# FABRICATION AND CHARACTERIZATION OF WHEY PROTEIN ISOLATE - ALGINATE HYBRID HYDROGELS USING *HIBISCUS SABDARIFFA*

by

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(Under the Direction of Anand Mohan)

## ABSTRACT

Hibiscus is a rich source of bioactive compounds that are known to have beneficial effects on human health. Encapsulating and protecting these compounds in appropriate carriers facilitates their incorporation into functional foods and health-related products. The instability of these compounds under typical food processing conditions, including exposure to high temperatures, pH changes, and the presence of light and oxygen, has necessitated using encapsulation techniques to increase their stability and applicability. This study examines the interactions between alginate and whey protein isolate, as well as strategies for encapsulating composites of these polymers. Alginate and whey proteins interact powerfully to form hydrogels, as results demonstrate. Future research must elucidate the interactions between alginate, whey proteins, and the encapsulated bioactive compounds, as well as the stability of these structures in food matrices.

INDEX WORDS: Polyphenols, microencapsulation, *in vitro* digestion, bioavailability

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B. Tech. Anna University, India, 2020

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment

of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2023

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August 2023

# DEDICATION

To my wonderful sister Niyati, who has been a constant source of support and encouragement for me during this journey. Your confidence in me has given me the courage to face challenges and strive for excellence. This thesis is dedicated to you.

To my incredible parents, who have always believed in me and provided unfailing love and guidance. Your devotion and sacrifices have laid the groundwork for my achievement. This thesis is proof of your unwavering support.

To my cherished grandparents, whose wisdom and stories influenced my worldview. Your affection and unflinching faith in me have been an endless source of motivation. This dissertation is dedicated to the principles you instilled in me.

Finally, to my future self, I wish you the continued pursuit of knowledge, progress, and making a positive difference in the world. This thesis serves as a reminder of your dedication and perseverance that has brought you this far. Accept the future with open arms!

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to all those who have supported and contributed to the completion of my thesis. Without their guidance, encouragement, and assistance, this endeavor would not have been possible.

First and foremost, I would like to extend my heartfelt thanks to my supervisor, Dr. Anand Mohan, for their invaluable guidance and mentorship throughout this research journey. Their patience and continuous support have been instrumental in shaping the direction and quality of this thesis.

I am grateful to my committee members Dr. Fanbin Kong and Dr. Suraj Sharma for their insightful feedback, encouragement, and for providing a nurturing academic environment that fostered my intellectual growth. Dr. Suh, Dr. Kerr, and Dr. Singh for their invaluable guidance and support throughout my thesis journey providing me with valuable insights and expertise that have greatly contributed to the successful completion of my research.

I would like to extend my appreciation to my family for their unwavering love, encouragement, and understanding. Their constant support and belief in my abilities have been a source of inspiration and motivation.

I am deeply indebted to my friends - Mounika G, Kezia Sharon, Shyam, Shriram, Aadeya, Ujjal, who have provided their valuable encouragement throughout my graduate school experience. Their feedback and camaraderie have greatly enriched my work.

Once again, I extend my sincere appreciation to all those mentioned above and others who have played a role, however small, in supporting me during this academic journey. Your contributions have been invaluable and have helped shape this thesis into what it is today.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

# CHAPTER 1

## INTRODUCTION

The food industry has been seeking alternatives to produce food items rich or fortified in natural antioxidants, as they may play an essential role in preventing cardiovascular diseases, cancer, and neurological disorders. Furthermore, there is a huge challenge in designing commodities that provide nutritious quality, naturalness, convenience, sensory acceptance, and attractive pricing. The need for food to be considered not only as a source of nutrients with sensory appeal but also as a source of well-being and health is a global trend (World Health Organization, 2018). Functional foods that target digestive health, child nutrition, geriatric nutrition, weight management, obesity prevention, and attractiveness enhancement are gaining popularity in the market. Alkaloids, anthocyanins, carotenoids, flavonoids, glucosinolate, isoflavone, phenolic acid, tannin, and terpenic phytochemicals, as well as protein hydrolysates, have qualities that support these health goals.

The genus Hibiscus contains more than 250 species, and various studies have shown that these plants contain bioactive substances that could be exploited to create functional meals. Encapsulation techniques have been used to increase these compounds' stability and application because they are unstable under typical food processing circumstances, such as exposure to high temperatures, pH fluctuations, and the presence of light and oxygen. Incorporating encapsulated bioactive chemicals from plants in the genus Hibiscus into food formulations holds significant technological promise to enhance food system stability and serve as a model for developing novel

functional foods. Techniques for encapsulation increase the stability of bioactive chemicals while maintaining their effectiveness. During encapsulation, a solid, liquid, or gaseous active chemical is enclosed in a polymeric matrix or wall to shield it from the elements and interactions with other ingredients and regulate its release into foods or customers. The following qualities are essential for a bioactive chemical encapsulation system: Produce food-grade products; incorporate bioactive compounds into food matrices with high physical-chemical stability and minimal impact on the product's sensory properties; safeguard encapsulated compounds from interactions with other food ingredients and degradation due to temperature, light, or pH; maximize encapsulated compound absorption after consumption while ensuring controlled release in response to a specific stimulus; and scale operations quickly.

Alginate, a naturally occurring anionic polymer commonly generated from brown seaweeds, has been thoroughly investigated and exploited in recent years for tissue engineering, in vitro cell culture, wound healing, medication delivery, and covering up the flavor of food. Alginates are most frequently immobilized using an extrusion procedure, in which a syringe injects an alginate solution containing an active ingredient into a gelation solution primarily made up of calcium ions. However, extrusion-based ionic gelation of alginate is a quick and easy way to create delivery systems for encapsulation. To overcome this drawback, certain infill materials are usually utilized to increase the porosity of alginate gel, stop and postpone the quick release of entrapped chemicals from a gel into surrounding media, and increase encapsulation effectiveness. For instance, alginate and whey protein isolates can be combined as an additive to encase polyphenols and anthocyanins.

The main elements of *Hibiscus sabdariffa*, useful for food and medicinal purposes, include organic acids, anthocyanins, polysaccharides, and flavonoids. Numerous studies have determined quercetin, delphinidin-3-sambubioside, and cyanidin-3-sambubioside to be the highest anthocyanins and phenolic compounds concentrations in hibiscus calyx extract. The calyx is the plant part most commonly used in the culinary business. It can make several goods, including teas, fermented drinks, juices, preserves, ice cream, chocolate, and pastries.

The purpose of this research is to produce and characterize whey protein-alginate hydrogels with the intention of encapsulating hibiscus bioactive components. The hydrogels had their physical and chemical properties examined, and an investigation into the release of bioactive chemicals during in vitro digestion was carried out. These findings help to a better understanding of the method of encapsulation as well as its prospective applications.

# CHAPTER 2

# LITERATURE REVIEW

## *Hibiscus sabdariffa*

As an edible flower, roselle (*Hibiscus sabdariffa* L.) has long been known to have several beneficial impacts on human health. This advantage is a result of bioactive compounds, which are naturally present primarily in the calyx. The investigations of phenolic chemicals in recent decades are consistent with the availability of numerous medical treatments and functional meals based on this flower. This literature review comprehensively details the chemical makeup, biological consequences, and health benefits of phenolic chemicals found in roselle (Hapsari & Setyaningsih, 2021). A perennial plant belonging to the genus Hibiscus (family *Malvaceae*), roselle is also known as jamaica (in Spanish), red sorrel (in English), and karkadeh (in Arabic). It is indigenous to India and Malaysia, but cultivation has spread to many tropical and subtropical areas, including China, Thailand (these two nations are major global suppliers), Indonesia, Saudi Arabia, Vietnam, Sudan, Egypt, Nigeria, and Mexico because it can thrive in marginal soils with low fertility and low moisture retention (Patel, 2014).

*Hibiscus* var. *sabdariffa* (Hs) has short, bushy growth and has been classified into four races: Bhagalpur Ensi, Intermedius, Albus, and Ruber. *Hibiscus* var. *sabdariffa ruber* is the most frequently cultivated species. It is distinguished by ordinarily smooth, cylindrical, and crimson stems. Its lush leaves grow between 7.5 and 12.0 cm long. It has up to 5-inch (12.5 cm) wide, yellow flowers that may become pink when they fade. The calyx, stems, and foliage of Vaccinium species all have an acidic taste and a flavor similar to that of blueberries. The history of Roselle is a subject of intense debate among academics. According to Cobley (Cobley, 1976; Singh et al., 2017), Roselle originated in India and Saudi Arabia and was later transported to other parts of the world, including Asia and America.

**Morphology of *Hibiscus sabdariffa***

More than 300 species of annual or perennial plants, shrubs, or trees belong to the *Malvaceae* genus. Common names for hibiscus include roselle, Jamaica sorrel or red sorrel (in English), and karkadeh (in Arabic). While Murdock presented evidence that Hs was domesticated by the black inhabitants of western Sudan (Africa) sometime around 4000 BC, some argue that Hs is from India or Saudi Arabia. These days, both tropical and subtropical regions, including India, Saudi Arabia, China, Malaysia, Indonesia, the Philippines, Vietnam, Sudan, Egypt, Nigeria, and México, are widely cultivating it. *Hibiscus* var. *sabdariffa ruber* is an annual, erect, bushy, herbaceous subshrub with smooth or nearly smooth, cylindrical, usually red stems. It can reach heights of up to 8 feet (2.4 meters). The leaves are alternate in arrangement, between three and five inches (7.5-12.5 centimeters) in length, green with crimson veins, and may have long or short petioles. Young seedlings' leaves and the upper leaves of older plants have plain margins; lower leaves have heavily lobed three to five or even seven lobes. Flowers up to 5 in (12.5 cm) wide,

with a rose or maroon eye, are borne singly in the leaf axils and become pink as they wither in the evening. At this time, the typically red calyx, consisting of 5 large sepals with a collar (epicalyx) of 8 to 12 slim, pointed bracts (or bracteoles) around the base, begins to enlarge, becomes fleshy, crisp but juicy, 1 1/4 to 2 1/4 in (3.2-5.7 cm) long and fully encloses the velvety capsule, 1/2 to 3/4 in (1.25-2 cm) long, which is green when immature, 5-valved, with each valve containing 3 to 4 kidney-shaped, light-brown seeds, 1/8 to 3/16 in (3-5 mm) long and minutely downy. When mature and dried, the capsule turns brown and cracks open. According to Da-Costa-Rocha et al. (2014), the calyx, stems, and leaves have an acidic flavor similar to the cranberry (*Vaccinium* spp.) flavor.

**Composition of *Hibiscus sabdariffa***

Its main cultivation points are the three calyx variants of roselle—green, red, and dark red. The most prevalent calyx color is red, and red calyxes stand out due to their high anthocyanin concentration. There are two main anthocyanins: cyanidin 3-sambubioside and delphinidin 3-sambubioside. The number of organic acids, minerals, amino acids, carotene, vitamin C, and total sugar in the calyx, leaves, and roselle seeds varies depending on the type and region. Flavonoids, anthocyanidins, triterpernoids, steroids, and alkaloids, among other substances, have reportedly been isolated and described from Roselle (Singh et al., 2017; Gangrade et al., 1979).

Flavonoids and phenolic acids are phenolic chemicals that are naturally present in roselle. The phenolic acids mentioned in roselle include ethyl chlorogenate, methyl chlorogenate, coumaroylquinic acid, dihydroferulic acid-4-O-glucuronide, neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, and 5-O-caffeoyl shikimic acid. The roselle calyx contains at least 95 flavonoids, according to research. Anthocyanins, which mostly contain delphinidin-3-O-

sambubioside and cyanidin-3-O-sambubioside, are the most prevalent of the discovered flavonoids. Quercetin-3-glucoside, methyl epigallocatechin, myricetin, quercetin, and kaempferol are other flavonoids that are frequently mentioned. In roselles, there are also other significant substances, such as organic acids that include hydroxy citric acid, hibiscus acid, hibiscus acid hydroxyethyl ester, and hibiscus acid dimethyl ester (Hapsari & Setyaningsih, 2021).

**Traditional culinary use of *Hibiscus sabdariffa***

*H. sabdariffa* calyces fresh or dried, are used to make herbal beverages, heated and chilled beverages, fermented beverages, wine, jam, jellied confections, ice cream, chocolates, flavorings, puddings, and cakes. The fleshy calyces are used to make "cacodyl tea" and other fermented drinks in Egypt (Kochhar, 1986), while in Sudan and Nigeria, they are boiled with sugar to make "Karkare" or "Zoborodo." This alcoholic beverage is referred to as Jamaica, Jamaica water, or Jamaica tea in Mexico. In the West Indies, calyces can also be employed as a flavoring and coloring component in alcoholic beverages (Da-Costa-Rocha et al., 2014).

Because of its high fiber, calcium, and iron content, baked goods incorporating roselle calyces have been used in the baking and confectionery sectors. Studies have shown that adding powdered Roselle calyces to chocolate cake increases its calcium, iron, and crude fiber content. In Turkey, Roselle calyces are a naturally occurring antioxidant component in Kavurma, a dish made of beef, beef fat, and salt. Another group of researchers looked into and evaluated the antioxidant properties of Roselle calyces, rosemary leaves, and BHT (butylated hydroxytoluene) in Kavurma (Cid-Ortega & Guerrero-Beltrán, 2015). While BHT was added at a concentration of 3.3 g/kg of meat, roselle calyces (milled dried calyces) and rosemary (pulverized dry leaves) were added at a concentration of 1 g/kg of meat each. Pizza with iron added as a fortifier and aqueous extract of

Roselle calyces as an antioxidant in a previous study (Daramola and Asunni, 2006; Cid-Ortega & Guerrero-Beltrán, 2015). The extract was prepared by mixing 31 g of Roselle calyces with 200 mL of water. The mixture was cooked for 20 minutes before being filtered and concentrated. In the recipe for the pizzas, the authors used three concentrations of the concentrated extract of Roselle calyces: 0:280 (control), 6:274, and 25:255 (v/v). Despite the product's stern look, the Roselle calyces extract improved its taste and texture. The three different pizza kinds all received a comparable level of sensory acceptability. The peroxides index was used to gauge the antioxidant effect. Pizzas with Roselle extract showed a lower peroxides index. Energizing foods and drinks Roselle calyces are frequently employed in producing soft drinks from their aqueous extracts. In this connection, considerable research has been done in which authors examined the production methods, dietary components, and bioactive substances (mostly anthocyanins) of various types of Roselle (red, burgundy, and yellow). Research has been done to investigate what happens when Roselle calyces extract is added to yogurt. Iwalokun and Shittu (2007) investigated the effects that adding aqueous extracts of *Hibiscus sabdariffa* at a concentration of 20 milliliters per liter of yogurt had on the yogurt's pH, titratable acidity, syneresis, ash content, and moisture levels (Iwalokun & Shittu, 2007).

**Anthocyanin content of *Hibiscus sabdariffa***

The dried flowers *of Hibiscus sabdariffa* include a group of pigments called anthocyanins; a group of flavonoid derivatives that change color based on pH. Hibiscin (delphinidin-3-sambubioside), gossypicyanin (cyanidin-3,5-diglucoside), delphinidin (anthocyanidin), and other anthocyanins are based on delphinidin and cyanidin. "Hiviscin," also known as "hibiscin," was the first anthocyanin removed from the calyx of Hs. It was later referred to as delphinidin-3-sambubioside and given the structural designation cyanidin-3-glucoside before being renamed

delphinidin-pentoside-glucoside (Da-Costa-Rocha et al., 2014). Three different anthocyanins, delphinidin-3-sambubioside (hibiscin), delphinidin-3-glucoside, and cyanidin-3-glucoside (chrysanthenin), were extracted from roselle pigments using material from Taiwan and Trinidad. Gossypicyanin, also known as cyanidin-3-sambubioside, was found in the prior study (Khoo et al., 2017). Later, it was found that the flower pigments of *H. sabdariffa* varieties included cyanidin-3,5-diglucoside and cyanidin-3-(2G-glucosylrutinoside). Cyanidin-3-sambubioside and cyanidin-3-glucoside were found to be the primary compounds in a study with five different strains of *Hibiscus sabdariffa* varieties. One strain (the Senegalese strain) did not contain delphinidin glycosides. The anthocyanin content of all strains in this investigation varied from 1.7% to 2.5% by dry weight. Another study discovered a comparable anthocyanin content of about 1.5 g per 100 g of dry weight of *Hibiscus sabdariffa* calyces for delphinidin -3-sambubioside. (Da-Costa-Rocha; Carvajal-Zarrabal et al., 2012)

**Phenolic content of *Hibiscus sabdariffa***

Research into traditional remedies as sources of bioactive molecules has been driven in recent decades by the growing desire to improve health through more natural approaches. As a result of the extensive research on the medical uses of plants and the connection between those uses and the chemical makeup of the plants, they have been used for purposes other than sustenance for ages. They, therefore, provide fascinating sources of physiologically active chemicals with great potential as bioactive components (Duque-Soto et al., 2023). Hibiscus comprises simple or polymerized flavonols and polyphenols of the flavanol type. The flavonoids hibiscitrin (hibiscetin-3-glucoside), sabdaritrin, gossypitrin, gossytrin, and other gossypetin glucosides, quercetin and luteolin, as well as the sterols ergosterol and -sitosterol, have all been

identified in Hs extracts. The phenolic acid chlorogenic acid is another that can be found in both leaf and Hibiscus extracts. Hs extract contains Hibiscus protocatechuic acid, a crucial phenolic acid.

**Health benefits of *Hibiscus sabdariffa***

Several researchers have assessed the antioxidant properties of Roselle calyces extracts. Usoh et al. (2005) examined the antioxidant effects of desiccated *Hibiscus sabdariffa* ethanol extracts on specific biomarkers of oxidative stress in Wistar rats. Oral administration of the extract (200 and 300 mg/kg body weight) substantially reduced the formation of malondialdehyde in the liver (the primary compound used as an indicator of lipid peroxidation). The fact that sodium arsenite-induced peroxidation suggests that the extract may be protective against the induction of cell damage. This treatment with extracts resulted in a significant increase in rat liver weight and a decrease in superoxide dismutase and catalase enzymes in rat blood; this may indicate the extract's efficacy as an antioxidant and protector against other metabolic disorders (Cid-Ortega & Guerrero-Beltrán, 2015; Usoh et al., 2005). Additionally, the effects of *H. sabdariffa* calyces extracts on certain types of malignancy have been investigated. Akim et al. (2011) assessed the antioxidant capacity of commercial juice of Roselle calyces stored for one week, one month, or one year, as well as the antiproliferative effect on the breast (MCF-7 and MDA-MB-231), ovarian (Caov-3), and cervix (HeLa) cancer cells. The antioxidant activity of Roselle calyces juices preserved for various durations was generally high (Akim et al., 2011; Cid-Ortega & Guerrero-Beltrán, 2015). Extracts of *Hibiscus sabdariffa* have also been examined for their chemoprotective and cytotoxic properties. Ologundudu et al. (2009, 2010) examined the impact of *Hibiscus*

*sabdariffa* extracts on the oxidative stress induced by 2,4-dinitrophenylhydrazine in rabbits over four months. Results indicated that anthocyanins in the extract protected blood against oxidation, induced by 2,4- dinitrophenylhydrazine, hemolysis, and cytotoxic and lipid peroxidation. The aqueous extract of *Hibiscus sabdariffa* in rodents (Rattus norvegicus) with potassium bromate-induced tissue damage (brain, kidney, stomach, spleen, heart, and liver (Cid-Ortega & Guerrero-Beltrán, 2015; Ologundudu et al., 2009).

Calyces can help you get rid of water in your body. Márquez-Vizcaino et al. (2007) studied male albino rats with Roselle calyces extracts that were gotten in different ways and then lyophilized. They used lyophilized aqueous extracts (20, 200, and 400 mg/kg body weight), lyophilized ethanol extracts (20 and 200 mg/kg body weight), and non-lyophilized aqueous extracts (20 mg/kg body weight). The most diuretic effects were seen at 20 mg of lyophilized ethanol extract and non-lyophilized water extract per kg of body weight. Under the same conditions, the first two medicines had similar effects on controlling urine volume (Cid-Ortega & Guerrero-Beltrán, 2015; Vizcaino et al., 2007). The antipyretic effect of Roselle is another thing that has been investigated. Reanmongkol and Itharat tested the effect of Roselle water (800 and 400 mg/kg) and ethanolic (400 and 800 mg/kg) extracts on rats and mice to see if they lowered their body temperature (Reanmongkol & Itharat, 2007). They saw that both the water-based and alcohol-based solutions lowered the yeast-caused fever in Swiss albino mice and Wistar rats. (Cid-Ortega & Guerrero-Beltrán, 2015). It has also been shown that the juice from Roselle can lower blood sugar. Wen-Chin et al. (2009) tested the Roselle extract on glucosamine-nitrosourea, which is harmful to beta cells, which are responsible for making insulin. They found that glucosamine-nitrosourea had a good effect. Sini et al. (2011) got the same results when they gave male albino

rats an oral amount of Roselle calyces extract (0.5 mg/mL), ascorbic acid (0.2 mg/mL), and glibenclamide (0.08 mg/mL) as a control (Lee et al., 2009; Sini et al., 2011).

**Effect of processing and encapsulation**

Bioactive compounds derived from plant species are used in the commercial manufacture of functional meals to enhance color, taste, texture and provide health advantages such as antioxidant capabilities (Daz-Bandera et al., 2015). Under typical processing circumstances, such as exposure to high temperatures, pH fluctuations, and the presence of light and oxygen, these compounds are frequently unstable (Etchepare et al., 2015). Because of this, manufacturing valuable compounds is difficult. The molecule responsible for the nutraceutical activity must be shielded from the time of ingestion until the bioactive component reaches the target tissue to boost the concentration of these chemicals. The action of the phytochemicals and their potential health benefits may also be constrained by polyphenols' instability under the conditions found in the gastrointestinal tract or during food processing and storage (pH, temperature, oxygen, light, and oxygen content). The concentration of polyphenols can be increased while attenuating their instability by using carriers to protect them rather than giving them as free compounds (Chen et al., 2006). These food systems may be made more stable by including extracts from plants in the genus Hibiscus that are rich in encapsulated bioactive compounds; this could also result in the creation of novel functional foods.

Bioactive compounds are easily broken down and chemically unstable despite their many health advantages. Due to their quick release, low solubility, and poor bioavailability, pure bioactive compounds are only occasionally used in food and medication compositions. According to Shishir et al. (2018), encapsulation can enhance physicochemical properties, shield bioactive molecules from environmental hazards, and increase the benefits of these substances on human

health and disease prevention. Extracts from Hibiscus plants rich in encapsulated bioactive components are a promising alternative for boosting the stability of these food systems. They may serve as an inspiration for the creation of novel functional foods (Martin et al., 2017).

Strategies for encapsulation increase the bioactive chemicals' stability while preserving their functional potential. Encapsulation is a process that isolates an active ingredient (solid, liquid, or gas) within a polymeric matrix or wall to shield it from the elements, prevent it from interacting with other ingredients, or regulate how much of it is released into food or the consumer's body.

There is a long history of eating edible fruit, vegetable, or aromatic flowers. Due to their aesthetic appeal and delectable nature, edible flowers are believed to include various bioactive components. People who promote eating wholesome food and paying attention to healthy foods have made flower foods a new diet fad. Despite having a significant sensory appeal, fresh vegetables, fruits, and edible flowers are more frequently used in meals. Edible flowers have a surprisingly short shelf life, restricting their economic application.

**Hydrogels**

Hydrogels' softness, elasticity, absorbency, flexibility, and hygroscopic properties make them suitable for various food applications. Due to their hydrophilic qualities, compatibility with food, and non-immunogenic nature, polysaccharide hydrogels are particularly well-suited (Rodriguez & Hemar, 2020). According to Manzoor et al. (2002), these hydrogels have a wide variety of practical applications in food preservation, medicines, agriculture, and food packing. In addition, polysaccharide hydrogels are crucial in creating food flavor carrier systems, opening up fresh advancements in the food processing industry (Manzoor et al., 2022). The four main issues in hydrogel science are the polymer source, structure formation, gel function, and gel applications. It is then possible to divide the phrase "structure fabrication" into structure kinds

and crosslinking techniques (Klein & Poverenov, 2020). Two conditions are required for the creation of hydrogels. First, the polymer molecules (natural or synthetic) must have hydrophilic groups on the main or side chain. Second, to create gel network designs, a certain crosslinking intensity between the molecules is also required (Klein & Poverenov, 2020). Polysaccharide hydrogels are created when natural polymers such as chitosan, pectin, starch, and hyaluronic acid are physically or chemically crosslinked (Kim et al., 1992; Liu et al., 2012). Broader uses in a variety of industries are made possible by hydrogels' improved mechanical characteristics. Finding novel methods to strengthen hydrogels has recently attracted much attention. In addition, the raw components and the production processes used to create food gels must be edible (Khalesi et al., 2020). A suitable encapsulation method for water-soluble components, hydrogel is a soft, moist substance that is simple to make and has a three-dimensional porosity network that allows it to absorb up to several thousand times its dry weight. Using hydrogels as encapsulating systems in food has been the subject of many recent papers (Khalesi et al., 2020).

**Applications of hydrogels**

Hydrogels are regarded to be particularly effective in improving food quality, modifying nutrients, optimizing sensory perception, delivering and protecting targeted nutrients, calorie control, risk monitoring for food safety, and food packaging in the subject of food science. According to Batista et al. (2019), hydrogels are three-dimensional, hydrophilic networks of polymeric chains joined by physical or chemical linkages. Hydrogels offer much potential as carriers for bioactive ingredients or in food packaging systems. This paper (Liu et al., 2012; Rahman et al., 2016) discusses the nature of hydrogels, their 3D network structure, functional capabilities, and potential uses in food packaging systems. Regarding potential uses for food

packaging, hydrogels may have a conformation that enables their use as a component of a packaging system to control the humidity brought on by foods with high water content. Hydrophobic bioactive compounds can be incorporated into hydrogels and delivered to specific locations through food. Hydrophobic bioactive ingredients may contribute to vitamins, dietary fiber, fatty acids, proteins, probiotics, polyphenols, carotenoids, and other nutrients when added to functional food products. On the other hand, factors such as low water solubility, crystallinity, chemical deterioration, biochemical instability, and low bioavailability commonly prevent their efficient delivery to the target locations. To solve these issues, encapsulation technologies that help incorporate bioactive chemicals into foods and can protect these substances throughout processing, transport them through the gastrointestinal tract, and release them precisely where they are needed are of great interest. Numerous delivery systems have been developed to control the stability and release of bioactive components throughout the gastrointestinal tract. The creation of low-fat or low-starch foods is urgently needed. Hydrogels can also assist in calorie reduction by boosting satiety or lowering intake. To create hydrogel particles with a suitable texture, Wu et al. used protein and dietary fiber as soft matter approaches (Wu et al., 2023). These particles may be a better alternative to starch granules. A comparable study was described by McClements et al. to reduce the caloric density of pancakes cooked at temperatures significantly higher than the boiling point of water.

**Alginate**

Brown seaweed is the source of alginate, an anionic polymer that occurs naturally. It has received extensive research because it is biocompatible, has low toxicity, is affordable, and forms a mild gel when divalent cations like Ca2+ are added. It is used in numerous biomedical applications (Tocha et al., 2022). Alginate is believed to be a form of a linear copolymer made up of connected

blocks of the molecules -d-mannuronate (M) and -l-guluronate (G) (1,4). G residues (GGGGGG), M residues (MMMMMM), and alternating M and G residues (GMGMGM) make up the blocks. Over 200 distinct types of alginates are produced nowadays, and they come from various locations with varying percentages of M and G and block lengths (Nezamdoost-Sani et al., 2023). One of the most significant natural polysaccharides is alginate because it may be used to create food delivery systems for bioactive ingredients. This is so that bioactive substances can be released gradually over time thanks to ionic crosslinking, pH responsiveness, high biocompatibility, and shell particles. Additionally, the alginate-based delivery technique has good encapsulation effectiveness (EE) and loading capacity (LC) of bioactive compounds and may shield delicate bioactive components from harsh environmental conditions (such strong as acidity, oxygen, bacteria, UV light, etc.). Because of this, developing delivery systems based on alginate is becoming increasingly popular.

**Whey protein isolate**

Whey is a globular protein used to make casein and cheese in the dairy industry, among other products (Smithers, 2008). It is made up of several different protein/peptide components, including lactoperoxidase, lysozyme, and lactoferrin, as well as four different classes of globular proteins, including -lactalbumin, -lactoglobulin, bovine serum albumin, and immunoglobulins (Weinbreck et al., 2003). Whey proteins are used mainly as hydrogels and in nanoparticle systems for the regulated encapsulation and release of various active components. According to Devi et al. (2017), it also creates complex coacervates with various polysaccharides. Due to their high nutritional content and capacity to create gels, emulsions, and gelled emulsions, whey proteins are widely employed as food additives. Whey proteins have been shown to offer potential as microsphere wall materials for applications requiring controlled release (Chen & Subirade, 2006).

Many dietary bioactives, including fat-soluble compounds, have been encapsulated in whey protein hydrogels. The cold-set gelation of whey protein hydrogels is especially beneficial for heat-sensitive bioactives (O'Neill et al., 2015). Whey protein (WP) is a dietary protein that is frequently used in the creation of gels. WP hydrogels have been the subject of numerous studies because of their potential for controlled-release applications. WP can create heat-set gel matrices that entrap active ingredients for delivery and absorb significant amounts of water. The globular proteins -lactoglobulin (-Lg, 65% w/w) and -lactalbumin (-25% w/w), which are in charge of WP's gelling, emulsifying, foaming, and hydration capabilities, make up the majority of its composition.

**Ionic gelation**

The method used to prepare the microencapsulation must be considered because different methods result in varied compositions and architecture. Controlling the alginate particles' physical structure is important since it directly impacts their digestibility, susceptibility to degradation, permeability, and porosity (Choudhury et al., 2021). Furthermore, the preparation method has been found to affect the releasing properties of chitosan-alginate beads (Abreu et al., 2008; Alexe & Dima, 2014).

The method used to create whey protein-alginate hydrogels is known as ionotropic gelation, and it entails the cross-linking of alginate chains with cations (Burey et al., 2008). According to Kurozawa and Hubinger (2017), exterior (or diffusion) gelation and internal gelation are the two main types of ionotropic gelation (Kurozawa & Hubinger, 2017).

**External Gelation**

According to Kurozawa and Hubinger (2017), external gelation is the development of gels after adding a hydrocolloid solution to an ionic solution. The primary mechanism of gelation is the

diffusion of ions, which leads to inhomogeneous gel particles, with surface gelation occurring before core gelation (Kurozawa & Hubinger, 2017). Due to the difference in gelation rates, the interior is more flexible and has a lower cross-link density than the surface (Burey et al., 2008; Leong et al., 2016).

Extrusion is a method frequently used in conjunction with external gelation, in which the encapsulation matrix (alginate) solution is loaded into a syringe and then injected drop-by-drop into the ionic solution (calcium chloride) to induce gelation (Joye & McClements, 2014; Weisany et al., 2022). A benefit of extrusion is that the size of the beads is determined by the syringe needle gauge size (Paredes Juárez et al., 2014). Extrusion can also be easily scaled up for large-scale industrial production (Paredes Juárez et al., 2014).

One advantage of extrusion is that the syringe's needle gauge determines the size of the alginate bead. Extrusion can also be easily scaled up for large-scale industrial production (Paredes Juárez et al., 2014).

**Internal Gelation**

The hydrocolloid and inactive ion solution is the basis for internal gelation (Burey et al., 2008). Then, ion activation, such as a pH shift, induces cross-linking and forming a hydrogel matrix, leading to gelation (Lupo et al., 2015). Internal gelation produces more homogenous beads due to the diffusion of ions before gelation, which does not affect the surface and core gelation rates. Thus, the cross-linkages are more evenly distributed throughout the beads, resulting in less dense matrices with larger pore diameters, more excellent permeability, and quicker release rates (Alexe & Dima, 2014).

***In vitro* digestion**

Food digestion is a complex process involving numerous components, which has aroused the interest of the food industry due to the growing relationship between food and health and, consequently, the prevention of the onset of certain chronic diseases (Bornhorst et al., 2016). Human digestion is a complex, continuous, and dynamic process influenced by the chemical or physical properties of the food ingested and the psychological state of the host (Xavier & Mariutti, 2021). During digestion, food is converted into nutrients that the body can use for energy, growth, and cell repair. Food digestion involves two major concurrent processes: (i) mechanical transformation, in which more significant food is broken down into smaller pieces, beginning in the mouth and continuing in the stomach; and (ii) enzymatic transformation, in which several different enzymes break down macromolecules into smaller molecules that can be absorbed into the bloodstream, beginning in the mouth, and continuing in the intestines. *In vitro* digestion experiments replicate the physiological conditions of *in vivo* digestion. They are valuable tools for studying and understanding the changes, interactions, and bio-accessibility of nutrients, medicines, and non-nutritive substances (Lucas-González et al., 2018). Nutrition, medicine, and food chemistry are all domains where the approach is commonly employed. *In vivo*, experiments define bioavailability as the proportion of an ingested component available at the site of action for use in normal physiological activities (Verhoeckx et al., 2015). Bioavailability results from three main processes: element digestibility and solubility in the gastrointestinal tract, element absorption by intestinal cells and transfers into the circulation, and element incorporation into the functional entity or target from the circulation. Tomás-Barberán and Mine (2013) defined bioaccessibility as the proportion of a molecule liberated from its food matrix within the gastrointestinal tract. Thus, it becomes available for intestinal absorption (typically determined *in vitro*). It involves the sequence of activities that occur during food digestion to transform it into potentially bioavailable

material. It excludes absorption/assimilation via epithelial tissue and pre-systemic metabolism, both intestinal and hepatic (Landberg et al., 2019; Galanakis, 2021). Conversely, bioactivity includes processes associated with how nutrition or bioactive substance is transported and reaches the target tissue, how it interacts with biomolecules, any metabolism or biotransformation that occurs, the development of biomarkers, and the physiological responses caused. Bioavailability includes bioactivity (Etcheverry et al., 2012). Bioaccessibility and bioavailability are frequently used interchangeably, but it is essential to note that bioavailability includes bioactivity. In some diets, anthocyanins are present in extremely high concentrations. Consequently, a dosage of several hundred milligrams would not be deemed exceptional. The mean dietary intake in Finland is estimated to be 82 mg/day, with berries, red wine, beverages, and the coloring agent E163 as the primary sources (Chiang et al., 2013; Manach et al., 2005).

Epidemiological studies have conclusively demonstrated that plant-based diets protect against degenerative diseases like cancer and cardiovascular disease. In addition to fiber, vitamins, phytosterols, sulfur compounds, carotenoids, and organic acids, which contribute to the health effects of plant foods, they also contain a variety of polyphenols, which are increasingly regarded as effective protective agents (Comunian et al., 2022). Polyphenols are abundant constituents in our diet, and mounting evidence shows they play a role in preventing degenerative diseases. Bioavailability varies widely between polyphenols, so the polyphenols with the highest concentrations of active metabolites in target tissues are not necessarily the most abundant in our diet (Manach et al., 2005).

**Oral digestion**

Food digestion takes place continuously and dynamically within the human gastrointestinal tract. Salivary and masticatory functions complement oral systems. When solid foods are

processed orally, the particle size is reduced, and the bolus is created (Engelen et al., 2003). On the other hand, liquids must only be adjusted to body temperature and diluted with saliva before being consumed (Engelen et al., 2003). The perception of ending changes in bolus characteristics is thought to inform the central nervous system (CNS) that the bolus is prepared to be swallowed without pain, discomfort, or risk of dysphagia. According to Li et al. (2020), the swallowing threshold has been hypothesized and described in terms of particle size, maximum cohesive force, degree of lubrication, and shape of food boluses.

**Gastric digestion**

Esophageal peristalsis, which performs the storing, mixing, grinding, and emptying motor tasks, transports the resulting food bolus into the stomach. The human stomach has four structural divisions: the fundus, body, antrum, and pylorus. Its resting (fast state) volume is 25 mL, but it can expand to 1.5–4 L to hold much food (Norton et al., 2014). While the distal stomach (antrum) acts as a food processor, mixer, and distributor, the proximal stomach (fundus and body) primarily serves as a storage area for undigested material. The antrum acts as a pump for the stomach emptying of solids by pushing activities, allowing small particles to pass over the pyloric sphincter and into the duodenum (Li et al., 2020; Kong and Singh, 2008).

**Intestinal digestion**

Digestion occurs continuously and dynamically inside the human GI tract. Mastication and salivation are complementary oral mechanisms used at the beginning of everything. The production of food bolus is the outcome of the reduction of particle size during the oral processing of solid foods (Engelen et al., 2003). On the other hand, liquids are essentially prepared for consumption and only need to be warmed to body temperature and diluted with saliva (Engelen et al., 2003). The sense of final changes in bolus characteristics is believed to signal to the central

nervous system (CNS) that the bolus is prepared to be swallowed without pain, discomfort, or risk of dysphagia. The 'swallowing threshold' has been hypothesized and described as a function of particle size, maximal cohesive force, degree of lubrication, and meal bolus shape (Li et al., 2020).

**Static vs. Dynamic model**

A static or dynamic in vitro digestive model should be used, depending on the bioavailability to be determined. Static digestion models simulate the physicochemical changes during digestion (pH, salt concentrations, enzymes) without attempting to replicate peristalsis, fluid flow, or mixing (Guerra et al., 2012). As selection criteria, the extent of the measurement and the digested sample are used. Peristalsis dramatically accelerated the release of oil-enhanced almond butter in the dynamic model, which was used to examine the bioavailability using both static and dynamic models.

**Previous studies on the encapsulation of Hibiscus**

High-pressure homogenization was used to create translucent liposomes containing anthocyanin-rich hibiscus extract. Liposomes were formed using various amounts of soy lecithin and hibiscus extract, with mean particle sizes similar to control liposomes. The hibiscus extract encapsulation efficiency in liposomes ranged between 63% and 72% w/v (Gibis et al., 2014).

A dry powder of red-colored roselle calyces was also produced using microencapsulation technology, which can be used as a natural colorant and functional food component. The encapsulation investigation included four different matrices, including maltodextrin, gum Arabic, a mixture of maltodextrin and gum Arabic, and soluble starch. Anthocyanins in the encapsulated

roselle were identified and quantified using high-performance liquid chromatography. (Idham et al., 2012).

The stability of the encapsulated pigments was studied over a 105-day period at three distinct temperatures (4°C, 25°C, and 37°C). The matrix employed for encapsulation greatly improved the pigments' half-life during storage, especially at 37°C. The color values were not considerably affected by storage temperature; however, the type of encapsulating agent and storage time were. During storage, the L* and a* values declined, whereas the b* value grew. When compared to the other matrices, the combination of maltodextrin and gum Arabic had the highest encapsulation efficiency, the lowest degradation rate at 4°C, and the smallest variations in a* and b* values.

Furthermore, the release of roselle polyphenols in whey protein, Carboxyl methyl cellulose (CMC), and pectin combinations was studied. The study discovered that CMC inhibited polyphenol release, but whey protein and pectin had the reverse effect. These findings were attributed to differences in the swelling rate because of the mixture's composition. The study concludes that by altering the content of the mixtures, polyphenols release can be controlled (Serrano-Cruz et al., 2013).

Finally, the microencapsulation of anthocyanin-rich hibiscus extract by ionic gelation was examined utilizing two approaches (dripping-extrusion and atomization). The encapsulation efficiency and antioxidant capacity of atomized particles were found to be higher, whereas the particles formed via dripping-extrusion were found to be more stable (de Moura et al., 2018).

# CHAPTER 3

# MATERIALS AND METHODS

## Materials

Whey protein isolate was sourced from Davisco Food Ingredients, USA. We obtained porcine mucin, porcine alpha-amylase, porcine pepsin, porcine pancreatin, sodium alginate, and calcium chloride from Sigma-Aldrich (St. Louis, MO). All remaining substances were of chemical grade. Throughout this investigation, deionized water was utilized. Kong and Singh (2010) provided the method for preparing simulated saliva, while Hur and colleagues (2009) provided the method for preparing simulated gastric and intestinal secretions. Table 1 presents the compositions of the stock solutions prepared and preserved at room temperature for each digestive juice. On the day of *in vitro* digestion trials, enzymes were added to the appropriate volume of stock solution and the pH was adjusted with 1 N HCL (Table 2) to produce the simulated fluids.

## Methods

### 1. Fabrication of Hibiscus-alginate-whey protein hydrogels:

### Preparation of Hibiscus extract

Dried *Hibiscus sabdariffa* calyces was bought from Amazon marketplace (Suchiate Hibiscus - Jamaica) and refrigerated at 4 °C until further use. A total of 10 g of dried flower was steeped in 100 mL of distilled water before being boiled for 30 minutes and left undisturbed for an hour before being filtered through sterile filter paper. The extract was obtained using a strainer, centrifuged for 5 minutes at 10,000 rpm, and the supernatant was kept at 4 °C for future use.

### Preparation of Hibiscus-alginate-whey protein hydrogels

With a few minor adjustments, previous work was used to create these microbeads. The Hibiscus extract was combined with sodium alginate (3% w/v), then heated to 80°C while continuously stirred with a magnetic stirrer. The sodium alginate solution was homogenized and then allowed to stand for around 2 hours to remove any air bubbles. The WPI powder was next rehydrated at 8% w/v in deionized water with agitation for 1 hour at room temperature, and then the proteins were given 2 hours to fully hydrate. The whey protein isolate solution was heated at 80°C for 30 min after pH-adjusted to 8.0 with 1 N NaOH to completely denature the proteins. The core solution was extruded into calcium chloride (2% w/v) using a 22-gauge needle to create

hydrogel beads. 10 cm was chosen as the distance between the needle's tip and the surface of the collection solution. The beads produced during this process were kept in the gelling bath for 30 min while being stirred to harden them. They were then passed through Whatman #1 paper for filtering. Two rounds of distilled water washings were performed on the hydrogels. The beads were air dried at 25°C for 24 hours before storage (O'Neill et al., 2015).

**Content of Total Phenolic Compounds:**

The total phenol of *Hibiscus sabdariffa* calyx extract content was determined using the Folin Ciocalteau procedure and method outlined by Delia et al. (2022). Briefly, the Folin Ciocalteau reagent (2 ml), deionized water (6 ml), and the test sample (100 µL) were collected and mixed in a test tube. After 4 min of rest, the mixture was mixed with 1.5 mL of 0.71M $Na_2CO_3$ and incubated in the dark for 90 min. After incubation, the absorbance of the mix was measured using a UV/VIS spectrophotometer (Shimadzu UV-1800, IL) at 760 nm as mg/g of Gallic acid equivalent.

$$TPC = (Abs - b/m \times d)/10$$

Where Abs is the absorbance value at 760 nm, b is the y-intercept of the linear equation, m is the slope of the regression line, and d is the test sample dilution factor.

**Determination of Anthocyanin Content**

The amount of total monomeric anthocyanins was calculated using the pH differential method. Utilizing a UV/VIS spectrophotometer (Shimadzu UV-1800, Illinois), absorbance was measured at 520 nm and 700 nm in buffers at pH 1.0 and 4.5 using the formula A = $(A_{510})–A_{700})$ $pH_{1.0}–(A_{510}–A_{700})$ $pH_{4.5}$.

$$= (\mathrm{A\ x\ MW\ x\ DF\ x\ 1000}) / \varepsilon \mathrm{\ x\ 1}$$

where A is $(A_{510})–A_{700})$ $pH_{1.0}–(A_{510}–A_{700})$ $pH_{4.5}$, DF is the dilution factor, l is the cuvette's path length, ε is 26, 900 molar extinction coefficients, in L & $mol^{-1}$ & $cm^{-1}$ and MW is the molecular weight of cyanidin-3-glucoside (449.2).

**Color Measurement**

Hunter *L**, *a**, and *b** values were measured using a Hunter colorimeter (LabScan XE, Hunterlab, Virginia). White and black ceramic plates were used each time to standardize the instrument. Lightness, greenness (a)/redness (+a), and blueness (b)/yellowness (+b) correspond to the Hunter *L**, *a**, and *b** values, respectively. Triplicate measurements were taken at ambient temperature (25 °C).

**2. Characterization of hydrogels**

**Hydrogel bead imaging**

A high-resolution digital microscope on a movable stand was used to examine randomly chosen hydrogel beads' surface morphology and appearance. The samples ' microstructure was

studied using confocal laser microscopy with a 60 objective lens and ten eyepieces. After being captured, the microstructure images were examined with the aid of image analysis tools.

**Water activity**

A Novasina (TH-500, Pfaffikon, Lachen, Switzerland) water activity instrument was used to assess the water activity (Aw) levels of the samples. The device was calibrated using typical patterns at 25 °C before measuring Aw. The sampling chamber's center was filled with 1 g of hydrogel for the measurements, and readings were taken repeatedly until steady Aw values were attained.

**Determination of encapsulation efficiency**

The efficiency was carried out in accordance with a prior study that was described. 1g of hydrogels was divided into aliquots, dissolved in 5 mL of sodium citrate (5% w/v), and then sonicated for 30 minutes. The following equation was used to compute the encapsulation efficiency:

$$EE\ (\%)\ =\ TPCe/TPCi$$

$$EE\ (\%)\ =\ TACe/TACi$$

Where $TPC_e$ is the total phenol content/total anthocyanin content encapsulated in beads, while $TPC_i$ is the total phenolic content/total anthocyanin content in the initial extract solution used for the encapsulation.

**Texture analysis**

In this experiment, a TAXT2i texture analyzer from Stable Micro Systems Ltd., Surrey, UK, with a 40 mm-diameter glass probe was utilized. The purpose of the investigation was to examine the sample's texture under specific conditions. The sample was positioned directly below the instrument on a plate, and a 50% compression was applied to a single particle. The tests were carried out at room temperature with a constant test speed of 10 mm s-1 and a trigger force of 5 grams. To assure accuracy, the test was conducted in duplicate with six measurements taken for each replicate. Subsequently, the collected data from the twelve measurements were averaged to account for any potential anomalies or measurement errors.

**SEM analysis**

Prior to analysis, the hydrogel beads were freeze-dried using a Labanco lyophilzer at -54 °C. The freeze-dried beads were secured with conductive tape to the sample holder of a scanning electron microscope at Georgia Electron Microscopy, University of Georgia. Then gold powder was sprayed with a tiny ion-sputtering device. A 13 kV acceleration voltage was used to capture pictures of the cross-section and surface topography at a magnification of 78x and 400x.

**Swelling capacity**

**S**welling degree (SD) measurements of hydrogel beads were performed in pH 1.2 and 7.4 solutions at room temperature. The hydrogel beads were allowed to swell in equilibrium in the buffer solution before being removed from the solution, excess surface-adhered buffer solution was carefully cleaned with tissue paper, and the swollen hydrogel beads were weighed correctly using an electronic scale. Beads were withdrawn from solutions at specified intervals and weighed to evaluate the increase in mass. The weight growth of the beads was calculated as a function of time. The percentage of bead growth was represented as:

$$Swelling\ degree\ (\%)\ = (Ms - Md)/Md$$

where $M_s$ and $M_d$ are weights of swollen beads and dry beads, respectively.

***In vitro* digestion - release**

A model that simulates digestion in the mouth, stomach, and intestines has been modified (Hur, Decker, & McClements, 2009). The compositions of simulated saliva, gastric and intestinal secretions are presented in Table 2.2. A 1-M HCl was used to alter the pH of the simulated digestive fluids. Samples of 5 g) were deposited in 125 ml Erlenmeyer flasks, incubated at 37 °C in a water bath equipped with orbital agitation, and set to 120 rpm. The samples were sequentially digested as follows: mouth: addition of 5 ml of salivary juice and mixing for 5 minutes; stomach: addition of 10 ml of gastric juice and mixing for 60 mins; and intestines: addition of 20 ml of intestinal juice and mixing for 2 hours.

**Statistical analysis**

The findings were evaluated using descriptive statistical analysis (mean ± SD), one-way ANOVA, and post hoc comparison using the Tukey least significant difference (LSD) test to discover significant changes between experiments (p-value ≤0.05). All analyses were performed using JMP analytic software (SAS Institute Inc, Cary, NC, USA).

Table 1: Composition of alginate and whey protein isolate in the preparation of hydrogel beads*

| **Code** | **AL: WPI (%)** | **Stirring speed (rpm)** |
|---|---|---|
| H1 | 100:0 (ALG) | 600 |
| H2 | 67:33 | 600 |
| H3 | 50:50 | 600 |
| H4 | 33:67 | 600 |
| H5 | 0:100 (WPI) | 600 |

Table 2: Composition of digestive juices

| | Saliva juice | Gastric juice | Duodenal juice |
|---|---|---|---|
| Stock solution | 0.117 mg/mL NaCl | 5.504 mg/mL NaCl | 14.024 mg/mL NaCl |
| | 0.149 mg/mL KCl | 1.648 mg/mL KCl | 1.128 mg/mL KCl |
| | 2.1 mg/mL NaHCO | 0.532 mg/mL $NaH_2PO_4$ | 6.776 mg/mL $NaHCO_3$ |
| | | 0.798 mg/mL $CaCl_2 \cdot 2H_2O$ | 0.16 mg/mL $KH_2PO_4$ |
| | | 0.612 mg/mL $NH_4Cl$ | 0.1 mg/mL $MgCl_2$ |
| Organic components | 0.4 mg/mL urea | 0.17 mg/mL urea | 0.2 mg/mL urea |
| Add to the stock solution | 1 mg/mL mucin | 6 mg/mL mucin | 18 mg/mL pancreatin |
| | 2 mg/mL α-amylase | 5 mg/mL pepsin | |
| pH | 6.8 | 1.3 - 3.0 | 7.4 |

*- (Apoorva et al., 2020)

# CHAPTER 4

# RESULTS AND DISCUSSIONS

## Characterization of hydrogel beads

Water is the primary solvent determining food hydrogel processing, quality, textural qualities, and stability. According to the results of this parameter research, 50:50 (alginate and whey protein isolate) and 100% WPI hydrogel beads have the lowest water activity. The hydration limits the amount of available free water, resulting in a decrease in water activity. Proteins feature hydrophilic areas that attract and bind water molecules, lowering the system's water activity.

Polysaccharides, such as starches and gums, can absorb and bind water similarly. They generate a gel-like structure that traps water, lowering water activity and limiting the amount of free water in the system. WPI may have little effect on water activity at lower concentrations. However, it can bind more water at greater concentrations or when WPI forms gels or aggregates and contributes to a modest drop-in water activity. The remaining two gels have greater values for this parameter; additionally, no significant changes were identified between 100% alginate beads and with varied whey protein additions 67:33 and 33:67 (alginate and whey protein isolate).

WPI-coated microcapsules exhibited a whiter hue, demonstrating WPI's good effect on masking the Hibiscus color. Green and red hues are displayed by parameter *a**. The higher the

value, the more inclined the hue is to be red. The previous results suggest a significant difference between beads containing Hibiscus (de Moura et al., 2019). It's interesting to notice that the alginate beads have the greatest *a** among the samples. It is possible that encapsulation did not affect the redness of capsules in alginate. However, the presence of WPI reduces the red hue of 100% WPI microencapsulated samples. The blue and yellow colors are indicated by parameter *b**. The higher the degree of *b**, the more yellow the color. The results in Table 2 show that all samples exhibited substantial differences in their *b** parameters. Beads produced a yellow hue with a larger percentage of WPI (Chikhoune et al., 2017; Nourmohammadi et al., 2020). Additionally, the output flow impacts bead uniformity because hand extrusion-dripping results in beads with poor uniformity, whereas pump-dripping results in better consistency. The beads used in this study had a mean diameter that ranged from 2.50 to 5.26 mm and were made with a 22-gauge needle. Chan et al. also had results that were comparable. Quantifying the crystal size is essential when adding an addition to a product to ensure correct distribution and satisfy customer demands. The outcomes showed that the beads had a spherical morphology except those made at lower alginate concentrations. It is well known that the viscosity and fluidity of the dripping process are influenced by the alginate concentration, changing the shape of the pearls. As a result, if the drop viscosity and/or surface tension are insufficient to overcome the surface tension of the gelling solution, beads may deform in a solution with a lower viscosity. For the procedure to be effective, the optimal concentrations of the encapsulating agents must be established (Chan et al., 2006). Chan et al. claim that ionic gelation often results in spherical particles of all sizes. The sphericity of alginate beads affects both their mechanical and chemical stability, which may encourage

reduced strength in less spherical beads, encouraging accidental rupture, for example, and may also affect controlled release.

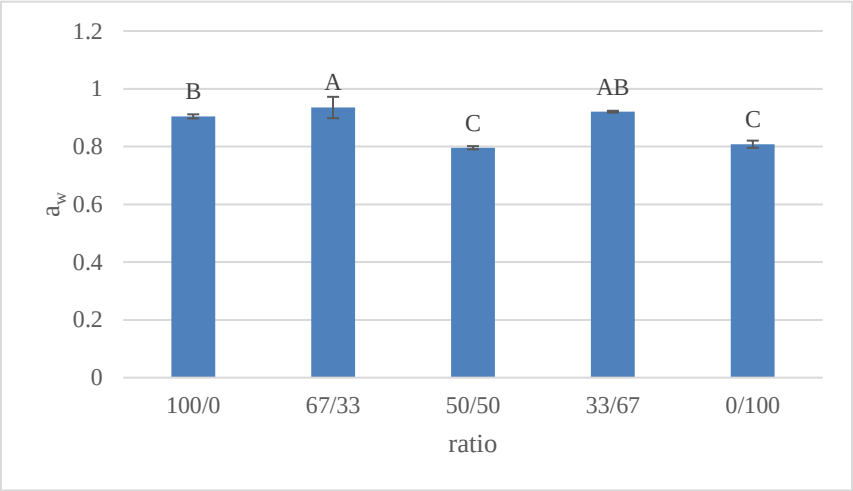

Figure 1: $a_w$ levels of different alginate - whey protein isolate hydrogel beads

Table 3: The water activity, mean diameter, and color of the different hydrogel beads of alginate - whey protein isolate beads

| Ratios | Water activity | Mean diameter | *L** | *a** | *b** |
|---|---|---|---|---|---|
| H1 | 0.904±0.007 | 2.56± 0.04 | 56.98±0.08 | 16.97 ± 0.43 | 8.27 ± 0.20 |
| H2 | 0.93±0.03 | 5.26±0.28 | 70.76±0.34 | 6.51 ± 0.13 | 1.59 ± 0.13 |
| H3 | 0.79±0.005 | 3.68±0.16 | 68.18±1.22 | 2.48 ± 0.04 | 1.59 ± 0.13 |
| H4 | 0.92±0.003 | 3.28±0.19 | 66.97±0.55 | 3.52 ± 0.03 | 8.70 ± 0.04 |
| H5 | 0.80±0.01 | 5.17±0.22 | 62.03±0.57 | 2.58 ± 0.13 | 8.35 ± 0.14 |

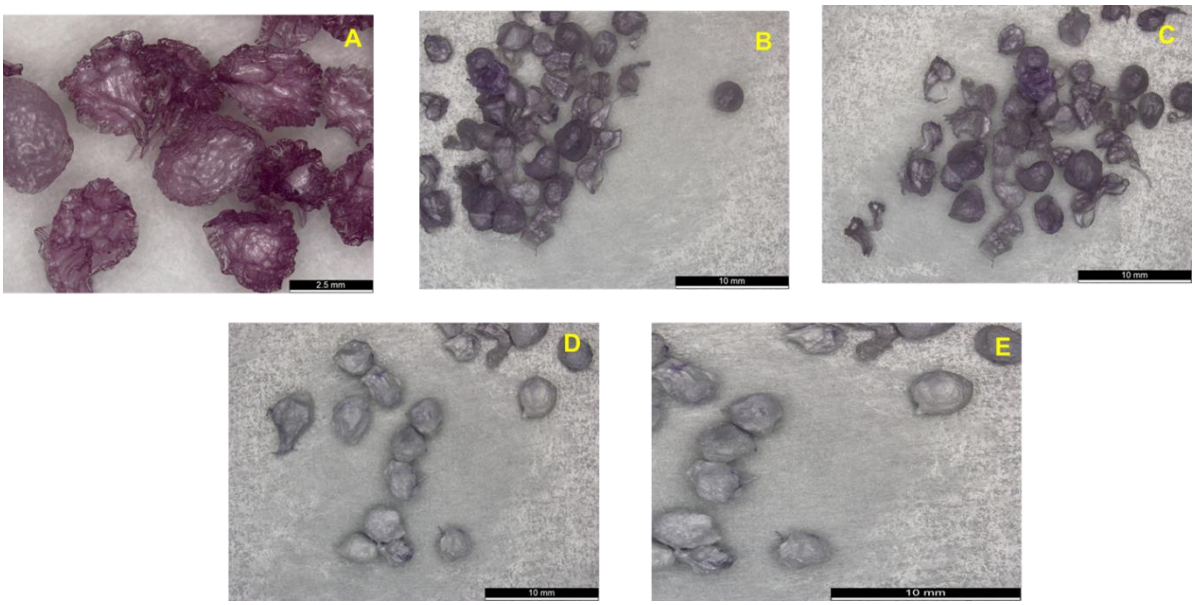


Figure 2: Digital microscopy images of freeze-dried beads prepared with different alginate - whey protein isolate concentrations A) 100% alginate B) 50:50 alginate - whey protein isolate, C) 67:33 alginate - whey protein isolate, D) 33:67 alginate - whey protein isolate, E) 100 % whey protein isolate beads

**Surface morphology (SEM)**

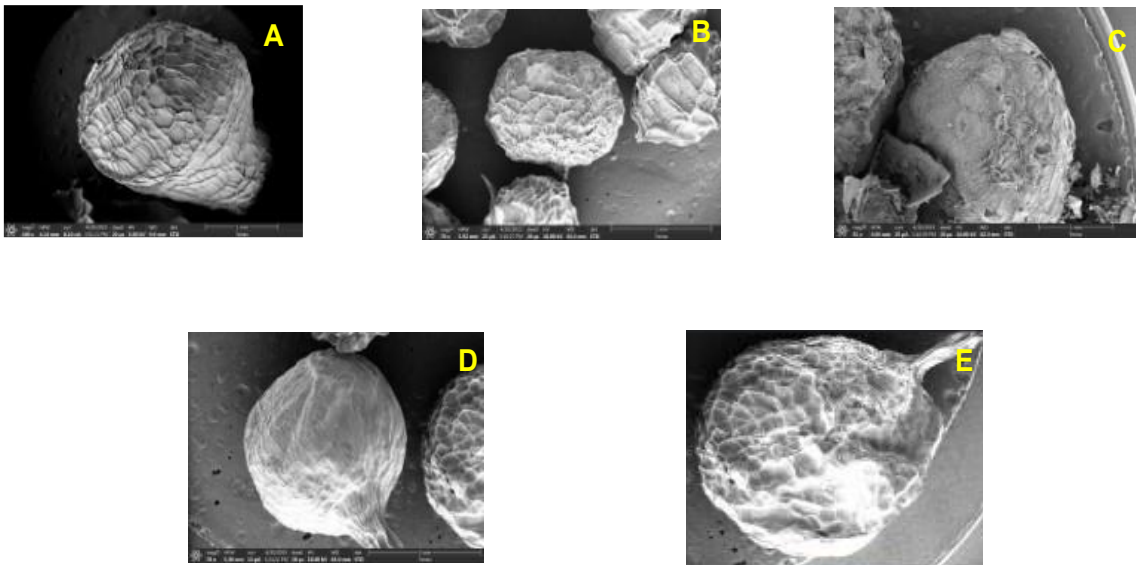


Figure 3: SEM of freeze-dried beads prepared with different alginate - whey protein isolate concentrations A) 100% alginate B) 50:50 alginate - whey protein isolate, C) 67:33 alginate - whey protein isolate, D) 33:67 alginate - whey protein isolate, E) 100 % whey protein isolate beads at 78x magnification

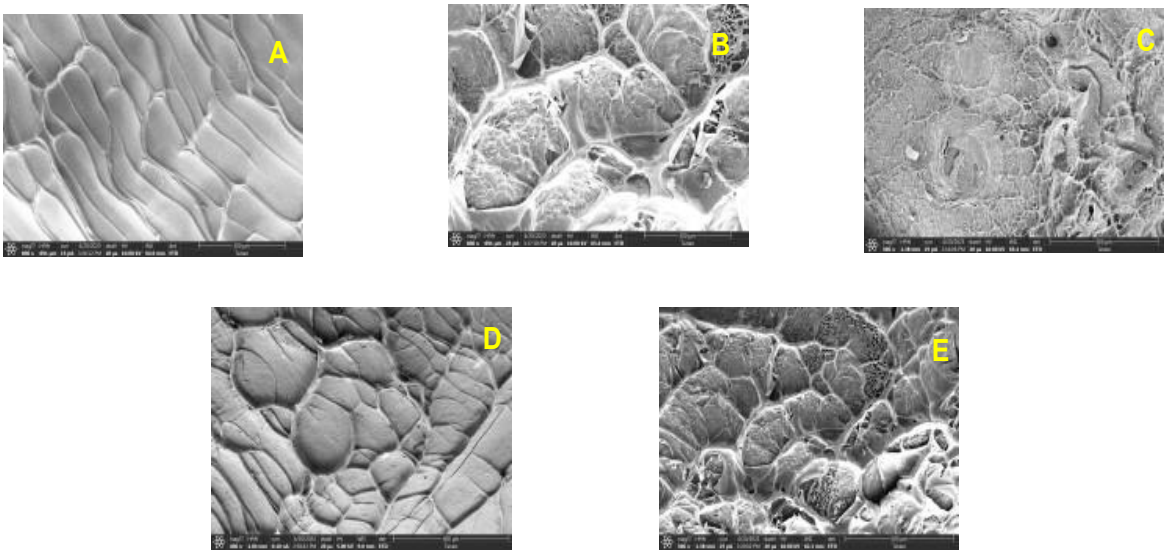


Figure 4: SEM of freeze-dried beads prepared with different alginate - whey protein isolate concentrations A) 100% alginate B) 50:50 alginate - whey protein isolate, C) 67:33 alginate - whey protein isolate, D) 33:67 alginate - whey protein isolate, E) 100 % whey protein isolate beads at 400x magnification

Using a scanning electron microscope to look at the beads' surface morphology (Fig. 3). Compared to whey protein isolate beads, the combination of alginate and whey protein isolate beads were smoother and rounder. Due to the controlled and prolonged release of encapsulated substances made possible by microbeads with precisely defined geometries, creating homogeneous spherical microbeads is preferred for drug delivery applications. A microscopic examination revealed that the beads' surface was wrinkled and wavy. Curiously, wrinkles were more prevalent in formulations with higher SA levels. All the examined beads had small holes and rough outside surfaces. The morphology of the beads produced in this study is consistent with earlier studies that showed alginate gels have a distinctive porosity that allows for the diffusion of tiny molecules into or out of them. It was discovered that as WPI content in bead compositions increased, so did bead size. Anionic polysaccharides are included in sodium alginate formulations to increase the size of the beads, per earlier findings (Mazza et al., 2023; Apoorva et al. 2017). According to these measurements, the WPI droplets were resistant to aggregation.

**Encapsulation efficiency**

The amount of bioactive chemicals encapsulated within the beads correlates with the efficacy of encapsulation. It is a critical feature, for example, for determining the amount or concentration of additives needed for use in a food product. Phenol encapsulation efficiency ranged from 12.6% to 50.0%. The beads with the best encapsulation performance 50:50 (alginate to whey protein isolate) and were substantially different from the other ratios ($p \leq 0.05$), followed by 100% alginate and 33:67 (alginate to whey protein isolate) hydrogel beads. Although encapsulation efficiencies vary widely, alginates have an encapsulation efficiency of approximately 42%. These findings support the notion and are consistent with previous research

on polymers encapsulated in alginate and how WPI aids efficiency improvement. Hibiscus extract's hydroxyl groups enhance hydrogen bond formation with the free carboxyl end groups of the added polysaccharides. Alginate and WPI had better encapsulation effectiveness at equivalent concentrations, which made them more useful. Future FT-IR research may reveal interactions between polyphenols and the used carriers, which could help explain why the encapsulated bioactive compounds have less phenolic activity. These interactions arise because the encapsulated bioactive compounds are involved in binding with the carriers. The polymer composition and structure, the diameter of the dripping needle employed in emulsion dispersion, and the distance between the needle's point and the gelling solution can all affect the size and shape of the beads.

Anthocyanins can degrade due to interactions with oxygen, high temperatures, light, and changes in pH values. Compared to whey protein concentrate (WPC) dispersions, beads containing whey protein isolate (WPI) resulted in a lower loss of monomeric anthocyanins throughout the coating process. This finding demonstrates that choosing the right coating agent is critical in anthocyanin encapsulation. Furthermore, as the content of alginate declines, the content of anthocyanin tends to increase. This suggests that there is a correlation between alginate content and encapsulated anthocyanin levels, implying that lowering alginate concentration can result in higher encapsulated anthocyanin levels (Robert & Fredes, 2015). Alginate is a hydrogel-forming polymer that can establish a physical barrier around anthocyanins, preventing them from being released. The diffusion of anthocyanins from the encapsulating matrix becomes more efficient as the alginate content drops. This increased diffusion allows for more anthocyanin uptake and encapsulation within the beads. Anthocyanins are water-soluble pigments that have improved solubility. As a hydrophilic polymer, alginate can interact with anthocyanins, potentially diminishing their solubility. As the concentration of alginate declines, so do the interactions

between the anthocyanins and the polymer, resulting in increased anthocyanin solubility and encapsulation efficiency.

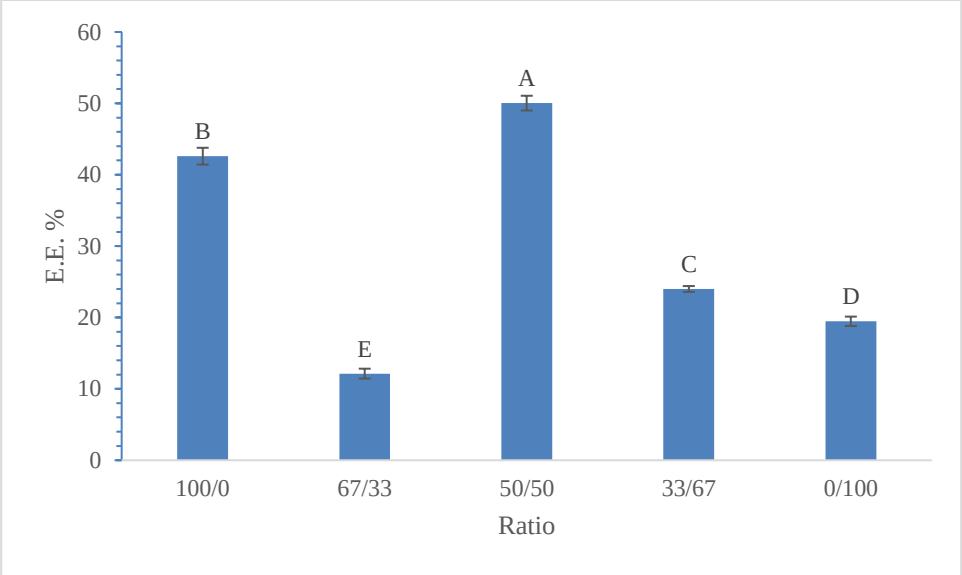


Figure 5: Effect of total phenolic content on different polymer combinations on hydrogel beads' encapsulation efficiency (Total Phenolic content) Error bars show the standard deviation value. Different letters over error bars indicate statistically significant results. E.E. %: Encapsulation efficiency.

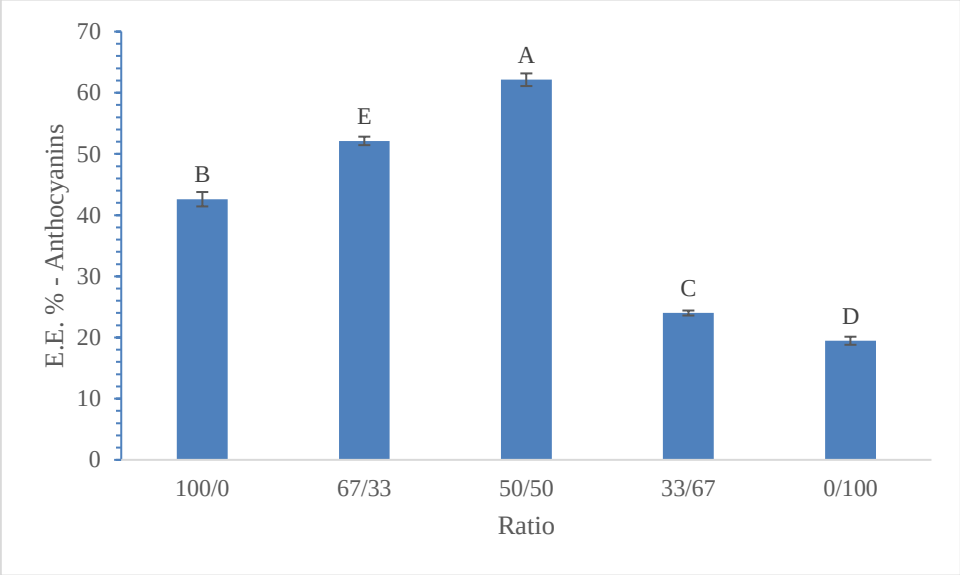


Figure 6: Effect of different polymer combinations on hydrogel beads' encapsulation efficiency (Total Anthocyanin Content). Error bars show the standard deviation value. Different letters over error bars indicate statistically significant results. (E.E. %: Encapsulation efficiency)

**Textural properties**

The textural properties of hydrogel particles are substantially impacted by biopolymer composition and concentration and the kind and concentration of cross-linking agents. This study examined the textural profile (hardness (N) and adhesiveness (N/s)) of a batch of bulk hydrogel particles, as well as the rupture force of a single hydrogel particle. The gel hardness decreased considerably from 408.68 to 340.61 g when the WPI concentration reached 67:33 (alginate to whey protein isolate) and 100% whey protein isolate hydrogel beads. The significant decrease in hardness seen at high concentrations could be attributed to the breakdown of the alginate network and WPI aggregation. WPI-dominated gels developed when WPI concentrations were high, breaking the continuity of the SA network (Wu et al., 2023). Textural properties suggested that a suitable concentration of WPI and Alginate at 50:50 could boost hydrogel strength. The adhesiveness values ranged from -0.978 to -4.11 N x s when WPI was added to alginate (Fig 7). The formation of a thin liquid bridge across the surfaces of the hydrogel beads is most likely responsible for their adherence. There is no significant difference between the adhesiveness of the different hydrogel beads due to several reasons: Temperature, humidity, surface roughness, and the presence of other compounds can all influence the adhesive qualities of a substance. If the experimental circumstances in the research comparing WPI and alginate were equivalent, the reported absence of substantial difference in adhesiveness might be explained. Adhesiveness varies according to the application or intended use. While there may be no discernible difference in overall adhesion capabilities, WPI and alginate may demonstrate differences in adhesive behavior under specific conditions or in specific applications but not in the different ratios used. When comparing the two substances, it is critical to examine the context and goal of the adhesion.

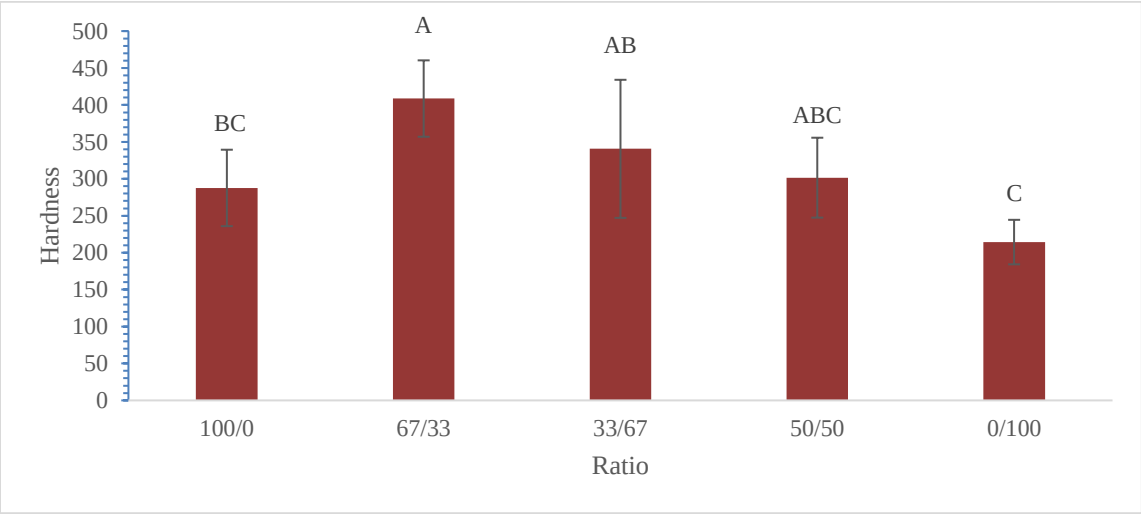


Figure 7: Textural properties of Alginate - Whey protein isolate hydrogels. Hardness (N)

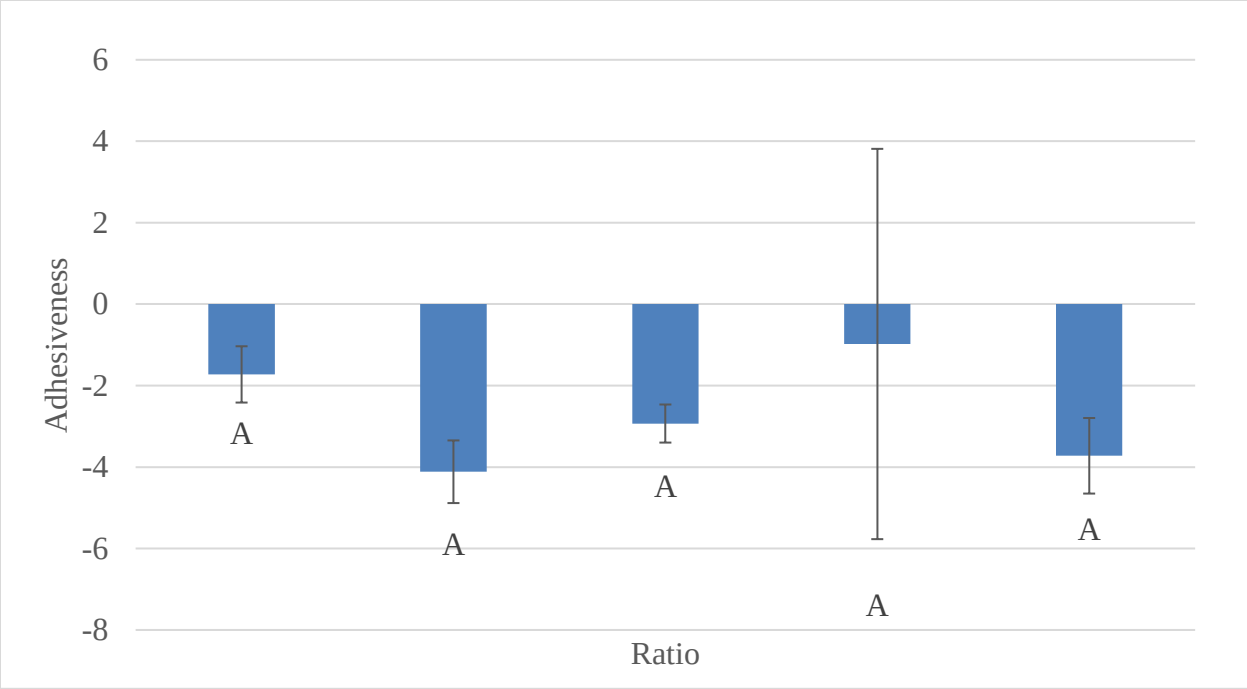


Figure 8: Texture properties of alginate -whey protein isolate hydrogels. Adhesiveness (N/s).

**Swelling properties**

As hydrogels are sensitive to changes in pH, the swelling medium is of the utmost importance in the process of hydrogel swelling. The swelling ratio can alter the amount of release in hydrogel beads. Hydrogel beads are crosslinked polymer networks that can absorb and hold huge volumes of water or other solvents. When the hydrogel beads come into touch with a solution or a medicine, they can swell and absorb the surrounding fluid, causing their size to expand. The release of chemicals from hydrogel beads occurs via diffusion, degradation, erosion, or pH release mechanisms. In the event of diffusion, the drug molecules spread through the swelling polymer network and are released into the surrounding medium. The swelling ratio of the hydrogel beads determines the degree of swelling and the size of the linked pores inside the polymer network.

A higher swelling ratio can result in larger pore diameters and increased diffusion paths, resulting in a greater amount of the encapsulated material being released. A decreased swelling ratio, on the other hand, may restrict diffusion paths and diminish release quantity. As a result, the swelling ratio of hydrogel beads can have a direct effect on the number of chemicals enclosed within them. The encapsulated compounds that are released are significantly affected by swelling. To evaluate the swelling characteristics of alginate - whey protein isolate beads, we employed SGF (pH 1.2) and SIF (pH 6.8) as our test solutions. As seen in Figure 9, the pH of the medium significantly impacts the degree to which the beads swell. We can observe that 100% WPI, 100% alginate and 33:67 (alginate to whey protein isolate) ratios showcased a positive swelling ratio. A pH-sensitive swelling behavior was observed for 100% alginate since the swelling degree of the beads in a buffer with a pH of 1.2 was lower than that in a buffer with a pH of 7.4. Because most of the -COO groups in SA converted into -COOH groups in the low pH zone (pH 1.2), the results obtained can be attributable to this transformation. The weight of the beads gradually decreased during the

subsequent incubation period, which took place in the acidic environment of SGF. According to Pasparakis and Bouropoulos (2006), both 67:33 and 50:50 (alginate to whey protein isolate) hydrogel beads shriveled when exposed to the pH of the intestinal environment. This was most likely caused by the extreme sensitivity of alginate to pH. On the other hand, the stability of 100% WPI was not affected by the pH of the gut (Ouwerx et al., 1998; Remondetto et al., 2004). However, we observed negative swelling ratio in 50:50 and 67:33 (alginate to whey protein isolate). We hypothesis that the phenol compounds were inhomogeneously dispersed within the matrix and organized in such a way that interfacial protein layers formed. We also anticipate that the effect of protein concentration on liberation will be dictated by two opposing tendencies: one is increased release with increased protein content due to swelling, and the other is decreased release with increased protein content (Volić et al., 2018).

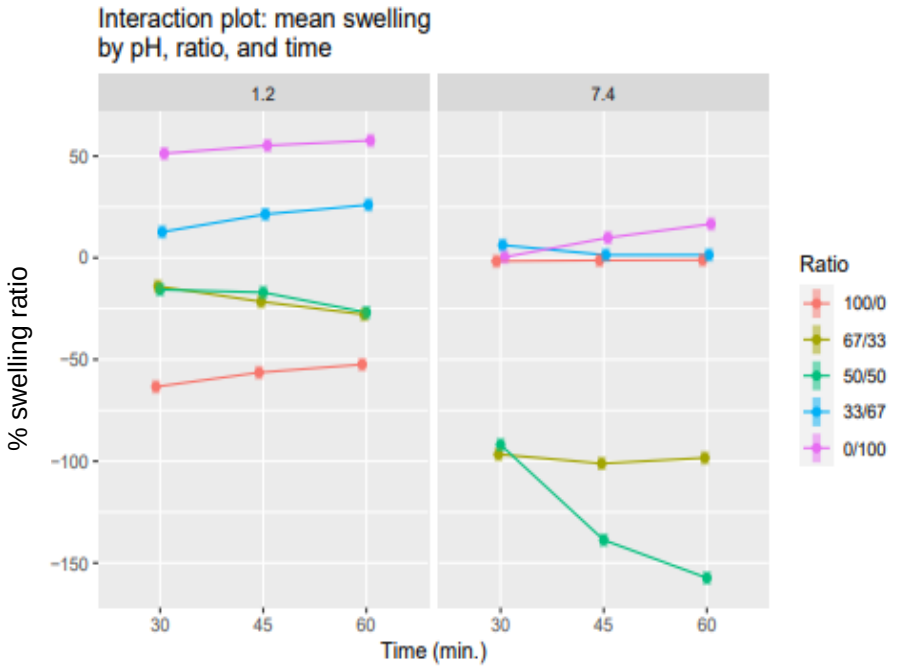


Figure 9: Swelling ratio represented as % of hydrogel beads at pH 1.2 and 7.4

***In vitro* release**

In this investigation, we assessed the release properties of hydrogels that have been enclosed. Phenolic levels rose during stomach to intestinal digestion before plateauing. Digestion can be divided into stomach (60 mins) and intestinal (120 mins) phases based on *in - vitro* release qualities. However, in our analysis, digestion of all samples resulted in a significant release of phenolics (14–44%), as previously found (Flores et al., 2014). At gastric pH, a substantially higher fraction of phenols was released, whereas a smaller amount % was released at intestinal pH. The 50:50 beads released profiles resulted in approximately 40% being released within the first 60 minutes followed by a stagnant release in the intestinal phase. We observed negative swelling ratio, higher release could be due erosion or degradation of the hydrogel beads. Apart from that looking at the SEM images we can notice some wrinkles on the surface of the hydrogel beads as well. The sudden release of phenols seen may have some connection to the electrical repulsion between polymers and phenols (Déat-Lainé, 2012). However, the release stagnated in the intestinal system after 100 mins. The co-precipitation of phenolics with whey proteins and other constituents in the digesta may have contributed to the low levels in the intestinal system. Like the phenolic release in 100% WPI, where we steep decrease in the release profile, a prior study found that the pH and simulated salts alter the release of phenols from whey protein hydrogel beads. Based on this criterion, according to the study, hydrogel bead sample 50:50 (alginate to whey protein isolate) had a more significant concentration of phenolics released after digestion than samples 100% alginate and 67:33 (alginate to whey protein isolate) hydrogel beads, which had higher alginate concentrations. Alginate is a biopolymer derived from brown algae that, in the presence of divalent cations such as calcium ions, forms a gel-like structure. The gel matrix provides a barrier and delays the release of encapsulated compounds. In the case of 100% alginate beads, the absence of

WPI could result in a more robust and denser polymer network, thereby decreasing the diffusion and release of phenolic compounds. In comparison to the 50:50 (alginate to whey protein isolate) beads, this can result in a lower phenolic release (Golmohamadi & Wilkinson, 2013).

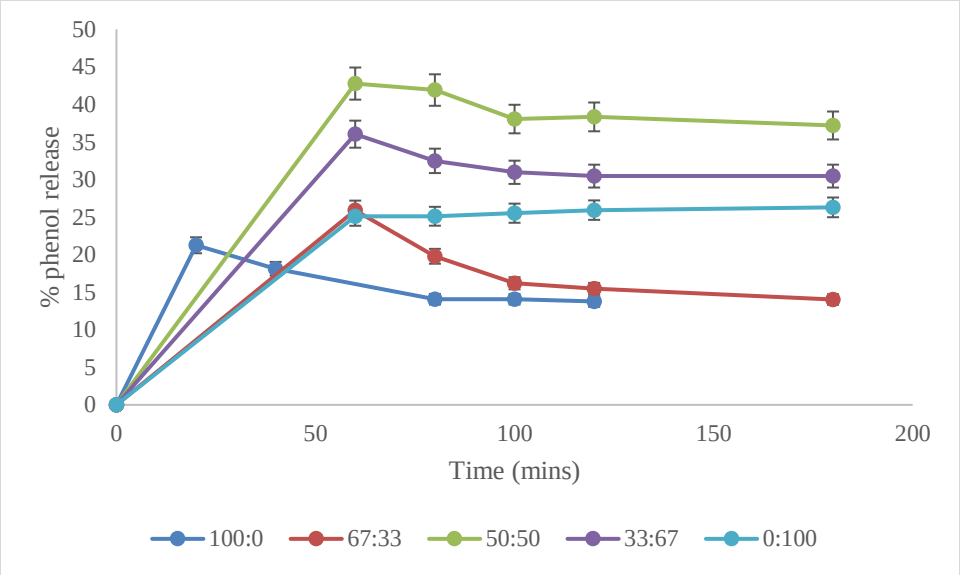


Figure 10: *In - vitro* release of anthocyanins from Alginate - Whey protein isolate hydrogel beads in SIF (Ratios of alginate: whey protein isolate - 100:0, 67:33, 50:50, 33:67 and 0:100)

# SUMMARY

- The lowest water activity $a_w$ was observed in 50:50 alginate and whey protein isolate hydrogel beads.
- The highest encapsulation efficiency % was observed in terms of total anthocyanin content and total phenolic content in 50:50 (50% alginate and 50% whey protein isolate) hydrogel beads.
- No significant difference was observed between the adhesiveness of different hydrogel beads.
- The strongest gel strength was observed in 67:33 (67 %alginate and 33% whey protein isolate) hydrogel beads.
- The presence of wrinkles on the hydrogel surface is often associated with higher alginate content.
- As the alginate content decreases, the hydrogel becomes more flexible and less prone to surface wrinkling.
- The highest % phenol release in the gastrointestinal tract was released by 50:50 (50% alginate and 50% whey protein isolate) hydrogel beads.

# CONCLUSION

Microencapsulation is a potential approach for encapsulating bioactive chemicals at the same time. Ionic gelation encapsulation is one of the most promising microencapsulation technologies for protecting and delivering polyphenols and anthocyanins because it can achieve relatively high encapsulation efficiency, stability, and effective controlled release under certain conditions. A 50% alginate and 50% whey protein isolate combination gave the highest encapsulation efficiency with regards to total phenolic content and total anthocyanin content. The % phenol release was also observed to be higher in the gastrointestinal system.

Researchers can gain a deeper understanding of the structural and chemical properties of polymer combinations by utilizing advanced characterization techniques such as spectroscopy, microscopy, and rheology. This can aid in determining the optimal combinations for applications. Future work may involve the design and synthesis of novel polymers with specialized properties for encapsulation applications. This may include polymers with precisely controlled solubility, surface properties, or sensitivity to external stimuli, allowing for precise encapsulation control.

# ABBREVIATIONS

- E.E - Encapsulation efficiency
- Hs - Hibiscus
- ALG - Sodium alginate
- WPI - Whey protein isolate
- ALG: WPI - Alginate: Whey protein isolate
- TAC - Total anthocyanin content
- TPC - Total phenolic content
- SIF - Simulated intestinal fluid
- Hardness (N) – Newton, unit to measure hardness of hydrogel beads
- Adhesiveness (N/s) – Newton per second, unit to measure adhesiveness of hydrogel beads

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