Formulation and characterisation of novel edible-packaging for fruits and vegetables

Degree Project Report

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Abstract

This master's thesis investigated the potential of algae raw materials, specifically k-carrageenan and to a lesser extent alginate, as film-forming ingredients for the development of a novel edible packaging solutions. The project involved the testing and characterisation of several active ingredients of algal and plant origins both as extracts and as ingredients in films. The effect of these films on the shelf life of cucumbers was evaluated over the course of a 14-day shelf-life study where coated samples were compared to plastic film-wrapped and unwrapped controls, respectively. The project involved the development and characterisation of different coating formulations, and the subsequent evaluation of their effectiveness in prolonging shelf-life by through weight loss, scavenging activity (DPPH), colorimetry and relative electrolyte leakage (REL) measurements. A The results indicated that coatings did not significantly extend shelf-life in coated cucumbers when compared to uncoated and plastic-wrapped samples. The rate of weight loss of coated and uncoated cucumbers was similar, and plastic-wrapped samples lost significantly less weight ($p<0.05$). REL values were lower for uncoated and wrapped controls than for coated samples. Results from DPPH-assay and colorimetric measurements were inconclusive, and it is suggested that these experiments be repeated with some modifications. Overall, results provide important insights into the suitability of carrageenan-based coatings as edible coatings, with and without the addition of active ingredients. The results of this study demonstrated that further research on formulation and development of films is necessary to ensure satisfactory mechanical and barrier properties, and thereby quality retention in coated produce. Future outlooks include the continued evaluation of k-carrageenan formulations, different antioxidant sources and incorporation methods. Replicating the study (with modifications) with a secure access to instruments and resources may also provide further insights.

Acknowledgements

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[1] To preserve secrecy, the true name of the company has been anonymised and is referred to as "The Company" throughout this report.

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List of Abbreviations

- **BHA:** Butylated hydroxyanisole

- **EOs:** Essential oils

- **EU:** European Union

- **LEC:** Long English Cucumber

- **GBP**: Gas barrier properties

- **WVBP**: Water vapour barrier properties

- **OSA:** Octenyl Succinic Anhydride

- **OSA-starch**: OSA-modified starch

- **dwb**: Dry weight basis

- **ROS**: Reactive oxygen species

- **RH:** Relative humidity

- **Tg:** Glass transition temperature

- **WVTR:** Water vapour transmission rate

- **WVP:** Water vapour permeability

- **PS:** Puncture strength

- **DPPH:** 2,2-Diphenyl-1-picrylhydrazyl

- **LFU/DFU:** "Light" / "Dark" [algae extract], Filtered and Unconcentrated

- **LFC/DFC:** Light / Dark [algae extract], Filtered and Concentrated

1.   Introduction

The growing length of the food supply chains as a consequence of the substantial changes in consumption patterns has introduced time-consuming steps between the harvesting, processing and consumption of perishable products (ING, 2019). The elongation of their shelf-life is of utmost importance in order to reduce food waste by maintaining their quality and nutritional value for an extended period (Ghafoor et al., 2022).

Multiple preservation methods are implemented, including use of pesticides, cold storage, or physical techniques such as packaging (Ghafoor et al., 2022). Conventional packaging materials such as plastic, are fossil-fuel derived and do not readily degrade, leading to a significant increase in environmental pollution and accumulation of synthetic preservatives such as BHT in soil and water (Wang et al., 2022). Its use is widespread as it accounts for 40% of all packaging materials in the food and beverage industry, a percentage that increases annually at a rate of 2% in EU (ING, 2019).

As plastic utilisation has become a major concern, its gradual replacement with natural or renewable sources is of importance and has highly motivated this study. The use of bio-based packaging materials made from natural resources such as algae can be a promising alternative to existing solutions. The incorporation of active ingredients, such as antioxidants, into the edible film formulation may be another beneficial feature that can enhance the quality and shelf-life of the coated product (Pham et al., 2023; Kaur et al., 2023; Singh et al., 2022).

This project investigated the use of carrageenan-based edible coatings with the addition of extracts as a tool for extending the shelf life of cucumbers. In addition, the antioxidant properties of extracts and prepared films were studied and quantified.

To our knowledge, there have been no similar studies performed using algae-based coatings with the incorporation of the selected active ingredients in cucumbers. Therefore, with this work we aspire to provide the field with new information regarding the effects and properties of edible carrageenan-based films and coatings as a way of preventing post-harvest losses of cucumbers by extending their shelf-life and overall sensorial acceptance.

2. Aims and objectives

2.1 Aims

This thesis project had two aims:
- The first was to develop and characterise edible film-forming formulations using commercially available algae raw materials
- The second was to assess the effect of these formulations on shelf-life of cucumbers

2.2 Objectives

The specific objectives related to the first aim of the project are the following:
- Development of at least one edible film formulation based on commercially available algae raw material
  - incorporation of two selected active ingredients
- Evaluation of the physicochemical properties of all prepared films
  - Thickness
  - Water vapour permeability
  - Puncture force
  - Colorimetry
- Evaluation of film's anti-oxidative active properties

The specific objectives related to the second aim are the following:
- Evaluation of effect on shelf-life of cucumbers via selected parameters
  - Weight loss

- REL
- Antioxidative Activity of skin samples
- Colorimetry

3. Literature Study

3.1 Formulation

This section summarises the current landscape of knowledge found in the literature regarding various aspects of the formulation of edible films, as well as a brief summary of factors associated with the shelf life of cucumbers and produce in general.

The shelf life (or amount of time for which produce is considered safe to eat while remaining attractive to consumers) and quality of all fresh produce is influenced by a variety of pre-, and post- harvest factors.

Pre-harvest factors include cultivar type, fruit morphology, position on the plant, plant health, growing conditions etc. and play a decisive role in the future health and senescence of resulting fruits by influencing biological mechanisms (Jolliffe & Lin, 1997; Schouten et al., 2002, 2004). From a practical perspective, senescence leads to a general loss of quality in the product and is observed through the appearance of scalding, yellowing, pitting of the skin, lesions and/or loss of turgidity (ed. Kader, 2002).

In the case of the Long English Cucumber[2] (LEC), the effect of these pre-harvest factors can be observed relatively easily: smaller fruits will lose water faster than larger ones, and fruits which were partially shaded by leaves during growth will have a lighter colour and have a shorter shelf life than darker LECs of equivalent size. These examples highlight two crucial parameters that have been identified as shelf-life indicators in LECs: chlorophyll content (or colour intensity), and water/weight loss (Jolliffe & Lin, 1997; Schouten et al., 2002). Although other parameters such as total soluble solids (Valverde-Miranda et al., 2021) are also good indicators of senescence progression, the monitoring the colour and weight of fruits have the advantage of being quick, easy, and non-destructive methods. The conditions of storage, or post-harvest factors of LEC also play a critical role in predicting shelf life: relative humidity, temperature, air flow and atmospheric composition are generally considered to be most closely associated with keeping quality in LECs (Kaur et al., 2021).

Edible films and coatings, produced from materials with film-forming capacity, can act as a barrier to the environment and thus provide physical protection to the coated product. The coating of fresh produce such as cucumbers can be beneficial to their quality parameters and enhance their stability throughout their shelf-life (Kocira et al., 2021). Edible films can reduce moisture migration and thereby minimise weight loss, as well as alter gas composition within the packaging by for instance restricting access to oxygen which slows down respiration, a principal factor in senescence (Hodges et al., 2004). Slowing down respiration also minimises weight loss. Generally, films derived from only polysaccharides exhibit satisfactory gas barrier properties, but inadequate barrier properties for water vapour, allowing water to leave the system and eventually leading to weight loss (Kocira et al., 2021). In contrast, (hydrophobic) protein-based and lipid-based films and coatings provide a better barrier against moisture and gas exchange but tend to be less flexible. Lipid-based formulations may also improve the general appearance of the coated products providing them with a polished surface (Kong et al., 2022).

The presence of reactive oxygen species (free radicals, peroxides etc.; ROS), which are naturally produced during normal cell function and stimulated by environmental stress, requires the presence of antioxidant enzymes and antioxidants such as polyphenols or organic acids for deactivation. Although ROS do play a role in cell signalling pathways, they are also partly responsible for the deterioration of

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[2] Long English Cucumbers, also known as Dutch or greenhouse cucumbers are the type of cucumber most commonly found in European supermarkets.

quality in LECs by breaking down pigments such as chlorophyll (colour loss), causing cell damage which can result in leakiness (weight loss, loss of turgidity). As both the antioxidative enzymes and antioxidants endogenous to the fruit are progressively inactivated over time, the limited ability of cells in post-harvest fruits to replace them leads to an accumulation of ROS which results in accelerated senescence via several mechanisms (Das & Roychoudhury, 2014).

Attempts have been done to solve this problem via the inclusion of antioxidants in edible coatings in the form of so-called "active ingredients" or other antioxidant-rich extracts. Indeed, a variety of extracts have been shown to increase shelf-life and overall keeping quality of LECs and other fruits or vegetables, including cinnamon oil, pomegranate peel extract (Gupta et al., 2016; Nair et al., 2018; Zhang et al., 2015) and ascorbic acid (Lee et al., 2003). The hypothesis behind one mode of action of these extracts is that the inclusion of antioxidants in the coating reduces oxidative stress by reacting with ROS produced by chloroplasts and respiration of cells in the skin, thus preventing ROS accumulation and preserving chlorophyll, among other effects (Das & Roychoudhury, 2014).

3.2 Composition of edible coatings & properties

This section focuses more closely on the ingredients used in the edible films produced as part of this thesis. It is further divided into three subsections: the first describes film-forming ingredients, while the second focuses on additives that support film-formation. The last section discusses active ingredients added to the films to enhance its properties.

3.2.1 Structural ingredients

Natural materials with film-forming properties that can be used as edible coatings include polysaccharides (ex: alginates of various types), proteins (ex: zein), lipids, starches (ex: corn starch) or a combination of these compounds (Mouzakitis et al. 2022; Suhag et al., 2020). Hydrocolloids can be used to form films with good GBP (O_2 and CO_2), as well as desirable mechanical properties that can have a protective effect when applied to delicate produce (Krochta et al., 1994). Their ability to form films when hydrated varies based on the particle's structure as well as other parameters such as ionic strength, pH, and temperature (Krochta et al., 1994).

Polysaccharides from brown algae – A. nodosum

Most typical carbohydrates found in algae cannot be digested by humans. However, many have found their way into the food industry: indeed, some of these

polysaccharides readily form films when hydrated, or can act as stabilisers, thickeners, or additives. Many such useful polysaccharides can be found in *Ascophyllum nodosum*, a type of brown algae found in the North-Eastern Atlantic Ocean (Pereira et al., 2020). Of these there are numerous types, the ones relevant to this work being shortly described below:

Alginates (alginic acids) are linear co-polymers of β-D-mannuronic acid and α-L-glucuronic acid residues linked via a (1→4) bond which are arranged in hetero-polymeric (M-G) and/or homo-polymeric (M/G) blocks. They are present in several types of algae, including *A. nodosum* where it represents 20-29% of total content (Pereira et al., 2020). Their ability to form water-soluble networks in the presence of divalent (ex: Ca^{2+}) ions has attracted much interest for commercial applications, both in the food industry as an additive and in the pharmacological industry as a component in drug-delivery systems. It is interesting to note that aside from the ionic strength of the medium, the relative proportion of M and G blocks within alginic acid influences the physical properties of the gel: stronger gels are obtained when G-blocks are present in higher amounts (Santos & Melo, 2020).

Laminarin**s** are glucans present in varying amounts (2-36% total) in *A. nodosum*. They are classified as a branched low molecular glucose polysaccharide (or glucan), with β(1→3) linked backbones and branching via β(1→6) bonds (Stone, 2009). They are soluble in water of varying temperatures depending on the form they are found in. If found in a soluble form, they readily form water-soluble gels.

k-Carrageenan

Useful compounds can also be found in red algae, as illustrated by one of its most famous products: carrageenan. Carrageenan is a natural polysaccharide with multiple applications in the food industry. Its use in edible film formulation has attracted considerable interest due to its low cost, nontoxicity, biodegradability, film-forming properties, and renewability (Teixeira-Costa & Andrade, 2021; Cazón et al., 2017; Larotonda et al., 2016). The term carrageenan refers to a family of anionic polysaccharides composed of 3-O and 4-O galactopyranosyl units with bound half-ester sulphate groups at positions 2C or 6C. Carrageenan occurs mainly in three forms called kappa (k), iota (i) and lambda (l), respectively, all of which have their uses within the food industry. Collectively, they and make up to 80% of the cell walls in members of the Rhodophyceae family. These forms of carrageenan are differentiated by their respective sulphate content as well as the presence of 3,6-anhydrous galactopyranosyl units which give rise to gelation (BeMiller, 2019a). Generally speaking, properties such as solubility, texture and protein reactivity are associated with the degree of sulfation in the manner illustrated by the table below:

Table 1. Relation between structure and seaweed polysaccharides (in BeMiller, 2019, pp- 283.)

Gum	3,6-Anhydro rings	Sulfate[a] half-ester content	Solubility[a,b]	Gelling ability and gel strength[a,c,d]
Agar	+			
Furcellaran	+			
κ-Carrageenan	+			
ι-Carrageenan	+			
λ-Carrageenan[e]	−			

[a]Arrows point in the direction of increases in the constituent or property.
[b]Of the sodium salt of the carrageenan in water.
[c]Water gels.
[d]See Table 13.3.
[e]Nongelling.

In the presence of monovalent (Na^+ and K^+) ions, k-carrageenan forms strong, brittle thermo-reversible gels. The mechanism by which gelation occurs is hypothesized to be as follows: dissolved coiled polysaccharide strands will reversibly form double helices as they begin to cool. As the cooling process progresses, the thermodynamic equilibrium of the double helix conformation is favoured to lower the overall Free energy of the system. The presence of K^+ ions lower this energy further by nesting between two separate double helices, forming a rigid tertiary structure, illustrated in figure 1 below, featured on page 286 of BeMiller's textbook (2019b).
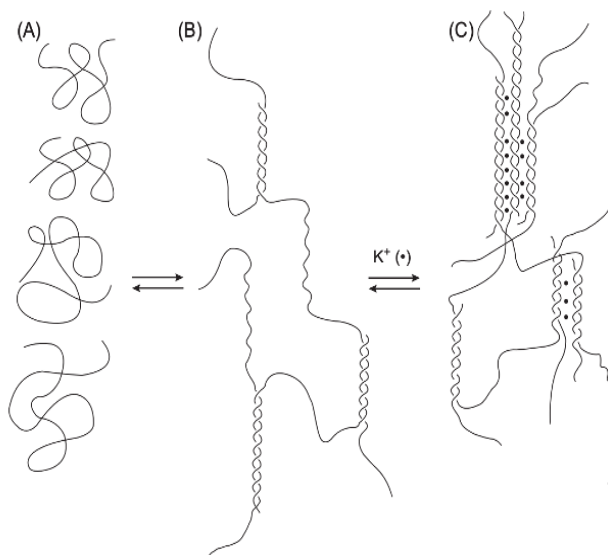
Fig. 1: Hypothesized mechanism for gelation of carrageenan - "egg-box" model.

The relationship between the thermo-reversibility of k-carrageenan gels and ionic strength was described in 1982 by Cyrille Rochas, a doctoral student from the University of Grenoble, France. In his work, he describes how this sol-gel transition from a disordered state (A in fig X above) to an ordered one (C) occurs at a specific temperature known as T_{d-o}; the value of which varies depending on the total concentration of ions in solution (C_T). This includes both ions originating from added salts such as K^+ and ionic groups found in the k-carrageenan itself[3].

The relationship between C_T, the concentration of sulphated groups in k-carrageenan (C_P), the concentration of dissolved ions (C_S) is described by the equation below:

$$C_T = C_s + \gamma C_P$$

where γ is the average activity coefficient. For potassium/k-carrageenan complexes, C_T is 0.007 mmol/dm^3 and γ is 0.55 (Tecante et al., n.d.). This formula was used to help determine the relative amounts of k-carrageenan and KCl in the formulation stage of this work (see section 5.3.1).

Starch & OSA-modified starch

Starches of various kinds are readily used as thickening agents in various industries due to their availability, price point and versatility. Their ability to stabilise colloidal emulsions and form films upon cooling following gelatinisation are some of many attractive features they possess.

The gelatinisation process is of great significance within the field of edible packaging: upon heating in aqueous solution starch granules swell significantly and a portion of free amylopectin is released. Swelling continues until the breaking point is reached and more amylopectin is released into the solution, crosslinking into a 3-D network as it cools. If a relatively thin layer of this starch solution is cast onto a surface and allowed to dry, a thin, opaque film will form. The resulting film possesses high GBP but low WVBP, which limits the use of starch as a primary ingredient in the formulation of edible films. To improve the film's properties, overall hydrophilicity (and as a result water vapour permeability, although other factors are also involved) must be lowered, which is commonly done through chemical modification using octenyl succinic anhydride (henceforth OSA; Fuentes et al. 2023). The chemical reaction results in the substitution of OSA groups onto

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[3] Dr Rochas produced a phase diagram illustrating the different states of k-carrageenan and the relationship between all the factors described in this section. To see this diagram, we refer you to appendix I.

the glucose units of starch[4] at carbons 2, 3 or 6 (Nilsson & Bergenståhl, 2006). The method used to obtain OSA-starch in this work is based on Bajaj et al., 2019, and is described in detail in section 5.2.1.

The resulting starch possesses many attractive characteristics including increased amphiphilic behaviour, surface activity and emulsifying properties, all useful in food formulation applications. Indeed, the ability of OSA-starch to stabilize oil-in-water emulsion was demonstrated in Nilsson & Bergenståhl, 2006. This makes the modification of starch a useful tool in food systems with dispersed oil droplets, as well as in edible film formulation where the inclusion of oil in the packaging can be used to increase WBP further (see section 3.2.2). The authors also point out that the addition of these charged groups result in the molecule becoming charged at low pH, an advantage compared to proteins which tend to lose their charge in such conditions, losing their surface activity. The extent to which these properties emerge depends on the degree of substitution of the starch, which tends to be rather low[2].

Blends of starch and k-carrageenan and/or other non-protein hydrocolloids such as agar or xanthan gum are commonly found in many food products such as dairy desserts, mashed potatoes (Fernández et al., 2009a) or mayonnaise (Magnusson & Nilsson, 2011) and the resulting rheological properties and effect on mouthfeel and creaminess have been studied. Results suggest that the addition of k-carrageenan may be beneficial in reducing synerisis in starchy systems and improves sensory properties when present in a carrageenan/xanthan gum blend (Fernández et al., 2009b).

Zein

Zein is a major storage protein found in the corn endosperm (Mouzakitis et al., 2022b). Films and coatings formulated with proteins as the main component, or composite films made by combining polysaccharides and proteins, are usually expected to possess better mechanical and water vapour barrier properties than films derived from polysaccharides (Mihalca et al., 2021; Chen et al., 2019). The above is most likely a result of the multiple functional properties of the 20 different amino acids that proteins consist of. A higher proportion of nonpolar and a lack of charged amino acids increases the hydrophobicity of the protein molecule, as seen in zein (Mouzakitis et al., 2022c; Cabra et al., 2005). Edible coatings formulated from zein

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[4] the reaction may occur at several sites in both amylose and amylopectin, although it was stated in Larsson & Bergenståhl 2006 that it preferentially occurs at the amorphous branch points of amylopectin. They also clarify that a typical degree of substitution for food applications is 0,01-0,03.

are reported to act as good barriers for the transfer and absorption of moisture, oxygen and the migration of lipids (Mouzakitis et al., 2022d).

3.2.2 Additives

The principal additives tested throughout the course of the project are introduced here. Edible films and coatings can facilitate the addition of various components that aim to give films auxiliary functionalities, such as antimicrobial or antioxidant capacities or to improve their mechanical or barrier properties by the inclusion of plasticisers or oils. Additives may therefore extend the applications of edible films and potentially have a positive effect on the coated products (Kong et al., 2022b; Atarés & Chiralt, 2016).

Plasticisers

Plasticisers are usually included in film formulations with the aim to enhance their mechanical properties, reducing brittleness and increasing flexibility (Kocira et al., 2021b). Compounds that are usually added to act as plasticisers are low molecular weight carbohydrates, polyols, lipids and their derivatives, and water (Chen et al., 2019b). The plasticisers enter the polymeric structure and by reducing the intermolecular forces between the polymeric chains, increase the latter's mobility (Sothornvit & Krochta, 2005).

Three theories can explain their effect (Sothornvit & Krochta, 2005):

- Lubrication theory: the plasticiser acts as a lubricant to promote the movement of polymers
- Gel theory: the plasticiser prevents the reactions between monomers
- Free volume theory: the plasticiser may lower the Tg by increasing the free volume of the polymer

The incorporation of plasticisers does not come without disadvantages. Their ability to reduce the intermolecular attraction of polymer chains may decrease the barrier properties of the film against gases, water vapour and other compounds such as aromatic molecules (Rodríguez et al., 2006).

Glycerol

Glycerol is a commonly used plasticiser in the production of edible films. Due to its high hydrophilicity, its incorporation in film formulations may increase the amount of moisture in the end films and enhance their water solubility, as observed in the publication of Nouri et al. (2018). Depending on the concentration of

incorporation, its effect on the mechanical properties of the films such as plasticity, brittleness, fragility, and permeability vary (Tarique et al., 2021).

Lipids

The incorporation of a lipid component into the film formulation has positive effects the WVBP of the film and may help increase resistance to water in soluble films. Best results in increasing WBP were observed with addition of lipids originating from solid (saturated) sources, or in intermediate (C14-C18) hydrocarbon chains from liquid sources (ex: olive or rapeseed oil) or in acid salt-form (ex: oleic acid) (Krochta et al., 1994). The reason for the increased effectiveness of saturated fatty acids is that they generate less disturbances in the network structure, thereby resulting in a tighter, less permeable network. Lipids in the form of waxes are also commonly used as an additive to increase pliability, and shellac for its G/WBP and for the glossy finish it produces in confectionary goods especially. (Krochta et al., 1994)

Salts

Calcium chloride ($CaCl_2$) is a divalent metal ion that was used for the initiation of ionic crosslinking reaction with sodium alginate. The presence of divalent ions such as $CaCl_2$ is necessary for the formation of strong heat-stable alginate gels. The alginate gelation is not a fully understood process, however, the mechanism to obtain a sol/gel transition is dependent on the ion exchange process between the counter-ions of sodium alginate and the Ca^{2+}. A cavity of two diaxially linked guloronic residues is formed which is a binding site for the calcium ions. This arrangement is portrayed in figure 1 (see section 2.3.1) and its commonly referred to as the eggbox model, with a similar structure to that seen carrageenan (Cuadros et al., 2012).

The mixing of sodium alginate and calcium ions leads to a rapid and irreversible bonding between the two compounds and is commonly conducted following the diffusion method. In this method, crosslinking first occurs on the film's surface when ions meet alginate, inducing crosslinking. As a result, the migration of $CaCl_2$ deeper into the film is restricted, which creates a Ca^{2+} concentration gradient in the gel (Tavassoli-Kafrani et al., 2016). This may result in uneven properties in thicker gels but has not been commented on as affecting the properties of resulting films once dried. The concentration of the $CaCl_2$ solution also influences the structure of the alginate gel, where increased concentration leads to a denser and less porous network where the permeability of the film is decreased (Aslani & Kennedy, 1996).

The role of potassium ions in carrageenan gelation are detailed in the section named "k-carrageenan" above.

3.2.3 Active ingredients

The incorporation of different antioxidants into the film formulations is of great interest with the aim to investigate their effect on the quality and shelf-life of products by reducing oxidation phenomena. Vitamins C and E, essential oils and phenolic compounds are among the most used antioxidants in the production of active edible packaging (Manzoor et al., 2023; Salgado et al., 2015b).

Ascophyllum nodosum & algae extracts

Algae of various types contain a variety of potentially useful components other than alginate or carrageenan: indeed, many species including *A. nodosum* contain antibacterial and anti-fungal molecules of various classes, including polysaccharides with advantageous rheological and biological properties such as fucoidans and ascophyllan (Pereira et al., 2020). Additionally, extracts often contain considerable amounts of antioxidants, mainly found in the form of phlorotannins which have shown promise in various study in combatting oxidation in food products and improving plant growth (Cassani et al., 2020; Fan, 2010). These features make the use of algal extracts to edible packaging formulations an attractive proposition, although attention must be paid to ensure the stability of these antioxidants present in amounts ranging from 4-13% of dry weight basis (dwb) in *A. nodosum* (Agregán et al 2018).

The review by Cassani et al. (2020) highlights the many factors influencing the stability of phlorotannins, and the attempts which have been made to preserve them through microencapsulation to protect them from oxidation inducing factors such as oxygen. The extraction method used to obtain the extract also has a significant impact on phlorotannin content of the extract, and thereby on resulting antioxidative capacity.

Pennyroyal

Mentha pulegium L., is an aromatic herb member of the *Lamiaceae* family (El-Gazar et al., 2022). Various *Mentha* species are extensively employed in the food and pharmaceutical industry as food flavourings and ingredients in pharmaceutical formulations (Anwar et al., 2019; Esmaeili et al., 2006).

The EOs and extracts obtained from this plant exhibit various physiological benefits and demonstrate several bioactive properties, such as antioxidant, antimicrobial, anti-inflammatory, and antiallergic activities (Teixeira et al., 2012). The extracts deriving from this plant exhibit antioxidant activities, which may be attributed to their phenolic-rich chemical profile, such as rosmarinic acid, caffeic acid and flavonoids (Teixeira et al., 2012). The antioxidant activity of these compounds

derives mainly through a redox mechanism. The components act as reducing agents that can scavenge free radicals, neutralise reactive oxygen species (ROS), and prevent lipid peroxidation (Kotha et al., 2022). The antioxidant activity of Pennyroyal has potential applications in the food industry as a natural preservative. The plant's essential oils and extracts have been shown to inhibit the growth of microorganisms, which can cause spoilage and oxidation of food products therefore prolong the shelf-life of food stuff (Domingues, 2019).

Greek Mountain tea

Sideritis scardica (family *Lamiaceae,* genus *Sideritis*) also known as (Greek) mountain tea is a perennial herbaceous plant growing in the central Balkan peninsula (Yanchev et al., 2022). Traditionally, the aerial parts of the plant are infused into a fragrant tea that is known to have healing properties against respiratory diseases in addition to their anti-inflammatory and gastroprotective action (Żyżelewicz et al., 2020).

Associated with the phenolic compounds present in the plant (phenolic acids, flavonoids, lignans, stilbenes, tannins), alcohol extracts obtained from the mountain tea offer a protection against oxidation. More specifically, the basic bioactive compounds found in *S. scardica* with proven antioxidative properties, are phenylethanoid glycosides, namely verbascosides and forsythosides, flavonoids such as apigenin and luteolin and phenolic acids (mainly caffeoylquinic acid, p-coumaric acid 4-O-glucoside and feruloylquinic acid) (Żyżelewicz et al., 2020).

The extraction of phenolic compounds from *Sideritis scardica* is highly influenced by the extraction method, the conditions of the extraction such as temperature and other parameters such as the solvent used and the plant to solvent ratio. According to the review of Todorova and Trendafilova (2014), the maximum content of polyphenols is obtained with extraction by maceration with water/ethanol ratio at 20:80.

Spirulina

Spirulina platensis is a species of cyanobacteria that has been gaining increasing attention due to its potential health benefits (Wu et al., 2016). One of the most notable properties of spirulina is its high antioxidant activity, which has been attributed to its abundant content of various bioactive compounds (Kumar et al., 2022). Phytonutrients and protein-pigment complexes such as phycocyanins and β-carotene are the usual mediators for the antioxidant protective effects (Hossain et al., 2016; Wu et al., 2016). The former act by scavenging ROS while the latter inhibits singlet oxygen-mediated lipid peroxidation (Wu et al., 2016). Studies on

the incorporation of Spirulina biomass and extract into films, have indicated positive results on the antioxidant capacity of films and the shelf-life of products (Karimzadeh et al., 2023; Kontogianni et al., 2021).

Methods of incorporation of active ingredients

Active ingredients such as antioxidants can be incorporated into edible coatings to enhance the nutritional and sensory qualities of food products. The most common methods used for this addition as explained by Yemenicioğlu (2022), are:

- Incorporation, which involves the solubilisation, dispersion, or emulsification of free or encapsulated, hydrophilic or hydrophobic agents.
- Encapsulation of active agents can improve their stability and maintain their release rates throughout the shelf-life of the product.
- Impregnation by dipping of film into a solution of active ingredients or coating by spreading, spraying, and brushing of active agent solution onto the edible film surface are other methods with an effect both on and below the food contact surface.
- Immobilisation of active ingredients can be achieved by the creation of charge-to-charge interactions, covalent crosslinking or hydrogen bonding between active agents and hydrocolloids. The effects of this technique can be observed only on the food contact surface.

Alternatively, hydrocolloids with antioxidative properties may be used for the formulation of active packaging as their ability to bind with negatively charged bacterial surfaces leads to the inactivation of microbial cells (Yemenicioğlu, 2022). The various methods used are summarised in table 2 below:

Table 2: Methods of active ingredients' addition in film formulations, from Yemenicioğlu (2022).

Description	Method	Comments
Solubilization/dispersion/ emulsification of free or encapsulated active agent	Incorporation	hydrophilic: solubilised into formulation, hydrophobic: homogenised with film-forming hydrocolloid and emulsifier effect both on the food surface and below food surface - depending on their capacity to diffuse into depths of food without losing their antimicrobial activity
Dipping into solution of active agent	Impregnation	effect both on and below food surface

Spreading/spraying/brushing of active agent solution on packaging surface	Coating	effect both on and below food surface
Creation of charge-charge interaction, covalent cross-linking, hydrogen bonding, etc. between active agent(s) and hydrocolloid that form packaging	Immobilisation	effect only on the food surface
Use of inherently antimicrobial (e.g. chitosan) or antioxidant (e.g. milk proteins) hydrocolloids in development of packaging	-	effect only on the food contact surface binding of chitosan on negatively charged bacterial surfaces → cell inactivation

3.3 Edible films with antioxidant properties

As highlighted previously, reducing oxidative stress is of interest when formulating edible films for fresh produce with the aim of increasing their shelf life. Table 3 below summarises some of the relevant research that has been done on films with antioxidative properties:

Table 3. Effect of edible coatings on quality of produce (mod. from Miteluț et al., 2021)

Film/Coating Matrix (Coating Method)	Functional Compound (Role)	Coated Fruits or Vegetables	Advantages of Coating Technology and Main Results of Study	Reference
Polysaccharides and their derivatives-based matrix (starch and its derivatives, cellulose and its derivatives, alginate, pectin, chitosan, and gums)				
Methyl cellulose (MC) (Dip coating)	Palm Oil (PO) (anti-browning agents, antioxidants, and antimicrobials)	Sapota fruits (a large berry)	Decrease PO, PPO, PME activity and discoloration; Increase anti-browning effect and retention of ascorbic acid; Delay the loss of total phenolic content; Extend the shelf life by three days	Vishwasrao et al., 2017
Methyl cellulose (MC) (Dip coating)	Curcumin; Limonene (antioxidants, antimicrobials)	'Chandler' strawberries	Decrease fungal growth; Increase TPC, TA	Dhital et al., 2020
Carboxymethyl cellulose (CMC) (Dip coating)	Aloe vera (anti-browning agents, antioxidants, and antimicrobials)	Apple slices	Decrease PO and PPO activity Lower microbial load; Better firmness; Anti browning effect.	Kumar et al., 2018
Chitosan (Dip coating)	8% and 12% blueberry (*Vaccinium* spp.) fruit and leaf extracts (BLE) (antioxidants, antimicrobials)	Blueberries (*Vaccinium* spp.)	Decrease microbial growth and decay rate; Increase shelf life	Yang et al., 2014
Chitosan and alginate (Coating)	Pomegranate peel extract (PPE) (anti-browning agents, antioxidants, antimicrobials)	Capsicum	Decrease loss in weight, firmness, color, and ascorbic acid content	Nair et al., 2018
Sodium alginate (Dip coating)	Eugenol (Eug) and Citral (Cit) (anti-browning agents, antioxidants, antimicrobials)	*Arbutus unedo* fruit (red berry)	Decrease microbial growth and weight loss; Improve physicochemical and biochemical parameters: color, firmness, AOC, and sensorial attributes	Guerreiro et al., 2015
Sodium alginate (Dip coating)	Essential Oil extracted from sweet orange (antimicrobials)	Tomatoes	Decrease weight loss up to 3-fold lower than uncoated samples; Decrease bacterial growth; Increase the firmness with up to 33%	Das et al., 2020
Modified starch from sweet potatoes (Dip coating)	Cumin essential oil (antimicrobials)	Pears	Suppress the respiration rate and delay the weight loss and maintain flesh firmness	Oyom et al., 2022
Starch and nystose (Dip coating)	Nystose (antioxidants, antimicrobials)	Blackberries	Positive effects in delaying the increase in pH, maintaining the firmness and anthocyanin content	Bersaneti et al., 2021

3.4 Characterisation of films and evaluation of shelf life

This section summarises the current landscape of knowledge found in the literature regarding various aspects related to the techniques and methods used over the course of the project.

3.4.1 Colorimetry

The colour of the films is a parameter which can have a substantial impact on perceived quality, which may influence the extent of their applications as food packaging materials. Differences in colour are most often investigated using colorimetry, the study of interactions between matter and light from the visible part of the spectrum. Typically, measurements aimed at comparing two colours are done using handheld-spectrophotometers, otherwise known as colorimeters (Konica Minolta, 2023). Such instruments measure the intensity of wavelengths reaching the lens of a camera after having interacted with the sample rather than measuring how much light is absorbed by the sample at one or several wavelengths, as is done with spectrophotometers. Hence, when trying to compare the colours of different samples, using a colorimeter is a more sensitive technique.

The $L^*a^*b^*$ coordinate system, defined by the Commission Internationale de l'Eclairage (CIE) in 1976 and was designed to quantify different aspects of light using a model based on human vision. The model is based on colour opponent theory, which states that two colours cannot be red and green or blue and yellow at the same time (Konica Minolta, 2023). Based on this theory, the following three orthogonal rectangular planes are used to communicate colour measurement data as coordinates with either positive or negative values: lightness (L^*), red/green (a^*) and blue/yellow (b^*). The CIE-Lab colour space is illustrated in figure 2 below:
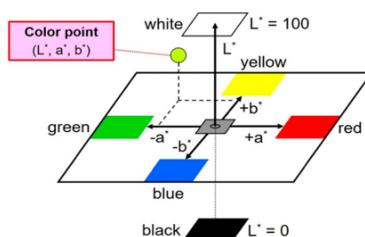
Figure. 2: Illustration featured in <u>online article</u> by Joshum Beetsma, 2011 Prospector Knowledge Center. Accessed 7/3/23

The difference (Δ) between values from a chosen sample and another sample or control is then used for comparison. Commonly, the total difference factor (*ΔE**) is calculated, the equation for which (eq. 1) is shown below (Konica Minolta, 2023). Another useful value that can be calculated (eq. 2) is the yellowness index (YI) which can be used to track the progression of senescence in cucumbers as their colour changes from green towards yellow over time (Sandoval et al., 2019):

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$$\Delta E^* = \sqrt{[(L2^* - L1^*)^2 + (a2^* - a1^*)^2 + (b2^* - b1^*)^2]} \qquad (1)$$

$$YI = 142,86 \times (b^* / L^*) \qquad (2)$$

Where L^* is lightness, a^* is the red/green parameter and b^* is the blue/yellow parameter.

3.4.2 Antioxidative Capacity – DPPH assay

Several methods have been developed for the quantification of antioxidants, although few are as fast and as convenient as using a DPPH assay. This spectrophotometric method involves the making of a 2,2-Diphenyl-1-picrylhydrazyl (DPP) solution using either ethanol or methanol, producing a deep purple colour. Upon dissolution, DPPH reacts with the solvent to produce stable free radicals which are then quenched by antioxidants found in the sample according to the following reaction pathways (Amarowicz & Pegg, 2019):

$DPPH° (violet at 517nm) + AROH \rightarrow DPPH(H) + ARO°$ $\qquad$ HAT pathway (1)

$DPPH° (violet at 517nm) + AROH \rightarrow DPPH^{.} + ARO^{°+}$ $\qquad$ SET pathway (2)

Whether one, both or a mix of the HAT and SET pathways are used, the overall quenching reaction causes a quantifiable loss of colour which can easily be measured using a spectrophotometer following an incubation period (usually 30 min). The decolourisation displays a linear relationship with the concentration of concentration of free radicals (Kedare & Singh, 2011), allowing for the final absorbance, to be compared to an unreacted blank, and the concentration of antioxidants present in the sample to be calculated using a standard curve (usually ascorbic acid or Trolox).Despite the ease and convenience of this method, a certain amount of consideration must be paid when designing the experiment, as highlighted in (Apak et al., 2013):The identity, pH and concentration of the solvent has a significant impact on the results due to factors including the production of endogenous radicals (in the case of ethanol), and its ability to solubilise sample antioxidants. Additionally, the sensitivity of DPPH to light, oxygen, and pH as well as its reaction kinetics must be considered when preparing, using, and subsequently storing DPPH solutions (Blois, 1958).

3.4.3 Electrolyte Conductivity

Ionic solutions have the ability to conduct electricity once dissolved in aqueous solvents such as water (Zhuiykov, 2018). Solution conductivity is based on the following parameters: ionic mobility, charge carrier concentration, elementary charge, and the magnitude of the mobile ionic charges (Muzaffar et al., 2023). The higher the concentration of ions in the solution, the higher the conductivity of electricity at a constant temperature, as the number of ions per unit volume that carries the current in a solution increases (Bigman & Reinhardt, 2018). This effect continues until the solution gets to a maximum value, after which, the conductivity may actually decrease with increasing concentration. The basic unit of conductance is expressed in Siemens (S) per unit distance, typically micro-Siemens/cm (μS/cm) (Bigman & Reinhardt, 2018). Additionally, the conductivity of a solution is temperature dependent, as it influences the solubility and ionic mobility of salts and minerals, as illustrated by the Nernst-Einstein and Stokes-Einstein equations respectively (Aqion). Increasing the temperature of the solution, under the same salt concentration, may have an effect on the dissociation of weak acids and base and therefore lead to a change in EC. This effect is commonly modest, around 2% per degree Celsius (°C) (Aqion).

Relative Electrolyte Leakage (REL)

REL is a well-established and commonly used method for assessing the integrity of cellular structures in response to environmental stresses, namely: salinity, pathogen attack, cold or heat stress, drought etc (Demidchik et al., 2014). As cell membranes degrade in response to stress, their integrity becomes compromised leading to the efflux of potassium cations (K^+) and accompanying counter-ions. This efflux induce stress in itself, promoting yet more leakage of electrolytes (Demidchik et al., 2014; Shabala, 2017).

REL measurements can also serve as an index of the plant's response to stress during storage. As a result of the stress experienced by the produce related to senescence and storage factors, an increase in leakage is observed over time as cells degrade. Electrolytes which are usually restrained within membrane-bound compartments may leak out to the apoplast as the cells' integrity decreases and/or the membrane's permeability increases (Rolny et al., 2011). Electrolyte leakage can be quantified by incubating samples in a medium (usually water) and measuring electrical conductivity (Hatsugai & Katagiri, 2018). The relative electrolyte leakage (REL value) is obtained by taking a second measurement after exposing the sample to extreme stress, usually by freezing or boiling. This destroys the cells and releases all unbound electrolytes into the medium, and a value between 0 and 1 is obtained

when the conductivity prior to this treatment is divided by that following the treatment.

The progressive increase in conductivity observed with increased storage time has been associated with a decline in the vegetables' freshness and an increased susceptibility to decay (Hatsugai & Katagiri, 2018). Hence, monitoring the relative electrolyte conductivity of cucumbers during storage can serve as a valuable index to assess their quality. As edible coatings are intended to have a positive effect on the shelf-life of fruits and vegetables, providing a physical barrier which may reduce oxidative stress through several mechanisms (modulation of respiration/photosynthesis, oxidation and more), the change of REL values during storage may give an indication of film performance.

3.4.4 Water Vapour permeability

Water vapour permeability (WVP) is an important property of edible coatings and films used for the packaging of vegetables (Sánchez-Tamayo, 2020). Water in its free form is the basis for many chemical reactions, supporting microbial growth and enzymatic changes. Thus, its role in food preservation is significant (Amit et al., 2017). The ability of a coating to hinder the transmission of water vapour may have an impact on the longevity of fresh produce by adversely affecting their quality, texture, and other sensory properties. The barrier properties of the coatings are determined by numerous factors such as the physicochemical composition of the formulation, including the presence of plasticising agents, and the thickness of the coating or film (Caicedo et al., 2022; Sánchez-Tamayo, 2020).

WVP is a value which quantifies the amount of water that permeates through a material per unit of area over a given period (g/m^2s is often used for films). Measurements are taken under constant relative humidity and temperature, and the water vapour pressure difference between the two sides of the film is also taken into consideration (American Society for Testing Materials, 2013). The permeation process of water vapour can be described in three successive steps (Sánchez-Tamayo, 2020):

1) Water vapour molecules (permeated molecule) in the fluid phase penetrate the surface of the film
2) The permeated molecules that are adsorbed on the film diffuse through the film. This is facilitated by a concentration gradient that causes the molecules to move from an area of high to an area of low concentration according to Fick's law ($c_1 > c_2$; see figure 3)

3) Once the molecules have crossed through the film and have reached the opposite interface, they diffuse into the adjacent continuous phase and leave the film
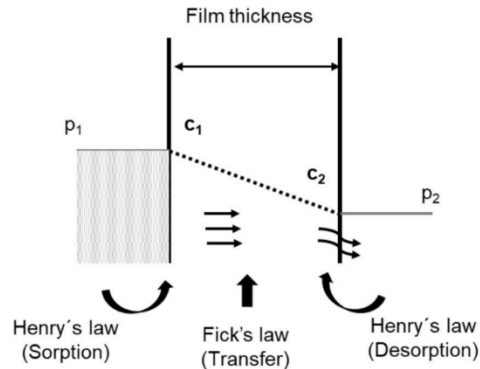
Figure 3: Permeation mechanism in edible films obtained from Sánchez-Tamayo, (2020).
Where: p_1 is the partial pressure of gas molecules with concentration c_1 and, p_2 is the partial pressure of permeated gas on the opposite side of the film with a concentration c_2.

WVP could be affected by the main components used to produce films and coatings and their intrinsic physicochemical properties. The permeability may be affected by the interactions between compounds within the film matrix, affecting their mobility and diffusion rates (Tavassoli-Kafrani et al., 2016b). The choice of solvent may modify the structure of the film matrix and influence the diffusion of molecules which show affinity to the chosen solvent. In the case of carrageenan, a polar polysaccharide, the film structure and diffusion rates are highly influenced by water (Sedayu et al., 2020). Incorporation of a hydrophobic compound such as lipids, may cause discontinuities in the hydrophilic matrices or create tortuous diffusion paths of water molecules, reducing the rates of diffusion thereby improving the water vapour barrier properties of the films and coatings (Ghanbarzadeh & Almasi, 2011, Pérez-Gago & Krochta, 2001).

4. Experimental Overview

The project was approached in three separate phases:

The first phase, the formulation phase, consisted of the development of the base formulation after assessing different structural ingredients that could result in an edible film with the desired properties. For this, the targets, needs and requirements of the film were determined after attending to the shelf life limiting parameters for the cucumbers. During the preliminary trials, thoroughly researched ingredients were selected and assessed at different ratios and concentrations. By the end of the preliminary trials, a main structural ingredient, kappa-carrageenan, had been selected and the concentrations of all the film forming ingredients (w/v%) were decided after a series of sensory evaluations. As the incorporation of active ingredients with antioxidant capacities into the formulation was part of the aim of this project, various plant extracts were also evaluated in formulations during the first phase. Those include: brown algae (*A. nodosum*) extracts, green spirulina, Greek mountain tea (also known as *Sideritis syriaca*) and Pennyroyal (*Mentha pulegium*).

The second phase - the film characterisation phase included the development, adjustment, and improvement of methods and protocols to characterise the physical and chemical properties of the film. These include colour, tensile strength, water permeability and scavenging activity. The antioxidant capacity and electrolytic conductivity of each extract were also assessed during the second phase.

The last phase of the project consisted of the shelf-life study, where the three final formulations (the base formulation and two others containing active extracts – *A. nodosum* and Pennyroyal respectively) were used to coat cucumbers. The effects of the films on the cucumbers were assessed over the course of a 14-day shelf-life trial using a variety of methods (section 5).

An overview of the distinct phases of the project is presented in the diagram below:

1st phase	2nd phase	3rd phase
Formulation	Characterisation	Shelf-life Study
Formulation trials (targets, needs, requirements) Structural ingredients, Active Ingredients	Development of protocols Characterisation of physical and chemical properties	Testing of effect of 3 different formulations on cucumbers over a 14-day period
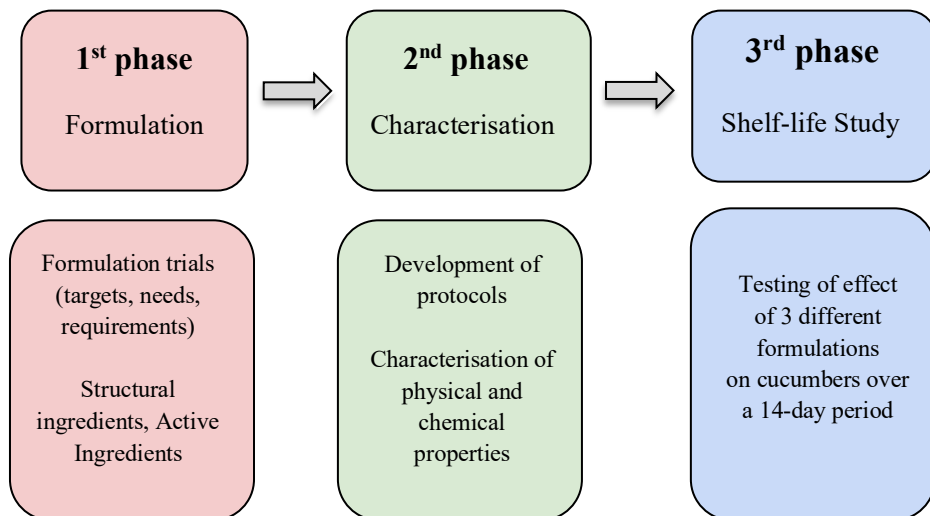
5. Materials and methods

5.1 Chemicals

Commercial kappa carrageenan (200 kDa, CAS 11114-20-8), zein (22 kDa, CAS 9010-66-6), corn starch (CAS 9005-25-8) and sodium oleate were purchased from Sigma-Aldrich Sweden. Sodium alginate was obtained from Alfa Aesar. Potassium chloride was obtained from BergmanLabora AB and calcium chloride was purchased from Acros Organics. Glycerol was purchased from Apoteket AB. Sunflower lecithin was purchased from RawFood Shop, Malmö. Food-grade acetic acid (24%) was purchased at ICA Tuna (Lund). Macroalgae extracts, spirulina powder and rapeseed oil were provided by The Company. Ethanol (95%) was of analytical grade. Unless otherwise indicated, deionised water was used for all formulations. DPPH (2,2-diphenyl-1-picrylhydrazyl) was supplied by Sigma-Aldrich. L-ascorbic acid was purchased from ABX advanced biochemical compounds. All chemicals and solvents were of analytical grade namely, sodium hydroxide (NaOH), hydrochloric acid (HCl). 5.2 Preparation of ingredients for the edible film

5.2 Preparation of Ingredients for films

5.2.1 Octenyl succinic anhydride (OSA) starch modification

A starch suspension of 20% (w/w) was prepared by mixing 150g of corn starch in 750 mL deionised water using a magnetic stirrer for 10 min. The pH of the suspension was adjusted to 8 using 1M NaOH solution. 3% OSA (dwb) of the total weight of the suspension, was slowly added and was let to react for a duration of 3h. After 3h, the pH of the solution was adjusted to 6.5 using 1M HCl solution and was then centrifuged (details of centrifugation). The supernatant was discarded, and the pellet was rinsed three times using 70% aqueous alcohol. The OSA modified starch was dried in a conventional oven at 40 °C for 22 h. The dried starch was then finely ground using a pestle and mortar, sieved, and stored in an airtight plastic container.

5.2.2 Algae extract preparation

The frozen "dark" (D) and "light" (L) algae extract (50mL portions) was first thawed before centrifuging using an Allegra® X-15R Benchtop Centrifuge (Beckman Coulter, Brea, CA, USA) at 10200 rpm for 10 min. The supernatant was kept for further processing while the pellet was spread onto a petri dish and allowed to dry at air temperature before storage at –20 °C for later analysis.

After centrifugation, the algae supernatant was distributed equally in pre weighed metal receptacles and was transferred to a drying oven (Termaks) set at 40 °C with a fan setting 4 for approximately 3h or until a 50% weight reduction was observed. The receptacles were weighted during and after drying and the total percentage of water loss was calculated from the weight difference. Both filtered (LFU/DFU) and concentrated (LFC/DFC) extracts were stored at –20 °C until further use or analysis.

5.2.3 Spirulina, Mountain tea and Pennyroyal extract preparation

Spirulina extract
For obtaining the ethanol extracts of spirulina, 1% w/v of dried biomass of *A. platensis* was mixed with 250 mL of solvent (1:1 H2O:EtOH) and was stirred at 400 rpm for 3h at room temperature. The extract was distributed in plastic centrifuge tubes.

Mountain tea extract
A maceration method with 70% v/v ethanol was used for the ethanol extraction, as reported by Yanchev et al., 2022. Five g of previously grounded mountain tea using an electric blender followed by a pestle and mortar were mixed with 200mL of ethanol in a 1:40 ratio. The solution was placed on a stirrer for 3 h at 1400 rpm for the completion of the extraction. After the extraction, the mixture was filtered and transferred to plastic centrifuge tubes.

Pennyroyal extract
Aerial parts of *Mentha pulegium* were grinded using a standard blender followed by pestle and mortar. Five g of the powdered plant were macerated in 200 mL of 80% v/v ethanol in a 1:40 ratio as in the previous method. The pH was adjusted to 6.5 using acetic acid. The extraction was completed after 3h while keeping the mixture under stirring at 400 rpm on a magnetic stirring plate.

After maceration, all extracts were centrifuged at 10200 rpm for 10 min. The supernatant was collected and used as active extracts in the films. Aliquots were frozen (-18° C) or stored in the fridge at 4° C.

5.3 Formulation of the edible film

5.3.1 Preliminary Trials

A total of 77 different formulations were tested over the course of the formulation phase, the majority of which were carrageenan-based.

Zein-based formulation

Zein films were prepared by mixing 2, 5 and 15g respectively of zein protein with 100 mL of 95% v/v ethanol under constant stirring using a magnetic heating plate and heating the mixtures up to 80 °C for 30 min. Glycerol was added at the quantities of 10g and 20g/100g zein and oleic acid was added at 40g and 70g/100g zein. After the addition of glycerol and oleic acid at their respective quantities, stirring of the mixture was kept for another 5 min. The mixtures were removed from the heating plate and 4mL of each formulation was casted on a glass petri dish (ø 9 cm) and were allowed to dry on the lab bench (20 °C ± 1 °C, RH 55% ± 2%) overnight. The formulations were repeated with the addition of the last step of homogenisation using the Ultra Turrax T-25 (Ika, Staufen, Germany) at 15,000 rpm for X min. The film forming formulations were then cast on petri dishes and were allowed to dry as described above.

Carrageenan - Alginate based formulation

Film forming solutions were prepared by mixing different amounts of kappa-carrageenan and alginate (0.3-1.2g respectively, total mass 1.5g) in 100 mL of water adjusted to pH 4 using acetic acid. The polysaccharide blend was first hydrated separately with a portion of water before being added to the rest of the water, to which OSA starch (1-2g), glycerol (0.4-2g) and KCl (0.1-0.15g) had been added. The solution was kept under continuous stirring and subjected to a heat treatment at 85 °C for 10 min. For the formulations containing algae extracts, those were added at percentages of 2 or 20% respectively for the "light" extract[5] and 5, 15 or 25% for the "dark" extract[3]. Solutions were stirred for an additional minute and a two-step

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[5] the descriptors "light" and "dark" used here refer to the relative appearance of the two extracts as no information other than the source organism, *A. nodosum*, was provided.

casting procedure was followed. This included the casting (4mL of solution) on petri dishes and partial drying of the film solution, followed by exposure to a calcium chloride solution (2% w/v) solution for 5-15 minutes. Excess solution was removed, and the films were allowed to dry in the petri dish.

For the coating of cucumbers, they were first dipped into the aqueous polysaccharide solution, allowed to dry, and then submerged into the solution for 15 minutes before being hung to dry.

Carrageenan based formulations

Suspensions of carrageenan at different concentrations (0.5-1.5% w/v) were prepared by mixing the polysaccharide with 100 mL of water at pH 4. Under continuous stirring, OSA starch, potassium chloride and glycerol were added to the solution. As previously, the polysaccharide was hydrated separately before being introduced to the rest of the solution once it had reached 55 °C. For the trials containing rapeseed oil and sunflower lecithin, these were added before the initiation of the heat treatment. All prepared solutions were heated, and the temperature was maintained at 85 °C for 10 min, to induce gelation. Formulations containing oil and lecithin were homogenised after the HT using the Ultra-Turrax (2 min, 10500 rpm).

The amount of 4 mL of the film-forming solution was pipetted onto glass petri dishes (∅ 9 cm) and allowed to dry for 10 min in a convection oven at 40 °C with the fan setting set at 4. The partially dried films were carefully peeled off the dishes and were left to condition for 24 h at 20 °C and 55% relative humidity in a temperature-controlled room before further analysis.

Five different amounts of carrageenan, presented in table 4a, were tested during the formulation phase. The different amounts of the remaining ingredients added to the formulation are shown in the complementary table 4b.

It should be noted that the tables should not be read row by row, as they do not correspond to one specific formulation. Rather, each amount of carrageenan (table 4a) should be considered together with one cell from each ingredient column (table 4b). This format was selected to present the data in a more concise fashion than a table detailing each formulation's composition, as so many (77) were tested.

Table 4a: amounts of carrageenan tested (V=100mL)

Carrageenan (g)				
0.5	0.12	0.15	1	1.5

Table 4b: amounts of other ingredients tested (V=100mL)

KCl	OSA starch	Glycerol	Oil	Lecithin
% wt carrageenan[6]				
0.44	66	0	0	0
0.66	133	26	6.66	66
3.33	200	33	25	-
6.66	-	50	40	-
8	-	66	-	-
10	-	-	-	-

5.5.2 Selection of extracts

A variety of different extracts were investigated and tested in formulations, including the aforementioned DFC extract and pennyroyal extract. Other options that were rejected include light algae extract (LFC), the dried residue following centrifugation of LFU/DFU (see section 5.2.2), mountain tea and spirulina extracts, respectively. These decisions were made based on the performance of each extract and using the following criteria:

- colorimetry – extracts included in formulations (5% solvent) should not noticeably change colour over time, which could indicate a deterioration of antioxidants or could negatively impact the appearance of the cucumber
- antioxidative capacity – the presence of a significant reducing species within the extracts is desirable as one of the aims of the films is to provide produce with some protection against oxidative stress.
- conductivity – high conductivity (compared to deionised water) is associated with high salinity, an undesirable feature in an edible film in

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[6] It was observed in the literature that amounts of non-film-forming ingredients were frequently reported in relation to the amount of film-forming ingredients (ex: carrageenan), usually as percentage mass for a defined volume (% wt for vol X). The same was done in table 4 to facilitate comparison with other studies.

direct contact with the surface of produce as it may increase the rate of water loss and contribute to quality deterioration.

Accordingly, LFC extract was rejected due to performing worse than DFC on all three parameters[7]. The other active extract was selected by using a successive elimination approach where the three remaining extracts were compared: none of them displayed significant difference in colour over time, but spirulina yielded no scavenging activity and increased conductivity compared to either pennyroyal or mountain tea and was thus eliminated. Conductivity (see appendix II for data) was similar in both pennyroyal and mountain tea, although the pennyroyal extract displayed a modestly higher scavenging activity than the mountain tea extract and was thus selected as the second active ingredient to feature in the shelf-life study.

The scaling-up of the formulation selected for the shelf-life study (see table 5) was achieved by increasing the total volume to 1.8-2.5L and increasing the amounts of other ingredients accordingly. Instead of using a stirring magnetic plate, mixing and the heat treatment was achieved with the use of the Thermomix (Vorweck, Wuppertal, Germany) set at a mixing speed of 2.5 and programming the heat treatment into the integrated system. Other than this modification, batches were produced following the method described previously (see "carrageenan-based formulations").

5.5.3 Final Base formulation

Following evaluation of the preliminary formulations via colorimetry, stretching and mechanical resistance tests and sensory methods, the final amounts of the ingredients were decided. The final base formulation (B39) was prepared by first hydrating the kappa-carrageenan with a portion of deionised water. The rest of the ingredients namely potassium chloride, OSA starch and glycerol were added to the remainder of the water and were heated up to 50 °C under constant stirring before the carrageenan was added. The film-forming solution was heated up to 85 °C and kept there for 10 min. The formulation was then ready to be used for the coating of cucumbers or to be casted on petri dishes. In formulations containing extracts, these

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[7] The agreement made at the beginning of this project stipulated that at least one of the macroalgae extracts (LFC/DFC) must feature in a formulation used during the shelf-life study. The two were therefore analysed and compared to understand whether one might perform better than the other.

were added to the formulation after the completion of the heat treatment and the solutions were stirred for an additional minute before casting.

The final formulations used in the shelf-life study are presented in table 5, using 100mL as the volume to facilitate comparison with earlier formulations.

Table 5: <u>Final formulations used in shelf-life study (V =100mL water, all solids in grams)</u>

Formulation	Carrageenan	KCl	OSA starch	Glycerol	Extract (%v) mL	Comment
B39	1.5	0.01	1	0.5	0	-
B63	1.5	0.01	1	0.5	5	DFC[1]
B67	1.5	0.01	1	0.5	5	Pennyroyal

[1] : Dark [algae extract], Filtered and Concentrated

5.4 Film characterisation

5.4.1 Film Thickness

A pair of digital micrometre vernier-callipers (Belika) were used to measure the films' thickness at three different points of the samples. Each film was characterised by measuring 5 different replicate films that were previously cast on petri dishes (∅ 9-14cm). An average thickness value was calculated for each film. Additionally, LDPE plastic cucumber wrappers removed from ordinary wrapped cucumbers were used to produce samples for the experiment.

5.4.2 Water vapour permeability

 The water vapour permeability (WVP) of the films was determined according to ASTM standard method E96-80 (ASTM International, 2017), with some modifications. Anhydrous $CaCl_2$ (ca 20g) was placed inside small (50mL) standard glass bottles, which were then covered with different film samples so as to seal the opening. Sealing was performed by using vacuum grease to carefully attach the film into place, making sure no folds or air channels were present. The inner diameter of the bottle opening was 1.6 cm, which is equivalent to an area of 2.1 cm^2. All bottles, film samples and the amount of calcium chloride in each bottle were weighed separately before the beginning of the experiment. The total weight of each bottle was recorded (avg 104.3 ± 0.9 g). All films were tested in triplicates. The bottles

were placed into desiccators, which were filled with a saturated NaCl solution achieving a relative humidity of 75% RH. The desiccators were kept in a temperature-controlled room at 20 °C for the duration of the experiment (48h). The bottles were weighed every few hours using the scale available in the room, to measure the weight gain over a 51h time period.

For the calculations of the water vapour transmission rate (WVTR), the weight increase of the desiccant overtime ($\Delta W/\Delta t$) was divided by the film area (A) as shown in Eq. (3):

$$WVTR = \Delta W \ / \ (\Delta t \times A) \tag{3}$$

Where, ΔW is the weight of water absorbed in the glass bottle (g), Δt is the time for weight change (h), A is the area exposed to moisture transfer (m^2). WVTR is expressed in unit: ($g \ h^{-1} \ m^{-2}$).
The water vapor permeability (WVP) was calculated using Eq. (4) below:

$$WVP = (WVTR \times L) \ / \ \Delta p \tag{4}$$

Where, L is the thickness of the film (mm) and Δp is the difference of partial water vapour pressure across the film ($\Delta p = p \ (RH_2 - RH_1)$), (kPa), where p is the saturation vapor pressure of water at 25 °C, $RH_1 = 0\%$, $RH_2 = 75\%$. WVP values are expressed in $g \ mm \ hour^{-1} \ kPa^{-1}$. Equations were obtained from Cazón et al. (2022).

5.4.3 Puncture force

The mechanical properties of the prepared films, namely puncture force was measured by puncture using a texture analyser (Perten TVT 6700, PerkinElmer, United States). Samples, approximately 10-14 cm in diameter were loaded in the equipment and secured using vacuum grease. A spherical probe (⌀25.4mm) was used, and the Heavy Duty Stand BB (height 65mm) was used as a rig to position the samples appropriately. Plates with a hole (⌀50mm) were utilised to keep the films secure in place. Before the initiation of the experiment, the scale and probe position were calibrated. The test mode was set as a single cycle. The test parameters were as follows: initial and test speed set to 0.5 mm/s, retract speed 10 mm/s, sample height at 2 mm, starting distance from sample 15 mm and compression 45 mm. The compression force applied was plotted as a function of time (s).

5.4.4 DPPH - radical scavenging activity

The antioxidant activity of the films was determined using a DPPH assay. The method was based on Marinova & Batchvarov (2011) although volumes and concentrations were adjusted to ensure sensitive results for all samples as preliminary tests done following the publication showed that the concentration of antioxidants in cucumber extracts were too low to be accurately detected. DPPH concentration was consequently adjusted to 1.04 mmol/mL to achieve a blank absorbance within the 0.5-1 range.

Homogenised liquid samples were prepared by weighing and mixing each film with 50% ethanol in a 1:3 ratio and stirring on a magnetic plate for 10 minutes at 14,000 rpm. The mixtures were then placed in 50 mL tubes and centrifuged at maximum speed for 15 minutes before separating the supernatant which was used for analysis.

For each standard and sample, 100μL and 200 μL of the dilutions were mixed with 1800μL and 1900 μL DPPH, respectively. The tubes were then vortexed and allowed to incubate in darkness for 30 min before measuring the absorbance with a spectrophotometer at 517 nm. Each film was analysed using three replicates, and standards were prepared using an ascorbic acid dilution series ranging from 2×10^{-3} to 2×10^{-5} mM/mL. Blanks were prepared using 100/200 μL 50% ethanol.

5.4.5 Colorimetry

Colorimetry measurements were carried out on both films and coated cucumbers using a colorimeter (CR-400 Chroma Meter, Konica Minolta, Tokyo, Japan) with a D65 illumination source as a standard at a 10∘ viewing angle, calibrated at a white background. The $L^*a^*b^*$ coordinate system defined by the Commission Internationale de l'Eclairage was used. Numerical values of L^*, a^* and b^* were used to calculate the total difference factor (ΔE^*), yellowness index (YI), ratio b*/a*, total difference in greenness (Δa^*) and total difference in yellowness (Δb^*). All above colorimetry properties were calculated on the basis of comparing coated and uncoated cucumbers on each storage day and additionally throughout the shelf-life study of coated cucumbers. ΔE^* was calculated using equation (1), yellowness index using equation (2) (see section 3.4.1). The colorimetric assessment was performed both on films cast on petri dishes and on coated cucumbers. For the petri dishes. five different points were measured (1 point on the centre and four points on the outer parts of petri dishes) while nine different points were measured from the cucumber samples (top, middle and bottom part and rotating the vegetable twice).

5.5 Extract characterisation

5.5.1 Electrolyte conductivity

The conductance of the prepared extracts was measured using a benchtop conductivity metre (Orion 150Aplus, Thermo Electron Corporation, U.S.A) in µS/cm. Each extract was measured in duplicates and the average conductivity was calculated. Results (see appendix II) were used as part of criteria to select extracts for the shelf-life study (see section 6.3).

5.5.2 DPPH free radical scavenging activity

The antioxidant activity of the extracts was evaluated according to the DPPH assay. Aliquots of 190µL of extract were mixed with 1810µL of the reagent solution DPPH. For the controls, the volume of extracts was replaced by a blank composed of 50% EtOH. All samples were vortexed and let to incubate in the dark for 30 min at room temperature. The absorbance was measured as described in section 5.7.4.

5.6 Plant material: handling and storage

Fresh cucumbers (*Cucumis Sativus* L.), with an average weight of 0.31 ± 0.02 kg were harvested in Helsingborg and delivered by the company on the same day. Cucumbers wrapped in LDPE were obtain from the same producer to serve as a control for the study. A total of 150 cucumbers were triaged, 110 of which were selected after visual inspection according to various quality parameters namely, firmness, colour, size, and skin defects for the shelf-life study. All cucumbers were washed, disinfected with 70% v/v ethanol, and surface-blotted before being weighed and stored. All cucumbers were labelled with randomly generated numbers.

Well-shaped and practically straight cucumbers with no visible unhealed skin damage, bruises or discoloration were selected for the study. Cucumbers whose circumference was uneven, and which displayed significant skin defects or other signs of disease were rejected. The average cucumber weighed 315± 26g.

Depending on the phase of the project, samples were stored under different conditions: For the preliminary experiments, the cucumbers were stored in a climatic chamber (Climacell Evo-Line 111L, MM Group) where the environment was controlled with the temperature and relative humidity set at 12 °C and 65% respectively. Cucumbers intended for the shelf-life study were received, triaged,

and coated the day before the beginning of the study, and then stored in stackable cases in a temperature-controlled room set at 20 °C, simulating retail conditions. It is important to note that the conditions of storage and handling between harvest and delivery were unknown. All cucumbers were allowed to temper at room conditions before further analysis.

5.7 Application of edible coating on cucumbers

Following the heat treatment and addition of extract, the formulations were transferred to a stainless-steel container (GN 1/2 type, 265 mm x 325 mm x 65mm) set over a water bath to keep the coating warm (70-75 °C) throughout the procedure. The coating was stirred regularly to ensure a uniform viscosity. Cucumbers were immersed horizontally into the coating solution for about 3 sec and then removed from the solution by rotating them vertically, holding them by the stem end. The excess solution was allowed to drip off and the coated cucumbers were rapidly transferred to a non-stick silicone mat. Once all samples were coated, they were inspected, and more coating was applied to uneven or uncoated areas if necessary, using a silicone brush before transferring the batch to a Termaks drying oven set at 20 °C at fan speed 4. Drying was monitored closely, and each cucumber was turned to ensure even drying. The total drying time for each batch was estimated to 4h by visually inspecting their surface. Once dried, cucumbers were transferred to labelled stackable cases in the temperature-controlled room.

5.8 Shelf-life study

During the shelf-life study, the following five categories were assessed:
1. Uncoated cucumbers (control A)
2. Cucumbers coated with the base formulation (B39)
3. Cucumbers coated with the base formulation and DFC extract (B63)
4. Cucumbers coated with the base formulation and pennyroyal extract (B67)
5. LDPE wrapped samples (control B)

Category numbers were randomly assigned using a random number generator, which was also used on each analysis-day to select samples.

Practical considerations

The time frame for the shelf-life study was 14 days, and measurements were taken on days: 1, 3, 5, 7, 10, 12 and 14, resulting in seven measurements points in total. A diagram illustrating the data collecting process can be found in appendix VII.

Measurements performed during the shelf-life included weight loss, colorimetry, relative electrolyte leakage and the 2,2-diphenylpicrylhydrazyl (DPPH) assay. On each day of measurement, three cucumbers were randomly chosen from each category for the above measurements. As some of the measurements are non-disruptive, this allowed for the same three cucumbers to be used for all the measurements of the day. When a bacterial, fungal or yeast contamination was observed, the samples were immediately discarded. The stackable trays were rotated on each analysis day in an effort to minimise the effect of local atmospheric conditions, and the sensor was moved to always be placed in the middle tray and record conditions in the centre of the stack. This rotation was performed as it was theorised that samples placed in the top tray would be exposed to a different local atmosphere than those in trays further down the stack. As stacking was the only option available due to a lack of space, rotating the trays was the only available option to attempt to provide equal treatment to all samples (each tray had been on the top/bottom of the stack at some point in the study). The temperature and relative humidity were monitored using a battery-powered data logging system (DEM105, Velleman, Gavere, Belgium) that was placed inside the middle plastic tray with the assumption that the conditions are similar in all trays. Temperature and RH were recorded every 17 min for the 14-d shelf-life period. At the end of the period, the data logger was retrieved, and the data was downloaded.

Weight loss

Weight of three individual cucumbers from each category, was measured with a precision scale (SPECS). The weight loss observed for each category was measured by comparing the average weight of the three samples at each measuring day and comparing the results with the average weight of the coating day.

The overall weight loss during storage was also calculated for all categories using the equation below.

$$WL\% = 100 \times \frac{(Wavg\ day\ 1 - Wavg\ day\ 14)}{(Wavg\ day\ 1)} \qquad (5)$$

Relative Electrolyte Leakage

Cucumber's cell leakage before and after coating was tested by relative electrolyte leakage measurements (REL). REL was assessed as described by Gómez Galindo et al., 2005, with some modifications. Three slices were cut longitudinally from each cucumber and one disk per slice was excised from the mesocarp using the tip of a plastic pipette. The disks produced were of 1 cm in diameter and 1 cm in thickness. The disks were washed three times using deionised water and blotted dry with a paper towel after each rinse. The samples were transferred into 50 mL centrifuge tubes containing 20 mL of deionised water and were incubated at 20 °C for 3.5h. After the incubation, the conductance of the incubation medium was measured using a conductivity metre (details about conductivity metre). The tubes containing both the medium and the samples were then put in the freezer (-40 °C) in order to induce cell damage. After thawing and allowing the temperature of the medium to reach the value of the first measurement, the conductivity of the incubation medium was measured again. The REL was calculated as the ratio of the initial reading to the final one after thawing.

DPPH free radical scavenging activity on cucumber skin
The cucumbers were washed in order to remove the coating from their surface and approximately 10g of the skin was peeled off from each sample. The cucumber skin was homogenised using an Ultra Turrax at 20500 RPM for 2 min. A volume of 25 mL from each mixture was transferred into a plastic centrifuge tube and was centrifuged at 5000g for 20 min. The supernatant was transferred to 15 mL plastic tubes and was kept in the freezer until the day of the DPPH assay.

Statistical analysis
The experimental data were subjected to analysis of variance (single- and two-way ANOVA) and t-Test, performed using Microsoft Office Excel (Santa Rosa, California, U.S.A). This approach was chosen to test for key differences amongst the formulations produced and the five cucumber categories tested during the shelf-life study. Data were presented as the mean ± the standard deviation.

6.   Results and Discussion

6.1   Formulation of edible films and coatings

Based on early trials where both wrapping and dipping were tested as application methods, the conclusion was drawn that the dipping method was the most effective technique to achieve a thin but evenly thick layer on the surface of the product. Although common in the literature, the casting, drying, and peeling of films prior to wrapping proved too inefficient, and yielded uneven results in adhesion to the skin.

As a result, the production of films for the shelf-life study was achieved by dipping, and samples for characterisation of the films by casting. The method of casting is described in detail in the Materials and Methods section. The drying of cast films was expedited when a conventional oven was used instead of evaporation at room temperature, increasing the drying rate, and reducing the drying time from several hours to 10 min.

6.1.1 Preliminary Trials

Zein-based formulation
With the aim to achieve a hydrophobic film formulation, the use of proteins was investigated. As zein is soluble solely in aqueous ethanol, 95% v/v ethanol was used as a solvent for the film formulation (Meng and Cloutier, 2014). Glycerol and oleic acid were used as plasticisers, with the purpose of improving the film's flexibility and reducing its brittleness by decreasing the Tg of the protein (*Edible Food Packaging with Natural Hydrocolloids and Active Agents*, 2022). Heating at a temperature of 80 °C aims to enhance the formation of the zein network and induce the film formation, by accelerating hydrophobic interactions (Schober et al., 2011). Films obtained with the different zein percentages appeared waxy and peeling them off the surface of the petri dishes was unsuccessful. Their sensory properties were unfavourable as the colour of the films was strikingly yellow and opaque, they possessed a strong odour, characteristic of zein. The solution was sticky, and its viscosity increased rapidly after the removal from the heating plate, making its application impractical using the available setup. After drying, the surface of the films displayed cracks and fissures. These results led to the conclusion that this formulation was inadequate for the purposes of this project.

Carrageenan-alginate mixed formulations

An attempt to formulate a heterogeneous film with improved film properties was performed by combining the two algae-derived polysaccharides and benefiting from their different functional properties.

Resulting films were not assessed to possess superior properties (tensile strength appearance, mechanical resistance to rubbing, elasticity etc) to carrageenan-only films, and many of them were found to be composed of two layers when put under tensile stress: a stronger one (top), and a thin, delicate one (bottom) which began tearing first. This division is likely due to insufficient penetration of Ca^{2+} ions into the film, forming a cross-linked layer close to the surface and a much weaker layer on the under-side. This specific observation is in accordance with the literature, although carrageenan-alginate films have been reported as being stronger and more resistant than films made with carrageenan alone. This could be explained by incomplete cross-linking, which would result in weaker gels.

Carrageenan based formulation

This trial was performed in order to obtain films with improved water vapour barrier properties. Films and coatings appeared opaquer compared to the formulations not containing oil, although when the coating dried on the surface of the cucumber, no difference could be observed compared to the rest of the formulations. Considering the introduction of two additional ingredient combined with an extra processing step, that of homogenisation, the production cost for this formulation is increased. As the hypothesis that the water vapour properties of the film would be improved (see section 6.2.2) was not confirmed, this formulation was not among the ones tested during the shelf-life study.

      The use of glycerol as a plasticiser was investigated. based on comparisons between identical formulations except for varying amounts of glycerol, the amount in films with the best mechanical properties was found to be 33 %wt of carrageenan. This finding is in line with the literature, which reports successful carrageenan-films containing similar amounts of glycerol (Larotonda, 2007; Nair et al., 2020; Martiny et al., 2020)

6.2   Film Characterisation

6.2.1 Film Thickness

Film thickness is an important characteristic that influences film properties, such as the water vapour permeability (section 6.2.2) and mechanical strength. The thickness values of the films developed in this project and of the plastic wraps used commercially, are presented in table 6 (see 6.2.2).

The base formulation (B39) resulted in significantly thinner films when compared to the formulations containing extracts and plastic wrap ($p < 0.05$). The plastic wrap yielded the highest average thickness, as well the highest variability in thickness. This variability may be a result of the technique used when wrapping the cucumbers, or simply a result of the manufacturing process. It would suggest that permeability is also variable at different points in the packaging which could affect shelf life, although the effects of this were not directly observed. As mentioned above, the thickness of the formulated films increased with the addition of extracts, although there were no significant differences between the two formulations. A study done using a similar base formulation also reported a similar although larger increase in thickness when an extract (olive leaf extract) was added (Martiny et al., 2020).

The drying rate and environmental conditions during drying can highly influence the final thickness of a film (Campos et al., 2011). As the casting and drying of all films was performed under the same conditions, the attribution of the extracts to this effect should be considered. A possible explanation for this increased thickness could be the increase in solid content origination from the influencing the viscosity of the film-forming solution (Lin & Zhao, 2007). Pennyroyal and algal extracts are both rich in antioxidants which usually consist of large polyphenolic compounds containing multiple aromatic rings. Those molecules could interact with the polymer matrix and their bulky chemical structure could disrupt the carrageenan network, making it less compact. Another explanation could be that films containing the extracts contain more water than those without due to the presence of hygroscopic compounds (polyphenols, (phloro)tannins) which would influence the final water content of resulting films, although further research is needed to investigate both theories.

6.2.2 Water Vapour Permeability

As previously mentioned, water vapour permeability is an important attribute of films. Decreased WVP is positively associated to an increase in the shelf-life of

fresh produce by decreasing moisture migration phenomena (Mouzakitis et al., 2022e). In order to calculate the permeability of the films, results from the thickness measurements had to be combined with those of the experiment described in section 5.6.2, where the rate of water vapour transmission is measured. Data from this experiment is presented in figure 4. It should be noted that the y-axis displays the weight increase relative to the starting weight of bottle (see section 5.6.2) in grams rather than percent, for two reasons: the first is that films were not weighed after the end of the experiment, so the relative amount of water absorbed by the films and the $CaCl_2$ could not be quantified. The second is that sealing grease was used to immobilise the films, the amount of which was not recorded for each sample. It was therefore felt to be more prudent to present the data as an average weight increase (g) for each category.
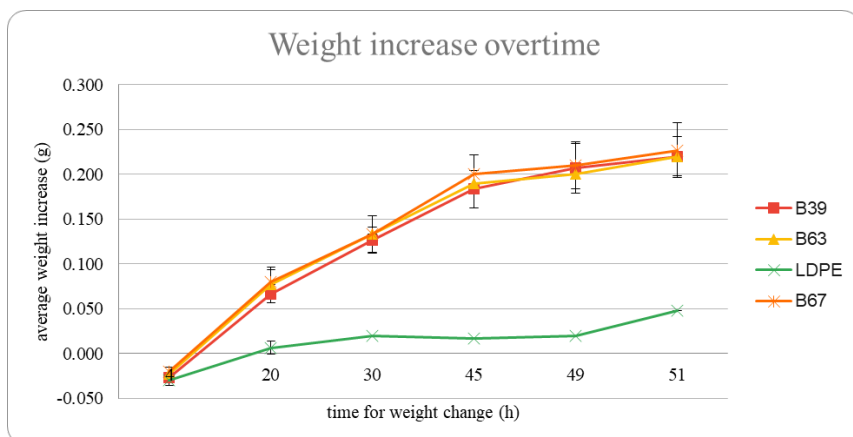

Figure 4: Overtime weight increase of formulated films and plastic wrap

Permeability was calculated from equations 3 and 4 (section 5.2.2) and the results are shown in table 6, in which the inverse relationship between WVP and film thickness is well illustrated by comparing extract (B63/B67) and non-extract (B39) containing coatings:

Table 6. Thickness, water vapour permeability of different formulations

Sample	ΔW (g)	Film thickness (mm)	WVP (gmmh^{-1}kPa^{-1})	Force peak (g)
B39	0.22 ± 0.04	$0.01 \pm 3.23 \times 10^{-3}$ a	$6.98 \pm 0.55 \times 10^{-3}$ a	519 ± 53 a
B63	0.22 ± 0.06	$0.02 \pm 3.35 \times 10^{-3}$ b	$1.44 \pm 0.08 \times 10^{-2}$ b	515 ± 201 a
Plastic	0.04 ± 0.01	$0.02 \pm 6.79 \times 10^{-3}$ c	$3.49 \pm 0.38 \times 10^{-3}$ c	N/A
B67	0.23 ± 0.07	$0.02 \pm 2.70 \times 10^{-3}$ b	$1.60 \pm 0.08 \times 10^{-2}$ b	641 ± 232 a

All values are given as mean ±SD. Please note that in this case, identical letters within a column indicate **no statistically significant differences (p ≥ 0.05).** ΔW is the average weight of water absorbed (g).

For the calculations of WVP the saturation vapour pressure of water (p) value used was 3.17 kilopascals (kPa), at 25 °C, based on the the Antoine semi-empirical equation (Pamuła, 2023). The vapour pressure at RH 75% and 0% was calculated at 2.38 kPa and 0 kPa respectively. The water vapour transmission rate (gh^{-1}m^{-2}) for all samples was calculated for all categories using t=51h. It yielded values of 2.05 gh^{-1}m^{-2} for B39 and B63, 2.11 gh^{-1}m^{-2} B67 and 0.37 gh^{-1}m^{-2} for plastic, respectively.

The water vapour transmission rate was not found to be statistically different between coating formulations, despite there being differences in thickness between B39 and the other two formulations containing extracts. When compared to the plastic wrap, all formulations resulted in films with significantly higher WVP (p<0.05), which indicates less effective barrier properties. The effects of this higher WVP were directly observed in the shelf-life study through higher weight loss of coated samples.

These findings generally seemed to be in line with the literature, as polysaccharide-based films tend to have higher water vapour transmission rates and lower WVP than LDPE, and films containing additional extracts tend to have lower transmission rates than films without.

As presented in Table 6, films deriving from the base formulation possessed significantly higher water vapour barrier properties than films containing the algal and plant extracts (p<0.05), although the difference in thickness of the films (a factor used in calculating WVP but not transmission rate) should be noted. This increase in water vapour barrier properties suggests that extract incorporation, at least in amounts used in the experimental trials (5% V_{tot}) influenced the WVP of

the films. A possible explanation for this could be the addition solids particles from extracts and their interactions with the polysaccharide network may lower its density by increasing the distance between the carrageenan helixes. This would create cavities and channels favouring the passage of water vapour through the film matrix.

It is worth commenting on the shape of the curves see in figure 4, however, which might suggest another explanation. For all formulations, the rate of weight increase decreases after 45h and extrapolating suggests the curves may be heading for a plateau, an effect which is not seen in LDPE. On the contrary, following a stable trend from t=0h the rate suddenly increases after 49h, suggesting that a longer experiment time may have been needed to gain a true understanding of how the respective films perform over time. This plateau described earlier could suggest that it was in fact the films rather than the $CaCl_2$ inside the bottle which absorbed water, a decrease in rate of weight increase being seen after a certain level of absorption. As seen in the introduction, carrageenan is known for being strongly hygroscopic, and preliminary trial confirmed the tendency of resulting films to partially-rehydrate when exposed to water which would support this hypothesis. Moreover, literature states that films made from carrageenan will retain their ability to interact with water once dry and rehydrate redily, resulting in films with higher plasticity where mass transfer is affected (Kocira et al., 2021).

To investigate the trends seen in these results, the experiment should be repeated using films of standardised thickness over a longer time-period and under different RH% conditions. Films of different thicknesses could also be used to investigate the rate of water migration through the film, and weighing the films before as well as after the experiment could provide information on whether or not they absorb water, and if so, how much is absorbed. The effect of the chemical reaction performed on potato starch using OSA, which was supposed to increase hydrophobicity, was not investigated. The degree of substitution is expected to be quite low (0.02 according to Nilsson & Bergenståhl) but should be measured to ensure the success of the reaction. At the very least, a direct comparison of base formulations using potato- and OSA treated potato starch should be done.

Amounts of starch included in the formulation should also be investigated further, as the increase in opacity associated with higher amounts of starch may be tolerable if the benefit to barrier properties is large enough. Further experiments on formulations containing higher levels of oil or, better yet, solid sources of fatty

acids should also be investigated as they may also help to limit weight loss through hydrophobic interactions, as well as reduce respiration by better restricting access to oxygen. Lastly, the investigation of coatings including additional hydrophobic layers (such as the inclusion of a second zein-based layer) should be considered.

Upon review of the literature study performed in preparation for this project, it was noted that carrageenan-based films were very rarely used for this type of application generally, especially on produce where weight loss is a primary shelf-life factor. Although no explicit statements were found in publications justifying this general avoidance of carrageenan as the main film-forming ingredient in edible films, the results obtained during this study seem to shed some light on this: there simply seems to be other more performant options available which may require less additives and chemical modifications to achieve similar or more performant films.

6.2.3 Puncture Strength

Edible films should exhibit an adequate mechanical strength to ensure that their integrity, on the coated products, would be maintained throughout the different stages of handling and transportation.

Table 6 in section 6.2.2 displays the values of the greatest puncture force that the films were able to sustain before breaking. The force peak values were not significantly different among the formulations ($p > 0.05$). In addition, representative graphs deriving from the puncture test for all final film formulations can be found in Appendix VI. The time of the break point is different among the samples, but no difference is observed in the behaviour of the curve. The force applied is increasing steadily until the break point which can be recognised as the point where the force decreases abruptly.

Results suggest that the differences in composition of the film formulations (incorporation of different extracts) did not influence the mechanical strength of the films ($p > 0.05$), although it should be noted with the exception of B39, SDs are rather high suggesting possible methodological problems that will be discussed below.

Since puncturing was observed in all formulated films but not in plastic wrap samples, no force peak value could be obtained for the latter samples. Plastic films exhibited a higher elasticity and flexibility as none of the samples were broken when force was applied. This gives an indication that the formulated films were more brittle than the commercial (plastic) ones. The addition of glycerol, as a

plasticiser, was expected to increase the flexibility of the films but as observed, not to the extent that their performance would be comparable to the plastic.

It is relevant to mention that a series of limitations may have influenced the results. The distance of the probe from the rig was calibrated to be in range for measurements on formulation samples and could not be re-configured at the time, so puncture strength of plastic films could not be measured. Problems with keeping the carrageenan films in place also contributed to making measurements more difficult, and although the experiment was repeated several times in various forms (vertical elongation test, puncture test using different probes) and several measures were taken (use of vacuum grease to stabilise the films, elastic bands around the rig and top plate, production of larger films), some films may still have been dragged along with the probe which could contribute to larger standard deviations in results.

To draw more solid conclusions about the formulations generally, is recommended that this experiment be repeated, perhaps using thicker films. For plastic film wraps the experiment may be repeated, but the length of the probe should be increased. Additionally, using a sharper probe (ex: a cone P-COXXS type) may yield better results especially in plastic samples, although attention should be paid to the sensitivity of the instrument. These modifications could unfortunately not be tested due to a lack of time.

6.2.4 Radical Scavenging Activity of films

The antioxidative capacity of the active extracts were first characterised individually (figure 5). This was done both as a way of characterising the extracts themselves and to make informed decisions regarding the extracts to be used in the final films used for the shelf-life study. Films made using these extracts in the formulations were then analysed (figure 6) according to the method described in section 5.6.4.

As seen in figures 5 and 6, the antioxidative capacity of the extracts is partially reflected in that of the resulting films made using those extracts:
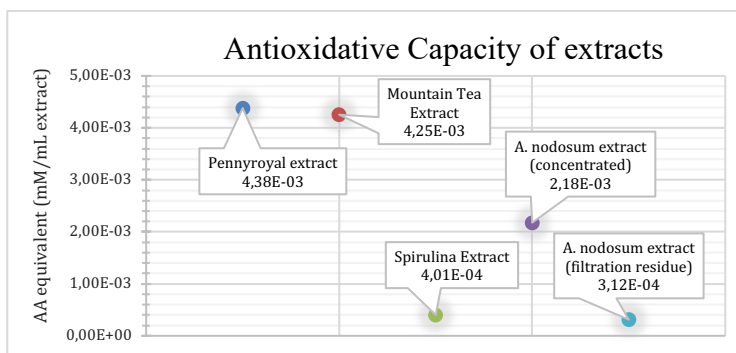
Figure 5: Antioxidative capacity of pure extracts considered for the shelf-life study. Standard deviations are not included as resulting error bars are too small to be visible.
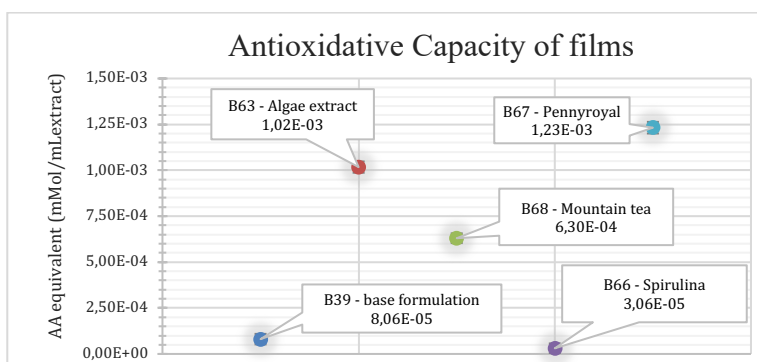

Figure 6: Antioxidative capacity of films made using the extracts featured in figure 5 (where "algae extract" in formulation B63 refers to the concentrated *A. nodosum* liquid extract, not the solid residue).

Results from this analysis suggest that films formulated using the extracts display antioxidative activity in all cases, except for films made using the spirulina extract. This was not especially surprising as the antioxidative activity of the spirulina extract alone was, although significant, is relatively low compared to that of other extracts analysed. Films containing pennyroyal, mountain tea and *A. nodosum* extracts all displayed significant scavenging activity, especially for pennyroyal films (figure 6). As oxidative stress constitutes a major negative influence on the shelf life of produce, films displaying significant radical scavenging activity are desirable as they may help counteract oxidative stress. Scavenging activity for films containing the pennyroyal extract was twice that of those containing mountain tea extract (1.23×10^{-3} and 6.30×10^{-4} AAE mMol/mL extract respectively). Values

obtained for films containing *A. nodosum* were similar to those containing pennyroyal.

Based on these results, alongside other practical considerations (see appendix II), the decision was made to include *A. nodosum* (B63) and pennyroyal (B67) films in the shelf-life study along with the control formulation (B39).

It should however be noted that a similar analysis performed using a different method (ex: Folin-Ciocalteu assay, a method used to determine total polyphenol content) may have yielded different results. Indeed, a variety of parameters may have affected the results such as antioxidant solubility in the reagent used (50% ethanol in the case of spirulina), particle size and density. Processing prior to extraction may also play a significant role, as the spirulina powder was obtained as a commercial supplement which had likely undergone prior heat/chemical treatments whereas both pennyroyal and mountain tea samples had only been minimally processed (minimal chopping and air drying prior to extraction).

The extraction method used may also have affected results. The method was based on similar ones found in literature and adapted using a systematic trial and error approach where film amounts, soaking and mixing times were tested until values within range were obtained. Perhaps with more time, the method could have been adapted further and more useful results could have been obtained.

Direct comparison of our results with data found in literature has proven difficult and should perhaps cautiously be avoided, as methods used are far from standardised: initial DPPH concentrations and ratios to standards/ samples wildly, as well as solvents used to create the DPPH solution (water/ethanol ratios or use of methanol) and incubation time. The effect of DPPH concentration on scavenging activity was illustrated rather extensively by Fadda et al., 2014: the study compared the DPPH decay rates at varying initial DPPH concentrations ranging from 25mM to 200mM and observed that the chemical composition of the sample as well as the initial concentration greatly impacted the rate of reaction. Although conversion equations have been devised to compare results produced using different standards (Hwang & Lee, 2023), less attention has been paid to correcting for DPPH concentration and its effect on reaction kinetics. As a result, Fadda et al. advised, perhaps wisely so, to be weary of comparing inter-lab results.

6.2.5 Colorimetry

The films' colorimetric parameters (L^*, a^*, b^*) and total colour difference (ΔE^*) as measured on petri dishes, are presented in Appendix IV. All formulations resulted homogeneous films which were mostly translucent in appearance.

It was expected that the differences in the chemical composition (oil, glycerol, lecithin etc) of the base formulations would influence the colour and opacity of the films, although this effect was not noticeable to the naked eye. The only exception to this was OSA starch content: as all formulations tested using colorimetry contained the same amount of starch, no differences were measured. Previous formulations containing more or less starch varied visibly in opacity, as can be expected when the number of large particles is varied within a material.

Regarding the lightness (L^*) values, no significant differences were observed in the following samples: B39 (base formulation), B65 (oil, lecithin, and dark algae extract) and B67 (pennyroyal extract). All three formulations appeared the lightest among all samples (higher L^* values). Samples B61 and B62 resulted in the lowest L^* values, both containing the highly pigmented ingredients rapeseed oil and lecithin. As the sample B65 also contains those two ingredients in the same quantities, the differences in the L^* parameters may be attributed to the presence of the extract, which may have diluted the formulation somewhat, thereby reducing the concentration of pigments.

No significant differences were observed on the results of the b^* index. Sample B65 showed the highest yellowness value (b^* positive parameter), possibly because of the presence of pigmented phlorotannins and other pigments. Samples B39 and B64 had similar b^* values (lowest among the samples) possibly due to the lack of additional ingredients or extracts.

Films produced from the formulations B65, B67 and B68 resulted in the lowest a^* values therefore appeared as the greenest. The above samples contain the distinctively green coloured algae and plant extracts, so this result was not particularly surprising. Interestingly however, films produced from formulation B66 (spirulina), although also containing a highly pigmented extract, did not influence the a^* value to the same extent. This could be caused by thinner films, or a loss of colour following plating.

Lastly the films were compared to the one deriving from the base formulation which acted as a control, allowing for total difference in colour *(ΔE*)* values to be compared. Higher *ΔE** values indicate greater overall colour differences when compared to the control formulation.

All films resulted in similar *ΔE** values (around 2), except for B66, containing spirulina. Balti et al. (2017) reported that incorporation of spirulina extract at a concentration of 5%, significantly influenced the colorimetric parameters, decreasing the L* and increasing the a* and b* values, respectively. Their use of chitosan instead of carrageenan as a film-forming ingredient, which likely results in differences in the chemical interactions between the ingredients and differences in thickness of the resultant films, may be the reasons for the differences found between their results and those found in this experiment.

6.3   Shelf-life study

The results of the measurements performed during the shelf-life namely weight loss, relative electrolyte leakage, DPPH assay and colorimetry are presented and discussed in the following sections.

6.3.1 Weight loss

Results from the weight loss study are presented in figure 7. Standard deviations were not included in this figure to simplify viewing, but a column diagram version which includes standard deviations can be found in appendix V. Please note that the y-axis displays data in terms of average weight loss (g) rather than percentage weight loss (%): this is because the small number of replicates (3 per data point) did not allow for accurate normalisation of the data.
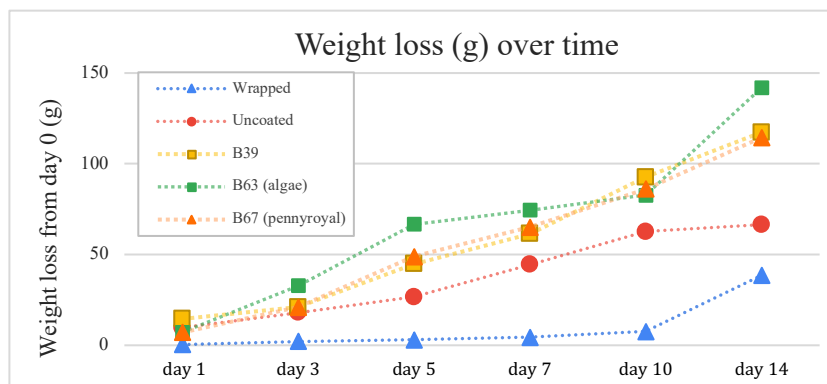

Figure 7: average weight loss of cucumber samples over course of shelf-life study

General trends

As seen in figure 7, prolonged storage at room temperature led to a continuous weight loss, observed in all categories, reaching maximum values at day 14. The data obtained from this experiment did not support the hypothesis that the edible films, with or without the addition of extracts, may preserve the moisture content of cucumbers when compared to uncoated ones. When standard deviations were considered, cucumbers from all categories but those wrapped in plastic lost a similar amount of weight. Wrapped cucumbers lost significantly less weight than other categories for all days, although a dramatic increase was observed between days 10 and 14. This was also observed in cucumbers coated with the B63 formulation.

Conversely, uncoated cucumbers lost weight at a slower rate over this period. These changes in rate of weight loss are likely connected to the natural progression of senescence, where cells degrade over time and become leaky, releasing their contents, contributing to the migration of water to the surface which finally evaporates resulting in net weight loss. The increased weight loss seen in coated samples compared to wrapped with plastic support the WVP differences between the materials. This could be explained by structural reasons (the structure of the carrageenan matrix was disturbed by other components such as large antioxidants or starch particles which could affect crystallisation) (Kocira et al., 2021).

Generally, our findings were not in accordance with reports found in literature suggesting that similar edible coatings applied to cucumbers, may act as a sufficient barrier against water evaporation reducing water loss rates (Olawuyi et al., 2019). Both the differences between results seen in plastic-wrapped cucumbers in this experiment and with edible coatings in similar experiments could be explained by differences in thickness: as previously mentioned, thickness is an intrinsic parameter of a material's permeability and that of the edible films was measured to be significantly thinner than that of plastic (especially B39), yielding higher respective WVP which likely contributed to more weight loss over time. Coating thickness of the edible films may also have been unevenly applied during sample preparation, as samples were dipped vertically into the liquified coating before being allowed to dry on their side. Although the coating seemed to solidify relatively fast (within a few seconds), some drops could be observed running down along the sides of the cucumber, suggesting a migration of excess coating towards the tip of the fruit and thereby an accumulation towards the flowering end. This may have led to an uneven thickness across the length of the fruit, protecting one end more than the other, potentially leaving the latter less protected against water loss.

It was also observed that standard deviations increased with the number of days, and that smaller cucumbers lost more weight and displayed more wrinkling and dramatic losses in turgidity than larger ones, effects which were in line with descriptions found in literature.

Barrier properties of LDPE

In wrapped cucumbers, the lower rate of weight loss can be explained by the excellent WBP properties of the plastic which prevents water from leaving the system, trapping it within the packaging.

It must also be noted that the plastic did not prevent water from migrating from the inside of the cucumber to the outside: this resulted in the progressive accumulation of water droplets on the inside of the packaging and on the surface of the cucumber. The high-humidity atmosphere this created lead to the apparition of mould on a notable number of samples, which was not observed in coated cucumbers (control and pennyroyal) and uncoated cucumbers. Cucumbers coated with the algae formulation were also prone to mould, but to lesser extent than wrapped ones. This observation is important to note, as it highlights the lack of sensitivity of the experiment which was unable to provide an accurate picture of the amount of water lost by the cucumbers themselves. To remediate this, wrapped cucumbers could be weighed twice: once with the packaging, and once more after removing the packaging and carefully drying the surface.

A difference in the treatment of wrapped and unwrapped samples could also have contributed to the differences seen in results: unwrapped samples (all formulations as well as B39) were all disinfected using 70% ethanol prior to coating (except uncoated controls) as described in method section 5.8. This may have had negative consequence on skin permeability, although this was not investigated in this study.

6.3.2 Relative Electrolyte Leakage

Results from this experiment are presented in figure 8.
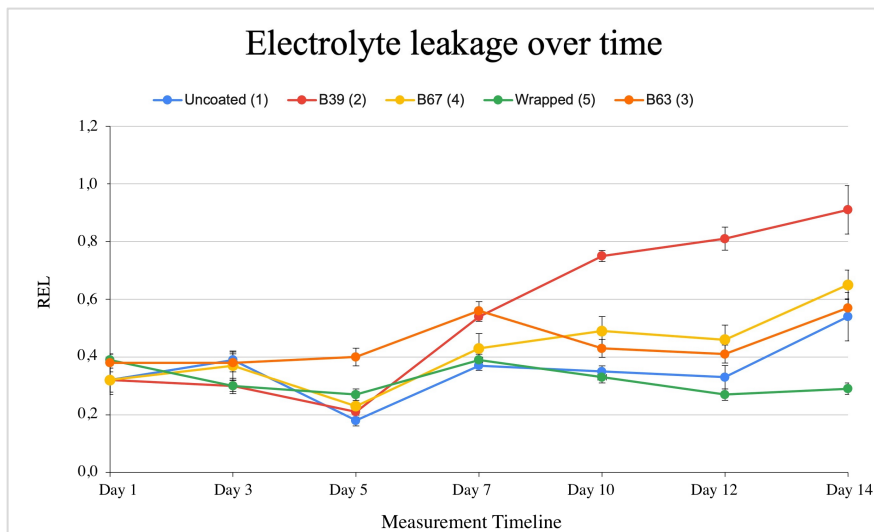
Figure 8: Electrolyte leakage over time of cucumber samples

Results from this section of the shelf-life study showed no clear differences between categories, as a result only trends can be discussed. The trends of curves from formulations B67, B63 and Uncoated samples are nearly identical, although REL values varied between categories up until day 14.

Overall, Wrapped and Uncoated cucumbers displayed lower REL values than coated ones, and samples coated in the base formulation behaved worst of all. Fluctuations in REL may reflect the biological variability in samples caused by pre-harvest factors such as plant stress or position in the green house (access to light). The similar shapes of the curves (minima/maxima on same days) suggest that the progression or rate of senescence was not affected by the coatings (similar changes in rate), but the number of cells affected was increased in coated samples. Samples coated with B39 behave the same as other formulations until day 5, where a dramatic increase is seen up until day 14.

Higher REL values are associated with increased cellular damage due to stress or senescence (Demidchik et al., 2014; Rolny et al., 2011). As samples were taken from the mesocarp tissue (the pale, firm tissue found between the skin and the wet seed-containing tissue in the centre of cucumbers), K^+ and other ions contained within the formulation itself are unlikely to have contributed to the readings. However, it may be that these ions or any other compound, migrating from the coating, contributed to stressing the cells indirectly, by influencing the metabolic

activity of the latter. Indeed, ionic strength and the formation of gradients is only relevant in an aqueous medium. Such condition may have occurred twice during the study:

- First when cucumbers were coated and dried, where an ionic gradient is formed between the inside of the cucumber and the outside (the wet coating). This gradient may have caused K^+ and other ions in the coating to migrate into the cucumber, potentially stressing cells and resulting in more water loss and increased REL values down the line. Literature suggests that high K^+ concentrations outside of cells, normally accompanied by oxidative stress, often promotes programmed cell death or catabolic processes in stressed plants (Demidchik et al., 2014) although other compounds may also play an effect.

- A second instance where ions in the coating may have been put in contact with water is when the latter migrates out of cells and out of the cucumber, where it must make its way through the coating to exit the system. As water \interacts with the film, the excess ions may dissolve in the water and stress the metabolism of cells further.

This increased stress caused by migration of salts and other ionic compounds from the coating may explain why, although weight loss results were similar for formulations with and without extract, they were not for REL as antioxidants in the coatings may have helped to manage possible metabolic consequences of this stress.

The potential effect of the ionic strength of the coating should be investigated by repeating the experiment, using coatings where different amounts of KCl and extract respectively are used. If there is a link between ionic strength of the coating and metabolic stress, curves with similar trends and varying REL values should be observed. If there is a link between ionic strength and the presence of antioxidants, curves should also reflect this.

6.3.3 Radical scavenging activity

The results of the DPPH radical scavenging activity of cucumber peel did not exhibit clear trends that could give valuable information on the effect of coatings on the bioactive properties of the samples. Several factors could have contributed to these results, including:

- **Biological Variability** – cause by pre-harvest factors such as position in greenhouse, health status of the mother plant, infections within the fruit (of which there were signs, both during preliminary and the shelf-life trial[8]), soil health and more. These factors may affect antioxidant production by cucumber fruits. As some skin samples harvested within the same category on the same day resulted in supernatants of different colour intensity, it is believed biological factors may have been involved.

- **Sample handling** – the harvesting of cucumber peel samples became more difficult with time, as LECs lost turgidity and their skin became wrinklier. The method devised obtaining peel samples using a vegetable peeler had to be modified in some cases, using a scalpel, or by carefully pulling on the skin. This may have led to variations in the skin to mesocarp ratio obtained. The next step, homogenisation, may also have been affected as it was observed that some samples required longer homogenisation times than others to obtain similar particle sizes.

- **Chemical factors –** reactions performed using DPPH are sensitive to temperature. Although efforts were made to remove both the reagent and samples from the refrigerator to temper before use, temperature was not recorded and may therefore have contributed to unclear results. As previously highlighted in the discussion pertaining to the antioxidative capacity of extracts, the reaction kinetics are also sensitive to concentration. As more DDPH assay had to be made several times over the course of the project, small variations in concentration may have also played a role even though great care was taken when preparing the solutions. This effect on kinetics is doubly important, as the standard used to calculate antioxidative activity (ascorbic acid) may not be influenced in the same way as antioxidants present in our samples. This effect is illustrated well in Fadda et al., 2014 cited previously. The temperature of the 50% ethanol solution used during homogenisation was not controlled either, which could influence the extraction rate.

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[8] A significant number of cucumbers displayed strange deformities (see appendix VIII) after few days of storage. Advice was thought from an expert at SLU Alnarp, who concluded they were due to a mixed fungal infection. LECs from all categories had to be removed from the shelf-life trial due to this, although samples not yet displaying obvious symptoms may have been analysed which could have influenced the results.

It should also be noted that the method selected for this analysis, although very common due to its speed and ease of execution, is not without controversy. As previously mentioned, DPPH is sensitive reagent, and its kinetics are easily affected by external factors. Additionally, reactions may be affected by internal factors such as the conformation and availability of antioxidants within the sample: if they are present but the reaction site is unavailable or unreachable, no reaction will occur.

Irrespective of the cause, the experiment should be repeated. It is recommended that a larger number of replicates per category be analysed, to reduce standard deviations. Skin samples could also be obtained using another method to standardise the skin/mesocarp ratio, and reagents could be taken out the day before to minimise the effect of temperature on reaction kinetics. Rotating cucumber plants around the greenhouse to ensure even access to light could help to limit disease and the effect of other pre-harvest factors on results, although cucumbers would have to be grown on site to ensure this was done in a satisfactory manner. Other larger studies have also repeated the experiment twice, using LECs cultivated during two different growing seasons within a year or cultivated during the same season on two different years, as a way of reducing the influence of diseases or abnormal weather conditions.

6.3.4 Colorimetry

Through colorimetric measurements, it would be possible to monitor changes in the coated cucumbers' L^*, a^*, and b^* parameters over the course of their shelf-life.
The measurements could only be performed on samples from the uncoated, base formulation and pennyroyal categories respectively for days 1 through 5. In an unforeseen strike of bad luck, the lamp of the only colorimeter available at the department stopped working, requiring to be sent away for service. As this event occurred on day 7 (Easter Sunday), the fault could not be reported until the following Tuesday, and the instrument was not put back into use before the end of the study. As the shelf-life study began one week later for wrapped samples and algae coated samples[9], no colorimetric measurements could be taken.

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[9] Wrapped samples could not be obtained in time for the beginning of the study, and an unsuccessful initial coating attempt for the algae category led to the decision of starting the study for these categories one week later. See appendix VIII for timeline illustration.

Attempts to find a solution were made, including trying to find another colorimeter to borrow and using a digital camera. Pictures were taken using the department's camera setup, equipped with bright lights in a non-reflective environment and a high-end camera from which RGB (another system used to record colorimetric data) values could be obtained and translated into $L^*a^*b^*$. Unfortunately, as the light source of the lamps was not the same as that of the colorimeter (different light production systems produce light with different qualities) and the environments were too different, data could not be compared to data obtained using the colorimeter. Having exhausted all other options, the decision was made to take pictures of samples in the lab using a smartphone, using as similar a setup as possible each time (blinds shut, ceiling lights on, white paper under samples for contrast). A representative picture can be found in appendix VIII.

Although this could not be measured, it was noticed that samples coated with extracts seemed to remain darker and more evenly green than unwrapped and wrapped samples (see appendix VIII). Many wrapped samples especially displayed typical yellowing at the flowering end of the fruit after 7-10 days of storage, this was not seen in algae and pennyroyal coated cucumbers. This would suggest, in accordance with the literature, that coatings containing antioxidants may contribute to preserve colour in produce (Miteluț et al., 2021) although this should of course be verified by repeating the experiment using a colorimeter.

7. Conclusions

The outcomes of this research project have yielded several key findings that advance the knowledge of the effectiveness of carrageenan-based films, their suitability for application on cucumbers as shelf-life extending edible coatings.

Our findings suggest that k-carrageenan formulations can be used to produce film and coatings that are transparent and palatable, although its highly hydrophilic and brittle nature may make it unsuitable for use on produce with a high water-content. Algal and plant extracts were successfully characterised and incorporated into formulations as secondary, active components which influenced the properties of the resulting film: differences in thickness and WVP were observed when compared to a control, but no differences in colour and mechanical strength were observed. Compared to plastic, formulated films possessed less effective water vapour barrier properties, and extract-containing films were less effective than the control film. All carrageenan films tested behaved similarly, and results suggest the material interacts with water much more than the traditional plastic wrap although the exact mechanism is unclear. Their use as a packaging alternative should therefore be further investigated, and other formulations should be tested.

The effect of the coatings was assessed via selected parameters during the shelf-life study, with the results suggested that carrageenan films did not enhance the keeping quality of LECs and did not extend their shelf life. Despite the higher antioxidative activity of samples B63 (*A. nodosum*) and B67 (pennyroyal) compared to B39 (control), DPPH-assay results from the shelf-life study were inconclusive, therefore, their performance over the course of the storage time is unknown and their potential beneficial effect in counteracting oxidative stress and preserving skin antioxidant content could not be seen. Colorimetric measurements did not produce definite results, but films containing extracts may have had some protective effect on the chlorophyll content of cucumbers based on the pictures taken. Weight loss and REL results showed that the coated samples' moisture content was decreased (increased weight loss and wrinkling on surface), and that they may have experienced increased oxidative stress (REL values were generally higher) when compared to wrapped and uncoated controls. REL was lower in samples containing extracts than in those coated in the base formulation, suggesting extracts may have had a positive

effect on reducing oxidative stress after day 5 although more data is needed to confirm.

While this study provides important insights into the effectiveness of this novel edible packaging alternative, it is important to highlight several limitations. These include the use of a single method for measuring the antioxidative capacity of the formulated films (DPPH assay) which may be unsuitable for this type of characterisation, and the inability to proceed with the colorimetric measurements during the shelf-life study. The biological variability in samples caused by non-controllable pre-harvest factors may also have influenced our results significantly. Evidently, time limitations did not allow for shelf-life related experiments to be repeated with the suggested changes and an increased number of replications.

8. Future Research

Several suggestions for future research opportunities have been highlighted throughout this report. A better general understanding of the effects of k-carrageenan based coatings may be obtained by repeating all experiments featured in this study using more replicates. Other suggestions are summarised in this section:

- Investigation into the degree of substitution of OSA-starch and its effect on the properties of resulting films (tensile strength, thickness, WBP/GBP, opacity).

- Investigation of salt and extract content and ratios in formulations, and of their effect on weight loss, permeability, scavenging activity and REL on LECs. This may contribute to a better understanding into the relationship between coating's salt content and oxidative stress, as well as the potential influence of extracts on salt-induced deteriorations in quality.

- Formulation and characterisation of hybrid and composite films using k-carrageenan together with other allergy-friendly hydrocolloids (polysaccharides from algae or plant origin, proteins such as zein or chitosan of fungal origin, starches from tubers and others) and oils. An additional point of interest might be the investigation of multi-layer coatings, also with the aim of improving WBP and GBP.

- Characterisation of antioxidant content and activity of films featured in this study using other methods (ex: Folin-Ciocalteu, FRAP, ORAC). The effectiveness of the films in protecting LECs from oxidative stress may be enhanced by combining different antioxidant sources (synergy) which may be effective at different points during senescence.

- Evaluation of effect of pre-harvest factors and biological variability on shelf-life of coated LECs by comparing results obtained from commercially available samples (obtained from a retailer, as was done in this project), from samples grown on site in optimised condition (no

crowding, even light distribution etc.) and samples from different growing seasons.

Overall, this research project has been a valuable learning experience, providing a significant insight into the complexities of conducting scientific research and familiarity with a variety of techniques used in the characterisation of foodstuffs and edible packaging. We sincerely hope that this work will contribute to the ongoing efforts in developing environmentally sustainable food packaging alternatives, and that it will inspire future research in this area.

Appendix

Appendix I.

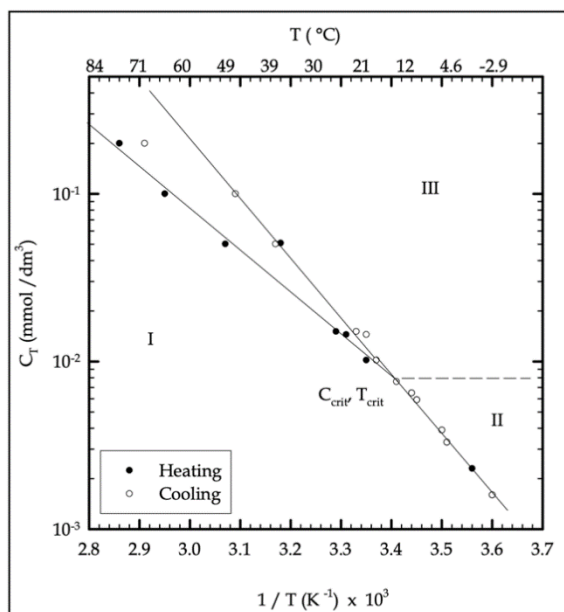Sol-gel transition diagram of k-carrageenan as seen in Rochas (1982):

Fig. 3. Sol-gel transition diagram of the potassium salt of κ-carrageenan (adapted from Rochas & Rinaudo, 1982). The dotted line represents the division between zone I and II.
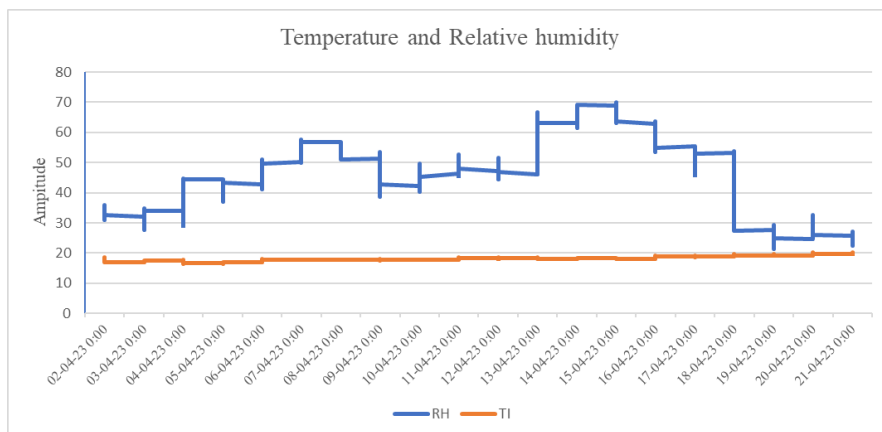
Appendix II.

Table II: Conductivity of extracts considered for incorporation into formulation

Sample	Measurement 1	Temp °C	Measurement 2	Temp °C	Average ± SD	Unit
LUU	24.1	20.7	24	20.6	24.1 ± 0.07	mS
DUU	14	20.3	15.6	21	14.8 ± 1.13	mS
DFC	26.7	20	26.6	19.9	26.7 ± 0.07	mS
Pennyroyal	287	20	287	20.1	287	μS
Spirulina	464	20	468	20.1	466 ± 2.83	μS
GMT	380	25.4	375	25	378 ± 3.54	μS

Where LUU & DUU are the "light" and "dark" extracts before filtration/ concentration, DFC is the filtered and concentrated "dark" extract, and GMT is the Greek Mountain Tea extract.

Appendix III.

Temperature and relative humidity conditions during the storage of cucumbers, as recorded by the sensor.
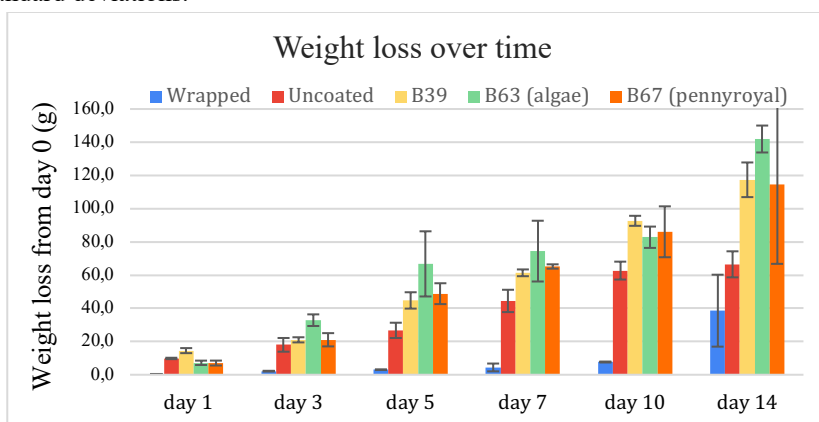
Appendix IV.

Table IV. <u>Colour values of films in preliminary trials (day 1)</u>

Film Sample	Parameters				
	L*	a*	b*	ΔE	Comments
B39	85.35± 1.2	-0.65± 0.10	4.67 ± 0.17	N/A	control
B61	83.37± 1.39	-0.65± 0.14	5.81 ± 0.22	2.87 ± 2.05	Oil and lecithin
B63	84.14± 0.93	-0.51± 0.08	5.88 ± 0.53	2.28 ± 1.68	DFC
B65	85.72± 0.5	-1.24 ± 0.05	6.80 ± 0.4	2.76 ± 0.21	Oil, lecithin, DFC
B66	84.37± 0.98	-0.69 ± 0.07	5.38 ± 0.1	0.84 ± 0.42	Spirulina
B67	85.72± 1.82	-0.95 ± 0.15	5.98 ± 0.16	2.07 ± 0.81	Pennyroyal
B68	83.77± 1.72	-0.82 ± 0.08	6.01 ± 0.15	1.56 ± 0.31	Mountain tea
B62	82.82± 1.35	-0.73 ± 0.04	6.25± 0.41	4.06	Oil, lecithin, LFC
B64	84.34± 1.11	-0.54± 0.03	4.70± 0.19	2.31	Increased glycerol

Where L* corresponds to lightness (positive values, or +), a* is the red (+) /green (-) index, b* is the yellow (+) /blue (-) index and ΔE is the total colour difference compared to day 0. For a more detailed explanation, see section 3.4.1 (Colorimetry).
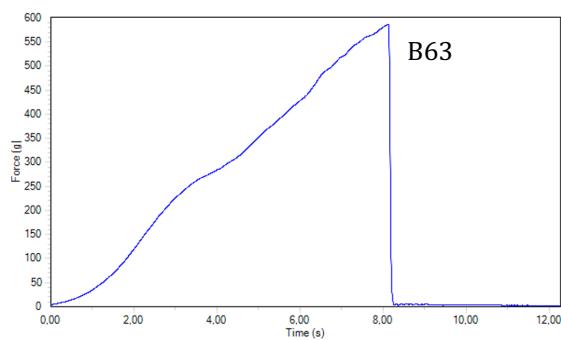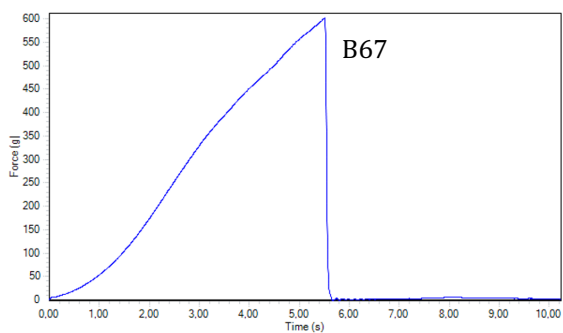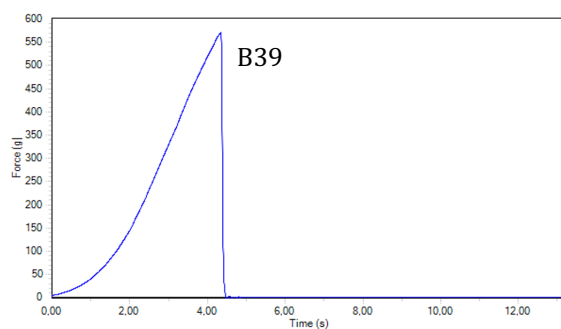
Appendix V.

Weight loss results from shelf-life study presented in a column diagram, including standard deviations.
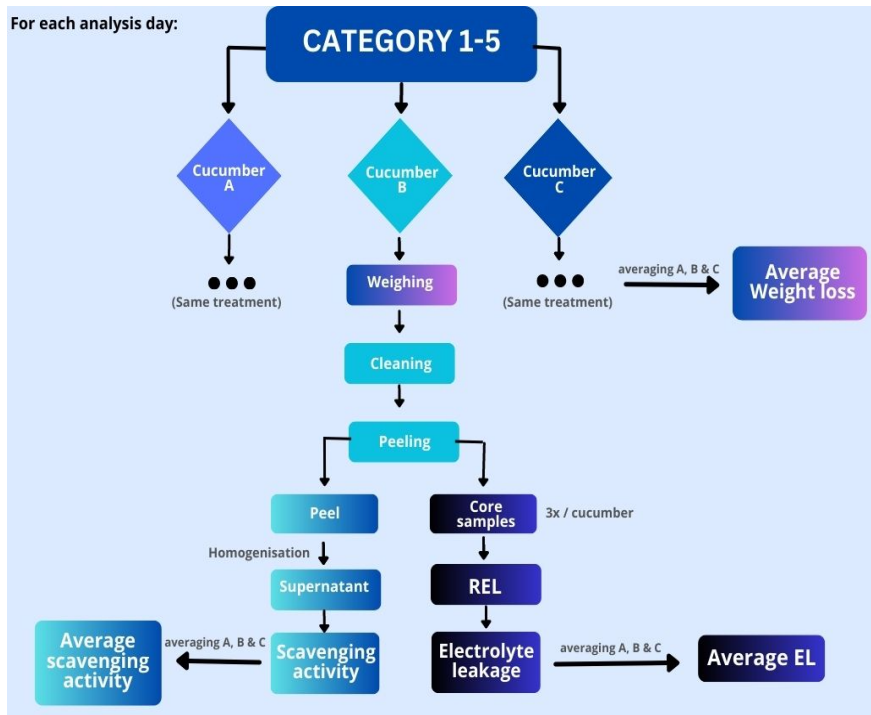
Appendix VI.

Representative puncture test graphs for final film formulations B39, B63 and B67, respectively:
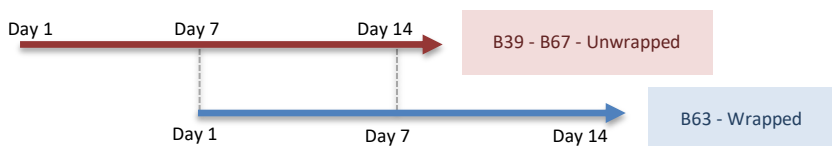
Appendix VII.

Diagram of data collection system used during shelf-life study:

And timeline used for respective categories:

Appendix VIII.

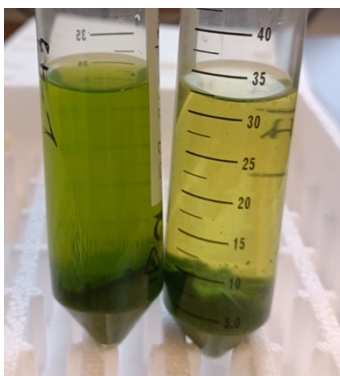Pictures taken during shelf-life study

Figure VIIIa: Representative example of colour differences in sample extracts for scavenging capacity experiment, following homogenisation and centrifugation

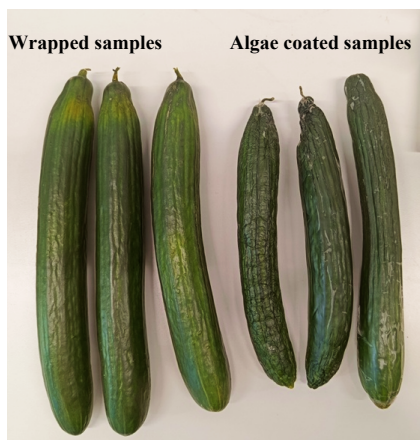**Figure VIIIb:** Evidence of mixed fungal infection in samples

Figure VIIIc: Representative illustration of colour difference between coated and wrapped samples on day 14

Figure VIIId: sample coated with B39

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