-pectin nanoparticle’s characteristics (particle diameter, PDI, and zeta-potential) that yielded the most minimal changes after 7-day storage in 4 °C, centrifugation after storage in 4 °C, and pH adjustment to zein nanoparticle’s pI at 5.0.

4.3.6. Chemical Characterization

4.3.6.1 Fourier Transform Infrared (FTIR) Characterization

The FTIR method was based on Smruthi et al. (2022) with some modifications. FTIR spectra of hesperetin, pectin and freeze-dried zein nanoparticles, zein/pectin nanoparticles, hesperetin-loaded zein (HZ) nanoparticles, and hesperetin-loaded zein/pectin (HZP) nanoparticles were read on a Thermo Scientific Nicolet iS10 scanner (Fisher Scientific, Chino, CA, USA). The freeze-dried samples were placed on to the instrument until its infrared sensors were fully covered by the samples. The scanning range was set to 4000 to 400 cm⁻¹ at 4 cm⁻¹ resolution.
4.3.6.2. Fluorescence Spectroscopy Characterization

The binding of polyphenols with zein protein was determined by fluorescence measurements based on the Cuevas-Bernardino et al. (2018) method with some modifications. The fluorescence emission spectra of 0.6 mg/mL zein solutions (w/v) (85% ethanol (v/v)) with different concentrations of hesperetin (0-100 µM) were recorded with a fluorescence spectrophotometer (Cary Eclipse, Varian NMR Instruments, Palo Alto, CA, USA) from 300 to 450 nm with an excitation wavelength of 280 nm at room temperature (25°C). Both the excitation and emission slit widths were set at 2.5 nm. Table 14 below represented the compositions of different hesperetin concentrations measured.

Table 14: Composition of different hesperetin concentrations for fluorescence spectrometer measurements with 85% ethanol (v/v) and 3% zein (w/v).

<table>
<thead>
<tr>
<th>Hesp. Conc. (uM)</th>
<th>Stock Hesp. (250uM) w/ 85% ethanol</th>
<th>85% Ethanol</th>
<th>30 mg/mL (25uM assuming 24kDa) Zein Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 uL</td>
<td>4.9 mL</td>
<td>100 uL</td>
</tr>
<tr>
<td>5</td>
<td>100 uL</td>
<td>4.8 mL</td>
<td>100 uL</td>
</tr>
<tr>
<td>10</td>
<td>200 uL</td>
<td>4.7 mL</td>
<td>100 uL</td>
</tr>
<tr>
<td>20</td>
<td>400 uL</td>
<td>4.5 mL</td>
<td>100 uL</td>
</tr>
<tr>
<td>40</td>
<td>800 uL</td>
<td>4.1 mL</td>
<td>100 uL</td>
</tr>
<tr>
<td>60</td>
<td>1.2 mL</td>
<td>3.7 mL</td>
<td>100 uL</td>
</tr>
<tr>
<td>80</td>
<td>1.6 mL</td>
<td>3.3 mL</td>
<td>100 uL</td>
</tr>
<tr>
<td>100</td>
<td>2.0 mL</td>
<td>2.9 mL</td>
<td>100 uL</td>
</tr>
</tbody>
</table>

The binding constant and number of binding sites of hesperetin with zein were calculated from maximum fluorescence of zein according to the Stern-Volmer equation. The equilibrium between the free and bound polyphenols was given by the equation below:

\[
\log \left( \frac{F_0 - F}{F} \right) = \log K_b + n \log[H]
\]
From this equation, Fo is the maximum fluorescence intensity of zein in the absence of hesperetin and F is the maximum fluorescence intensity of zein in the presence of hesperetin at different concentrations. Kb is the binding constant, [H] is the concentration of hesperetin, and n is the number of binding sites.

4.3.6.3. Antioxidant Activity

The antioxidant activity of 250 µg/mL hesperetin in deionized water, 250 µg/mL hesperetin in 85% ethanol (v/v), HZ nanoparticles, and HZP nanoparticles were measured using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) decolorization assay method from Floegel et al. (2011) with some modifications. A calibration curve with ascorbic acid was set as the standard and set up before running samples. A 58uM DPPH solution was created in 100% methanol and then wrapped in aluminum foil until ready to use. Then 60 µl of each sample, and control (60 µl of deionized water), was mixed with 2.94 mL DPPH solution. Once mixed, the samples were incubated in the dark for 30 minutes. The absorbance of each sample was measured at 517 nm using a UV-1600PC UV-Vis Spectrophotometer (VWR, Pennsylvania, USA). The radical scavenging activity was calculated with the formula below:

\[ \text{Radical Scavenging Activity (\%)} = \left( \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \right) \times 100 \]

The antioxidant activity was reported as Ascorbic Acid Equivalents (µg/mL AAE), after fitting the radical scavenging values into a calibration curve using Ascorbic Acid (5–65 µM).
4.3.7. Environmental Stability of HZ and HZP Nanoparticles

Incorporation of these nanoparticles into food products will be exposed to a variety of environments like long-term refrigerator stability, pH, high temperatures, and metal ions like sodium, (Liu & Qin et al., 2021; Pascoli et al., 2018). Stability tests were performed on HZ and HZP nanoparticles to measure their stability in a variety of environments found in foods. Due to HZP nanoparticles having a final concentration of 250 ug/mL hesperetin after the addition of pectin coating, HZ nanoparticles were diluted 1-fold with 5mM citrate phosphate buffer at pH 4.0 to also have the final hesperetin concentration at 250 ug/mL.

4.3.7.1. Storage Stability

The storage stability of HZ and HZP nanoparticles was evaluated based on changes in particle diameter, PDI, and zeta potential using the method described in section 4.3.2, on the day of production at room temperature and after each week within a span of one month of storage at 4°C.

4.3.7.2. pH Stability

The pH stability method was based on Dai et. al. (2019) with some modifications. Freshly prepared HZ and HZP nanoparticles were adjusted from pH=2 to pH=8 (2, 3, 4, 5, 6, 7, and 8) with 0.1 M hydrochloric acid and 0.1 M sodium hydroxide. Each of the samples at different pH were stored at 4°C overnight and then diluted in 5mM citrate-phosphate buffer adjusted to the respective pH for the measurements of their particle diameter, PDI, and zeta-potential.
4.3.7.3. Temperature Stability

The temperature stability method was based on Khan et. al. (2021) with some modifications. Freshly prepared HZ and HZP nanoparticles were incubated in a water bath at different temperatures (40, 50, 60, 70, and 80, 90 °C) for 60 mins and then stored in 4°C overnight. The nanoparticles were then evaluated for their particle diameter, PDI, and zeta-potential.

4.3.7.4. Salt Stability

The salt stability method was based on Khan et. al. (2021) with some modifications. Freshly prepared HZ and HZP nanoparticles were exposed to sodium chloride (25, 50, 75, 100, and 125 mM concentrations) under continuous mixing for 30 min. followed by a 5 min. rest. The samples were stored at 4°C overnight and then measured for changes in their particle diameter, PDI, and zeta-potential.

4.3.8. In Vitro Digestion

*In vitro* digestion followed an established protocol from Caballero (2021) for simulated gastric and simulated intestinal phases, which was adopted from Brodkorb et al. (2019) with some modifications. The oral phase was omitted from in vitro digestion because liquid solutions are expected to be present in the mouth for a short amount of time before traveling to the gastric phase (Guo et al., 2020). The simulated gastric phase consisted of simulated gastric fluid and pepsin enzyme (equal to 2000 U/mL) at pH=3 and 37 °C for 1 hour while the simulated intestinal phase consisted of simulated intestinal fluid, pancreatin (equal to 100 U/mL for trypsin) enzymes, and bile salts (equal to 52
mg/mL) at pH=7 and 37 °C for 2 hours. The compositions of the simulated gastric and simulated intestinal phases, along with the simulated gastric fluid and simulated intestinal fluid, are shown in tables 15 and 16, respectively. Once the simulated intestinal phase concluded, the digesta was centrifuged at 4000 rpm for 30 minutes to isolate the micelle phase.

**Table 15**: Composition of simulated gastric phase and simulated intestinal phase for in vitro digestion of nanoparticles (Caballero, 2021).

<table>
<thead>
<tr>
<th>Simulated Gastric Phase (adjusted to pH=3 with 0.5M HCl)</th>
<th>Simulated Intestinal Phase (adjusted to pH=7 with NaOH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mL sample</td>
<td>20 mL from simulated gastric phase*</td>
</tr>
<tr>
<td>8 mL simulated gastric fluid</td>
<td>8 mL simulated intestinal fluid</td>
</tr>
<tr>
<td>0.0005 mL CaCl$_2$·2H$_2$O</td>
<td>0.04 mL CaCl$_2$·2H$_2$O</td>
</tr>
<tr>
<td>1.147 mL pepsin (2000 U/mL)</td>
<td>5 mL pancreatin (100 U/mL)</td>
</tr>
<tr>
<td>0.448 mL deionized water</td>
<td>3 mL bile salts (52 mg/mL)</td>
</tr>
<tr>
<td>0.4 mL 0.5M HCl</td>
<td>3.96 mL Deionized Water</td>
</tr>
</tbody>
</table>

Note: Composition of simulated gastric and simulated intestinal phase is adopted from Caballero (2021).

*After the simulated gastric phase, a 3-mL aliquot of each sample was taken for analysis and replaced with deionized water, which was diluted by a factor of 1.2 (Caballero, 2021).
Table 16: Composition of simulated gastric fluid and simulated intestinal fluid for simulated gastric and simulated intestinal phase (Caballero, 2021).

<table>
<thead>
<tr>
<th></th>
<th>Simulated Gastric Fluid (SGF) (0.4L)</th>
<th>Final Salt Concentration in SGF</th>
<th>Simulated Intestinal Fluid (SIF) (0.4L)</th>
<th>Final Salt Concentration in SIF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M KCl</td>
<td>6.9 mL</td>
<td>6.9 mM</td>
<td>6.8 mL</td>
<td>6.8 mM</td>
</tr>
<tr>
<td>0.5 M KH₂PO₄</td>
<td>0.9 mL</td>
<td>0.9 mM</td>
<td>0.8 mL</td>
<td>0.8 mM</td>
</tr>
<tr>
<td>1 M NaHCO₃</td>
<td>12.5 mL</td>
<td>25 mM</td>
<td>42.5 mL</td>
<td>85 mM</td>
</tr>
<tr>
<td>2 M NaCl</td>
<td>11.8 mL</td>
<td>47.2 mM</td>
<td>9.6 mL</td>
<td>38.4 mM</td>
</tr>
<tr>
<td>0.15 M MgCl₂(H₂O)₆</td>
<td>0.4 mL</td>
<td>0.12 mM</td>
<td>1.1 mL</td>
<td>0.33 mM</td>
</tr>
<tr>
<td>0.5 M (NH₄)₂CO₃</td>
<td>0.5 mL</td>
<td>0.5 mM</td>
<td>0 mL</td>
<td>0 mM</td>
</tr>
<tr>
<td>6 M HCl</td>
<td>1.3 mL</td>
<td>15.6 mM</td>
<td>0.7 mL</td>
<td>8.4 mM</td>
</tr>
<tr>
<td>0.3 M CaCl₂*</td>
<td>0.005 mL</td>
<td>0.15 mM</td>
<td>0.04 mL</td>
<td>0.6 mM</td>
</tr>
<tr>
<td>Deionized Water</td>
<td>365.7 mL</td>
<td>0 mM</td>
<td>338.42 mL</td>
<td>0 mM</td>
</tr>
</tbody>
</table>

Note: Composition of simulated gastric and simulated intestinal phase is adopted from Caballero (2021).

*CaCl₂ was added right before the use of the simulated gastric and simulated intestinal phases.

4.3.9. Characterization of Nanoparticles During In Vitro Digestion

Aliquots were obtained before the gastric phase, after the gastric phase, after the intestinal phase, and the micelle phase. Each aliquot was analyzed for the particles’ size and zeta potential using LS230 Beckman Coulter (Beckman Coulter, Brea, CA, US) and Zetasizer Nano ZS 90 (Malvern Panalytical Inc., Westborough, MA, USA), respectively. For particle diameter analysis, samples were diluted in 5mM citrate phosphate buffers either at pH 4.0 for initial phase, pH 3.0 for gastric phase, or pH 7.0 for intestinal and micelle phase until obscuration reached 8% or PIDS reached 45%. For zeta-potential of samples was diluted 10-fold in the same buffers as described above.
4.3.10. Bioaccessibility of Hesperetin

The concentration of hesperetin was measured according to the procedure described in Section 4.3.3. This was achieved by diluting the micelle phase 40-fold with 85% ethanol (v/v) to release the entrapped polyphenols for measurement in HPLC. The bioaccessibility was calculated according to the equation below.

\[
\text{Bioaccessibility of Hesperetin} (\%) =
\left( \frac{\text{Concentration of Hesperetin in Micelle Phase}}{\text{Initial Concentration of Hesperetin Prior to Digestion}} \right) \times 100\%
\]

4.3.11. Data Analysis

One-way analysis of variance (ANOVA) along with Tukey’s post hoc test was performed using IBM SPSS statistics software for observing significant differences (p<0.05). Changes in the zein/pectin nanoparticle characteristics before and after centrifugation and pH adjustment to zein’s pI at 6.2 were determined using paired-sample t-tests. Data were presented as mean ± standard deviation as all data was conducted in triplicates.
5. RESULTS AND DISCUSSION

5.1. Optimal Hesperetin Concentration in Zein Nanoparticles

5.1.1. Particle Diameter (PD), PDI, and Zeta-Potential

Optimal hesperetin concentration was selected based on the particle diameter, PDI, zeta-potential, and highest encapsulation efficiencies on a broad range of final hesperetin concentrations (0, 166, 333, 500, 666, and 833 µg/mL). Figure 7 shows the particle diameter (a), PDI (b), and zeta-potential (c) of hesperetin-loaded zein nanoparticles at different final hesperetin concentrations (0, 166, 333, 500, 666, and 833 µg/mL).
Figure 7: Particle diameter (a), PDI (b), and zeta-potential (c) of hesperetin-loaded zein nanoparticles at different hesperetin concentrations. No significant differences (p<0.05) were observed between different final hesperetin concentrations.

Optimal hesperetin concentration was selected based on the particle diameter, PDI, zeta-potential, and highest encapsulation efficiencies on a broad range of final hesperetin concentrations (0, 166, 333, 500, 666, and 833 µg/mL). All of the nanoparticle
solutions at different hesperetin concentrations exhibited monomodal peaks in its particle diameter distribution while hesperetin-loaded zein nanoparticles showed smaller particle diameter from Figure 7a with the highest size at 179.23 ± 5.0 nm for 500 µg/mL hesperetin compared to zein nanoparticles without hesperetin at 190.13 ± 12.2 nm. This phenomenon might be due to the removal of water molecules from nanoparticles with the presence of a hydrophobic compound like hesperetin to create a more compact structure (Cuevas-Bernardo et al., 2018). This could be why there was a decrease in both particle diameter and PDI from 500 µg/mL to 833 µg/mL from figure 7b due to more water molecules expelled out of zein nanoparticles in the presence of hydrophobic compounds like hesperetin (Cuevas-Bernado et al., 2018).

All particles showed a polydispersity index (PDI) below 0.3, PDI is an indicator that represents how homogenous the particle diameter distribution is and it is presented as a range from 0 for perfectly uniform particle diameters to 1.0 with a very diverse multiple particle diameter population, which ideally should have a value less than 0.3 to show excellent homogeneity (Pathak & Nagarsenker, 2019; Danaei et al., 2018). Low PDI generally shows monodisperse and evenly sized nanoparticles with great stability while high PDI shows lower nanoparticle stability with the presence of aggregated nanoparticles (Masarudin et al., 2016).

All zeta-potentials of the nanoparticles at different hesperetin concentrations yielded positive charges above 22.0 mV from Figure 7c. This is because the nanoparticle colloidal solutions were at pH 4.0, which is lower than the isoelectric point of zein at 6.2 and carries positive surface charges from the protonation of amino groups (Wang & Fan, 2019). Nanoparticles that are highly positively charged generate electrostatic repulsion
that overtakes attractions like hydrophobic interactions that would have caused aggregation (Hu & McClements, 2014).

These results were similar to other encapsulation systems based on zein nanoparticles like for apigenin at pH 4.0 (PD=189.3 ± 2.2 nm; PDI 0.43 ± 0.02; Zeta-potential=24.7 ± 1.6 mV) (Pourieini et al., 2021) and coumarin 6 at pH 4.0 (PD=168.6 ± 1.60 nm, PDI=0.044 ± 0.01, zeta-potential=25.5 ± 2.7 mV) (Li et al., 2017).

Though different hesperetin concentrations yielded different sizes, PDI, and zeta-potentials, there aren’t any significant differences (p<0.05) among each other. Thus, the optimal hesperetin concentration was determined based on the encapsulation efficiencies.

5.1.2. Encapsulation Efficiency

Figure 8 shows an example of a chromatogram of 12.5 µg/mL hesperetin through HPLC for the calibration curve of hesperetin. Hesperetin was noticed at the elution time of 13 minutes with no other elution peaks at that time and was used to identify the peak areas of hesperetin at different concentrations for the calibration curve shown in Figure 9.

![HPLC chromatogram of 12.5 µg/mL hesperetin for hesperetin calibration curve (figure 9).](image)

**Figure 8:** HPLC chromatogram of 12.5 µg/mL hesperetin for hesperetin calibration curve (figure 9).
Figure 9: HPLC calibration curve for the quantification of hesperetin.

The encapsulation efficiency for each hesperetin concentration measured both the total amount of hesperetin within the solution and the free hesperetin that passed through the centrifugal filters based on procedure 4.2.3 to determine how much hesperetin was encapsulated by the zein nanoparticles from figure 10.

Figure 10: Encapsulation efficiencies of different final hesperetin concentrations. Lowercase letters (a, b) represent significant differences between samples.
Table 17: Concentration of free hesperetin (filtrate) after centrifugation through centrifugal 10 kDa filter.

<table>
<thead>
<tr>
<th>Hesperetin Concentration (ug/mL)</th>
<th>Free Hesperetin After Centrifugation (ug/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>166</td>
<td>24.3 ± 1.5</td>
</tr>
<tr>
<td>333</td>
<td>33.7 ± 3.5</td>
</tr>
<tr>
<td>500</td>
<td>34.35 ± 3.0</td>
</tr>
<tr>
<td>666</td>
<td>34.1 ± 2.0</td>
</tr>
<tr>
<td>833</td>
<td>32.5 ± 1.5</td>
</tr>
</tbody>
</table>

From Figure 10, the encapsulation efficiencies increased as the hesperetin concentrations increased from 166 ug/mL hesperetin with 85.09 ± 1.5 % to 833 ug/mL with 95.59 ± 0.02 %. These results were also similar to other encapsulation systems like resveratrol-loaded zein nanoparticles 70.47 ± 2.5 % (Khan et al., 2019), (hesperetin-loaded polycaprolactone and capric/caprylic triglyceride nanoparticles 98.81%±0.28%) (Duranoğlu et al., 2018), and β-carotene-loaded zein nanoparticles ~52%) (Wang et al., 2018). Based on the measurements of the free hesperetin (Table 17), 166 ug/mL hesperetin had a concentration of 0.486 ± 0.03 ug/mL and then increased to 0.687 ± 0.06 µg/mL for 500 µg/mL hesperetin with the highest concentration of the most detected free hesperetin. However, the free hesperetin decreased to 0.682 ± 0.04 µg/mL and 0.650 ± 0.03 µg/mL for hesperetin concentrations at 666 µg/mL and 833 µg/mL, respectively. This decrease in detectable free hesperetin was possibly due to the formation of complex crystals with other hesperetin molecules which ultimately get trapped within the centrifugal filters during centrifugation, which caused less detectable hesperetin and likely increased encapsulation efficiency on 666 ug/mL and 833 ug/mL hesperetin concentrations (Meneguzzo et al., 2020; Zou et al., 2016). Based on the highest true encapsulation efficiency, without evident signs of crystallization trapped within the
centrifugal filter, 500 µg/mL hesperetin was the selected concentration for the fabrication of encapsulated zein nanoparticles.

5.2. Optimal Pectin Concentration for Coating on Zein Nanoparticles

5.2.1. Particle diameter, PDI, and Zeta-Potential

Dispersions of zein nanoparticles have shown poor colloidal stability by readily forming aggregates and precipitation, which decreases their functionality (Pascoli et al., 2018; Chen & Zhong, 2014). Thus, the addition of high-methoxyl pectin to zein nanoparticles could further improve their colloidal stability. Figure 11 represents the particle diameter (a), PDI (b), and zeta-potential (c) that were measured on zein nanoparticles mixed with different pectin concentrations (0, 0.5, 1, 1.5, 2, and 2.5 mg/mL) on both the day they were made and after 1 week in 4°C storage to find an optimal pectin concentration.
**Figure 11:** Particle diameter (a), PDI (b), and zeta-potential (c) measurements of zein nanoparticles coated at different pectin concentrations from the day they are made and after 1 week storage in 4°C. *represents significant difference (p<0.05) between week 0 and week 1 samples. Uppercase (A, B) and lowercase (a, b) letters represent significant differences (p<0.05) within day 7 and day 0 samples, respectively.

The addition of HM-pectin on bare zein nanoparticles significantly increased (p<0.05) its particle diameter from 190.13 ± 12.2 nm at 0 mg/mL pectin to 355.03 ± 3.4
nm for 0.5 mg/mL pectin and even to 393 ± 9.5 nm for 2.5 mg/mL pectin. This was due to higher concentrations of anionic pectin on the dispersed nanoparticles that form thicker shells on to the nanoparticle dispersions and increased its average diameter size (Huang et al., 2019). The PDI of zein nanoparticles without and with pectin (2 mg/mL) showed low PDI at 0.12 ± 0.02 and 0.11 ± 0.03, respectively with all samples that exhibited monomodal peaks in its particle diameter distribution. All of the nanoparticles were below 0.3, which is indicative of stable nanoparticles with their monomodal peaks and with great homogeneity (Li et al., 2022). These results on the zein/pectin nanoparticle’s initial average size were similar to other works like hordein/pectin nanoparticles at pH 4.0 (PD=~360 nm and PDI= ~0.32) (Zhang et al., 2023) and zein/pectin nanoparticles at pH 4.0 (PD=~350 nm and PDI= ~0.24) (Huang et al., 2023). The addition of pectin on these protein nanoparticles formed thicker shells which increased their average diameter and PDI.

The nanoparticles’ size and PDI showed no significant differences (p>0.05) after one week after storage at 4°C. However, there was a significant difference (p<0.05) in the zeta-potential of zein nanoparticles without pectin from 22.06 ± 12.2 mV to 19.63 ± 7.3 mV after 1 week of storage while no significant differences (p<0.05) were observed at different pectin concentrations after 1 week of storage. The decrease in zeta-potential for zein nanoparticles without pectin could be due to the partial unfolding of the zein over time and likely reduced the exposure of charged amino acids present at the zein nanoparticle’s surface (Xue et al., 2022). This supports Chen & Zhong’s (2014) claim that pure zein nanoparticles are not stable over time because the decrease in zeta-potential will cause them to be more neutral in charge and more likely to aggregate amongst each
other. The zeta-potential of zein nanoparticles also shifted from $22.06 \pm 0.7$ mV without pectin to $-23.56 \pm 0.6$ mV with pectin at 0.5 mg/mL. The negatively charged surface on the zein/pectin nanoparticles is indicative of their core-shell structure due to the adsorption of anionic pectin on to the surfaces of the cationic zein nanoparticles and that not all the anionic groups from the pectin were bound to the cationic groups from the zein nanoparticles (Hu et al., 2015; Smruthi et al., 2022). Zein has a pI at 6.2 while the carboxyl groups on the pectin have a pKa at 3.5, so at pH 4.0, zein had a net positive charge and pectin had a net negative charge with electrostatic attraction as a result between these two molecule interactions (Smruthi et al., 2022; Shukla & Cheryan, 2001; Jones et al., 2010). These results on their zeta-potentials were also similar to Zhang et al.’s hordein/pectin nanoparticles at pH 4.0 (~30.0 mV) (2023) and Huang et al.’s zein/pectin nanoparticles at pH 4.0 (~21.0 mV) (2023). This also supported that electrostatic attraction occurred between the cationic protein surface with the anionic pectin groups for the formation of a core-shell structure with an overall negative surface charge on the nanoparticles.

5.2.2. Characterization of Particles After Centrifugation

Centrifugation of nanoparticles after 4°C storage was conducted to remove large particles and aggregates that may have formed with hesperetin, zein, and pectin (Liang et al., 2022). With the removal of large particles and aggregates after centrifugation, the nanoparticles should exhibit significant differences (p<0.05) before and after centrifugation with their average size decreasing with more uniformity for a more stable nanoparticle colloidal delivery system (Feng & Lee, 2016).
**Figure 12:** Particle diameter (a), PDI (b), and zeta-potential (c) of zein nanoparticles at different pectin concentrations before and after centrifugation. *represents significant difference (p<0.05) before and after centrifugation. Uppercase (A, B) and lowercase (a, b) letters represent significant difference (p<0.05) within after centrifugation and before centrifugation, respectively.

From figure 12, no significant differences (p<0.05) were observed between different pectin concentrations for both particle diameter and zeta-potential. The only significant difference observed after centrifugation was the PDI of 2 mg/mL pectin from 0.098 ± 0.01 to 0.14 ± 0.01 figure 12b. There might have been plentiful number of larger particles and the removal of them after centrifugation may have caused more diverse sizes to be noticed (Liang et al., 2022). Even though there was a significant difference shown at 2 mg/mL pectin on its PDI, they still exhibited great homogeneity below 0.3. Since most pectin-coated zein nanoparticles did not show significant differences (p<0.05) at different pectin concentrations after centrifugation for particle diameter and zeta-potential, centrifuging was not conducted on zein nanoparticle samples throughout the experiments.
5.2.3. pH Adjustment to Isoelectric Point (pI) of Zein Nanoparticles (5.0)

The isoelectric point (pI) of zein nanoparticles (starting at 5.0) is the pH value where the zein protein exhibits a low net charge close to 0 and attractive forces like hydrophobic attraction overcome electrostatic repulsion and cause aggregation (Hu & McClements, 2015). Zein nanoparticles at different pectin concentrations (0, 0.5, 1, 1.5, 2, and 2.5 mg/mL) were adjusted from pH 4.0 to zein nanoparticle’s pI at 5.0 for significant differences in their particle characteristics before and after pH adjustment. This method was selected for choosing the optimal pectin concentration since zein nanoparticles aggregate at their isoelectric point and the pectin concentration was chosen that exhibited stability at the isoelectric point with the least number of differences in its particle characteristics between pH adjustments (Huang et al., 2019). Figure 13 shows the results for the nanoparticles’ particle diameter (a), PDI (b), and zeta-potential (c).
Figure 13: Particle diameter (a), PDI (b), and zeta-potential (c) of zein nanoparticles with different pectin concentrations before and after pH adjustment to zein nanoparticle’s pI (5.0). *Represents significant differences before and after pH adjustment to 5.0. ^Represents particle diameter >1000nm.

At 0 mg/mL pectin, the nanoparticles destabilized and aggregated when adjusted to pH 5.0 along with having a zeta-potential from 19.93 ± 0.3 mV at pH 4.0 to -4.23 ± 3.2 mV at pH 5.0. This result is comparable to Hu and McClements’s (2015) where zein
nanoparticles without coating destabilized at pH 5.2 and exhibited a neutral charge (~4mV) due to a lack of electrostatic repulsion between nanoparticles. The particle diameters have shown significant differences (p<0.05) among different pectin concentrations except at 0.5 mg/mL pectin where there was no significant difference from 359.6 ± 7.0 nm to 361.26 ± 0.5 nm. In addition to that, the PDI of zein nanoparticles with pectin coatings did not show significant differences (p<0.05) after pH adjustment to the pI at 5.0. The pectin coating on to the zein nanoparticles likely kept the great homogeneity that kept them below 0.3. As for their zeta-potentials, both 0.5 mg/mL and 2 mg/mL pectin concentrations did not show significant differences from -24.06 ± 0.1 mV to -23.5 ± 0.5 mV and -24.96 ± 0.9 to -24.9 mV, respectively. However, all pectin concentrations still yield sufficient zeta-potentials since they all are below -20mV, which are still more stable than nanoparticles that are considered neutral charges between -10 and 10 mV (Clogston & Patri, 2011). Another possible reason for the pectin coated zein nanoparticle’s stability, besides electrostatic repulsion, is steric hindrance. The long chain polymers from pectin acted as an obstruction between zein nanoparticle surfaces that caused steric repulsion and helped prevented van der Waals attraction and agglomeration between other zein nanoparticles (Liu et al., 2022).

Because 0.5 mg/mL pectin concentration was the only pectin concentration that did not show significant differences in its particle diameter, PDI, and zeta-potential before and after pH adjustment to zein nanoparticle’s pI, it was selected as the optimal pectin concentration to be coated on to the 500 µg/mL hesperetin-loaded zein nanoparticles. Also, 0.5 mg/mL pectin was the selected concentration because there will be less free pectin not bound to zein nanoparticles that are susceptible to changes in
particle characteristics when exposed to a variety of changes (Smruthi et al., 2022; Hu & McClements, 2015).

5.3. Characterization of Hesperetin-Loaded Zein (HZ) and Hesperetin-Loaded Zein/Pectin (HZP) Nanoparticles

5.3.1. Particle diameter, PDI, Zeta-Potential, and Encapsulation Efficiency of HZ and HZP

With the optimal hesperetin concentration at 500 µg/mL and pectin concentration at 10 mg/mL, both the hesperetin-loaded zein (HZ) nanoparticles and hesperetin-loaded zein/pectin (HZP) nanoparticles were measured on its particle diameter, PDI, zeta-potential, and encapsulation efficiency. Their results are listed on Table 18.

Table 18: Particle diameter, PDI, zeta-potential, and encapsulation efficiency of HZ and HZP nanoparticles. Lowercase letters (a, b) represent significant difference between HZ and HZP nanoparticle’s characteristics.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Particle Diameter (nm)</th>
<th>PDI</th>
<th>Z-Potential (mV)</th>
<th>Encapsulation Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hesperetin-loaded zein (HZ)</td>
<td>174.56 ± 2.3 a</td>
<td>0.132 ± 0.01</td>
<td>24.26 ± 1.1 a</td>
<td>92.78 ± 1.0</td>
</tr>
<tr>
<td>Hesperetin-loaded zein/pectin (HZP)</td>
<td>356.83 ± 10.7 b</td>
<td>0.119 ± 0.03</td>
<td>-22.56 ± 0.4 b</td>
<td>93.97 ± 0.2</td>
</tr>
</tbody>
</table>

With the optimal hesperetin concentration at 500 µg/mL and pectin concentration at 10 mg/mL, both the hesperetin-loaded zein (HZ) nanoparticles and hesperetin-loaded zein/pectin (HZP) nanoparticles were measured on its particle diameter, PDI, zeta-potential, and encapsulation efficiency. Their results were listed on Table 18. Both HZ
and HZP nanoparticles exhibited similar results from their respective measurements in Figures 7, 10, and 11. Even with the encapsulation of hesperetin, the addition of pectin coating increased its particle diameter from $174.56 \pm 2.3$ nm to $356.83 \pm 10.7$ nm and from $24.26 \pm 1.1$ mV to $-22.56 \pm 0.4$ mV. It was previously mentioned that these changes were due to the anionic pectin attraction to the cationic zein nanoparticles that gave their respective charges and pectin forming a thicker shell around the zein, which increased its diameter size (Smruthi et al., 2022). HZP nanoparticles also showed a higher encapsulation efficiency of $93.97 \pm 0.2\%$, compared to HZ nanoparticles without pectin at $92.78 \pm 1.0 \%$. This is likely due to the pectin coating increased the binding site of hesperetin along with a stronger bond between the pectin and zein nanoparticles, which makes hesperetin less likely to turn into crystalline form (Li et al., 2022; Zou et al., 2016).

5.4. Chemical Characterization

For the chemical characterization of the delivery systems, HZ nanoparticles were diluted 2-fold with 5mM citrate phosphate buffer at pH 4.0 to get the same final hesperitin concentration at 250 µg/mL as HZP nanoparticles.

5.4.1. Hesperetin and Zein Interactions through Fluorescence Spectroscopy:

The fluorescence signal of proteins can be quenched in the presence of polyphenols, which can be used to determine interactions between the protein and polyphenol (Keppler et al., 2014). The fluorescence spectrometer was used to observe the interaction between zein and hesperetin in 85% ethanol (v/v) solutions.
Figure 14: Fluorescence spectra of zein with different hesperetin concentrations from 0 to 100 µg/mL.

From Figure 14, a strong fluorescence emission peak for zein was observed at 310 nm when excited at 280 nm, which is where the tyrosine residues are present (Rihi & Cheryan, 2001). The fluorescence intensity of zein was increasingly quenched as the hesperetin concentration increased. In addition to that, the hesperetin caused the maximum in the emission peak of zein to shift to the right from 307 nm to 315 nm, respectively. The shift indicates that changes in the molecular environment of the tyrosine groups from the zein occurred when interacting with the polyphenol (Liang et al., 2021; Chen et al., 2018). Specifically, the hesperetin interaction with the tyrosine residues of the zein protein caused the tyrosine residues to be concealed, which resulted in stronger fluorescence quenching (Yu et al., 2022)
Figure 15: Stern volmer linear plot of log[(Fo-F)/F] vs log [hesperetin (M)] for obtaining its binding constant and number of binding sites.

The binding constant of hesperetin to the fluorescent zein (K) was determined based on the equation below for static quenching (Joye et al., 2015; Acharya et al., 2013).

\[
\log \left( \frac{F_0 - F}{F} \right) = \log K + n \log[R]
\]

Both the apparent binding constant K and the number of available binding sites n were provided by plotting log[(F0-F)/F] against log[R] in figure 15 and using its intercept with the y-axis and its slope, respectively (Joye et al., 2015). The binding constant and number of binding sites were shown to be 4.94 x 10^5 M^-1 and 1.2126, respectively, which is similar to other polyphenol interactions with proteins like propolis+whey protein isolate (1.738x10^5 M^-1 + 1.30 and at 298K) (Shakoury et al., 2022) and natamycin+zein (1.147x10^5 M^-1 + 1.134 at 303K) (Wu et al., 2021). The high binding constant between hesperetin and zein likely showed a high binding affinity between hesperetin and zein within the protein’s binding site (Cueva-Bernardino et al., 2018; Joye et al., 2015). These
results likely showed that there were strong interactions involved between hesperetin and zein nanoparticles, the nature of the chemical interactions was further explored using FTIR in section 5.4.2.

5.4.2. Hesperetin, Zein, and Pectin Interactions through FTIR

Fourier-Transform Infrared (FTIR) analyses were performed to further understand the chemical bonds that occurred between hesperetin, zein, and pectin as nanoparticles. Figure 16 shows the spectra of pure hesperetin and pectin with Z, HZ, ZP, and HZP nanoparticles, respectively.
Figure 16: FTIR spectra (from top to bottom) of hesperetin, pectin, zein nanoparticles, hesperetin-loaded zein nanoparticles, zein/pectin nanoparticles, and hesperetin-loaded zein/nanoparticles.

From the spectra of hesperetin, the -OH stretching peak at 3495 cm⁻¹ shifted to 3292 cm⁻¹ for HZ and 3295 cm⁻¹ for HZP, which indicated the formation of hydrogen bonds between hesperetin, zein, and pectin (Wang et al., 2022). As for the spectra of zein, the shift in peak from 1651 cm⁻¹ (amide I band) to 1647 cm⁻¹ in HZ showed that...
electrostatic interaction between the polyphenol and zein occurred (Wang et al., 2020; Dai et al., 2018). In addition to that, characteristic hesperetin peaks at 3495, 2955, 1634, 1281, and 866 cm\(^{-1}\) were absent in HZ and HZP spectras. The absence of these peaks suggested that hesperetin was encapsulated in the nanoparticles through hydrogen bonding and hydrophobic interactions (Wang et al., 2020; Dai et al., 2017).

As for the spectra of pectin, carbohydrate peaks were present on 3331 cm\(^{-1}\) (-OH stretching), 1733 cm\(^{-1}\) (C=O stretch), and 1635 cm\(^{-1}\) (carboxylate bond), which were similar to the spectra in Smruthi et al. (2022) work. As for the spectra of zein, characteristic peaks were noticed at 3292 cm\(^{-1}\) (-OH stretching), 1651 cm\(^{-1}\) (C=O stretching), and 1539 cm\(^{-1}\) (N-H bending). The hydroxyl group stretching from pectin was shifted to 3291 cm\(^{-1}\) for ZP and 3295 cm\(^{-1}\) for HZP, respectively. This also indicated that hydrogen bond formation occurred between the hydroxyl or carboxyl groups from pectin with the amide group from zein and hesperetin as part of the formation of core-shell nanoparticle structure (Smruthi et al., 2022). In addition to that, the carboxylate bond peak from pectin shifted to 1652 cm\(^{-1}\) for ZP and 1651 cm\(^{-1}\) for HZP, respectively. This suggested that electrostatic interactions have occurred between pectin and zein nanoparticles (Smruthi et al., 2022). Based on these results, this suggest that the driving force for the formation of protein nanoparticles was non-covalent interactions (Wang et al., 2020).

### 5.4.3. Antioxidant Activity

Hesperetin is a trihydroxyflavone with three hydroxy groups and a methoxy group within its structure, which allows it to become a strong antioxidant flavonoid that relieves
oxidative damage (Kara et al., 2014; Khan et al., 2020). The hydroxyl groups from hesperetin can cause free radicals to become more stable and less reactive to other body cells that could otherwise cause damage (Panche et al., 2016). Due to hesperetin being a potent antioxidant flavonoid, a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to measure the antioxidant activity of HZ and HZP nanoparticles. Figure 17 shows the antioxidant activity calibration curve for ascorbic acid while Figure 18 shows the antioxidant activity of the samples as ascorbic acid equivalent (AAE) (µg/mL) per 1 µg/mL hesperetin of encapsulated (HZ + HZP) and unencapsulated (hesperetin+deionized water (w/v) and hesperetin+85% ethanol (v/v)) samples.

**Figure 17**: Calibration curve of ascorbic acid for free radical scavenging % equivalence to hesperetin in figure 18.
The DPPH scavenging activity of hesperetin solubilized in 85% ethanol (v/v) showed to be higher (0.13 ± 0.001 µg/mL AAE) than the encapsulated hesperetin with pectin (0.09 ± 0.008 µg/mL AAE) and without pectin (0.10 ± 0.006 µg/mL AAE). This is because some of the hesperetin molecules were bound to the biopolymers within the nanoparticles and not free to scavenge the free DPPH radicals (Arts et al., 2002; Liang et al., 2021). Based on FTIR results from 5.4.2., some of the hydroxyl groups from hesperetin were likely still bound to the zein protein and unable to scavenge the free radicals from DPPH. Both HZ and HZP nanoparticles also showed significantly higher AAE (p<0.05) compared to hesperetin mixed with water (0.03 ± 0.02 µg/mL AAE) because hesperetin has poor water dispersibility due to its insoluble crystallization in water, which leads to poor functional properties like free-radical scavenging for its antioxidant activity (Gu et al., 2019). Encapsulation of hesperetin into HZ and HZP

Figure 18: Ascorbic acid equivalence (µg/mL) per 1 µg/mL of hesperetin in a variety of hesperetin samples. Lowercase letters (a, b) represent significant differences (p<0.05) between samples.
nanoparticles have greatly improved its free-radical scavenging as opposed to hesperetin without encapsulation in water.

5.5. Environmental Stability of HZ and HZP Nanoparticles

For the environmental stability assays of the delivery systems, HZ nanoparticles were diluted 2-fold with 5mM citrate phosphate buffer at pH 4.0 to get the same final hesperetin concentration at 250 µg/mL as HZP nanoparticles. Tests were performed (4°C storage, pH, temperature, and NaCl) to show HZ and HZP nanoparticle’s potential stability under common food processing conditions.

5.5.1. Storage Stability

A storage stability test was conducted to observe the shelf-life of both HZ and HZP nanoparticles at 4°C for 28 days with weekly measurements on their particle diameter, PDI, and zeta-potential. The temperature was set to 4°C because the nanoparticles can go through destabilization at a faster rate when stored in temperatures at or higher than room temperature (25°C), which causes particle agglomeration (Xue et al., 2022; Hong et al., 2020). This temperature was also selected at 4°C to slow the growth of fungi and spoilage bacteria onto the colloidal delivery system (Erkman & Bozoglu, 2016). With this storage stability test, Figure 19 shows the particle diameter, PDI, and zeta potential of HZ and HZP nanoparticles in 7-day measurement intervals.
Figure 19: Particle diameter (a), PDI (b), and zeta-potential (c) of HZ and HZP nanoparticles at 4°C at different days. Uppercase (A, B) and lowercase letters (a, b) represent significant differences (p<0.05) within HZP and HZ samples, respectively.

Both HZ (182.76 ± 1.4 nm) and HZP (352.96 ± 3.1 nm) samples showed significant differences (p<0.05) in particle diameter from day 0 to day 7 (HZ = 214.13 ± 3.1 nm; HZP = 371.13 ± 5.9 nm) but had no significant differences for HZP samples from day 7 to day 28 (369.6 ± 6.3 nm) while HZ samples also showed significant differences from day 7 to day 21 (232.7 ± 3.7 nm). The lack of pectin coating on the HZ sample could have caused the zein nanoparticles to coalesce and increase its average diameter size (da Rosa et al., 2015). This could be due to the partial unfolding of zein nanoparticles where the inter-particle hydrophobic attraction was dominant over inter-particulate repulsive forces, which will cause instability of the nanoparticles and aggregation over time (Xue et al., 2022; Donsi et al., 2017; Guo et al., 2008). This could also be the same for HZP nanoparticles where some of the zein nanoparticles might not have had a complete pectin coating and increased size in day 7. However, the HZP
nanoparticles did not show an increase in its diameter from day 7 to day 28 due to the pectin coating that ensured electrostatic and steric repulsion and its prevention of aggregation (Chang et al., 2017; Huang et al., 2016). Specifically, the anionic pectin coating produced a net negative surface charge onto the zein nanoparticles that produced electrostatic repulsion that overcame attractive forces like hydrophobic attraction while the long chain polymers from pectin obstructed the van der Waals and hydrophobic attraction between other nanoparticles due to steric repulsion (Huang et al., 2019; Liu et al., 2022) For the zeta-potential of HZ and HZP samples, both samples showed no significant differences from day 0 (HZ = 17.03 ± 1.6 mV; HZP = -22.83 ± 0.5 mV) to day 28 (HZ = 13.3 ± 3.6 mV; HZP = -22.23 ± 0.5 mV). The zeta-potential of HZ nanoparticles decreased with longer refrigeration due to a possible restructuration of zein and coalescence between other zein nanoparticles with lower zeta-potentials (da Rosa et al., 2015). Based on these results and visual observations, both HZ and HZP samples exhibited stability after 28 days. However, the pectin coating allowed the HZP samples to keep a constant particle diameter while the HZ samples will likely increase in particle diameter and eventually lead to coalescence and aggregation of the zein nanoparticles after longer storage periods.

5.5.2. pH Stability

Protein-based nanoparticles are liable to aggregation around the pI, which limits their application within foods (Patel et al., 2010). Figure 20 shows the particle diameter (a), PDI (b), and zeta-potential (c) of HZ and HZP nanoparticles at different pHs from 2.0-8.0.
Figure 20: Particle diameter (a), PDI (b), zeta-potential (c), and image (d) of HZ and HZP nanoparticles at different pH adjustments from initial pH 4.0. Uppercase (A, B) and lowercase letters (a, b) represent significant differences (p<0.05) within HZP and HZ samples, respectively. ^Represents particle diameter >1000nm and PDI at 1.0.

From figure 20c, HZ nanoparticles exhibited a positive charge (pH 2.0 = 30.73 ± 2.0 mV) below the pI of zein nanoparticles (6.2) while at pH above the pI of zein nanoparticles showed a negative charge (pH 8.0 = -27.9 ± 1.2 mV). This phenomenon occurred because a pH shift to acidic caused the amino groups in zein to be protonated for a more positive surface charge while the pH shift to alkaline caused deprotonation of
the carboxyl groups for a more negative surface charge (Doan & Ghosh, 2019; Wang & Fan, 2019). As for HZP samples, pH 2.0 showed a low negatively charged zeta-potential (-3.92 ± 0.5 mV) while pH 8 exhibited a more highly negatively charged zeta-potential (-32.66 ± 5.9). When the pH is below the pKa of pectin (3.5), the carboxylic groups from pectin became deionized and resulted in having less charges (Khan et al., 2021; Huang et al., 2019). This suggested that the hesperetin-loaded zein nanoparticles were coated with pectin because the measurement of zeta-potential relies on the electrical characteristics of the nanoparticles exterior surface (Liang et al., 2022). As for HZ and HZP nanoparticles, their particle diameters at different pH are shown on figure 20a. From figure 20a, there was a significant difference (p<0.05) in the particle diameters of HZP nanoparticles between low pH at 4.0 (PD=330.23 ± 11.0 nm) and high pH at 8.0 (PD=241.06 ± 14.4 nm). The HZP samples had a similar particle diameter and zeta-potential trends as Liang et al.’s (2022) work, where HZP sizes increased at pH lower than pI of Zein (6.2) while zeta-potential of the pure pectin was close to zero at low pH but becomes increasingly negative with increased pH. This could be due to the pectin having less charged groups when the pH is below its pKa (3.5), which can cause the loosely adsorbed pectin molecules to detach from the zein nanoparticle’s surfaces (Khan et al., 2021; Birch et al., 2014). Within the isoelectric point of zein nanoparticles at pH 5.0-6.0, HZ nanoparticles were destabilized while HZP was still stable at pH 5.0 (PD=290.23 ± 21.7 nm; PDI=0.151 ± 0.006; zeta-potential= -29.93 ± 0.8 mV) and pH 6.0 (PD=312.9 ± 2.82 nm; PDI=0.186 ± 0.03; zeta-potential=−26.0 ± 1.0 mV). The HZ nanoparticles destabilized because close to the pI their surface charge was close to zero charge (figure 20c.) and hydrophobic attractions became more favorable instead of repulsion, which lead to
instability and aggregation amongst other zein nanoparticles (Xue et al., 2022; Donsi et al., 2017). The pectin coating on HZP nanoparticles still retained strong zeta-potential and helped prevented aggregation through steric and electrostatic repulsion (Chang et al., 2017; Huang et al., 2016). At pH above the isoelectric point, both HZ and HZP nanoparticles exhibited similar particle characteristics at pH 7.0 (HZ=254.53 ± 22.4 nm; HZP=236.13 ± 14.6 nm) and pH 8.0 (HZ=231.46 ± 4.9; HZP=241.06 ± 14.4 nm). Both zein nanoparticles and pectin were negatively charged at pH above their isoelectric point and pKa, which caused electrostatic repulsion between zein and pectin and caused the pectin to desorb from the surface of zein nanoparticles (Huang et al., 2019). The higher pH also caused both HZ and HZP nanoparticles to have a higher particle diameter compared to lower pH likely because the rapid pH adjustment from the initial pH 4.0 to pH 7.0 and 8.0 may have caused some nanoparticles to slightly aggregate since the solution may have been briefly exposed to the isoelectric point of zein at 6.2 before the solution reached pH 7.0 and 8.0 (Wang & Fan, 2019). Even with slight aggregation, the zein nanoparticles have still shown stability in its size, PDI, and zeta-potential. In addition to that, HZ nanoparticles showed poor stability with high PDIs of 1.0 at pH 5.0-6.0 while HZP nanoparticles were stable at pH 2.0-8.0 with PDIs below 0.2.

These findings suggest that the hesperetin-loaded zein nanoparticles coated with pectin provided great protection against aggregation and precipitation at a range of pH 2-8, especially around the pI of zein nanoparticles, since the pectin coating increased its steric and electrostatic repulsion while also decreasing hydrophobic attraction that caused aggregation in HZ nanoparticles (Huang et al., 2019).
5.5.3. Temperature Stability

Nanoparticle delivery systems within foods could be exposed to a variety of temperature treatments during food production. Figure 21 shows the effect of temperature on the particle diameter (a), PDI (b), and zeta-potential (c) of HZ and HZP nanoparticles.
Figure 21: Particle diameter (a), PDI (b), zeta-potential (c), and image (d) of HZ and HZP nanoparticles exposed to different temperatures for 60 minutes. Uppercase (A, B) and lowercase letters (a, b) represent significant differences (p<0.05) within HZP and HZ samples, respectively. ^Represents particle diameter >1000nm.

HZ samples gradually increased in particle diameter from the control room temperature (198.56 ± 1.6 nm) to 70°C (358.66 ± 11.9 nm) and then completely destabilized at 80°C and 90°C with an increased PDI at 0.28 ± 0.2 and 0.70 ± 0.08,
respectively. The reason HZ nanoparticles destabilized was because zein denatured at higher temperatures, which caused the molecular structure of zein to expose more of its hydrophobic amino acids, caused hydrophobic attractions to overcome repulsions, and then collapsed the nanoparticle (Xue et al., 2022; Yu et al., 2021; Sun et al., 2016). As for HZP samples, its particle diameter stayed relatively the same from the control room temperature (333.0 ± 10.6 nm) to 90°C (344.16 ± 3.37) while its PDI was below 0.3 at all temperatures. For the zeta-potentials of both HZ and HZP samples, there were no significant differences (p<0.05) among each other at different temperatures. The pectin coating improved the colloidal stability of HZP particles by inhibiting the collision of the zein nanoparticles with more exposed reactive functional groups at higher temperatures, which prevented an increased particle diameter (Khan et al., 2021). Thus, the addition of pectin coating prevented aggregation of hesperetin-loaded zein nanoparticles at higher temperatures.

5.5.4. Salt Stability

Different ionic environments are present in a variety of foods that could inhibit the stability of zein nanoparticles. Due to this, the stability and characterization of HZ and HZP samples were observed after the addition of sodium chloride at different concentrations. Figure 22 represents the particle diameter (a), PDI (b), and zeta-potential (c) of HZ and HZP nanoparticles at different salt concentrations.
Figure 22: Particle diameter (a), PDI (b), zeta-potential (c), and image (d) of HZ and HZP nanoparticles in different salt concentrations. Uppercase (A, B) and lowercase letters (a, b) represent significant differences (p<0.05) within HZP and HZ samples, respectively. ^Represents particle diameter >1000nm and PDI at 1.0.

With the addition of 25 mM NaCl to HZ nanoparticles, the particles immediately destabilized and caused aggregation. Due to immediate destabilization of HZ nanoparticles at 25 mM NaCl, measurements on its particle diameter, PDI, and zeta-potential were halted at 50 mM. The mean particle diameter of HZP, however, increased from 332 nm to 359 nm with a slight increase of its PDI from 0.113 to 0.127 when the
NaCl concentration increased from 0 to 50 mM. As for the HZP nanoparticles, aggregations formed at higher salt concentrations starting at 75 mM NaCl possibly while complete destabilization occurred at 100 and 125 mM NaCl. This phenomenon is possibly due to the counter ion effect where the ionic salts reduced electrostatic attraction between the surface of zein and pectin and allowed the pectin to desorb from the nanoparticles, which caused bridging flocculation effects between uncoated zein nanoparticles (Liang et al., 2021; Yao et al., 2018). This was also why the zeta-potential of HZP nanoparticles was still negative due to the pectin still likely retaining its negative charge when measured (Dai et al., 2017). The change in HZ nanoparticle’s zeta-potential in figure 22c was evidenced by the counter ion effect where its zeta-potential was changed to being negatively charged.

The binding of ionic salts to the Zein’s nanoparticle’s positive surface influenced the ionic strength of the system. This allowed the attractive interactions like hydrophobic attraction from the zein nanoparticles to overcome electrostatic repulsion and promoted aggregation (Dai et al., 2017; Chen et al., 2014; Hu & McClements, 2015). These results were also observed in the previous study by Liang et al. with tannic acid-loaded zein/pectin nanoparticles (2021). This was also why the variability of the PDI on HZP nanoparticles increased with an increase of NaCl concentration as more pectin was desorbed from the nanoparticles and more zein aggregated amongst each other (Liang et al., 2021). These results suggest that zein/pectin nanoparticles may have limited use within food products that have high ionic strengths.
5.6. In Vitro Digestion

For the in vitro digestions assays of the delivery systems, HZ nanoparticles were diluted 1-fold with 5mM citrate phosphate buffer at pH 4.0 to get the same final hesperetin concentration at 250 µg/mL as HZP nanoparticles. Particle distributions were measured at different stages of in vitro digestion (initial, after gastric, after intestinal, and micelle phase) to observe particle size distribution (figure 22) and zeta-potential changes within HZ and HZP nanoparticles throughout different phases (figure 23) and their stability at these different environments (Zou et al., 2016). Their diameters were measured with static laser diffraction because it can more reliably measure and account for larger particle diameters that may be present during in vitro digestions from 0.04 µm – 2000 µm (Beckman Coulter, Inc., 2011) compared to dynamic light scattering where it can only measure particle diameter range of 0.3 nm – 10 µm (Malvern Instruments Ltd., 2010).
Figure 23: Particle size distribution (a) and zeta-potential of HZ and HZP nanoparticles at different phase of in vitro digestion. Uppercase (A, B) and lowercase letters (a, b) represent significant differences (p<0.05) within HZP and HZ samples, respectively.

5.6.1. Initial Phase Before Start of Digestion

From the initial phase of digestion, the particle diameter distributions of HZ and HZP nanoparticles were measured and shown in figure 23a. HZ nanoparticles were shown to have a broader size distribution compared to HZP nanoparticles, which could be due to the lack of repulsions from the pectin coating that made HZ nanoparticles more susceptible to van der Waals and hydrophobic attractions for agglomeration (Huang et al., 2019; Liu et al., 2022). This was evidenced in Table 18 where HZ nanoparticles yielded a higher PDI (0.132 ± 0.01) compared to HZP nanoparticles (0.119 ± 0.03) when measured with dynamic light scattering. The zeta-potentials from figure 23b of HZ (19.26 ± 0.8
87

mV) and HZP (-25.06 ± 0.9 mV) nanoparticles were also similar to HZ and HZP nanoparticles obtained from Table 18 due to the zein’s cationic surface and pectin’s anionic surface, respectively (Zou et al., 2016).

5.6.2. Gastric Phase

After the simulated gastric phase, the size distribution and zeta-potential were measured on HZ and HZP nanoparticles. Both HZ and HZP nanoparticles have similarly bimodal size distribution peaks where their first peaks were very broad within the 0.1 um region with HZP being a little more narrow than HZ and their second peaks were small and past 100 um. The pepsin enzymes may have partially hydrolyzed the HZ nanoparticles, which caused flocculation and coalescence of the nanoparticles and then resulted in precipitation during the gastric phase (Tan et al., 2022). For HZP nanoparticles, however, the size distribution of the first peak was a little more narrow than HZ and did not show signs of precipitation during the gastric phase. The zeta-potentials of HZ and HZP nanoparticles from figure 23b also resulted in being -3.6 ± 1.1 mV and -8.0 ± 1.4 mV, respectively. HZ nanoparticles exhibited low zeta potential due to the plentiful amount of salt ions present within the gastric phase, like the salt stability results shown in section 5.5.4, which promoted agglomeration. As for the HZP nanoparticles, the zeta-potential was higher than HZ but is still relatively close to neutral charge because the pH of the gastric phase was below the pKa of pectin at 3.5 (Jones et al., 2010). Even though the zeta-potential of HZP nanoparticles was close to neutral charge, the high-methoxyl pectin may have reduced proteolysis and extensive aggregation of HZP nanoparticles through steric repulsion (Huang et al., 2016). The
pectin coating adsorption onto the zein nanoparticles protected the nanocarriers from enzymatic degradation and ionic environments with steric repulsion where the long chain polymers from pectin overcame the attractive forces between zein nanoparticles that would have caused aggregation, similar to the results from the stability tests in section 5.5 (Liu et al., 2022; Xu et al., 2014).

5.6.3. Intestinal Phase

The simulated intestinal phase consisted of pancreatic enzymes (trypsin, amylase, and lipase) and bile salts. The size distribution and zeta-potential were measured on HZ and HZP samples after the intestinal phase (figures 23a and 23b). The size distribution of HZ nanoparticles were bimodal peaks with the first peak being more narrow and higher while the second peak was small and measured past 1 um. As for the size distribution of HZP nanoparticles, it exhibited multimodal peaks where the first peak had a smaller size distribution than HZ while its second and third peaks were small and near 1 um. This was similar to Zheng et al.’s (2016) results where average size distribution peak of curcumin-loaded zein nanoparticles increased from the gastric phase with a very broad monomodal peak to the intestinal phase with multimodal peaks and higher average particle diameter. This was also similar to Chen et al.’s (2015) results where the size distribution peaks of curcumin-loaded soy protein isolate nanoparticles within the intestinal phase have bimodal narrow peaks near 100 nm and 1000 nm. Lastly, the size distributions of soy isoflavones-loaded whey protein nanoparticles had bimodal peaks with the first peak close to 100 nm and the second peak past 1000 nm (Liu et al., 2022). The increase in particle diameter for both HZ and HZP nanoparticles from the gastric phase to the
intestinal phase was due to the pH adjustment from pH 3.0 to 7.0, which caused the
dissociation of the nanoparticles between anionic pectin and anionic zein nanoparticles
(Zou et al., 2016). At pH 7.0, this caused electrostatic repulsion between the pectin and
zein nanoparticles since zein becomes negatively charged above its isoelectric point
(pI=6.2) and pectin is more negatively charged above its pKa at 3.5 (Mirpoor et al., 2017;
Shukla & Cheryan, 2001; Khan et al., 2021). Even though nanoparticle diameter
decreased at pH 7.0 from the pH stability test in section 5.5.2, there was an increase in
salt concentrations from gastric phase to intestinal phase and the presence of other
enzymes present within pancreatin. The salts could have played a role in the counter ion
effects of promoting aggregation while the trypsin from pancreatin could have digested
more of the protein nanoparticles present within the digesta and also promote aggregation
(Zou et al., 2016; Hu & McClements, 2015). This was evidenced in Figure 23a where the
additional peaks from the intestinal phase for both HZ and HZP nanoparticles were
possibly the irregular particles from biopolymer aggregates and insoluble sediments (Liu
et al., 2018).

From figure 23b, the zeta-potentials from both HZ and HZP samples increased
from -3.6 ± 1.1 mV to -37.7 ± 5.3 mV and from -8.04 ± 1.4 mV to -36.5 ± 5.8 mV,
respectively. The reason for these changes was that within the simulated intestinal phase,
it contained anionic bile salt micelles that had highly negative surface charges (Chen et
al., 2022; Ni et al., 2014). These changes were evidenced in figure 20c where the zeta-
potentials of HZP nanoparticles were highly negative with a pH shift from 3.0 to 7.0 and
in figure 22c when the zeta-potential of HZ nanoparticles became negatively charged
with the addition of NaCL. After the zein nanoparticles were digested by the trypsin from
pancreatin, the amorphous hesperetin was released and then bound to the broken peptides from proteins and bile salt micelles so hesperetin could be solubilized and be more bioaccessible (Zou et al., 2021).

5.6.4. Micelle Phase

The micelle phase after digestion consists of compounds that were dissolved in the intestinal fluid, which can then diffuse through the gastrointestinal lumen and then be absorbed through the intestinal wall membrane (Yao et al., 2018). Specifically, for hesperetin to be absorbed within the intestinal wall membrane, it can either be incorporated into the hydrophobic interior of the micelles formed from bile salts or bound to the soluble portion of proteins or peptides that were released by hydrolysis from pepsin and trypsin (Yao et al., 2018). From the micelle phase on Figure 23a, both the particle diameter distribution peaks from HZ and HZP nanoparticles shifted to a smaller particle diameter size despite broader and multiple peaks present from the gastric and intestinal phases. Centrifugation of the simulated intestinal phase removed aggregates and larger particles as a result of the intestinal phase, which resulted in smaller particle diameters in the micelle phase. The supernatant presumably contained the particles that consisted of mixed micelles that contained the amorphous hesperetin incorporation into the hydrophobic interior of bile salt micelles and dissolved pectin for solubilization (Zou et al., 2021). This could be the likely reason why HZ nanoparticles from Figure 23a only had a monomodal particle dispersion where all of the larger aggregates were removed after centrifugation as opposed to HZP nanoparticles that likely still had pectin molecules not large enough to be removed during centrifugation (Verrijssen et al., 2016). In addition
to that, the centrifugation also increased the zeta-potentials of HZ and HZP samples within the micelle phase in figure 23b due to the higher presence of highly anionic bile salt micelles with the removal of larger particles and aggregates that likely had lower zeta-potentials (Chen et al., 2022; Ni et al., 2014).

5.7. Bioaccessibility

Bioaccessibility (%BA) after in vitro digestion is the concentration of hesperetin in the micelle phase after the conclusion of the simulated intestinal phase. The %BA of hesperetin encapsulated/unencapsulated in zein nanoparticles and coated/uncoated with pectin are shown in figure 24.

Figure 24: Bioaccessibility of 250 µg/mL hesperetin unencapsulated (H=hesperetin mixed with deionized water) and encapsulated (HZ and HZP nanoparticles) after the conclusion of in vitro digestion. Lowercase letters (a, b) represent significant differences (p<0.05) between encapsulated (HZ and HZP) and unencapsulated (h=hesperetin+deionized water) hesperetin samples.
Both bile salts and peptides within the intestinal phase play an important role in the formation of micelles or complexes for the solubilization of released polyphenols from the lumen to the bloodstream (Reboredo et al., 2022; Huang et al., 2019). The %BA (figure 24) of 250 µg/mL hesperetin in deionized water was at 11.49 ± 2.1 % while HZ and HZP nanoparticles had 64.32 ± 0.5 % and 64.55 ± 0.4 %, respectively. These encapsulated hesperetin results were similar to curcumin-loaded zein nanoparticles (51.5 ± 4.7 %) (Zheng et al., 2016), quercetin-loaded zein nanoparticles = 55.6 ± 0.8 % (Carrasco-Sandoval et al., 2021), and quercetin-loaded zein/ alginate nanoparticles = 57.0 ± 1.3 % (Carrasco-Sandoval et al., 2021). Both HZ and HZP nanoparticles exhibited similar %BA and hesperetin encapsulated in these delivery systems is close to six times more bioaccessible than the hesperetin dispersed in deionized water. This is because free hesperetin was in crystalline form within the aqueous digesta and their poor water solubility did not allow their ability to be bound to the hydrophobic interior of the bile salt micelles (Gu et al., 2019; Yao et al., 2018).

Amorphous hesperetin from HZ nanoparticles did not show a significant difference (p>0.05) in bioaccessibility in comparison to amorphous hesperetin from HZP nanoparticles, even though precipitation and agglomeration occurred during the simulated gastric phase. Polypeptide chains that were released during hydrolysis of the zein protein could have been bound to hesperetin as opposed to HZP nanoparticles where pectin may have shielded the nanoparticles from proteolysis, which resulted in prolonged release of amorphous hesperetin until the intestinal phase and then bound to the hydrophobic interior of the bile salt micelles (Li et al., 2022; Liang et al., 2021; Liu et al., 2021; Gu et al., 2019). Some of the zein protein agglomerates from gastric phase digestion may have
entrapped hesperetin throughout the digestion and would have been separated from the micelle phase during centrifugation (Li et al., 2022). Based on these results, both HZ and HZP nanoparticles were able to withstand the environmental conditions within in vitro digestion long enough for the amorphous hesperetin to be solubilized within the micelle phase.
6. CONCLUSIONS

Summary of Results: The optimal hesperetin concentration for its encapsulation in zein nanoparticles was selected at a final concentration of 500 µg/mL with its particle characteristics at PD=179.23 ± 5.0 nm, PDI=0.146 ± 0.02, zeta-potential=23.23 ± 0.8 mV, and encapsulation efficiency=92.78 ± 1.0 %. With the optimal pectin concentration (0.5 mg/mL final concentration pectin) for pectin coating onto hesperetin-loaded zein nanoparticles, HZP nanoparticles exhibited particle characteristics of PD=356.83 ± 10.7 nm, PDI=0.119 ± 0.03, zeta-potential=-22.56 ± 0.4 mV, and encapsulation efficiency=93.97 ± 0.2 %. The use of fluorescence spectrometer resulted in a binding constant of 4.94 x 10^5 M^-1 with the number of binding sites at 1.2126. FTIR confirmed that the interaction of hesperetin with zein was hydrogen bonding and hydrophobic interaction while the interaction between zein and pectin was electrostatic attraction. Hesperetin encapsulated in zein nanoparticles exhibited much higher free radical scavenging antioxidant activity with HZP=0.09 ± 0.008 µg/mL AAE per 1 µg/mL hesperetin and HZ=0.10 ± 0.006 µg/mL AAE per 1 µg/mL hesperetin compared to hesperetin mixed with deionized water at 0.03 ± 0.02 µg/mL AAE per 1 µg/mL hesperetin due to encapsulation that prevented hesperetin from crystallizing. HZP nanoparticles showed better stability through various environmental conditions compared to HZ nanoparticles because HZP’s pectin coating shielded hesperetin-loaded zein nanoparticles from aggregation and precipitation at pH 2.0-8.0, high temperatures at 90°C, and 50 mM NaCl concentration due to HZP’s steric and electrostatic repulsion. Lastly, both HZ and HZP nanoparticles exhibited higher bioaccessibility (64.32 ± 0.5 % and 64.55 ± 0.4 %, respectively) compared to hesperetin mixed with water (11.49 ± 2.1 %).
because both nanoparticle samples were able to prolong release of amorphous hesperetin into bile salt micelles within the intestinal phase for solubilization. Although both HZ and HZP nanoparticles exhibited similar bioaccessibility, the addition of pectin coating could protect the HZ nanoparticles from a variety of foods and beverage environments. The use of zein/pectin nanoparticles is a promising protein nanoparticle colloidal delivery system for hesperetin and potentially other polyphenols from orange pomace into a variety of foods and beverages as nutraceutical additives.functional ingredients. The potential use of this delivery system for other polyphenols within orange pomace would bring more value to the byproducts from the orange juicing industry as well as bring more purpose to the wastes of oranges other than cattle feed and pectin extraction.

Future Work: If additional work on this project could be possible for future projects, additional follow-up studies are recommended. One follow-up study could be the encapsulation of other orange pomace polyphenols not encapsulated in zein/pectin nanoparticles like nobiletin and neohesperidin. Co-encapsulation of these orange pomace polyphenols like hesperetin+naringenin or hesperetin+tangeretin could also be possible in zein/pectin nanoparticles.

Additional structural analysis on the hesperetin-loaded zein/pectin nanoparticles would be ideal for further characterization. One instrument could have been using a scanning electron microscopy (SEM) to observe their morphology and see if the particle diameter measured through SEM was similar to its measurement through dynamic light scattering. Other instruments that could be used would be a circular dichroism to observe secondary structure changes of zein within zein/pectin nanoparticles and x-ray diffraction...
for the verification of hesperetin’s physical state after encapsulation by zein nanoparticles.

Another follow-up study could be doing a 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) antioxidant activity study on hesperetin’s free radical scavenging activity when encapsulated and not encapsulated. The free radical scavenging could be improved on these nanoparticles since ABTS would be more suitable for hydrophilic solutions like the zein/pectin nanoparticles dispersed within aqueous water solutions.

Lastly, an in vivo digestion model could be conducted with rats where the gastrointestinal system is more similar to a human’s gastrointestinal system than in vitro model. Rats will ingest the nanoparticle solutions and then have their blood extracted after certain times to isolate plasma cells that should have hesperetin and measure through HPLC for its bioavailability. Bioavailability is a more accurate measurement of hesperetin already absorbed within the bodies that could act out its functional properties than bioaccessibility.
REFERENCES


https://doi.org/10.1016/j.foodhyd.2014.11.023


Duan, Dhar, A., Patel, C., Khimani, M., Neogi, S., Sharma, P., Siva Kumar, N., & Vekariya, R. L. (2020). A brief review on solid lipid nanoparticles: part and
parcel of contemporary drug delivery systems. *RSC Advances, 1*(45), 26777–26791. https://doi.org/10.1039/d0ra03491f

https://doi.org/10.1088/1361-6528/aad111

https://doi.org/10.1002/9781119237860.ch29


https://doi.org/10.1016/j.ijbiomic.2017.03.005


https://doi.org/10.1631/jzus.B1800346

DOI: https://doi.org/10.1016/j.jcis.2007.11.058

https://doi.org/10.1016/j.foodhyd.2020.105777

https://doi.org/10.3390/pharmaceutics12070604


https://doi.org/10.1016/j.foodchem.2015.03.009

https://doi.org/10.1016/j.lwt.2018.09.044


https://doi.org/10.3390/ph15020211


https://doi.org/10.3390/antiox10091476


https://doi.org/10.2147/IJN.S143733


https://doi.org/10.3390/molecules28083550


https://doi.org/10.1016/j.fshw.2023.03.034


https://doi.org/10.1111/1750-3841.15535


https://doi.org/10.3390/foods11213478

method. *Food Hydrocolloids, 119*, 106851–.

https://doi.org/10.1016/j.foodhyd.2021.106851


https://doi.org/10.1016/b978-0-323-88656-7.00013-1


https://doi.org/10.3390/foods10112773


https://doi.org/10.1016/j.lwt.2019.03.059


https://doi.org/10.1016/j.colsurfb.2011.11.027


Nallamuthu, Ponnumay, V., Smruthi, M. R., & Khanum, F. (2020). Formulation of Naringin Encapsulation in Zein/Caseinate Biopolymers and its Anti-adipogenic...


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https://doi.org/10.3390/pharmaceutics11090432


https://doi.org/10.4161/oxim.2.5.9498

https://doi.org/10.1002/ptr.5256


Producing Industry at Industrial Scale. *Molecules (Basel, Switzerland)*, 26(1), 246–. https://doi.org/10.3390/molecules26010246


bioaccessibility and antioxidant capacity under simulated gastrointestinal conditions. *Food Hydrocolloids*, 79, 262–272.


https://doi.org/10.1021/jf404420s


