University of Reading

SeaCoat - Marine based edible coatings for minimally processed fruit

A Thesis Submitted in Fulfilment of the Requirements
for the Degree of Doctor of Philosophy

Department of Food and Nutritional Sciences

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Declaration:

'I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.'

Ana Augusto

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Acknowledgements

"'Beginning at the beginning', the King said gravely, 'and go on till you come to the end: then stop'"

Lewis Carroll, Alice in Wonderland

During the last four years, I thought a lot about what to write once this stage was reached and one constant thing came to my mind in every one of my thoughts: it will be the end of a journey that I longed for. Now, is time to stop, breathe and enjoy the happiness of finishing this unique odyssey. Just as with many other aspects of my life, I wasn't alone in this journey and, since day one, there are Professors, friends, and family that I must acknowledge for their undeniable presence. My general gratitude to all those involved in this project, especially my supervisors Prof. Susana Silva, Prof. Keshavan Niranjan and Prof. Geoffrey Mitchell. I could never express enough gratefulness to Prof. Susana Silva that since the early stage of my academic career is always supporting me, letting me be part of her projects, and who always reminds me, that even working in science, we cannot forget that we are humans, and making mistakes is an indissoluble condition. To Prof. Keshavan Niranjan, always available and who accepted all my ideas, thank you for all the support especially in the composition of the manuscripts, transforming my weakness into hope. To Prof. Geoffrey Mitchell, always available and ready to discuss the work. I also leave here my strong appreciation to FCT, the Portuguese Foundation for Science and Technology, for the financial support given to the work developed.

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Tiago… I have no words for your tolerance in the last months, hope now we may have time to enjoy life. Thank you for letting me know that the world is not only black and white as I thought, but there are also grey zones, and that, it is just ok.

Abstract

Fresh-cut fruits and vegetables are in increasing demand, as these products comply with key consumer trends combining health promotion and convenience. Shelf-life of fresh-cut fruits as apples and pears is limited by the development of superficial browning, which causes product rejection by consumers. Fresh-cut fruits requirements limit the array of suitable conservation technologies, with edible coatings application as one of the most used and successful barriers to product degradation. Most of the commercially available solutions include synthetic substances that contribute to a shelf-life of up to 8 days. Options that simultaneously extend shelf-life and comply with consumer demand for natural-based additives will strengthen the resilience of fruits and vegetables value chain.

The present thesis aims to evaluate the potential of *Codium tomentosum* extract as an edible coating for fresh-cut apple and pear, extending fresh-cut fruit shelf-life through superficial browning regulation. For this purpose, *C. tomentosum* extract-based coating solution was applied as an anti-browning treatment to fresh-cut 'Rocha' pear and 'Fuji' apple produced under industrial conditions. During the storage period of 15 days at 4 °C, fresh-cut pears coated with the seaweed extract showed lower colour changes and browning index than the control and samples treated with a commercial solution. Fresh-cut apples, stored with modified atmosphere, and coated with the seaweed extract had identical browning index and colour change values to those samples coated with an ascorbate-based commercial additive, possibly because of lower oxidative enzyme activities observed in extract treated samples. For an understanding of the mechanism underlying anti-browning functionality of the extract, gene expression analysis was performed. It was observed that in fresh-cut apple coated with the seaweed extract, peroxidase (*mdPOD*) and phenylalanine ammonia-lyase (*mdPAL*) genes expression was particularly affected one hour after the coating application, followed by a reduction in polyphenol oxidase (*mdPPO*) expression. These results suggest that the

aforementioned genes are activated at a later stage, thus contributing to decreased enzymatic activity, and consequently, to a lower browning in treated samples. The effects of time, temperature and pH on the extraction of *C. tomentosum*, and their influence on the extract functionality, were evaluated. A Response Surface Methodology was applied, and it was observed that higher yields of extraction are obtained with longer extraction periods and neutral to alkaline pH. In terms of the functionality of the extracts, browning compounds absorbance and peroxidase activity were mainly influenced by changes in temperature, especially above room temperature.

The present thesis sets baseline key findings to support the use of seaweed extracts as edible coatings in the fresh-cut industry, validates *C. tomentosum* extract functionality as an anti-browning treatment for fresh-cut fruits and establishes the basis for a thorough understanding of its mechanism of action at a gene expression level.

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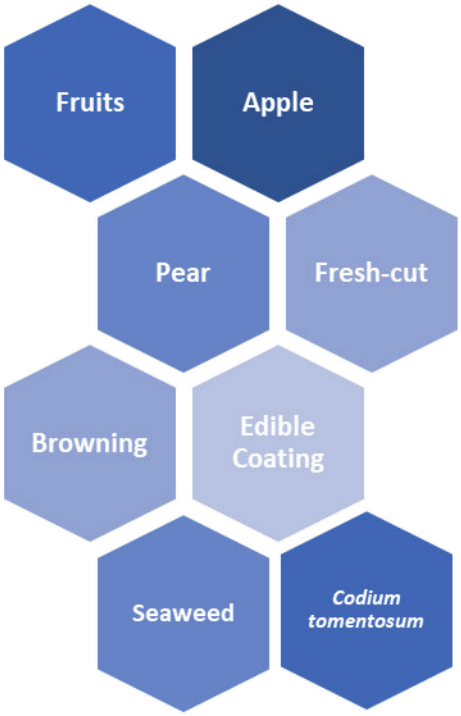

CHAPTER I

General Introduction and Objectives

1. General Introduction

1.1. Fruits

Fruits are considered to be an important part of a healthy and equilibrated diet, since their consumption and nutritional value are linked with health promotion. An insufficient intake of daily fruit portions is associated with an increase of about 11 % in cardiovascular diseases, type 2 diabetes, cancer and other less prevalent diseases (Stea et al., 2020; Sun et al., 2021).

The beneficial impact of fruit consumption is directly correlated with its nutritional value and composition, which is mostly composed of vitamins (vitamins A, C and K), minerals, fibre and phytonutrients or phytochemicals, also commonly referred to as bioactive compounds (Colabianchi et al., 2021; Sun et al., 2021). More than 5,000 types of phytochemicals exist in fruit, vegetables and grains, although only a fraction of these has been identified and studied (Yalcin & Çapar, 2017). The most abundant and extensively studied phytochemicals are the (poly)phenolic compounds, which are divided into two major groups- flavonoids and phenolic acids; and the carotenoids, responsible for the colour of many vegetables and fruits (Karasawa & Mohan, 2018; Yalcin & Çapar, 2017).

As a result of the nutritional value and biochemical composition of fruit and vegetables, the World Health Organization (WHO) recommend the consumption of 5 portions of fruit per day (approx. 400 g) (Stea et al., 2020). Stea et al. (2020) reported different consumption patterns in Europe. Eastern Europeans tend to consume fewer fruits and vegetables, while Southern and Northern Europeans tend to include more fruits and vegetables in their daily lives (Stea et al., 2020). In the US, fruit and vegetable consumption is considered an important health subject, whereas around 12 % of the adults meet the WHO fruit recommendations (Colabianchi et al., 2021).

To address the global demand for fruits, in 2019, around 883 million[1] tonnes of primary fruits[2] were produced worldwide, accounting for 80 million tonnes for Europe (FAOSTAT, 2021). East and South Asian countries, including China, are known to be the large producers of fruits (FAO, 2020a). Within Europe, where the numbers mentioned above represent a rise of about 15 % between 2010 and 2019 (FAO, 2020a), Spain, Italy and France are the most significant growers. Grapes is the dominant fruit grown in all three countries (total of 19 million tonnes), followed by apples in France and Italy (total of 4 million tonnes), and oranges in Spain (3 million tonnes) (FAOSTAT, 2021).

1.2. Apple

The apple *Malus domestica* Brokh, which replaced the previous species name *Malus pumila*, belongs to the Rosaceae family and is considered a modern cultivar derived originally from the wild cultivar *Malus sieversii* which is native to Central Asia (Musacchi & Serra, 2018). Apple is one of the most widely produced crops, in Europe, with a production of 17,094,614 tonnes in 2019, revealing its economic importance for the producing countries (Chen et al., 2021; FAOSTAT, 2021; Musacchi & Serra, 2018). In the European Apple Inventor are listed more than then thousand cultivars, exhibiting a broad range of variability in the quality traits (Musacchi & Serra, 2018). However, despite the vast number of known cultivars, very few are commercialized. About 25 apple varieties are sold all over Europe, which include 'Golden Delicious', 'Royal Gala', and 'Idared' (Almeida et al., 2017; Hecke et al., 2006). Apple cultivars can be roughly divided into two main varieties: I) red varieties such as 'Red Delicious', 'Fuji', and 'Royal Gala', and II) non-coloured or green varieties as 'Golden Delicious' and 'Granny Smith' (Chen et al., 2021). This division helps consumers in their purchase choice since vision is the first

[1] The following abbreviation was adopted: million = 10^6

[2] Primary crop definition by FAO: *Primary crops are those which come directly from the land and without having undergone any real processing, apart from cleaning. They maintain all the biological qualities they had when they were still on the plants*. Excluding citrus fruit.

sense that consumers use when shopping. Colour is therefore a major focus of apple breeders and researchers. Apple colour is determined by the type of anthocyanins, which are secondary metabolites depending on the pH in cells (Chen et al., 2021). Cyanidin-3-galactosidase is the main form of anthocyanin found in apples and the main responsible for the reddish colour present in apple fruits.

Nutritionally, apples are well-balanced, mostly due to their sugar and fibre contents, and phytochemical composition, which vary greatly between different varieties (Feliciano et al., 2010; Serra et al., 2010). Apples have approximately 84 % water and are a source of minerals such as K, Mg, Ca and Na, trace elements such as Zn, Mn, Cu, and Se and vitamins, mainly B complex. The concentration of proteins and lipids is reduced, however pectin, cellulose, and lignin contents make apple fruit an important source of carbohydrates and fibre (Feliciano et al., 2010). All these components together, make the sugar-acid ratio responsible for the taste and flavour of the apple fruit (Hecke et al., 2006). Apples have the second-highest level of antioxidants compared to other commonly consumed fruits such as grapes, strawberry, peach and pears (Kalinowska et al., 2014; Yalcin & Çapar, 2017). The main antioxidants are polyphenolic or phenolic compounds. Compared to vitamin C, which is widely known for its antioxidant activity, 100 g portion of apple has an antioxidant activity equivalent to 1,500 mg of vitamin C (Kalinowska et al., 2014). Amongst the phenolic compounds found in apples, chlorogenic acid is the main phenolic acid (Kalinowska et al., 2014), followed by quercetin glycosides, procyanidins, catechin, epicatechin and phloridzin (Feliciano et al., 2010; Lee et al., 2003); higher concentrations of these are found in the apple peel than in the flesh. Quercetin conjugates are found exclusively in the peels (Feliciano et al., 2010). Phenolic acids and flavonoids are also relevant as they determine fruit quality characteristics such as colour, flavour, bitterness, and astringency (Almeida et al., 2017). In addition to apple cultivars, environmental conditions and post-harvest handling practices are important factors which

determine the distribution of these compounds. These factors include fruit season, fruit maturity, light exposure, raining seasons, storage and processing conditions (Feliciano et al., 2010).

The most commercialized cultivars, such as 'Red Delicious', 'Golden Delicious', 'Granny Smith' and 'Fuji' are well characterized in terms of phytochemicals and nutritional composition. The whole genome sequence of apple ('Golden Delicious' cultivar) was first reported in 2010 and was conducted by the Italian Agriculture Research Institute and the 454 Sequencing Company (USA) (Velasco et al., 2010), which substantially improved our knowledge of apples.

Regular consumption of this fruit is widely accepted as a part of a healthy diet, with proven aid in the prevention of chronic diseases and maintenance of good health (Feliciano et al., 2010; Kalinowska et al., 2014; Sun et al., 2021).

Among the European fruits with protected denominations, there are 23 fresh apple cultivars (Almeida et al., 2017). In terms of the area restricted to apple orchards, Portugal has approximately an area of 14,313 ha, which in 2020, resulted in the production of 286,504 tonnes of apples (INE, 2021). Only in 2019, the Portuguese producers exported an average of 69,752 tonnes, a number which has been increasing especially in the last decade, as can be seen in Figure 1.1 (FAOSTAT, 2021).

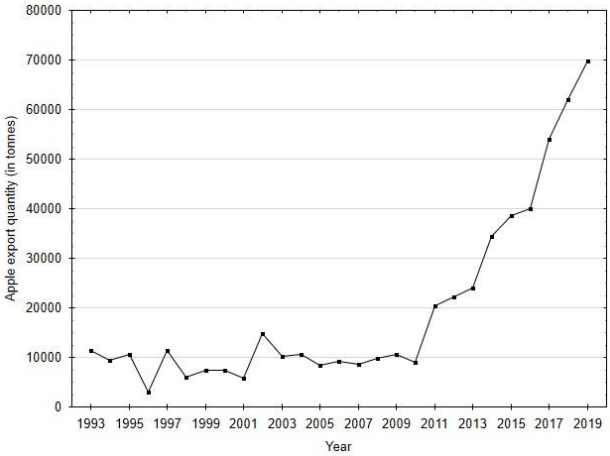

Figure 1.1. Apple export quantity in Portugal. Adapted from FAOSTAT (2021).

'Maçã de Alcobaça' is a Protected Geographical Indication (PGI) for apple cultivars grown in the Western Region of Portugal. This region, with natural conditions characterized by an average annual temperature of 15 °C, total rainfall ranging from 600 to 900 mm, high insolation, and high relative humidity from the Atlantic Ocean influence the phytochemical properties of 'Maçã de Alcobaça' cultivars. Within this PGI are included nine apple cultivars: 'Casanova', 'Golden Delicious', 'Red Delicious', 'Gala', 'Fuji', 'Granny Smith', 'Jonagold', 'Reineta' and 'Pink' (Figure 1.2).

Figure 1.2. 'Maçã de Alcobaça' apple varieties.

1.3. Pear

Pear (*Pyrus* spp.) is a climacteric fruit that belongs to the Rosaceae family and is considered one of the most important deciduous fruit trees in the world (Cheng et al., 2020). Worldwide, there exists about 22 pear species divided into 2,000 cultivars, however, only a small part is considered relevant in terms of production and commercialization (Brahem et al., 2017; Li et al., 2019a). In the last decade, pears have become an interesting crop and a way of economic diversification within modern fruit orchards (Deckers & Schoofs, 2008), especially in regions with temperate climates. Pear crops have better characteristics and growth rates in temperate climates, such as those prevalent in Europe, North America, and temperate regions of the Southern hemisphere (Brahem et al., 2017), which result in cultivars with distinct phenotypic and organoleptic characteristics. Pear cultivars commercially available derive from two main species: the Asian pear (*Pyrus sinensis*) and the European pear (*Pyrus communis* L.). In Europe, the most common cultivar is the *P. communis,* however, there are several pear varieties available for consumption (Deckers & Schoofs, 2008), which include 'Conference', 'William', 'Abbé Fétel', 'Blanquilla', 'Doyenne du Comice', 'Kaise', 'Dr. Jules Guyot' and 'Coscia' representing about 80 % of the production (Figure 1.3) (Dondini & Sansavini, 2012). Pear production is mainly used for fresh consumption, and in 2019, about 2,495,218 tonnes of pear were produced in Europe (FAOSTAT, 2021), mainly in Italy, Netherlands, Belgium, and Spain (in descending order of production).

Pear fruit is sought by consumers because of its pyriform shape which also characterizes the European cultivars, a weight average of 180 - 200 g, its their juicy and fine flesh possessing high-quality flavour, and good shelf-life (Dondini & Sansavini, 2012; Li et al., 2015b). Depending on the cultivar, pears possess different nutritional compositions and consequently distinct organoleptic properties such as aroma and taste. Pears are also considered a nutrient-dense fruit, they are rich in fibre, vitamins and minerals, and low in calories, which are common characteristics of nutrient-dense fruits (Kolniak-Ostek, 2016; Li et al., 2015b). These differences are mostly verified between the European and Asian pears. The former have higher calories and sugar content, while the latter are rich in water with a lower content of sugar and starch (Li et al., 2015b).

Focusing on the European cultivar, pears contain 85 % water, 14 % carbohydrates, 2 % of fibre, and very low protein and fat contents (0.3 % and 0.1 % respectively), accounting for a total of 54 calories of food energy in 100 g of edible portion (Kolniak-Ostek, 2016). Ascorbic acid - the main antioxidant of pears - is the major vitamin, accounting for 3 mg per 100 g of edible portion

(Li et al., 2015b). Mineral nutrients such as phosphorus, iron, potassium, calcium and magnesium can also be found in pears (Li et al., 2015b), as well organic acids, such as malic and citric acid. The sweetness in pear fruits is mainly due to the conversion of glucose, fructose and sucrose into sorbitol, conferring the sweet flavour (Li et al., 2015b). In terms of phenolic compounds, or phytochemicals, pears contain important triterpenoids that have anti-oxidative, anti-inflammatory and anticancer properties (Kolniak-Ostek, 2016). As in the case of a majority of fruit, a higher content of phytochemicals can be found in the peel than in flesh. Anthocyanins, flavan-3-ols and flavonoids are present in higher concentrations (Barroca et al., 2006; Li et al., 2015b).

Within modern cultivars commercially available, 'Pera Rocha do Oeste' or 'Rocha' pear (Figure 1.3) is an exclusive Portuguese pear variety, whose export market has been progressively increasing in recent years accounting for 95 % of the national pear production in Portugal (Deuchande et al., 2016). The production of this cultivar is concentrated in the Portuguese Western Region (Salta et al., 2010), and is largely exported to the United Kingdom, France, Brazil and Russia (Pessoa et al., 2021). Portugal contributes about 6 % of the total pear production in Europe (Deckers & Schoofs, 2008). With a harvest area of 11,325 ha, in 2020, Portugal produced an average of 131,004 tonnes of pears (INE, 2021), corresponding to a total of 124,000 tonnes of 'Rocha' pear and a value of 80,465,000 euros. In Figure 1.4, it is possible to observe the rise in pear exports from 1993 until 2019. Being a Protected Designation of Origin (PDO), their organoleptic properties are mainly due to the specific climacteric and soil characteristics that characterize the western region of Portugal, with higher cultivated area situated in the municipalities of Bombarral, Cadaval, Caldas-da-Rainha, and Lourinhã (Pedro et al., 2020). Curiously, these are the same municipalities where 'Maçã de Alcobaça' apples are produced (please see 1.1).

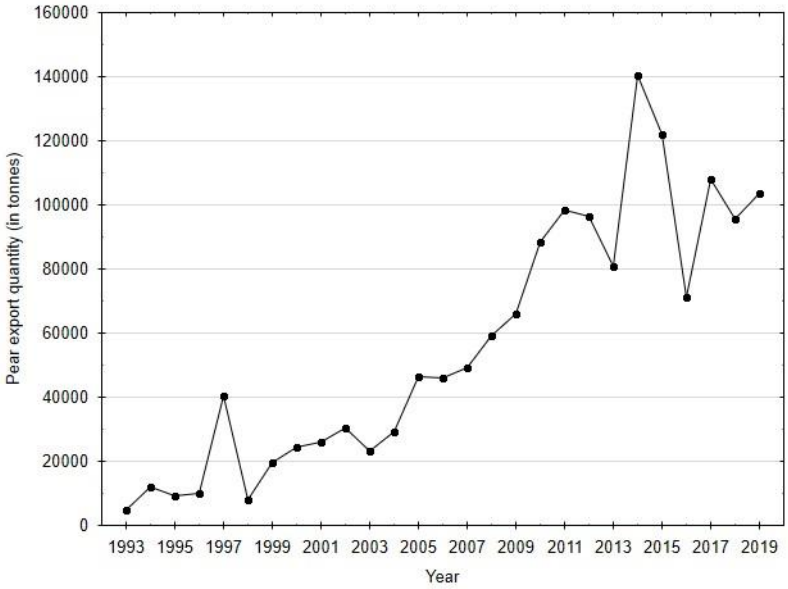

Figure 1.4. Pear export quantity in Portugal. Adapted from FAOSTAT (2021).

1.4. Fresh-Cut Fruits

Regular consumption of fresh fruits is directly correlated with health promotion (Stea et al., 2020). In the last decade, the consumption pattern of fresh products has been changing, and the market for freshly prepared fruit and vegetable products has grown (Oliveira et al., 2015). Consumers demand for fresh, healthy, convenient, and additive-free items increased, making this the main driving force for fresh-cut fruits (Botondi et al., 2021; Oliveira et al., 2015). According to the Euromonitor, between 2009-2014, the fresh-cut fruit and vegetable market experienced 19 % per capita volume growth (Anonymous, 2015). United Kingdom has the largest fresh-cut fruit and vegetable market, followed by Spain and Germany, two emerging markets (Baselice et al., 2017; Oliveira et al., 2015). In southeastern European countries, fresh-cut fruits and vegetables consumption is still negligible (Baselice et al., 2017). However, more literature is needed to understand consumers acceptance of fresh-cut fruits and vegetables across Europe, especially in countries where the fresh-cut market is starting to emerge, such as Portugal. Vegetable preparations are a clear preference in the fresh-cut market, where packaged lettuce account for 50 % of sales, and only 10 % is represented by fresh-cut fruits, and the remaining 10 % by ready-to-cook and crudités products (Botondi et al., 2021).

Fresh-cut fruits, also known as minimally processed products, are ready-to-eat products, where all the non-edible parts are removed and the fruit is washed, peeled and cut into bite-size or a 100 % usable form (Botondi et al., 2021). There are various steps involved in the preparation of fresh-cut fruits (Figure 1.5), however, each unit operation should be adapted according to the type of fruit being processed, ensuring satisfactory quality, shelf-life, and safety of the final product (Oliveira et al., 2015; Yousuf et al., 2018). Despite the convenience of these food products, fresh-cut fruits have a short shelf-life as a result of rapid microbial spoilage (Siroli et al., 2015). Compared to the original raw material, which has a storage life of weeks to months, fresh-cut products have a 4 – 10 days shelf-life (Prakash et al., 2018; Siroli et al., 2015). To guarantee the safety of the product, and according to the Commission Implementing Regulation (EU) number 1308/2013, fresh-cut products can only be stored at temperatures below 8 °C for a short period (ER 1308/2013).

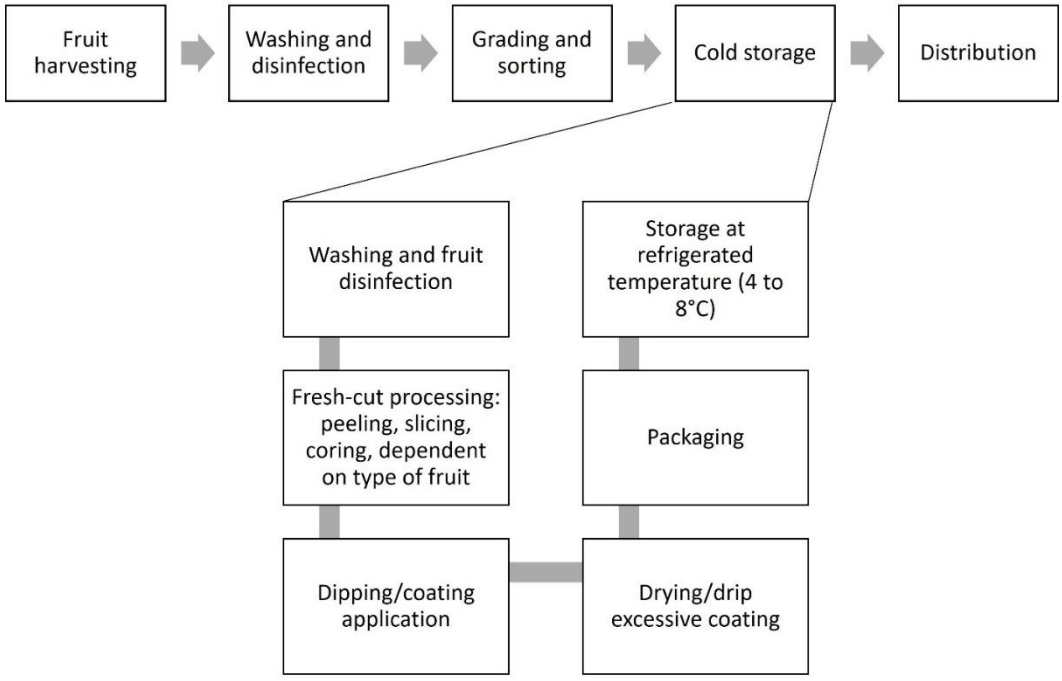

Figure 1.5. General flow diagram of fresh-cut fruit and vegetables processing. Adapted from Botondi et al. (2021) and Yousuf et al. (2018).

Contributing to the short shelf-life of the fresh products is not only the microbial spoilage, but also physiological ageing, biochemical changes, physical and chemical deterioration, which invariably result in colour degradation and texture and flavour changes (Oliveira et al., 2015). These changes in food quality and food safety lead to food rejection, which translates into increased food waste (Botondi et al., 2021). The physiology of fresh-cut fruits is related to the tissue injury during cutting/slicing, which can lead to excessive tissue softening and superficial browning (Augusto et al., 2016). In fruits rich in phenolic compounds such as apples and pears (Kalinowska et al., 2014; Li et al., 2015b), the processes of cutting, peeling and packaging trigger deteriorative processes led by enzymes and microbial contaminations (Putnik et al., 2017). In fresh-cut apple and pear, superficial colour is one of the first attributes that customers consider when buying. In both fruits, superficial browning may result from enzymatic and non-enzymatic reactions, although enzymatic browning is considered to be the main cause responsible for browning development (Putnik et al., 2017; Saba & Sogvar, 2016).

Superficial browning mediated by enzymatic reactions occurs mainly when the polyphenolic substrates come into contact with the enzymes responsible for their oxidation (Saba & Sogvar, 2016). In the presence of oxygen, the enzyme polyphenol oxidase (PPO, EC number 1.10.3.2) catalyses the hydroxylation of monophenols to *o*-diphenols, followed by the oxidation of *o*-diphenols to *o*-quinones (Rasouli & Koushesh Saba, 2018). These highly reactive *o*-quinones condense and react with amino acids and proteins, leading to the formation of heterogeneous black, brown, or red compounds – the melanin complexes (Li et al., 2017c), which are responsible for the superficial colour that is visible in oxidized fresh-cut apples and pears. A second enzyme is commonly assigned to the progress of superficial browning in fresh-cut apples and pears. Although less often referred, the enzyme peroxidase (POD, E.C. 1.11.1.X) can also enhance browning reactions in the presence of ongoing PPO-mediated browning (Chen et al., 2021). This enzyme catalyses the oxidation of phenolic compounds using hydrogen peroxide.

Herein, monophenols are hydroxylated by peroxidase into dihydroxy-phenols (Oliveira et al., 2021), contributing to quinones production and consequently to browning intensity.

To enhance fresh-cut apple and pear shelf-life and safety, and simultaneously, lower superficial browning, several technologies can be applied, from the most conventional methods, such as refrigeration and modified atmosphere packaging, to more modern methods such as the use of ozone technologies and edible coatings application (Botondi et al., 2021; Oliveira et al., 2015; Putnik et al., 2017).

1.5. Edible Coatings

The use of edible coatings to extend the keeping quality of fresh-cut fruits is a very common practice in the food industry (Zhao, 2018). Edible coating consists of applying a thin layer of edible materials on the surface of the food. With the formation of this thin layer, it is possible to control oxygen and carbon dioxide exchanges as well moisture and solute migration between the food product and the environment (Mitelut et al., 2021; Zhao, 2018). Depending on the fruit product, edible coatings can have in their composition antimicrobial and antioxidant compounds, working as additive carriers and providing a simultaneously attractive appearance of the food product and a longer shelf-life (Zhao, 2018). However, when developing a new formulation, edible coatings have to comply with several requirements, such as (Mitelut et al., 2021; Olivas & Barbosa-Cánovas, 2005; Zhao, 2018):

I. It must be a biocompatible material, respecting the physiological nature of the food product.

II. It must be a food grade material to maintain the edibility of the food product, i.e. the coating components must be food-grade quality and to be among the list of Novel Foods or Food Improvement Agents (in EU) or Food and Drug Administration's GRAS (generally recognized as safe) (in the USA).

III. It must easily adhere to the fruit to ensure durability, uniformity and appearance of the product and coating.

IV. It's application must result in the right sensory and organoleptic quality. As far as possible, it must be colourless and tasteless.

V. It must be physico-chemically and microbially stable.

A great diversity of materials can be used to produce edible coatings. These materials can be obtained from several sources: plants, animals and microorganisms being the most common (Galus et al., 2020). Within the wide range of materials that can be used in coating formulations, three types are highlighted: I) proteins, II) polysaccharides and III) lipids. Among the most popular proteins are soybean protein, gelatine, whey, casein, corn zein, sunflower protein and wheat gluten. Alginate, chitosan, pullulan, carrageenan, cellulose derivates and starch are the most popular polysaccharides (Galus et al., 2020; Olivas & Barbosa-Cánovas, 2005). Lipids are included in edible coatings formulations mostly to form a second layer and improve the water vapour barrier properties (Olivas & Barbosa-Cánovas, 2005). Beeswax, acetylated monoglycerides, fatty alcohols, and fatty acids, are some of the lipids that have been successfully employed to coat cut-fruits, always applied in combination with a polysaccharide or protein (Olivas & Barbosa-Cánovas, 2005).

Edible coatings can be applied in food by different methods like dipping or immersing, spraying, and brushing, the most popular being dipping and spraying, largely due to the low costs associated with their use (Galus et al., 2020; Mitelut et al., 2021).

Although the large number of coating options mentioned above, only a few are commercially available, especially those dedicated to fresh-cut fruits, where NaturSeal® and FOOD freshly® are the solutions most used by the industry (Olivas & Barbosa-Cánovas, 2005). Research and development efforts are leading to an improvement of coating functional characteristics, which depend on the fruit properties and regulatory authorizations for commercialization. In Table 1.1

are listed several edible coatings which are currently used by industry and formulations that are under study.

Table 1.1. Examples of commercialised available and under study edible coatings for fresh fruits. Adapted and modified from Olivas & Barbosa-Cánovas (2005) and Zhao (2018).

Authorized and commercialized coatings			
Coating name (Coating method)	Composition	Fruit application (Role)	Manufacturer (M) / Reference (R)
NatureSeal® (Dip coating)	Ascorbic acid, calcium chloride, hydroxypropyl methylcellulose	Inhibit enzymatic browning, maintain taste, texture, and colour of sliced apples, pears, avocados, and bananas	**(M)** Mantrose-Haeuser Co., USA
FOOD freshly® (Dip coating)	Naturally derived vitamins and minerals	Browning inhibition, taste and texture of sliced apples, pears, bananas, avocados.	**(M)** Food Freshly AFC, Germany
CITRASHINE ® (Spraying)	Wax-based solutions	Delay ripening, softening, maintaining firmness, colour, and flavour of fruits.	**(M)** Citrashine (Pty) Ltd. with Holding company Decco U.S. Postharvest, Inc., Monrovia, CA, USA
NATURCOVER (Spraying)	Sucrose ester-based solution.	Delay ripening in apples and pears.	**(M)** Decco Ibe'rica Post Cosecha S.A.U., Valencia, Spain
Semperfresh (Dip coating and spraying)	Sucrose ester-based coating	Maintains colour and fruit firmness in pears and other fruits.	**(M)** AgriCoat NatureSeal Ltd, UK.
Under development			
Biocontrol agents (Dip coating)	Solution containing *Lactobacillus casei*	Control of microbial spoilage of mixed salads.	**(R)** Siroli et al. (2015)
Antimicrobial nanoemulsions	Nanoemulsion-based edible coatings with	Fresh-cut apple reduction of	**(R)** Salvia-Trujillo et al. (2015)

(Dip coating)	lemongrass essential oil	browning and respiration rates.	
Alginate-based solutions (Dip coating)	Alginate-based solution with cinnamon and rosemary essential oils incorporation.	Colour preservation and enzymes inactivation of fresh-cut apples.	**(R)** Chiabrando & Giacalone (2015)
Seaweed-based coating (Dip coating)	Coating solution containing *Codium tomentosum* extract.	Colour preservation and enzyme activity reductions in fresh-cut apples and pears.	**(R)** Augusto et al., (2016)
Carboxymethyl cellulose coating and aloe vera (Dip coating)	Carboxymethyl cellulose and aloe vera coating in combination with anti-browning agents.	Colour preservation and enzyme activity reduction in fresh-cut apples.	**(R)** Kumar et al. (2018)
Hydrocolloid-based coating (Dip coating)	Gellan, alginate and pectin coatings combined with N-acetylcysteine and glutathione	Control of enzymatic browning, microbial safety, and ethylene production reduction in fresh-cut pears.	**(R)** Oms-Oliu et al. (2008)

1.6. Edible Seaweeds

Seaweeds, also known as marine macroalgae or marine vegetables, are mainly composed of three groups, divided by the predominance of pigments: green (Chlorophyta), brown (Phaeophyta) and red (Rhodophyta) (Cordero, 2019). In the last FAO report it was mentioned that, worldwide in 2018, around 32,386.2 tonnes of seaweeds were produced (FAO, 2020b), mostly used for hydrocolloids extraction, such as carrageenan (*Euchema* spp. and *Kappaphycus alvarezii*), and agar (*Gracilaria* spp.). For human consumption, the most prevalent seaweeds are *Saccharina japonica* (commonly called 'kelp'), *Undaria pinnatifida*, *Porphyra* spp. and *Sargassum fusiforme* (Buschmann et al., 2017). Traditionally consumed in Asiatic countries, it is only recently, that consumers started to pay more attention to seaweeds and the advantages of their

use in Europe (Fleurence, 2016). The use of seaweeds for food consumption remains marginal, and seaweeds are mostly used for the cosmetic and pharmaceutical purposes. In Europe, seaweeds are harvested mainly directly from the sea, an activity that is dependent on the climacteric conditions and seaweed growth rate, which strongly contrast with Asiatic countries, where seaweed farming is commonly practiced (Buschmann et al., 2017).

There are multiple ways of using seaweeds for direct human consumption (e.g. fresh, dried, powder, salted, canned, infusion or included in prepared foods), or added to formulations used for foods or nutraceuticals, feed, fertilizers etc (Buschmann et al., 2017; Fleurence, 2016). Seaweeds are claimed to be low in cholesterol content, and good to fight obesity, reduce blood pressure or even tackle free radicals and promote healthy digestion (Rodrigues et al., 2015).

The potential of using the whole seaweed or its extracts in food applications has been widely studied. Food hydrocolloids industry is the main market for the hydrocolloids extracted from seaweeds (Roohinejad et al., 2017). Alginate, agar, fucoidans, and carrageenan and other hydrocolloids are examples of compounds extracted, respectively, from brown and red seaweeds, which are widely used as texturing agents and stabilizers in foods (Augusto et al., 2018; Roohinejad et al., 2017). Seaweed composition varies significantly across species depending on the type of habitat. Typically, seaweeds are found in intertidal zones, where the consequences of tidal fluctuations are most visible. Here, seaweeds are subject to repeated immersion and emersion, exposing the organisms to air, nutrient limitation, intense light, rapid temperature fluctuation, osmotic stress and desiccation (Valentão et al., 2010). Unlike terrestrial plants that probably would not survive, seaweeds grow in very harsh conditions and are those conditions that allowed them to evolve and adapt creating defence mechanisms, such as antioxidative and antimicrobial activities (Charoensiddhi et al., 2017), making seaweeds a natural source of interesting bioactive compounds.

1.6.1. Codium tomentosum seaweed

Codium tomentosum (Stackhouse, 1797) is a green and edible seaweed (Figure 1.6) of the family Codiaceae. There are about 125 identified species of *Codium* sp. widely distributed through the ocean's, mainly found in temperate and subtropical areas (Costa et al., 2015; Fernández et al., 2014). Morphologically, these are organized in thalli, which are formed by long branches, the siphons, with a single cell containing multiple nuclei, forming a spongy, dark green mesh of siphons. These species can occur in a wide array of forms, sizes, and compositions (Fernández et al., 2014). The species *C. tomentosum* can be found on exposed rocks and deep rock pools on the seashore Northeast Atlantic Ocean, from the British Isles to Azores and Cape Verde (Silva et al., 2021) and is persistent throughout the year.

Figure 1.6. An exemplar of *Codium tomentosum* seaweed.

Like all fresh products, after harvesting, seaweeds deteriorate rapidly in a few days making drying an essential step to preserve their quality (Rodrigues et al., 2015). Thus, most studies use dried biomass instead of fresh biomass. The proximate composition of dried *C. tomentosum* is given in Table 1.2, where protein, fibre and carbohydrate contents may be highlighted by the high values. Despite its wide distribution and biomass availability, the existing studies with *C.*

tomentosum extracts or secondary metabolites are mainly limited to its potential for medical and biotechnological applications (Silva et al., 2021). More examples of the use of *C. tomentosum extracts* can be seen in Table 1.3. Briefly, *C. tomentosum* extracts have been studied in the context of their neuroprotective potential in Parkinson's and Alzheimer's diseases, environmental bioremediation, fish respiratory quality, and fresh-cut fruits preservation (see Table 1.3)

Table 1.2. Proximate composition of dried *Codium tomentosum* (own results).

Proximate composition (g 100 g^{-1} dry biomass)	
Total Protein	17.4
Total Fibre	24.4
Total soluble solids	5.5
Total Fat	3.3
Salt	22.78
Ash	38.5
Nutrients	
Calcium (mg 100 g^{-1} of dried biomass)	6900
Potassium (mg kg^{-1})	372.9
Magnesium (mg kg^{-1})	15000
Phosphorus (mg kg^{-1})	2000
Iron (mg kg^{-1})	710
Zinc (mg kg^{-1})	19
Copper (mg kg^{-1})	14
Iodine (mg kg^{-1})	130

Table 1.3. Example of studies conducted with *Codium tomentosum* extracts or compounds.

Field of research	Research	Reference
Pharmaceutical/ medicine	Neuroprotective and anti-inflammatory effect of *C. tomentosum* extracts and compounds.	Silva et al., (2020, 2021); Soares et al., (2021)
Pharmaceutical/ medicine	Extracts anti-oxidative, genotoxic and antigenotoxic potency in human lymphocytes.	Celikler et al. (2009)
Environment	Seaweed-mediated synthesis of silver nanoparticles using *C. tomentosum*, acting as a reducing and capping agent.	Murugan et al. (2016)
Aquaculture	Water-soluble seaweed extracts modulate the respiratory burst activity of turbot phagocytes.	Castro et al. (2004)
Food	Post-harvest treatment for fresh-cut fruits.	Augusto et al. (2016); Portuguese Patent number 107369
Food	Edible films containing *C. tomentosum* extracts.	Augusto et al. (2018)

Seaweeds employed for bioprospecting are commonly collected from the wild and may display contrasting chemical compositions over the year or different areas, which is a major problem to guarantee the consistent supply of target metabolites. The production of seaweeds in a land-based sustainable Integrated Multi-Trophic Aquaculture (IMTA) system allows to overcome the lack of supply and at the same time to monitor biotic and abiotic factors. With the monitoring of these factors, the variability associated with seaweed growth is diminished, presenting a sustainable solution, as well as replicability when larger volumes of biomass are required (Costa et al., 2015). In Portugal, it is possible to purchase *C. tomentosum* from ALGAplus, a Portuguese seaweed producer located in Ilhavo. Here, *C. tomentosum* is produced under controlled conditions in an IMTA system. It is therefore possible to purchase it throughout the year, which avoids supply issues for research or industrial applications.

1.6.2. Seaweed Legislation in Europe

Concerning the European Legislation on seaweeds for human food consumption, there is no harmonized regulation in Europe. The regulation, which has been applied, is the so-called "Novel Food" regulation (Regulation UE 2015/2283), which applies to food and ingredients that were not consumed in Europe before May 15th 1997 (CEVA, 2019). With this regulation, all seaweed-derived products, from fresh or dried seaweed to their extracts, need to be authorized by the EU before commercialization. Currently, there are several seaweeds authorized for human consumption following the Novel Foods regulation, among them brown seaweeds from *Fucus and Ascophyllum* genus, red seaweeds as *Palmaria palmata* and *Chondrus crispus*, or green seaweeds as *Ulva lactuca* and *Enteromorpha* sp.. Concerning authorized seaweed extracts, the list is considerably smaller, and include the commercialization of *Ecklonia cava* phlorotannins for food supplements, and fucoidan extracts from *Fucus vesiculosus,* and *Undaria pinnatifida* as novel food for use in food and food supplements. At the European level, countries have specific regulations for seaweed consumption and commercialization. In France, 22 seaweeds were listed so far as food vegetables or condiments (CEVA, 2019). Following the EU regulation number 1379/2013, the Portuguese Directorate-General for Natural Resources, Safety and Maritime Services (DGRM) issued a list containing a total of 41 seaweeds that can be commercialized in Portugal, which can be consulted in Table S1.1 (Supplementary data, Table S1.1) (EC 1379/2013). The species *Codium tomentosum* is included in this list, meaning that in Portugal the commercialization of *C. tomentosum* is authorized for human consumption, although any information concerning the use of *C. tomentosum extracts* for food applications is available. With this, the use of *C. tomensoum* extracts in the industrial environment requests previous authorization by the EU.

2. Aim of the thesis

The market for fresh-cut fruits and vegetables has progressively increased in the last decade and its value in the UK alone is worth around 3 Billion GBP. A key requirement in the technology employed to produce fresh-cut fruits and vegetables is the use of an anti-browning agent as a processing aid. Most of the anti-browning agents currently employed are synthetic, expensive and leave a high environmental legacy. There is a need to replace synthetic with natural and safe anti-browning agents. Certain seaweeds, which are harvested on the North Atlantic coasts of EU, are known to yield extracts that slow down browning. However, there are relatively few systematic studies to demonstrate their efficacy, especially on an industrial scale. Moreover, the mechanism of their action is also not clearly understood.

The present thesis aims to improve the knowledge on the potential of the seaweed extract of *Codium tomentosum* to enhance fresh-cut fruit shelf-life through superficial browning regulation. To bring more realism to this research, a pilot-scale production of fresh-cut 'Fuji' apple and industrial application of a coating formulated with *Codium tomentosum* seaweed extract was performed.

Thus, the following specific objectives were targeted:

I. Investigation into the effectiveness of *Codium tomentosum* seaweed extract for the inhibition of superficial browning in fresh-cut 'Rocha' pear (*Chapter II*).
II. Pilot-scale production of *Codium tomentosum* extract and potential to prevent browning in fresh-cut 'Maçã de Alcobaça' apple ('Fuji' variety) under industrial conditions (*Chapter III*).
III. Understand the mechanisms of action of *Codium tomentosum* seaweed extract in preventing browning in fresh-cut 'Fuji' apple (*Chapter IV*).
IV. Investigate the effects of extraction conditions, i.e. time, temperature, and pH on *Codium tomentosum* extract functionality as anti-browning agent for fresh-cut fruits (*Chapter V*).

This scientific approach was designed, not only to provide relevant information about the seaweed extract coating applicability under industrial conditions and its mechanism of action to prevent browning in fresh-cut apples, but also explore the possibility of extending its application for other fresh-cut fruits.

The results from this study will thus enable us to gain knowledge of the relevance and applications of seaweed *Codium tomentosum* which will also facilitate future authorization requests made either to the novel food seaweeds catalogue or to use the seaweed extract as a food additive. Ultimately, the present study aims to strengthen the bridge between academia and industry, which is critical for food industry development.

3. Scope of the thesis

In order to achieve the general goals, set out, the thesis is organized into the following six chapters:

Chapter I- General introduction and objectives

In this chapter, a general overview is given on the current status of fruits and fresh-cut fruits consumption, and how industry and academia are using edible coatings to preserve fresh-cut products. The importance of apple varieties belonging to 'Maçã de Alcobaça' and 'Rocha do Oeste' pear, both with high significance for the Portuguese economy, are also mentioned. Finally, a brief introduction is given to the seaweed *Codium tomentosum*, including the most relevant studies that have been conducted with this yet understudied seaweed.

Chapter II- Preservation of fresh-cut Rocha Pear using *Codium tomentosum* extract

A detailed experimental study exploring the efficacy of the aqueous extract of *C. tomentosum* in delaying superficial browning of fresh-cut 'Rocha' pear is presented here. The cultivar 'Rocha' pear was chosen given its economic relevance for the Portuguese Western Region, and the rapid deteriorative processes that occur with its manipulation, especially when sold as a fresh-cut product, which lead to product rejection. Fresh-cut pear slices were treated with a *C.*

tomentosum coating solution, and its effect on slices quality was investigated over a storage period of 15 days and compared with a commercial coating used by industry.

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Chapter III- A pilot plant scale testing of seaweed-based natural coating for shelf-life extension of fresh-cut apple

This chapter was designed as an extension of the previously published work of Augusto et al. (2016), where for the first time, seaweed-based coating solutions were used to delay browning in fresh-cut apples. In Chapter III, 'Fuji' apple from the Protected Geographical Indication 'Maçã de Alcobaça' were used for fresh-cut processing. The study was conducted under industrial conditions, employing a pilot plant for extracting the seaweed extract, coating the fruits and finally packaging prior to storage for a duration of 30 days. The extract functionality was assessed in ambient and modified atmosphere packaged samples. The extract functionality was compared with a commercial synthetic coating solution. This research validated the applicability of the seaweed extract under scaled-up conditions, justifying further studies, i.e. the mechanism of action and the reasons for the extract efficacy (Chapter IV) and the effect of distinct extraction conditions to increase the extract functionality (Chapter V).

This chapter was published as Augusto, A., Miranda, A., Costa, L., Pinheiro, J., Campos, M. J., Raimundo, D., Pedrosa, R., Mitchell, G., Niranjan, K., & Silva, S. F. J. (2022). A pilot plant scale testing of the application of seaweed-based natural coating and modified atmosphere packaging for shelf-life extension of fresh-cut apple. Journal of Food Processing and Preservation, 00, e16630. https://doi.org/10.1111/jfpp.16630

Chapter IV- An insight into the mechanisms underpinning the anti-browning effect of *Codium tomentosum* on fresh-cut apples

Following the natural sequence of events when a new food agent is being developed, and after the seaweed extract functionality validation at industrial conditions (Chapter III), the mechanisms of action of *C. tomentosum* extract for delaying browning in fresh-cut apples was investigated at a genetic level. Here, an experimental design similar to chapters II and III was used, and fresh-cut apples coated with the seaweed extract were kept under refrigerated storage over 15 days. The activity of browning-related enzymes (PPO and POD) was determined, as well as their gene expression using molecular tools such as RT-PCR. Earlier studies showed that this seaweed extract, with a relatively low antioxidant activity, could reduce PPO and POD activities. However, this efficacy was only confirmed *in vivo*, which leads to the hypothesis that the extract interferes with the processes of transcription and translation of enzymes.

This chapter will shortly be submitted for publication.

Chapter V- The effects of *Codium tomentosum* extraction conditions on anti-browning functionality

Based on the results of Chapters II – IV, where the effectiveness of the seaweed extract and the scaling-up process were validated, Chapter V was planned to explore the enhancement of seaweed extract functionality, by modifying the seaweed extraction conditions. In this chapter, three factors, considered critical in solid-liquid extractions, were chosen: time, temperature, and pH. The functionality of the extracts obtained under different conditions was validated by coating fresh-cut apples. Thus, this study gives insight into time, temperature, and pH effects on the extraction of *C. tomentosum*, a study that has never been reported for this seaweed. This provides key information to obtain the best performance of a marine-based coating to be used in fresh-cut fruit shelf-life extension.

This chapter will shortly be submitted for publication.

Chapter VI- General Conclusions and Future Perspectives

In this chapter, the results obtained from Chapters II-V are integrated and a general discussion of the seaweed extract functionality and overall findings of this work are addressed. The importance of the scale-up processes is investigated, and how these contribute to the knowledge transfer from academia to industry is also discussed.

Future perspectives on chemical characterization and validation of the seaweed extract, with the aim of classifying it as a food additive is discussed.

Appendix A. Supplementary data **Chapter I** (page 151).

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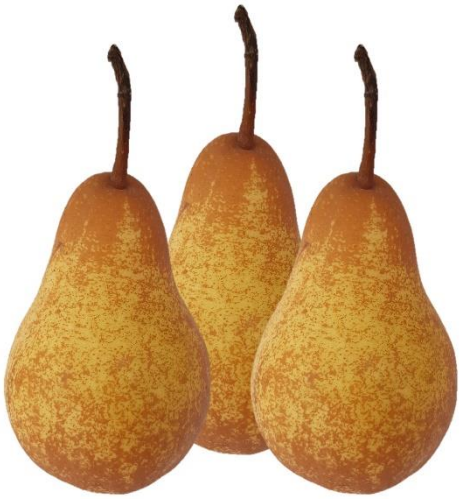

CHAPTER II

Preservation of fresh-cut Rocha Pear using *Codium tomentosum* extract

Preservation of fresh-cut Rocha Pear using *Codium tomentosum* extract

Abstract

'Rocha' is a Portuguese pear cultivar with high economic importance in the Portuguese Western Region. Degradative processes following its manipulation can quickly lead to product rejection, especially when sold as a fresh-cut product. The efficacy of a marine-based edible coating to inhibit superficial browning development in fresh-cut 'Rocha' pear slices was investigated over a storage period of 15 days. The aqueous extract of *Codium tomentosum*, an edible green seaweed, was incorporated in an edible coating (0.5 g 100 mL^{-1}) for fresh-cut 'Rocha' pear. This novel treatment effect on the quality parameters of the pears was compared with a commercial coating currently used by industry and a control (dipping in deionised water). After 15 days storage at 4 °C, samples treated with the seaweed extract exhibited fewer colour changes and lower rates of superficial browning than control and commercial samples. Seaweed extract treatment was also observed to inhibit yeast and mould development, which may further contribute to shelf-life extension.

Keywords: Edible seaweed; Fresh-cut fruit; Superficial browning; Fruit preservation

1. Introduction

Consumer acceptance of sustainable fresh fruits and vegetables depend on the freshness, quality, and convenience of the products available in the market. Fresh-cut fruits are a convenient option for daily life, in which the fruits are subjected to simple processes like disinfection, slicing and packaging (Augusto et al., 2016; Yousuf & Qadri, 2019). Given the processing steps involved (peeling, cutting, slicing), the shelf-life of fresh-cut fruits tends to be lower than the whole fruit, thus requiring additional preservation techniques (Ncama et al., 2018).

The use of edible coatings to extend keeping quality is economic and effective and widely used in the food industry (Ncama et al., 2018). Such coatings are applied by immersing the product into a coating solution or by spraying the solution, which, after drainage, generates a thin layer of surface coating. As the coating is consumed along with the product, its formulation must only contain a food-grade substance that doesn't interfere with the organoleptic quality of the fruit (Hassan et al., 2018). The use of edible coating in fresh-cut fruits reduces senescence, controls moisture loss and gas exchange between the food and environment, thereby acting similar to modified atmosphere packaging. It is also possible to modify the coating formulation, depending on the application objectives and the type of fruit (Yousuf & Qadri, 2019).

One of the dominant impacts of processing fresh-cut fruits is the dramatic increase observed in the fruit metabolic rate, which consequently enhances the rates of enzymatic browning reactions (Dellarosa et al., 2016). This necessitates the application of anti-browning postharvest treatments. Several anti-browning agents have been used to prevent tissue browning of fresh-cut fruits. Gomes et al. (2012) established the efficacy of calcium ascorbate solution with a pH of 7 as anti-browning in the case of fresh-cut 'Rocha' pear and showed that browning was more marked under acidic conditions. In the case of fresh-cut 'Granny Smith' apples, a combination of citric acid (4.0 - 4.5 %), ascorbic acid (3 - 4 %) and *N*-acetyl-*L*-cysteine (1.5 - 2.0 %) achieved a

reduction in *Listeria monocytogenes* and at the same time maintained colour parameters over 21 days of storage at 4 °C (Fan et al., 2018). The application of hydrocolloid-based coating consisting of gellan, alginate and pectin combined with *N*-acetylcysteine and glutathione, to fresh-cut 'Flor de Invierno' pears controlled enzymatic browning, promoted microbial safety and reduced ethylene production (Oms-Oliu et al., 2008). Guerreiro et al. (2017) tested different formulations of alginate and pectin coatings incorporated with citral and eugenol on 'Bravo de Esmolfe' fresh-cut apple. These authors reported that coating with a solution containing 2 % (w/v) sodium alginate and 0.1 % (w/v) eugenol, followed by dipping in ascorbic acid (0.1 %, w/v) resulted in improved anti-browning efficacy and sensory quality characteristics. Most of the substances used in the formulation of postharvest treatment solutions for extending the keeping quality of fresh-cut fruits are of synthetic origin and goes against current consumer trends, which favour the use of edible coatings and additives of natural origin (Mahajan et al., 2017). Salvia-Trujillo et al. (2015) studied the effect of an edible coating formulated with nanoemulsions of sodium alginate and 0.1 % (v/v) lemongrass essential oil (LEO) on the quality parameters of fresh-cut 'Fuji' apples. In the study, the authors observed higher *Escherichia coli* inactivation, slower microbial growth and lower browning in samples coated with a solution containing a low LEO concentration, instead of high LEO concentration (0.5 and 1 % v/v). Whereas Hashemi et al. (2017) studied the use of a basil-seed gum coating containing the essential oil of *Origanum vulgare* to treat fresh-cut apricots, which demonstrated to extend fresh-cut apricots shelf-life by decreasing microbial spoilage, keeping the apricot quality-related features. Seaweeds have been used as a natural and renewable source of bioactive compounds possessing antimicrobial and antioxidant activities and are strong candidates to serve as natural additives for food preservation (Cian et al., 2014; Ummat et al., 2020). *Codium tomentosum* is a green edible seaweed, native to the North East of the Atlantic Ocean (Costa et al., 2015). Augusto et al. (2016) developed a coating solution containing 0.5 % w/v of a hydroethanolic extract of *C. tomentosum* which delayed enzymatic browning of fresh-cut 'Fuji' apple stored under refrigerated conditions

for 20 days at a laboratory scale. The authors observed a reduction in superficial browning development of 26 % and attributed this observation to a significant reduction in the activities of enzymes like peroxidase and polyphenol oxidase caused by the coating. 'Rocha' pear (*Pyrus communis* L. cv Rocha) is an exclusive Portuguese pear variety, recognized as a Protected Denomination Origin and highly relevant to the economy of the Portuguese Western Region (Deuchande et al., 2016). In 2019, Portugal produced 153,000 te of 'Rocha' pears of which 103,000 te – worth ninety thousand million USD– were exported (FAOSTAT, 2021). Due to specific climacteric and soil characteristics, 'Rocha' pear has distinct organoleptic characteristics and natural antioxidant profile, which differentiate it from other pear varieties (Coelho et al., 2019; Salta et al., 2010). 'Rocha' pear flesh is particularly sensitive to environmental changes, especially to temperature and light, which makes it more vulnerable to a high rate of superficial browning, particularly after slicing, which is the main obstacle for the successful commercialization of fresh-cut 'Rocha' pear. Pear cultivars which browns easily, exhibit cell membrane denaturation within the first 30 min after cutting (Li et al., 2017). With cell wall disruption, browning-related enzymes like peroxidase (POD) and polyphenol oxidases (PPO) leak and act on phenolic substrates which are present in significant concentrations in 'Rocha' pear (164.3 mg 100 g^{-1} of fresh weight) (Salta et al., 2010). Given the pressing need for technological solutions to preserve fresh-cut 'Rocha' pear, especially for slowing down browning rates during storage, the present Chapter aims to assess the use of the seaweed *Codium tomentosum* extract for coating fresh-cut 'Rocha' pear.

2. Methods

2.1. Vegetable material

Campotec S.A. (Torres Vedras, Portugal), a local producer of fresh-cut fruits provided the 'Rocha' pear to be used in the experiments as well a commercial additive formulated with ascorbic acid that is currently used on their commercial products. Harvested pears were stored at room

temperature (21 ± 3 ˚C) protected from light until use. The dried seaweed *Codium tomentosum* (particle size of 1.5 mm, dried at 25 ˚C) was obtained from ALGAplus (Ilhavo, Portugal).

2.2. Seaweed extract production

The extraction conditions were determined based on the work described by Augusto et al. (2016), with slight modifications. A ratio of 6.6 % of seaweed to solvent (water) was stirred for 3 h at 15 ˚C to produce a batch of 5 L liquid extract using a solid-liquid extractor (Pilotdist SL5®, Meckenheim, Germany), which was subsequently freeze-dried (CoolSafe 55-4, LaboGene, Denmark) and stored protected from light at room temperature until use.

2.3. Fruit treatment and packaging

Before slicing, pears were sanitized with Amukina (Angelini pharma, Italy) following the manufacturer's instructions. A seaweed extract coating concentration of 0.5 % (w/v) was used after investigating four coating concentrations in a preliminary study: 0.25 %, 0.50 %, 0.75 % and 1 % (w/v). The concentration of 0.50 % (w/v) gave higher browning prevention efficacy following storage for 9 days at 4°C. An automatic slicer (Turatti, Italy) was used to produce pears slices, each weighing *ca*. 11 g. A 0.5 % (w/v) coating solution of the seaweed extract was prepared, and 100 g of fruit slices were immersed in 200 mL of the solution at 4 ˚C for 1 min. Likewise, a similar solution of the commercial ascorbic acid-based formulation was prepared and the same weight of cut fruit slices were similarly immersed in this solution. Control samples of pear slices were obtained by simply dipping the slices in deionised water. Comparisons had to be made under similar conditions and all factors had to be maintained – which included the water content of the fresh-cut samples. Therefore, deionised water was used as a dipping solution. After coating, the slices (86.01 ± 9.65 g) were packaged in 200 cm^3 plastic film bags (Pigmea, Jaén, Spain) under modified atmosphere conditions (1 - 8 % oxygen, 12 - 22 % carbon dioxide and 79 - 83 % nitrogen at 8 ˚C) using an Ulma packaging system (Oñati, Spain).

A total of 48 packages were prepared with each coating solution (seaweed extract and commercial coating solutions) and control and stored at 4 ˚C for 15 days. Every 5 days, 12 packages were used for assessing the fruit quality parameters, enzymatic assays and microbiology enumeration. A flowchart comprising fruit treatment and analysis performed during storage is given in Figure 2.1.

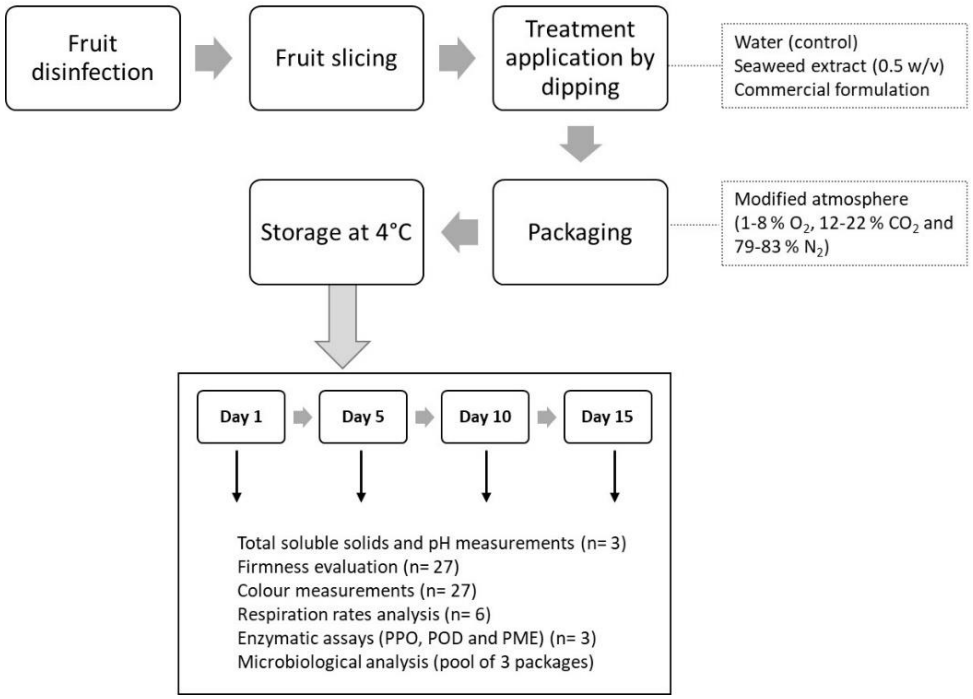

Figure 2.1. Flowchart including the stages from fruit-slicing, coating and storage of samples, as well as the analysis performed each time.

2.4. Physicochemical quality evaluation

Total soluble solids (TSS) were determined as the degree of brix (°Brix) using a digital refractometer (RFM340+, Paragon Scientific, Liverpool, UK). pH was directly measured in samples according to the procedure described by Augusto et al. (2016). Tests were performed using one slice per package and 3 packages per treatment (n = 3), per sampling time.

The firmness of samples was assessed by a compression test using a texture analyser TA.XT.Plus (Stable Micro Systems, Surrey, England). Firmness was considered to be the maximum force

measured when a 5 mm cylindrical probe penetrated a depth of 5 mm at a speed of 1.5 mm s^{-1} (Augusto et al., 2016). The test was performed using three slices per package and 3 packages per treatment. Further, each slice was pierced in three different areas (one central and two near the edges, thereby giving 27 readings for each storage conditions.

Colour was assessed using CIELAB system with a portable colourimeter (Konica Minolta, CR 400, Japan) as described by Augusto et al. (2016). The results were expressed as colour changes (ΔE*) and browning index (BI). The Euclidean distance of two points (ΔE*) was calculated between a fresh-cut sample without treatment on day 0 and the day of analysis according to the equation described by Lante, Tinello, & Nicoletto (2016). The browning index, BI, was estimated as Augusto et al. (2016):

$$\mathrm{BI} = \frac{x-0.31}{0.172} \times 100 \qquad \text{(eq. 1)}$$

$$\text{Where} \quad \mathrm{x} = \frac{a+1.75\,L}{5.645\,L+a-3.012\,b} \qquad \text{(eq. 2)}$$

Colour evaluation was conducted using three slices per package and 3 packages per treatment. Here too, the colour was measured at three locations on each slice, one at the centre and two near the edges of the slices, giving 27 sets of readings for each storage conditions.

2.5. Headspace gases

An OxyBaby® 6.0i (Witt, Witten, Germany) gas analyser was used to measure the percentage of oxygen and carbon dioxide inside the packaging, which enabled calculation of the respiration rates of the slices during storage, as described by Dellarosa et al. (2016). Six packages per treatment (n = 6) were analysed per sampling time.

2.6. Enzymatic assays

The extraction protocol of peroxidase (POD) and polyphenol oxidase (PPO) enzymes was performed as described by Augusto et al. (2016). The supernatant was divided in aliquots for the enzymatic assays and Bradford method for protein quantification (Bradford, 1976). The

determination of the enzymatic activity of both enzymes followed the methodology proposed by the same authors, adapted for a reaction volume of 300 µL in a 96-multi-well plate. POD activity was assessed at 470 nm for 10 min, and PPO activity at 400 nm over 2 min of reaction. Results were expressed as U mg^{-1} protein.

For pectin methylesterase (PME) extraction, the conditions for PPO and POD extraction, described above, were replicated but using 1.5 M of NaCl with 2.5 w/v of polyvinylpyrrolidone (PVP) as extraction buffer. The determination of PME activity was adapted from Liu et al. (2016) and Delgado-Reyes et al. (2001). To a 96-multi-well plate were added 15 µL of 0.01 % bromothymol blue (in 0.003 M sodium phosphate buffer, pH 7.5), 235 µL of 5 g L^{-1} citrus pectin (pH 7.5) and 50 µL of the extracted sample (pH 7.5). The reaction was conducted for 4 min at 35 °C and followed by spectrophotometry at 610 nm. Results were expressed as U mg^{-1} protein. Enzymatic activities were evaluated by pooling three slices per package and 3 packages per treatment (n = 3), per sampling time.

2.7. Microbiological analysis

The microbiological analysis followed the protocols approved and published by the International Organization for Standardization (ISO). Sample homogenization and decimal dilutions were made with buffered peptone water (ISO 6887-4: 2017). The ISO 4883-2: 2013 was followed for the enumeration of microorganisms at 30 °C and 4 °C, and the ISO 21527-1: 2004 for the enumeration of yeasts and moulds. A pool of three packages per treatment per sampling time was used for homogenization and further analysis. Even though the bacterial count exceeded safety limits after 10 days of storage at 30 °C and 4 °C, the evaluation of microbial counts and assessment of browning continued until day 15, in order to establish the relative efficacies of the seaweed extract and the commercial coating.

2.8. Statistical data analysis

For the data analysis, the influence of coating solution and storage time on the samples coated with the seaweed extract or the commercial solution were evaluated. R statistical and programming environment (R Development Core Team, 2012) was used with the *nlme* package (Pinheiro et al., 2017), to test independent regression models for each dependent variable (TSS, pH, firmness, ΔE*, BI, $RRCO_2$, POD, PPO and PME). When necessary, a Generalized Least Squares (GLS) procedure was used with the appropriate variance covariate structure (Pinheiro et al., 2017). This avoided data transformation by allowing residual spread to vary with the explanatory variables. CANOCO version 4.5 package was used for the Principal Component Analysis (PCA) design.

3. Results and discussion

3.1. Physicochemical quality evaluation and colour parameters.

The present chapter is the first study where a 100 % aqueous extract of *Codium tomentosum* is used for coating fresh-cut fruits. However, in the studies of Augusto et al. (2016, 2018) a hydroethanolic mixture containing 75/25 (v/v) of water to ethanol was used for *C. tomentosum* extracts production. Water was used as extraction solvent because the functional components responsible for lowering browning is water soluble. If organic solvent was used, then its application to fresh-cut fruits will require additional safety compliances (Directive 2009/32/EC). Total soluble solids (TSS) and pH are two indicators regularly monitored to assess product quality over storage. TSS and pH values of fresh-cut pear slices at day 0 were 10.91 ± 1.31 % and of 4.66 ± 0.30 respectively, values in line with those reported in the literature, where TSS values ranged from 10.40 % to 11.58 % (Carra et al., 2018; Saquet & Almeida, 2017) and 4.47 to 4.79 (Gomes et al., 2012). All these values were within the limit of 10 % of higher values considered for product quality acceptance (Saquet, 2019).

The statistical model confirmed that there were no differences between treatments in TSS during the experimental period (Table 2.1). Regarding pH, there was a significant effect of the interaction between treatment and days of storage (Table 2.1). However, the observed decrease in pH values was residual with no consequences on the physicochemical profile of the fresh-cut pear slices.

Table 2.1. Summary of regression models (GLS) to examine the effects of coating solution (treatment) and storage time (day) in the response variables for shelf-life quality and enzymes activity. For each model, are listed the main terms, variance-covariate, degrees of freedom (d.f.), likelihood ratio (L-ratio) and level of significance (p-value).

Dependent Variable	Significant terms / Main terms	Variance-covariate	d.f.	L-ratio	p-value
TSS	Day	-	9	23.35	0.0055
pH	Treatment x Day	Day	6	20.54	0.0022
Firmness	Treatment x Day	-	6	27.06	1e-04
ΔE*	Treatment x Day	-	6	33.72	<0.0001
BI	Treatment x Day	-	6	38.19	<0.0001
$RRCO_2$	Treatment x Day	Treatment x Day	6	23.92	5e-04
POD	Treatment x Day	Day	6	22.38	0.001
PPO	Treatment x Day	Treatment	6	27.54	1e-04
PME	Treatment x Day	Day	6	26.82	2e-04

TSS: total soluble solids; ΔE*: colour differences; BI: browning index; $RRCO_2$: respiration rate; POD: peroxidade; PPO: polyphenol oxidase; PME: pectin methylesterase.

Fruit firmness is another quality attribute determined. It has a high impact on the shelf-life of fresh-cut fruits, working as an indicator of fruit freshness, and internal attributes which can potentially influence a consumer's purchasing intention (Belay, Caleb, & Opara, 2019; Li et al., 2020). Firmness values for all treatments during storage are presented in Figure 2.2A.

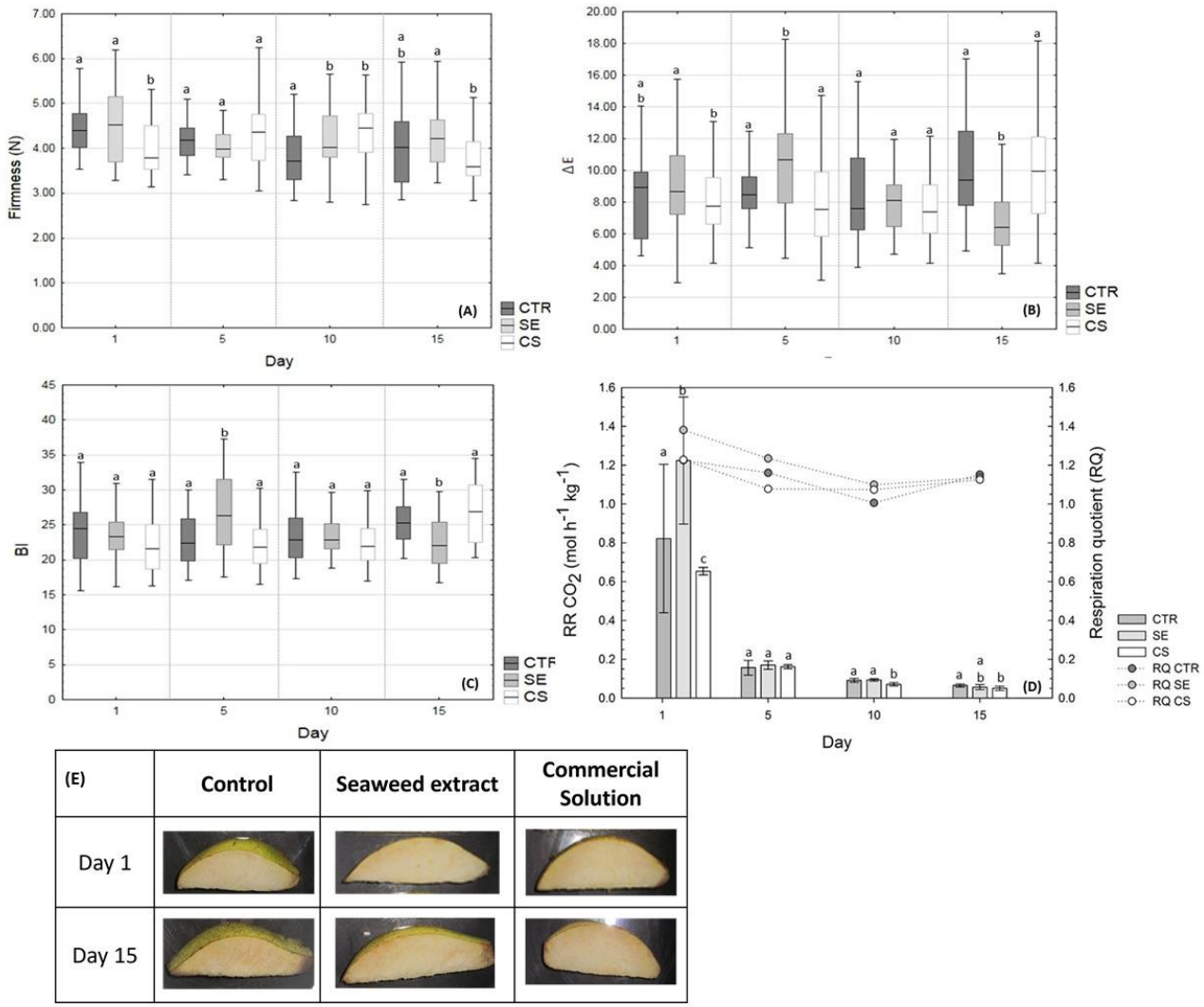

Figure 2.2. Boxplots of firmness (N) **(A)**, Colour differences (ΔE*) **(B)**, Browning index (BI) **(C)** and Respiration rate expressed as the production of carbon dioxide (RR CO_2), respiration quotient **(D)** and visual photographs **(E)** of fresh cut pears treated with seaweed extract (SE), commercial solution (CS) and control (CTR), and stored for 15 days at 4 ˚C. In (D), vertical bars represent mean ± standard error. On each day, boxplots or bars with different letters represent significantly different values (ANOVA, GLS, $p < 0.05$).

Firmness was influenced by the interaction coating solution *x* storage time (Table 2.1), and the variable with a higher impact on its values was storage time (L-ratio = 35.278, df = 9, p = 1e-04), with a smaller contribution to the statistical model of the coating solution (L-ratio = 30.998, df = 8, p = 1e-04). A slight difference, although significant, was detected at day 1, between commercial solution and control (t = 2.125, p = 0.034), and seaweed extract coated samples (t = -2.652, p = 0.008), with the commercial coated samples presenting lower values. Over storage time, variations were detected namely on days 10 and 15. At day 10, control samples showed lower values - around 11.5 % and 16.5 % - than seaweed extract (t = -2.486, p = 0.013) and commercial solution (t = -3.465, p = 0.001) samples. Although, at the end of the study, control samples had similar values to samples coated with the seaweed extract (t = -1.264, p = 0.207) and commercial solution (t = 1.719, p = 0.087). However, when comparing coating solutions at day 15, it is possible to observe that seaweed extract samples had 10 % higher firmness values than those treated with the commercial solution (t = -2.957, p = 0.003), thereby indicating efficacy of the seaweed extract to preserve fresh-cut pear slices firmness during cold storage. A hydroethanolic extract *of C. tomentosum* was also reported to efficiently preserve firmness of fresh-cut apple slices, after 20 days at 4 °C (Augusto et al., 2016). The firmness maintenance of the fresh-cut pear slices coated with the seaweed extract was also confirmed by the unchanged firmness between days 1 and 15 of storage (t = 1.363, p = 0.174) (Supplementary data, Table S2.1). This effect may be due to the incorporation of bioactive compounds from the seaweed which enhance cell integrity, lower component leakage from the cell and prevent flesh softening (Fundo et al., 2016). In the case of *C. tomentosum* extract which is poor in antioxidant molecules (Augusto et al., 2016; Silva et al., 2020), the cell wall protection by the extract may be specifically related with the presence of sulphated polysaccharide in the extract composition (Augusto et al., 2018). Although with lower values of firmness, commercial coated samples also did not show a significant decrease at the end of the experiment (t = 1.363, p = 0.174). At the end of the study,

a loss of about 6 % in firmness was observed in control (t = 2.117, p = 0.035) (Supplementary data, Table S2.1).

Colour changes are a good indicator of the freshness of fresh-cut fruits, the emergence of brown colour is commonly associated with the decrease in freshness and quality, which also represents the limiting factor for consumer acceptability (Belay et al., 2019). The determined colour parameters – colour differences (ΔE*) and browning index (BI) are presented in Figures 2.2B and C, respectively. As observed in the case of the quality parameters presented above, the variable that influences colour results the most is storage time (ΔE*: L-ratio = 39.009, df = 9, p <0.0001; BI: L-ratio = 46.539, df = 9, p <0.0001), when compared with coating solution (ΔE*: L-ratio = 34.907, df = 8, p <0.0001; BI: L-ratio = 39.176, df = 8, p <0.0001). This is an expected result, since shelf-life decays significantly over time even when preservatives such as ascorbic and citric acids, among others, are applied (Zheng et al., 2019). Determining colour changes (ΔE*) in fresh-cut fruits is a simple measure of the ability of a coating to preserve colour: higher values of ΔE* usually denote a lower product efficacy. In the present work, it was possible to observe that samples coated with seaweed extract had lower colour differences than control (t = 3.751, p <0.0001) and commercial solution treatment (t = 4.325, p <0.0001) (Figure 2.2B), after 15 days of storage. The visual photograph of the fruit can be observed in Figure 2.2E. In a previous study, it was demonstrated that the hydroethanolic extract of *C. tomentosum* was also able to prevent colour changes in fresh-cut apples (Augusto et al., 2016). The results observed in the present chapter indicate a potential functionality of the extract in delaying colour changes in fresh-cut pear, widening the application of *C. tomentosum* extract to fresh-cut fruits. The initial variations in ΔE*, with statistical significance, namely between commercial solution and seaweed extract coated samples observed at day 1 (t = -1.759, p = 0.08), can be explained by the instant browning which occurs immediately after the cutting process (Zheng et al., 2019). The browning index results (Figure 2.2C), follow the trend reported for ΔE*. On the last day of the experiment, day

15, the seaweed extract treated samples had a BI value 15 – 16 % lower than control (t = 2.235, p = 0.026) and commercial solution (t = 4.075, p = 0.00) samples. Besides, the samples treated with the seaweed extract had a similar BI value at days 1 and 15 (t = 0.687, p = 0.493), while samples coated with commercial solution presented an increase of about 20 % in BI values over storage time (t = -4.131, p = 0.00) (Supplementary data, Table S2.2). In pears, especially in 'Rocha' pear cultivar, the high polyphenol content makes it susceptible to oxidation and leads to rapid and severe browning after cutting (Gomes et al., 2014; Zheng et al., 2019). This makes it challenging to formulate a coating with high efficacy to reduce browning. Nevertheless, as reported above, seaweed extract treatment maintains colour after 15 days of storage. The browning inhibition efficacy may be attributed to the presence of polysaccharides in the extract composition (Augusto et al., 2018), which, once in contact with pear slices, forms a protective barrier between the cells and the environment. This barrier reduces oxygen gas exchange, and lowers the oxidative stress and the levels of peroxide anion (O_2^-) and hydrogen peroxide (H_2O_2), which are necessary for browning development (Wang et al., 2021).

3.2. Respiration rates

Like all other fruits, the respiration rate of fresh-cut pear slices increases significantly in response to the wounding of tissues which is caused during slicing. The use of modified atmospheres packaging along with other postharvest techniques is employed by industry to control the effects of high respiration (Gomes et al., 2014). The respiration rates expressed as CO_2 production and the respiration quotient results are presented in Figure 2.2D. There were interactive effects of coating solution and storage time on CO_2 production (Table 2.1), where days of storage was the variable with a higher influence on the respiration rates (L-ratio = 144.583, df = 9, p <0.0001, in comparison with coating solution: L-ratio = 34.672, df = 8, p <0.0001). With time, the composition of the modified atmosphere changes as consequence of

gas exchange and respiration rates, which leads to a sharp decrease in CO_2 production in all samples after 15 days of storage (Figure 2.2D).

Since respiration quotient values ranged between 1.00 and 1.38 (Figure 2.2D), it is possible to hypothesise that fresh-cut pear slices used organic acids as the major respiration substrate (Fonseca et al., 2002), a fact also observed by Gomes et al. (2012) in a study with fresh-cut 'Rocha' pear.

3.3. Enzymatic activities

As mentioned before, browning is one of the driver factors determining consumer acceptance in the case of fresh-cut fruits. The two main enzymes responsible for this phenomena are polyphenol oxidase (PPO) and peroxidase (POD) (Kou et al., 2015). These enzymes accelerate pear slices deterioration, leading to the advent of browning spots on the surface. Therefore, it is essential to monitor POD and PPO activities during storage.

POD activity suffered the interactive effect of coating solution *x* storage time (Table 2.1 and Figure 2.3A). In relation to the results obtained for POD activity, the variable which predominantly contributed to these results was storage day (L-ratio = 34.993, df = 9, p = 1e-04, vs coating solution: L-ratio = 23.170, df = 8, p = 0.003). It is possible to observe that, on the first day of storage, all analysed samples had similar values of enzymatic activity (Figure 2.3A, $p > 0.05$, Supplementary data, Table S2.3). However, after 5 days, the samples coated with the commercial solution presented lower values of POD activity when compared with control (T = 2.243, p = 0.034), and no significant differences were found between commercially coated samples and those coated with seaweed extract (t = -1.48, p = 0.152). At day 10, the samples coated with the seaweed extract solution gave a higher POD activity when compared to CTR (t = -3.784, p = 0.001) and CS samples (t = -4.676, $p < 0.0001$). In an earlier study, Augusto et al. (2016) found that the POD activity of apple slices was lower in the control sample than in slices coated with a similar seaweed extract. In the present case, after 15 days of storage, the

commercial coating and seaweed extract coating had the same effect on POD reduction ($p > 0.05$, Supplementary data, Table S2.3).

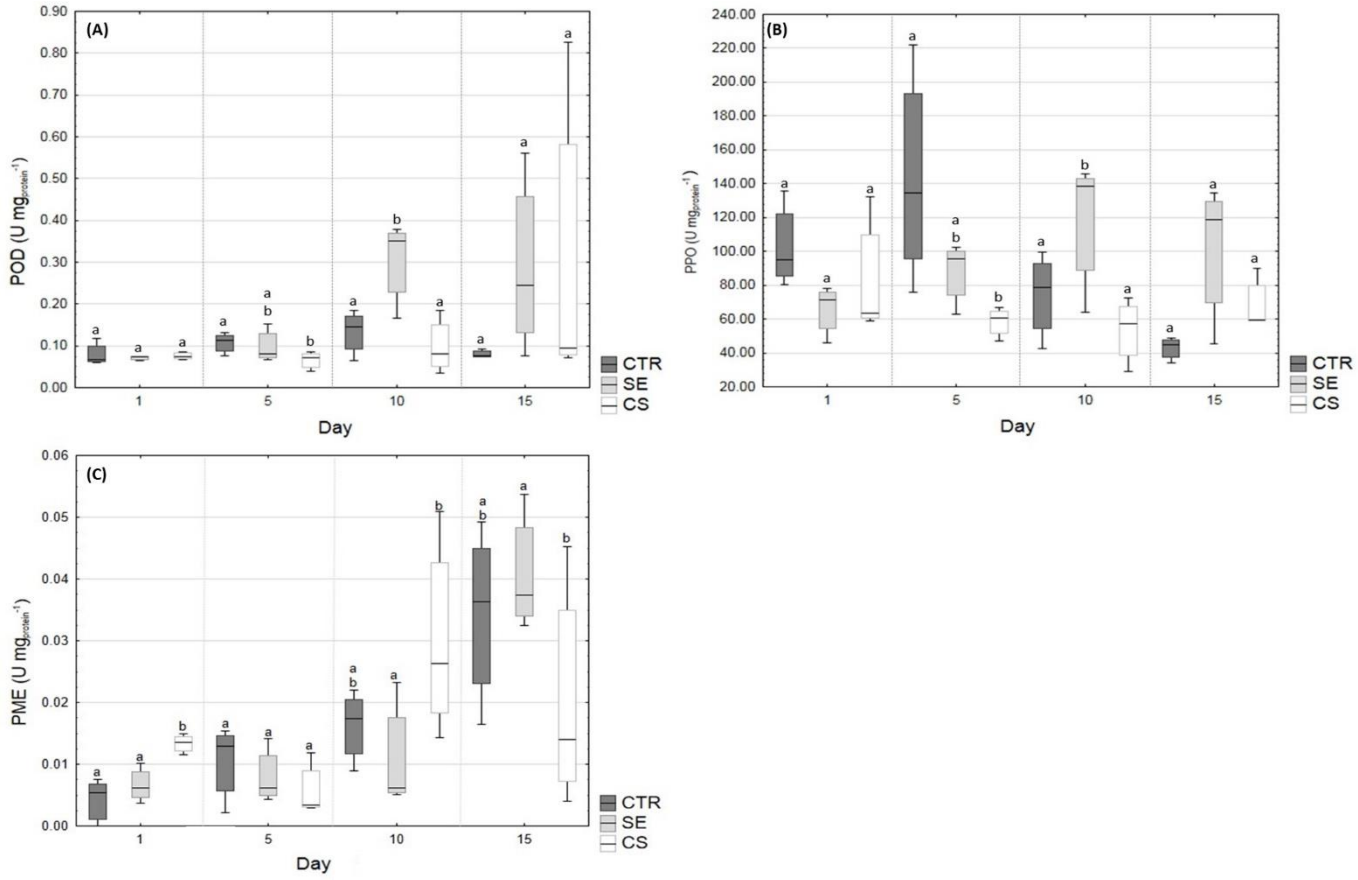

Figure 2.3. The activities of **(A)** peroxidase (POD), **(B) p**olyphenol oxidase (PPO), and **(C)** pectin methylesterase (PME) of fresh-cut pears treated with seaweed extract (SE), commercial solution (CS) and control (CTR), and stored for 15 days at 4 ˚C. On each day, boxplots with different letters are significantly different (ANOVA, GLS, $p < 0.05$).

In the case of PPO activity, the coating solution employed was the variable with the highest influence on the results despite there being significant interaction with storage period (Table 2.1) (L-ratio = 38.072, df = 8, $p < 0.0001$, vs storage time: L-ratio = 28.122, df = 9, p = 9e-04). On day 1, no differences between treatments were detected ($p > 0.05$, Supplementary data, Table

S2.4). Only after 5 days, the changes in PPO activity were detected (Figure 2.3B). The control showed higher values of PPO when compared with commercial solution treatment ($t = 2.243$, $p = 0.034$). No differences in PPO activity were detected between seaweed extract and commercial solution coated samples, indicating a similar effect on PPO activity ($t = -1.48$, $p = 0.152$). After day 10, the seaweed extract treatment showed lower efficacy in respect of decreasing the PPO activity when compared with the commercial solution treatment ($t = -4.676$, $p = 0.000$) and control ($t = -3.784$, $p = 0.001$). Also, on day 15, there were no statistical differences between the coating solutions ($p > 0.01$, Supplementary data, Table S2.4), suggesting comparable efficacies of the seaweed and commercial extracts.

It is interesting to note that the enzyme activities of PPO and POD are greater after 15 days of storage than after day 1 for the same treatment, a trend that is not in accordance with colour results (Figure 2.2B and C). In terms of colour parameters, seaweed extract application led to slower browning over time, and these results were not correlated with PPO and POD activities. Despite the significance of these enzymes in pear browning processes, several authors have noted that PPO and POD activities are not the only key factors for brown colour development in sliced pear (Gomes et al., 2014; Li, Zhang, & Ge, 2017; Liao et al., 2020). Maillard reaction is known to cause significant levels of non-enzymatic browning, especially in high sugar containing fruits (Paravisini & Peterson, 2018). The results obtained in this work on 'Rocha' Pear also seem consistent with these authors. However, more studies on the mechanism of action of PPO and POD are needed to understand the efficacy of the coatings.

With storage time, several cell wall-related enzymes, such as pectin methylesterases (PME), have an important role in fruit postharvest softening by regulating cellular wall degradation (Liu et al., 2016), including the hydrolysis of the galacturonic acid, a major component of pectin, inducing pectin solubilisation and consequently firmness loss (L'Enfant et al., 2015).

Figure 2.3C shows that the PME activity increases with storage time for all samples. The statistical model showed a significant interaction between coating solution and storage days (Table 2.1), with a higher contribution of (L-ratio = 45.047, df = 9, p <0.0001, vs coating solution: L-ratio = 33.004, df = 8, p = 1e-04). After day 1, the samples coated with the commercial solution had higher values of PME when compared with control (t = -4.914, p = 0.00) and seaweed extract treatment (t = 3.762, p = 0.001). However, at day 5 a different trend was observed, with the increase of PME activity in control and seaweed extract samples, although without significant differences (p > 0.05, Supplementary data, Table S2.5). After 10 days a switch in PME was observed: samples treated with seaweed extract had significantly lower values than those treated with the commercial solution (t = 2.778, p = 0.01), and no differences to control (t = 0.977, p = 0.338). At the end of the storage period, although significant, the seaweed extract coated samples had a slightly higher value of PME than the samples treated with the commercial solution (t = -2.30, p = 0.03). Despite the initial PME activity in samples treated with commercial solution, after 15 days the values remained stable (t = -0.805, p = 0.429) (Supplementary data, Table S2.5), and in contrast, the control and seaweed extracted samples gave significantly higher PME activity [683 % (t = 4.563, p = 0.00) and 511 % (t = -5.026, p = 0.00), respectively] (Figure 2.3C; Supplementary data, Table S2.5).

3.4. Microbiological analysis

After 10 days of storage, the mesophilic and psychrotrophic bacteria counts of all samples exceeded the legal criteria (i.e. 6 log CFU g^{-1} recommended by the Portuguese government (Santos et al., 2005) (Table 2.2). Similar results were obtained by Gomes et al. (2012) in a study of fresh-cut 'Rocha' pear packaged under modified atmosphere. Neither the seaweed extract nor the commercial coating solution showed activity against mesophilic and psychrotrophic bacteria, although further studies are needed to detect the efficacy of the seaweed extract against specific pathogenic microorganisms, e.g. *Escherichia coli*, *Salmonella enterica* and

Listeria spp., especially since 'Rocha' pear is known to be a good substrate for the survival of pathogenic bacteria (Graça et al., 2017).

Table 2.2. Total mesophilic bacteria count (log CFU g^{-1}), psychotropic bacteria (log CFU g^{-1}), yeasts, and moulds (log CFU g^{-1}) of fresh-cut pears subjected to different treatments and stored for 15 days at 4 ˚C.

Storage day	Control	Seaweed extract	Commercial Solution
	Mesophilic bacteria (log CFU g^{-1})		
1	4.35	4.20	4.02
5	5.24	4.86	4.45
10	7.06	7.39	7.01
15	7.21	7.36	7.10
	Psychrotrophic bacteria (log CFU g^{-1})		
1	3.97	3.91	3.73
5	5.3	5.69	5.40
10	7.27	7.35	6.94
15	7.16	7.36	7.16
	Yeasts and moulds (log CFU g^{-1})		
1	2.04	2.02	2.00
5	N.D.	N.D.	N.D.
10	N.D.	N.D.	2.10
15	3.75	1.80	3.10

ND. Not detected

In relation to yeasts and moulds, all the samples analysed had counts below the recommended threshold limits (3 – 5 log CFU g^{-1}) (Santos et al., 2005) (Table 2.2). It is also worth highlighting that the seaweed extract treated samples showed lower counts of yeasts and moulds than control and commercial solution treatment, which suggests that the seaweed extract may possess antifungal activities not yet reported. Only a few studies have reported on antimicrobial

activity of this seaweed extracts, however using organic solvents in the extractions (Silva et al., 2020). In Chapter III (section 3.3), the antimicrobial activity of the extract was reported, here, a solution of *C. tomentosum* extract applied to fresh-cut apples showed inhibitory effect in the case of yeasts and moulds growth over 20 days of refrigerated storage (Chapter III, Table 3.3). Being these the first reports regarding *C. tomentosum* aqueous extract antifungal activity, more studies are necessary to identify the compounds responsible and then access the mechanism of action behind this action.

3.5. Principal component analysis

Principal component analysis (PCA) was used to assess how the measured variables influences samples to be similar to, or when or how they differ from each other (Sun et al., 2016). PCA was applied to evaluate qualitative differences between the two coating solutions and control samples. The principal component 1 (PC 1) and 2 (PC 2) accounted for 61.2 % of the total variance, with 41.0 % being explained by axis 1 (PC 1) (Figure 2.4)

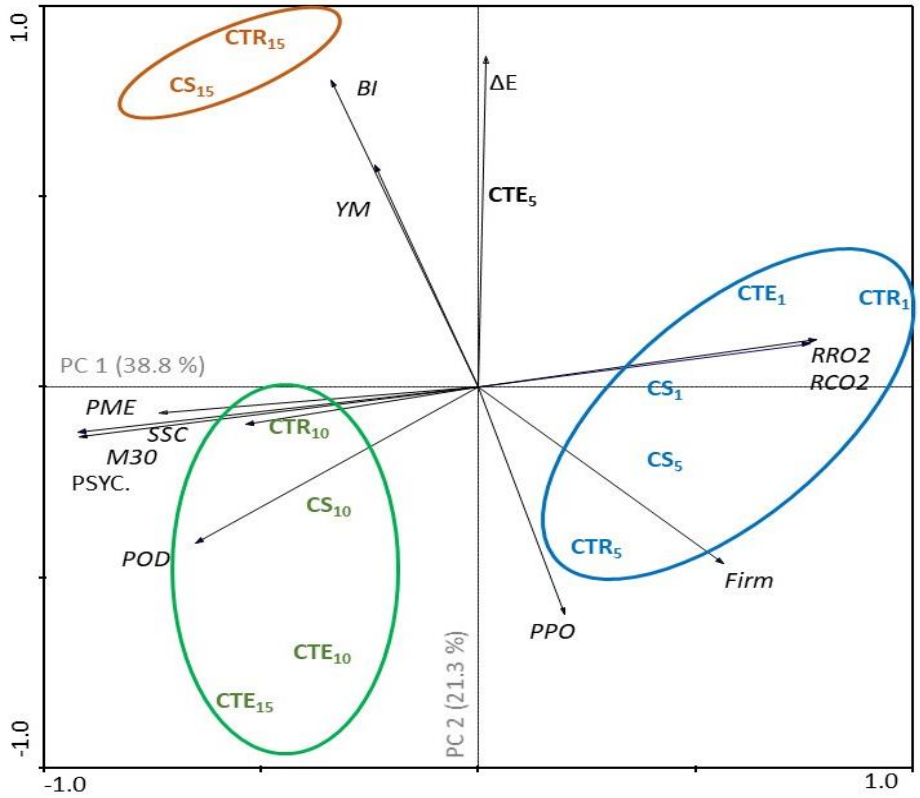

Figure 2.4. Biplot from principal component analysis integrating all physical and chemical parameters, enzymatic activities and microbiological analysis in fresh-cut pears treated with seaweed extract (CTE), commercial solution (CS) and control (CTR) and stored at 4˚C for 1, 5, 10 and 15 days. From four latent variables calculated, two latent variables identified as the most representative are represented. Firm.- firmness, SSC- soluble solids concentration, RR CO_2- CO_2 production, RR O_2- O_2 consumption, ΔE*- colour differences, BI- browning index, PPO- Polyphenol oxidase, POD- Peroxidase, PME- Pectin methylesterase, YM- yeasts and moulds, M30- mesophilic bacteria, PSYC.- psychrotrophic bacteria.

After days 1 and 5, the coated and control samples showed similar results and formed a cluster (Blue line, Figure 2.4). In this cluster, respiration rates (RRO_2 and $RRCO_2$), pH, firmness and polyphenol oxidase activity are the main vectors influencing the results. Although there are

similarities between samples at day 5, the results of the seaweed extract coated samples were mostly differentiated by colour changes and browning index.

A second cluster emerged at day 10 (Green cluster, Figure 2.4), where the main vectors were peroxidase and pectin methylesterase enzymes, total soluble solids, mesophilic and psychrotrophic bacteria. Considering the proximity of these vectors to the control and commercial solution treatment at day 10, it is possible to conclude that the results of these samples were markedly influenced by those vectors. And looking for the samples coated with the seaweed extract solution, at days 10 and 15, and included in the same cluster, it is also possible to observe a relationship between the vectors mentioned above and the sample results. Also, the proximity of the seaweed extract sample vectors at days 10 and 15, and included in the same cluster, may represent lesser changes in the physicochemical properties at the end of the storage period.

A third cluster was defined, but only including control and commercial samples at day 15 (Orange line, Figure 2.4). The results of control and commercial solution treatment after 15 days of storage were mainly influenced by the high values of the browning index and colour changes. This influence in control and commercial sample results also evidence the superficial browning development observed after the 15 days of storage, while the samples coated with the seaweed extract (SE_{15}) were marginally influenced by colour parameters (observed by the 90° angle with colour vectors), justifying the browning delay of the seaweed coated samples.

4. Conclusions

The efficacy of the aqueous extract of *C. tomentosum* in delaying the superficial browning of fresh-cut 'Rocha' pear was evaluated. Samples treated with the seaweed extract showed considerable stability in terms of total soluble solids and firmness, after 5 days of storage. Samples treated with this solution showed lower colour change and browning index than control and commercial samples. Samples treated with the seaweed extract also showed lower

development of yeasts and moulds, indicating some antimicrobial activity. The anti-browning and antifungal actions, especially their underpinning mechanisms require further research attention.

Appendix B. Supplementary data **Chapter II** (pages 152 – 154).

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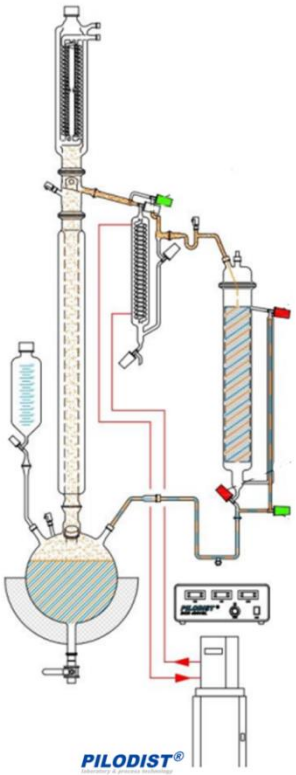

CHAPTER III

A pilot plant scale testing of seaweed-based natural coating for shelf-life extension of fresh-cut apple

A pilot plant scale testing of seaweed-based natural coating for shelf-life extension of fresh-cut apple

Abstract

Codium tomentosum hydroethanolic extract was obtained using a pilot solid-liquid extractor to validate the anti-browning functionality of the extract under industrial conditions. Fresh-cut apple slices were coated by immersion in: 1) a seaweed extract solution (0.5 % w/v) and 2) a commercial coating, and the two sets of samples were compared with a control (water). Packaged samples were stored, under ambient and modified atmosphere conditions at 4 ˚C for 30 days. After 15 days of storage, the samples which were coated with the seaweed extract and packaged under modified atmosphere, demonstrated lower peroxidase activity and polyphenol oxidation, when compared with the samples treated with the commercial additive. These results confirm, at pilot scale and under industrial production conditions, the efficacy of the seaweed extract as a bio-based substitute for the synthetic coatings which are currently used to prevent browning in fresh-cut apples.

Keywords: Natural additive; Macroalgae; Peroxidase activity; Browning; Quality parameters; Industrial application.

1. Introduction

The consumption of fresh-cut fruits is progressively increasing, and it accounts for a significant proportion of sales for horticultural processing companies (Putnik et al., 2017a). The cutting process inevitably triggers physiological responses (e.g. browning), which results in fresh-cut products having a significantly lower shelf-life than the whole fruit. Most fresh-cut produces currently available have approximately a 7-day shelf-life, and there is a need to improve upon this figure without compromising on product quality attributes (Prakash et al., 2018). Extending the keeping quality of fresh-cut produce will contribute to market expansion, increasing producers' competitiveness and reducing food waste and loss.

The application of edible coatings after cutting provides a physical barrier against moisture and solute migration and lowers respiration rates (Saba & Sogvar, 2016). In fresh-cut apples, substances like citric and ascorbic acids, calcium and thiol-containing compounds and enzyme inhibitors can be incorporated in the coating formulations to lower browning rate which is the limiting factor determining shelf-life (Krasnova et al., 2017; Siroli et al., 2015). Most components currently used in these coatings are synthetic and there is considerable interest in natural alternatives because it better aligns with consumer trends (Chen & Xu, 2019).

Salvia-Trujillo et al. (2015) observed a reduction in browning and respiration rates in fresh-cut apples coated with a nanoemulsion of alginate incorporated with lemongrass essential oil. Chiabrando and Giacalone (2015) observed that the presence of cinnamon and rosemary essential oils in alginate coatings preserves the colour of fresh-cut apples, by reducing the activity of polyphenol and peroxidase enzymes.

Seaweeds are a natural source of bioactive compounds with recognized food applications (Qin, 2018), possessing antioxidant and antimicrobial activities (Charoensiddhi et al., 2017). Several studies have been reported on the use of seaweed powder or extracts and highlighted their benefits in human and animal healthcare (Roohinejad et al., 2017). According to FAO, around

32,386.2 tonnes of seaweeds were produced for human consumption worldwide in 2018 (FAO, 2020). Recently, Augusto et al. (2016) developed a *Codium tomentosum* extract, which significantly inhibited enzymatic browning in fresh-cut apples even after 20 days of refrigerated storage under laboratory conditions. As these results were obtained at laboratory scale, and commercial acceptance of this extract requires the validation of its efficacy on a larger scale under industrial conditions, the validation of the extract functionality on a pilot scale is the main focus of the present chapter, allowing an indirect comparison between the extracts produced in the two different scales. The efficacy of this seaweed extract and an ascorbic acid based commercial formulation, which is currently used in industrial applications, has been carried out. The combined effect of modified atmosphere packaging on the shelf-life has also been investigated, to assess whether the application of the extract can contribute towards a reduction in product loss.

2. Methods

2.1. Materials and Chemicals

'Fuji' apple was obtained from a local supplier in Torres Vedras, Portugal (Campotec S.A.) and stored at 4 ˚C before use. Dried milled seaweed *Codium tomentosum* having a particle size of 1.5 mm was purchased from ALGAplus (Ílhavo, Portugal). The ascorbic acid based commercial formulation, currently used in fresh-cut fruit production, was provided by Campotec S.A.

2.2. Seaweed extract preparation

A batch of seaweed extract was prepared from the dried milled seaweed purchased, by using a solid-liquid extractor (Pilotdist SL5®, Meckenheim, Germany) operating in a batch mode. A total of 1,980 g of dried seaweed, sieved through a mesh of 1 µm, was added to 30 L of a mixture of water and ethanol (75/25 v/v) taken in the extractor and contacted for 3 hours at 15 ˚C. The sieving process allowed robust solid-liquid contact and a clear extract was obtained at the end of procedure. This process is a scaled-up version of the extraction described by Augusto et al.

(2018) and Augusto et al. (2016). After the contact time, 15 L of the liquid seaweed extract was collected and evaporated at 35 ˚C (90 mbar) (Evaporator IKA, HB10+RV10, Germany) to remove most of the solvent. The residue was frozen at -80 ˚C, freeze-dried (Scanvac, Cool Safe™, Lyng, Denmark), and stored protected from light exposure at room temperature until further use.

2.3. Immersion coating of cut apples

Slicing and immersion of 'Fuji' apples in the seaweed extract was performed in a controlled temperature facility (2 ˚C) at Campotec S.A.. Two dip solutions were prepared: 1) a 5 % aqueous solution of ascorbic acid based commercial formulation (currently employed in commercial products), and 2) a 0.5 % (w/v) aqueous solution of *Codium tomentosum* extract. Control samples of apple slices were obtained by simply dipping the slices in deionised water.

A total of 45 kg of 'Fuji' apples, with an average weight of 100 g, were automatically de-cored and sliced (Turatti, Italy), and 6.5 kg of slices were immediately placed on a conveyor belt running through 15 L of dip solution so that the slices were immersed for 2 minutes (Figure 3.1). The occluded dip solution was allowed to drain whilst the slices were still on the conveyor, following which the coated slices were transferred to the packaging system (Ishida, Kyoto, Japan and Ulma, Spain), where the slices were automatically divided into portions of 70.97 ± 10.72 g, packaged in plastic bags using a modified atmosphere (MAP) consisting of 1 – 8 % O_2, 12 – 22 % CO_2 and 70 – 87 % N_2 (for MAP samples) and air for ambient samples packaged only with atmospheric air followed by heat sealing. After packaging, the samples were transported under refrigerated conditions (5 ˚C) and protected from light exposure for 45 min from Campotec S.A in Torres Vedras (Portugal) to MARE- Polytechnique of Leiria in Peniche (Portugal), to simulate transportation between the producer and the consumer. A total of 534 packages of sliced apples, representing combinations of two dip solutions and two modified atmospheric conditions (89 packages per condition), were stored for 30 days at 4 ± 2 °C. The effects of treatment on fresh-cut apple quality were assessed every 5 days after storage. Fresh-cut apple

samples analysed immediately after cutting were used as a *gold standard* for comparing the treated and stored samples.

2.4. Physicochemical analysis

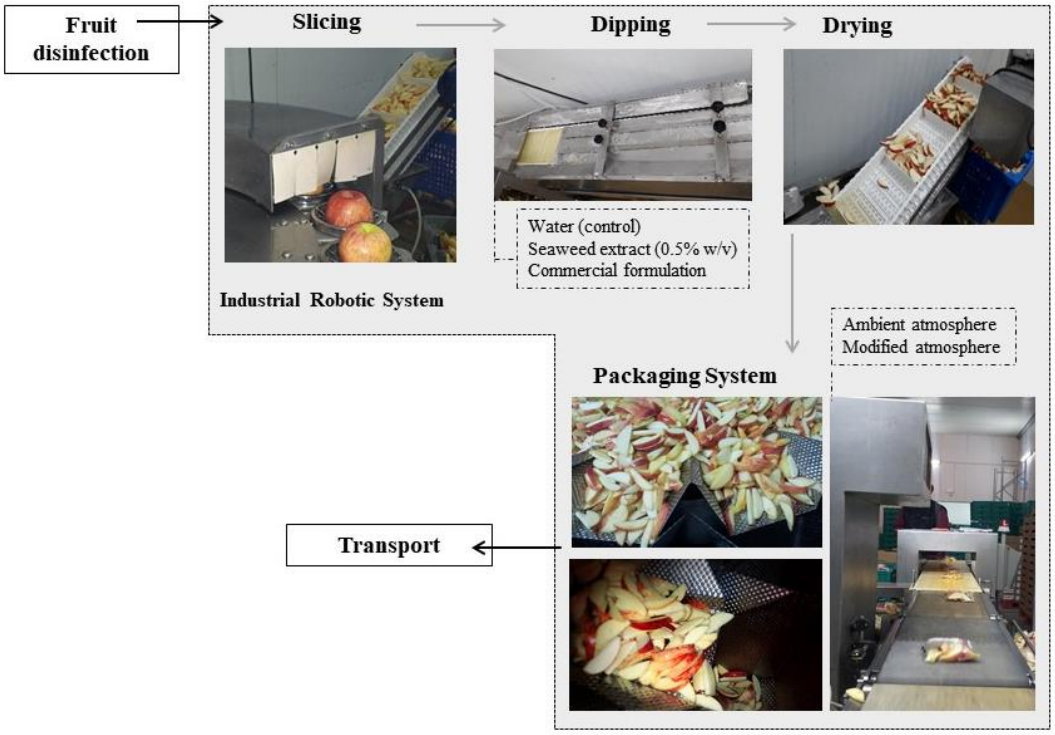

Figure 3.1. Schematic representation of coating process and packaging system.

Moisture content, water activity (a_w), soluble solids content (SSC), and pH were determined as described in Augusto et al. (2016). Three different measurements were performed (one per package) and the average difference between samples at day 30 and the *gold standard* was calculated.

A texture analyser TA.XT.plus (Stable Micro Systems, Surrey, England) was used to determine sample firmness as described in Augusto et al. (2016). Briefly, a 5 mm cylindrical probe was used to penetrate samples to a depth of 5 mm at a speed of 1.5 mms^{-1}, and each sample was punctured once. Firmness was defined as the maximum force required to perforate the apple

slice and is expressed in Newton (N). Fifteen measurements were taken for each condition (5 per package).

Colour parameters were analysed according to the CIELAB system as described in Augusto et al. (2016). Results were expressed as intensity of lightness (L* parameter) and browning index (BI) (Augusto et al., 2016). The Eucladian distance of two points (ΔE*) was calculated between an individual sample and *golden standard* according to the equation described by Lante et al. (2016). Fifteen measurements were performed for each condition (5 per package). *Gold standard* samples were also assessed (n = 3) in terms of moisture, a_w, SSC, pH, texture and colour.

2.5. Enzymatic assays

Polyphenol oxidase (PPO) and peroxidase (POD) activities were determined according to the procedure of Augusto et al. (2016). For both enzymes, the protocols were adapted for a reaction volume of 300 µL using a multi-well plate. Results were expressed as U mg^{-1} protein. Protein was quantified using the Bradford method (Bradford, 1976). Three different samples were analysed for each condition (1 per package) (n = 3).

For pectin methylesterase (PME) activity determination, the methodology was adapted from previous works of Liu et al. (2016) and Delgado-Reyes et al. (2001). The PME reaction was followed spectrophotometrically (Biotek, SynergyH1, USA) at 35 °C (610 nm, 4 min). To a volume of 50 µL of enzyme extract (pH 7.5), 15 µL of 0.01 % bromothymol blue (in 0.003 M sodium phosphate buffer, pH 7.5) and 235 µL of the substrate (5 g L^{-1} citrus pectin, pH 7.5) were added. Results were expressed as U mg^{-1} protein. Three different samples were analysed for each condition (1 per package) (n = 3).

2.6. Microbiological analysis

The mesophilic bacteria, Enterobacteriaceae, yeast and mould counts were determined by the procedure described in ISO 4833-1 (2013), ISO 21528-2 (2004) and ISO 21527-1 (2008),

respectively. Samples solutions and dilutions were prepared according to ISO 6887-4 (2017). Three packages for each condition were analysed for each sampling day.

2.7. Sensory evaluation

Two independent triangular tests were carried out according to the standard ISO 4120 (2004). First, three samples coated with commercial coating or seaweed extract solutions (see point 2.3) were presented to 21 untrained panellists. In the second test, the panellists were presented with untreated control samples and samples treated with seaweed extract. In both tests, the panellists had to identify the different samples. The sensory test was performed immediately after coating application (day 1) in a room complying with ISO 8589 (2007). Red lighting was used to avoid discrimination due to colour differences, and the sample presentation was randomized (Perez-Gago et al., 2006).

2.8. Statistical analysis

The results were statistically evaluated by one-way analysis of variance (ANOVA) with the Least Significant Difference (LSD) test for multiple comparisons of the means group. The evaluated variables were: coating solution, packaging and storage time. All data were checked for normality and homoscedasticity. The non-parametric test Kruskal-Wallis was used when the data did not meet variance or distributional assumptions. Differences were considered statistically significant at level 0.05 ($p < 0.05$). The software IBM SPSS Statistics 24 (IBM, New York, United States) was used for all calculations. Wherever suitable, results were expressed as mean ± standard deviation (n = 3).

3. Results and discussion

3.1. Physicochemical properties of fresh-cut apple during storage

Table 3.1 shows the results of moisture, water activity, pH and soluble solid content (SSC) variation (in %) of fresh-cut apples after 30 days of storage when compared to the gold standard.

No differences were observed for moisture content between treatments for each packaging type ($p > 0.05$). The observed moisture increment (an average of 3 %) is possibly a consequence of the low temperature and high moisture content in the storage environment which may have resulted in water vapour transfer from the surroundings to the packages (Augusto et al., 2018). An increase in water activity was observed for all samples. The lowest variation (approx. 50 %) was measured in control samples under modified atmosphere ($p < 0.05$) when compared with the commercial and seaweed extract coated samples. The pH value of untreated and treated samples ranged between 3.6 and 4, and no statistical differences were observed between samples ($p > 0.05$), indicating quality maintenance of the fruits over storage. The pH values noted were comparable to the study of Augusto et al. (2016).

Table 3.1. Variation (Δ) of moisture, pH, water activity and soluble solids content values between packaged samples (ambient and modified atmosphere) at day 30 and gold standard. Data are expressed as mean value ± standard deviation (n = 3). Values with the same packaging with different superscripts (A-B) are significantly different (LSD test, $p < 0.05$).

Packaging	Sample	Δ **Moisture (%)**	Δ **Water activity (%)**	Δ **pH (%)**	Δ **SSC (%)**
Ambient atmosphere	**Control**	2.44 ± 0.62^{A}	1.70 ± 0.22^{A}	-0.73 ± 2.90^{A}	-5.71 ± 4.17^{A}
	Commercial	3.72 ± 1.35^{A}	1.46 ± 0.21^{A}	1.19 ± 2.18^{A}	-14.15 ± 0.68^{B}
	Extract	3.82 ± 1.10^{A}	1.56 ± 0.18^{A}	-1.65 ± 0.88^{A}	-7.11 ± 1.12^{A}
Modified atmosphere	**Control**	2.11 ± 2.58^{A}	0.52 ± 0.28^{A}	1.74 ± 1.37^{A}	-17.09 ± 3.31^{A}
	Commercial	1.52 ± 2.71^{A}	1.60 ± 0.16^{B}	-2.01 ± 0.88^{A}	-10.73 ± 2.58^{B}
	Extract	3.02 ± 1.98^{A}	1.49 ± 0.16^{B}	-0.55 ± 4.41^{A}	-9.41 ± 3.73^{B}

The initial soluble solid content (SSC) of the gold standard samples and fresh-cut samples was 14.07 ± 0.94 $g_{sucrose}$ 100 $g^{-1}_{product}$, which is within the same value range stated in Augusto et al. (2016). For all samples, a decrease in SSC (Table 3.1) was observed. The greatest decreases in SSC was observed in the case of samples treated with the commercial solution and control, packaged with ambient and modified atmospheres, respectively. This may be attributed to the possibility of microbial metabolization of sugars (Putnik et al., 2017b). On the other hand, in the study developed by Augusto et al. (2016), an increase in SSC values, in coated and uncoated fresh-cut apples after 20 days of storage, was observed and attributed to moisture loss.

Ripeness occurs in climacteric fruits during storage, and one of the main consequences is firmness loss. This softening requires the use of techniques to prevent ripeness and consequent textural quality decrease (Guerreiro et al., 2017). Since texture is related to structural and mechanical food properties and an important parameter for consumer's acceptance, the effect of treatment type, package and storage time on texture parameters were evaluated. After 30 days of storage, most samples had a firmness decrease of about 17 % ($p < 0.05$). Samples treated with the seaweed extract stored under modified atmosphere were the only samples to increase firmness by 26 %. This is consistent with earlier research by Augusto et al. (2016) which showed that fresh-cut 'Fuji' apples coated with seaweed extract were firmer than water-treated control after 20 days of refrigerated storage. These observations establish the efficacy of the seaweed extract in maintaining textural attributes of fresh-cut apples even after scaling up the process to pilot scaling and under industrial conditions.

The luminosity (L*), browning index (BI) and colour differences (ΔE*) of stored apple flesh are shown in Table 3.2. A sharp decrease in L* values with storage was observed ($p < 0.05$) for all treatments. However, on day 1, samples coated with the seaweed extract and packaged with ambient atmosphere showed about 44 % higher luminosity (L*) ($p < 0.05$) than control and commercial samples. The difference in luminosity observed between samples on day 1 can be

explained by the rapid coating application and high efficacy of the seaweed extract during storage: browning is initiated on the surface of the fresh-cut apple during slicing (which induces enzymatic and non-enzymatic reactions leading to superficial darkening) with a consequent decrease in L* value (Shao et al., 2018). After 30 days of storage, for both types of packaging, no differences were observed between the commercial and seaweed extract treatments ($p > 0.05$), indicating similar darkening of tissues, which also suggests similar anti-browning protection offered by both the commercial extract as well as the seaweed extract.

Table 3.2. Colour parameters of Luminosity (L*), browning index (BI) and colour differences (ΔE*) of fresh-cut apples packaged with ambient and modified atmosphere at days 1 and 30 of storage at 4 ˚C, and gold standard samples. Data were expressed as mean value ± standard deviation (n = 10). Results with different superscripts are significantly different in each day^{A,B,C} in ambient atmosphere and ^{a,b,c} in modified atmosphere, and between days$^{1 \text{ and } 2}$ (LSD test, $p < 0.05$). n.a.: not applicable.

Packaging type/ Sample		Storage time (days)/ **L***		Storage time (days)/ **BI**		Storage time (days)/ **ΔE***	
		1	30	1	30	1	30
Gold standard		77.75±6.01^{Aa}		42.56±4.95^{Aa}		n.a.	
Ambient atmosphere	**Control**	65.56±3.59^{B,1}	47.72±4.57^{B,2}	116.14±19.97^{B,1}	147.79±45.98^{B,2}	23.04±4.30^{A,1}	32.26±5.22^{A,2}
	Commercial	69.24±4.26^{B,1}	68.76±5.36^{C,1}	52.31±8.05^{C,1}	64.17±21.17^{C,2}	9.20±4.01^{B,1}	12.44±5.11^{B,2}
	Extract	85.03±2.48^{C,1}	62.83±7.32^{C,2}	40.12±8.91^{A,1}	91.49±27.15^{D,2}	19.73±1.54^{C,1}	18.37±7.29^{B,2}
Modified atmosphere	**Control**	70.56±4.22^{b,1}	58.52±9.99^{b,2}	92.06±17.62^{b,1}	129.74±28.93^{b,2}	17.12±4.46^{a,1}	26.03±6.24^{a,2}
	Commercial	69.33±3.71^{b,1}	64.88±6.60^{b,2}	70.60±10.07^{c,1}	69.32±22.61^{c,2}	12.36±3.01^{b,1}	14.27±7.02^{b,2}
	Extract	70.35±3.61^{b,1}	65.96±8.38^{b,2}	69.94±8.25^{c,1}	70.95±17.91^{c2}	11.88±2.57^{b,1}	13.64±7.74^{b,2}

The development of brown colour on the surface of fresh-cut apples, indicating the occurrence of oxidative reactions and chemical reactions like Maillard reaction (Lante et al., 2016; Paravisini & Peterson, 2018), was assessed by monitoring the browning index (Augusto et al., 2016). On day one, samples treated with the seaweed extract and packaged under ambient atmosphere showed browning index values similar to the gold standard samples ($p > 0.05$) (Table 3.2), while significantly higher browning index values were measured in control and commercial samples ($p < 0.05$). In modified atmosphere packaged samples, a higher browning index, in all coated samples, was observed in comparison to the gold standard ($p < 0.05$), although lower than control samples ($p < 0.05$). The difference between the browning index values of the gold standard and other day 1 samples may be attributed to the emergence of brown spots immediately after cutting (Shao et al., 2018) which seems inevitable even on the industrial scale process, despite close monitoring and control of temperature.

All samples stored for 30 days showed higher values of browning index ($p < 0.05$), than gold standard samples – which is an expected result, considering the number of storage days and the natural development of apple browning (Fan et al., 2018). Surface browning is responsible for approximately 50 % loss in fruits (Shao et al., 2018), but, when individual treatments were compared and associated with ambient atmosphere, samples treated with the commercial coating showed the lowest values of browning index ($p < 0.05$), followed by seaweed extract. The observed efficacy of the commercial additive in preventing browning is due to the presence of ascorbic acid and calcium ascorbate, which are two commercial additives frequently used as anti-browning agents in fresh-cut apples (Fan et al., 2018). Based on the findings reported by Ramazzina et al. (2016), ascorbic acid protects the fruits against oxidative stress by several mechanisms like reactive oxygen species scavenging (ROS) and reduction of sugars. However, when in the presence of modified atmosphere, samples coated with the seaweed extract showed similar values of browning index as the commercial extract treatment ($p > 0.05$),

demonstrating the seaweed extract efficacy in preventing browning in fresh-cut apples under industrial conditions.

As mentioned in the studies of Musacchi and Serra (2018) and Shao et al. (2018), colour changes in food products are an important and decisive factor of consumers acceptance, and to estimate the changes in samples, colour differences (ΔE*) were determined (Table 3.2). According to Musacchi and Serra (2018) with the evaluation of ΔE* it is possible to understand colour changes that are perceived by human eyes. Observing ΔE* results, after 30 days, for both types of packaging, samples treated with the seaweed extract and commercial additive had similar values, with lower values of ΔE* than control.

All the results of colour parameters corroborate the earlier work of Augusto et al. (2016), and extend the effectiveness of the seaweed extract to a pilot-scale process when the cut slices are stored under ambient and modified atmosphere conditions.

3.2. Evaluation of enzymatic activities of fresh-cut apple during storage

Ripening associated processes originated by apple slicing are promoted by polyphenol-associated browning, which is essentially triggered by the contact between the enzymes polyphenol oxidase (PPO) and peroxidase (POD) and their corresponding substrates (Collazo et al., 2018; Paravisini & Peterson, 2018). These processes are accompanied by an increase in tissue respiration rate and an increase in enzymatic activity which in turn generate more browning products like *o*-quinones (Augusto et al., 2016). Figure 3.2 shows the effect of coating solutions on PPO and POD activities during storage of the fresh-cut apple samples. All samples showed significantly higher PPO activity after 30 days storage ($p < 0.05$) (Figures 3.2 A and B) which is considered the main enzyme responsible for the formation of *o*-quinones and other browning components (Collazo et al., 2018; Février et al., 2017). At the end of the 30 days storage period, samples coated with the seaweed extract and packaged under ambient atmosphere gave higher PPO activity when compared to control and the commercial coating ($p > 0.05$).

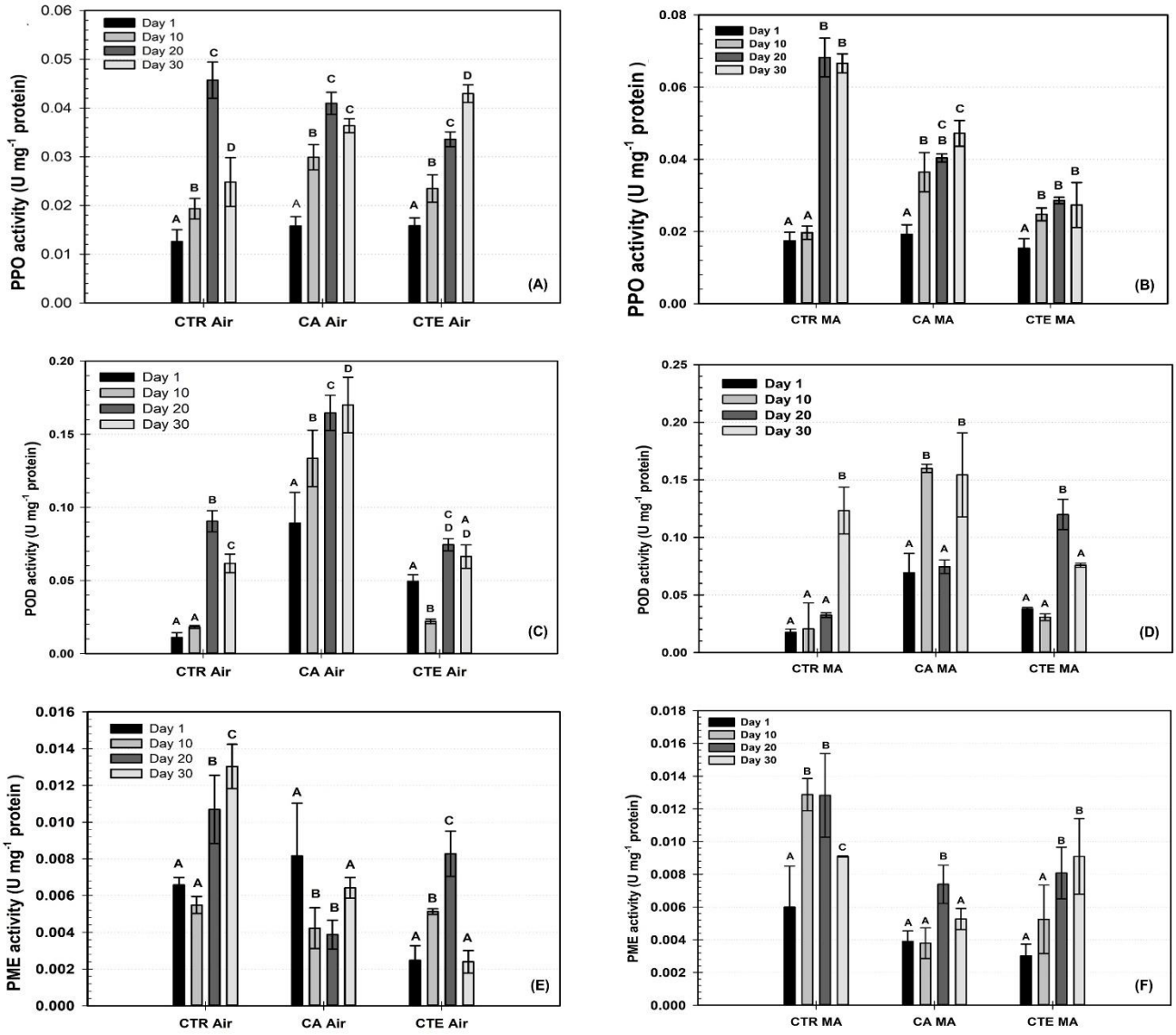

Figure 3.2. Polyphenol activity (PPO) **(A-B)**, peroxidase activity (POD) **(C-D)** and pectin methylesterase (PME) **(E-F)** of fresh-cut apples packaged with ambient (left) or modified atmosphere (right). CRT- control, CA- commercial additive, CTE- Codium tomentosum extract. (Mean value ± standard deviation, n = 3). Values with different letters (A-D) in the same treatment are significantly different (LSD test, $p < 0.05$).

In the case of samples stored under modified atmosphere, samples coated with the seaweed extract and stored for 30 days possessed the lowest PPO values (less 24 % to 58 % activity) ($p <$

0.05) (Figure 3.2B), while POD activity increased ($p < 0.05$) in samples stored under ambient and modified atmospheres (Figures 3.2C and D). Samples coated with the seaweed extract had similar values of POD activity after 30 days when compared to day 0, demonstrating the efficacy of the seaweed extract at a pilot scale and for a longer duration than that reported by Augusto et al. (2016).

In the samples coated with the seaweed extract, the POD activity was 50 % lower than the samples coated with the commercial extract – which was the case for all sampling days ($p < 0.05$). The confirmation of these results for ambient as well as modified atmosphere packaging reinforces the efficacy of the seaweed extract as an anti-browning edible coating which can be applied to fresh-cut apples processed under industrial conditions.

It may be noted that, in addition to oxidative enzymes, other enzymes like pectin methylesterase (PME) are also triggered into action soon after slicing. PME is involved in apple ripening process (Wang et al., 2018; Zambrano-Zaragoza et al., 2014) by influencing cell wall degradation and causing loss of tissue firmness. It was therefore thought desirable to understand if the seaweed extract could influence PME activity just as it influences the activity of oxidative enzymes. Figures 3.2E and 3.2F show PME activity for all samples during the storage period.

When slices are packaged under ambient atmosphere (Figure 3.2E) no specific trend is observed in PME activity over time. However, by comparing the results on days 1 and 30, a remarkable increase in activity of 49 % was observed in control samples ($p < 0.05$), while coated samples only showed slight variation in PME activity ($p > 0.05$). It can also be highlighted that after 30 days, samples coated with seaweed extract had the lowest value of PME activity ($p < 0.05$) – 81 % and 63 % lower activity than the control and samples coated with the commercial extract, respectively. This may be due to the presence of polysaccharides in the seaweed extract which was described earlier by Augusto et al. (2018). The polysaccharides can act to protect the cell membrane against external damages.

Concerning samples packaged under modified packaging, it was possible to observe a consistent increasing trend in PME activity with storage duration (Figure 3.2F), although only control and seaweed extract coated samples showed a major increment in activity values ($p < 0.05$) of 34 % and 67 %, respectively.

At the end of storage, and in contrast to ambient packaging results, the samples coated with the commercial additive presented significantly lower ($p < 0.05$) values of PME (0.002 U mg^{-1} protein). The possible presence of calcium in its formulation can explain this result, since calcium is known to stabilize the integrity of cell membrane and retard the action of PME (Aguayo et al., 2010).

3.3. Evaluation of microbiological counts in fresh-cut 'Fuji' apple during storage

Fresh-cut apple is susceptible to microbiological degradation mainly due to cutting processes which increases the surface area and therefore the probability of contamination (Holban & Grumezescu, 2018). The European Commission regulation (EC No 2073/2005) requires evidencing the absence of *Salmonella* sp, *Escherichia coli*, and *Listeria monocytogenes*. Portuguese government also recommends the control of mesophilic bacteria (less than 10^6 CFU g^{-1}), Enterobacteriaceae species (less than 10^4 CFU g^{-1}), and yeasts and moulds (less than 10^3 – 10^5 CFU g^{-1}) (Santos et al., 2005). The results of microbial analysis are shown in Table 3.3. After 15 days of storage, the mesophilic and Enterobacteriaceae bacteria counts in all samples were above the recommended threshold (10^6 CFU g^{-1}), so no further analysis was performed. However, the yeast and mould counts remained below the threshold values up to 25 days of storage. Regardless, after 15 days of storage, the samples coated with the seaweed extract had the lowest mesophilic and Enterobacteriaceae counts in both types of packaging, demonstrating a possible antimicrobial effect of the seaweed extract, a result also observed in Chapter II (see section 3.4) but not yet reported in literature. A study conducted by Padhi and Tayung (2015) reported that *Codium decorticatum* seaweed contained several symbiotic microorganisms with

antimicrobial activity which may also be present in the seaweed extract, thereby accounting for the observation. However, further studies are necessary to understand the role of seaweed extract as an antimicrobial component for preserving fresh-cut apples.

Table 3.3. Total viable counts (mesophilic bacteria, Log CFU g^{-1}), enumeration of Enterobacteriaceae (Log CFU g^{-1}), yeasts and moulds (Log CFU g-1) in fresh-cut apples. Microbial counts above permitted threshold values are reported in bold (Santos et al., 2005). N.P.- Not present.

	Ambient packaging			**Modified atmosphere packaging**		
Storage time (days)	**Control**	**Commercial**	**Extract**	**Control**	**Commercial**	**Extract**
Mesophilic bacteria (Log CFU g^{-1})						
1	4.2	3.9	4.5	4.2	4.2	4.1
5	5.4	4.2	4.7	5.0	5.8	4.4
10	5.4	6.7	6.2	6.5	7.1	4.5
15	**7.1**	**7.3**	**6.8**	**7.1**	**7.6**	**6.5**
20	**7.0**	**7.5**	**7.2**	**7.0**	**7.7**	**6.6**
25	**7.2**	**7.2**	**7.1**	**8.1**	**7.6**	**7.5**
30	**7.5**	**7.7**	**7.3**	**8.2**	**7.7**	**7.9**
Enterobacteriaceae (Log CFU g^{-1})						
1	N.P.	2.8	N.P.	N.P.	N.P.	3.4
5	4.4	4.4	3.7	3.2	4.2	3.7
10	3.2	6.2	5.1	**4.0**	**4.8**	2.6
15	**6.2**	**7.1**	**5.4**	**4.4**	**7.3**	**4.2**
20	**5.2**	**7.1**	**4.1**	**4.7**	**6.2**	3.3
25	**6.4**	**6.4**	**3.7**	**5.8**	**5.1**	4.0
30	**6.5**	**6.3**	**4.1**	**5.8**	**6.4**	**3.7**
Yeasts and moulds (Log CFU g^{-1})						
1	N.P.	N.P.	N.P.	N.P.	N.P.	N.P.

5	2.8	N.P.	2.7	2.9	3.1	N.P.
10	3.0	4.5	4.2	3.1	4.4	2.7
15	3.6	3.4	3.3	3.4	3.7	2.7
20	4.3	4.4	4.3	**6.5**	5.4	2.7
25	4.3	**5.3**	4.6	3.8	**5.2**	4.4
30	**5.2**	**5.4**	4.5	**5.6**	**6.3**	4.0

3.4. Sensory analysis of coated and uncoated fresh-cut 'Fuji' apple

The commercial application of this extract, as a new postharvest treatment, is viable only if its application does not compromise organoleptic profile. Therefore, two triangular tests were performed, helping understanding consumers preferences on the different samples.

In the first test, panellists were instructed to identify the different sample between the seaweed extract and the commercial additive coated samples, statistical differences were identified ($p < 0.05$), indicating that there are organoleptic differences between commercial and seaweed extract coated samples. From the consumers comments, an "off-flavour" was present in the commercial samples, which was not reported in seaweed extract coated samples. The same "off-flavour" was also reported in fresh-cut apples treated with a calcium-ascorbate solution (Aguayo et al., 2010).

In the second test, panellists were instructed to identify whether differences could be perceived between control and samples coated with the seaweed extract. No statistical differences were identified, supporting that the seaweed extract application doesn't significantly alter apple organoleptic attributes.

4. Conclusions

The results of the present chapter contributed to the understanding of the pilot plant scale testing of a seaweed extract coating to preserve fresh-cut apple, showing for the first time, the use of a natural coating applied under industrial conditions. A batch of 15 L of seaweed extract was produced using a pilot-scale solid-liquid extractor, and its functionality was assessed in

ambient and modified atmosphere packaged samples. Different effects were found according to coating and packaging type as well as storage duration, depicting different scenarios after 30 days of storage:

Samples stored under modified atmosphere and coated with the seaweed extract

i) had similar textural attributes to fresh-cut apples on day 1.

ii) gave identical browning index and colour change values to those coated with the commercial additive.

iii) showed lower oxidative enzyme activities when compared with the commercial coated samples.

iv) showed delayed microbial growth.

v) did not influence the organoleptic quality as evidenced through sensory triangular tests.

The results clearly show the benefits of seaweed extract coating, especially when associated with modified atmosphere packaging, thus establishing for the first time the efficacy of the seaweed extract under industrial conditions.

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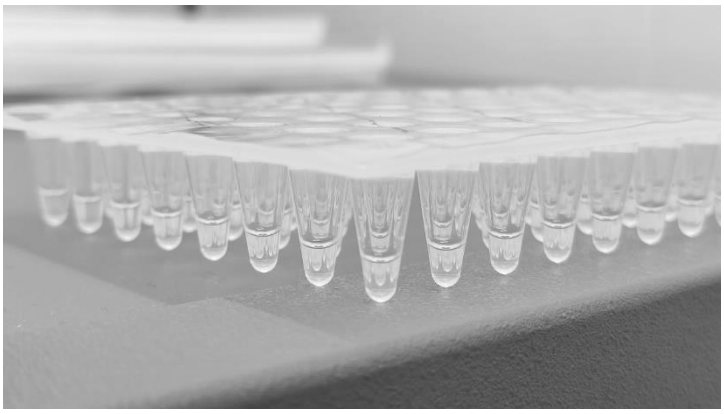

CHAPTER IV

An insight into the mechanisms underpinning the anti-browning effect of *Codium tomentosum* on fresh-cut apples

An insight into the mechanisms underpinning the anti-browning effect of *Codium tomentosum* on fresh-cut apples

Abstract

This chapter focuses on understanding the mechanism of action of a novel seaweed extract with anti-browning functionality in fresh-cut apples. Organic fresh-cut apples were coated by immersion in an aqueous *Codium tomentosum* seaweed extract (0.5 % w/v), packaged under ambient atmospheric conditions in plastic bags, and stored at 4 ˚C for 15 days. Browning-related enzymatic activities, as well as targeted gene expression related to superficial browning, were monitored immediately after coating, and followed at five-day intervals, until a final storage period of 15 days. Gene expression was particularly affected one hour after coating application (day 0), with no expression registered for peroxidase (*mdPOD*) and phenylalanine ammonia-lyase (*mdPAL*) genes in the coated samples. A reduction in polyphenol oxidase expression levels was also observed. After 15 days of storage, the coated samples developed lower browning levels and presented distinctly lower activities of polyphenol oxidase and peroxidase - the oxidative enzymes predominantly involved in enzymatic browning. The observed post-coating suppression of *mdPAL* and *mdPOD* expression, and reduction in *mdPPO* expression, suggest that the seaweed *C. tomentosum* extract delays the activation of these genes, and decreases enzymatic activity, which in turn accounts for the coating's anti-browning effect.

Keywords: Fresh-cut apple; *Codium tomentosum*; Post-harvest; Browning; Peroxidase activity.

1. Introduction

Apple (*Malus domestica*) is a fruit with a high economic value. Recently data suggests that 17 million tonnes of apples were produced in Europe in 2019 and the cost per tonne was 664 USD dollars (FAO, 2021). Apples are generally consumed as a whole fruit, but more recently there has been a rapid growth in the consumption of ready-to-eat fresh cut apples mainly due to the convenience it offers. Fresh-cut apples are made by washing the whole fruit followed by cutting, treating with a dip-solution and packaging (Tarancón et al., 2021; Yousuf et al., 2019). Fresh-cut processing demands a high standard of preparation and handling in order to prevent rapid deterioration due to microbial spoilage and browning (Yousuf et al., 2019).

In the production of fresh-cut apples, the mechanical damage induced by peeling and slicing operations triggers the onset of surface browning, which is a mechanistically complex process (Chen et al., 2021). Surface browning results in a loss of both nutritional and organoleptic quality, leading to a decline in consumer acceptance and commercial value (Rasouli & Koushesh Saba, 2018). Browning can be caused by enzymatic activity as well as by non-enzymatic reactions. The Maillard reaction, which is non enzymatic in nature and depends on the apple sugar content and ascorbic acid concentrations (Paravisini & Peterson, 2018), can potentially cause browning of fresh-cut apples but its contribution to browning in the case of fresh-cut apples is arguable (Paravisini & Peterson, 2018). On the other hand, enzymatic mechanisms which cause superficial browning in fresh-cut apples have been widely studied and reported in literature (Chen et al., 2021; Tang et al., 2020; Toivonen & Brummell, 2008). In whole apples, cellular organelles are compartmentalized, and metabolic pathways occur without external interference. However, cutting disrupts cellular membranes, causing enzymes like polyphenol oxidase (PPO, EC 1.10.3.1) and peroxidase (POD, EC 1.11.1.7) to come into contact with substrates thereby triggering browning (Toivonen & Brummell, 2008). More specifically, these enzymes catalyse the oxidation of phenolic compounds into quinones, which in turn undergo

condensation reactions which result in browning (Chen et al., 2021; Oliveira et al., 2021; Rasouli & Koushesh Saba, 2018; Toivonen & Brummell, 2008). The presence and concentration of phenolic compounds ranging between 60 and 220 mg per 100 g of apple are considered to be key factors for pro-oxidative enzyme activity (Zhu et al., 2020). It is therefore necessary to develop technological solutions to inhibit such enzyme mediated browning processes in fresh-cut apples.

Edible coatings formulated with active ingredients have been widely used as agents to prevent the development of enzyme-mediated browning (Maringgal et al., 2020). Active substances in the coatings may be chemically synthetic, like ascorbic acid (E300), calcium ascorbate (E302), and hydrogen sulphide or obtained from sources of natural origin, such as *Aloe vera*, lemongrass oil or edible seaweed extracts (Augusto et al., 2016; Carocho et al., 2018; Chen et al., 2021; Maringgal et al., 2020). Augusto et al. (2016) reported on the efficacy of a green edible seaweed extract - *Codium tomentosum* - to reduce browning in fresh-cut apples and pears. The activities of PPO and POD in fresh-cut apples were assessed over a storage period of 20- days and a reduction of 36 % and 87 %, respectively, was observed (Augusto et al 2016). In the case of fresh-cut pears, a study previous discussed in Chapter II, the samples treated with the seaweed extract solution were found to exhibit significantly lower rates of superficial browning than samples coated with an ascorbic acid-based synthetic formulation widely used in industry (see Chapter II, Figure 2.2). Even though the efficacy of seaweed extracts have been conclusively established in Chapters II and III, the mechanism of their action is not clearly understood. This chapter aims to assess the mechanism underpinning anti-browning functionality of this extract, exploring the relation between gene expression regulation, oxidative-enzymes activities, and browning development in fresh-cut apples.

2. Methods

2.1. Seaweed extraction and sample coating

An aqueous seaweed extract was produced as the anti-browning coating for fresh-cut apples. Dry *Codium tomentosum* (particle size with an average of 1.5 mm) was extracted in a 1:15 ratio of biomass: deionized water. The extraction was conducted away from light for 3 hours under constant stirring (1,500 rpm) at room temperature. Subsequently, the mixture was filtered, centrifuged and freeze-dried as described in Augusto et al. (2016). The extract obtained was kept away from light until use.

Organically produced apples (*Malus domestica*, var. 'Fuji') without any post-harvest treatment were supplied from a Portuguese producer - Campotec S.A. Prior to the coating application, fruits with similar weight and maturation stage (15.78 ± 0.40 g $_{sucrose}$ 100 g$^{-1}_{product;}$ and pH of 4.08 ± 0.14), were washed with deionized water and manually sliced. Each apple was cut into 6 to 8 slices, each slice weighing 16 g on average. The slices were coated by immersion for 5 min in a solution containing 0.5 % (w/v) of the previously prepared seaweed extract dissolved in deionized water. For comparison, a control treatment, comprising only immersion in deionized water, was adopted. After coating, the excess coating solution was drained, and samples were packaged in plastic bags. Each sliced apple was considered to be a replicate, giving in a total 6 replicates per treatment. To promote the natural development of browning, treated and control samples were stored for 15 days at 4 ± 2 °C, with analyses being conducted every 5 days (i.e. on days 0, 5, 10, and 15). The time taken to slice, coat, package and store apples was around one hour. The aforementioned "day 0" corresponded to the time point just before the slices went into storage. Physicochemical parameters were evaluated in samples every 5 days. The analyses of browning compound absorbance, enzyme activities, and gene expression were undertaken on samples which were removed from storage at the stipulated time and frozen at -80 ˚C.

2.2. Browning compounds absorbance

Browning compounds absorbance was determined according to Paravisini & Peterson (2018) and Shao et al. (2018). In brief, 2 g of frozen apple slice was homogenised with 14 mL of deionized water (×10/25 Homogenizer, Ystral, Germany), followed by a 1-hour incubation period at room temperature. The mixture was centrifuged at 1,000 g for 5 min, after which 5 mL was collected from the supernatant, mixed with 96 % ethanol and re-centrifuged. The absorbance of the supernatant was measured at 440 nm in a microplate reader (4 wells per sample) (Epoch2 Microplate reader, Biotek, USA). Results are expressed in absorbance units. Six control slices and six coated samples were withdrawn for analysis at each point of storage time (n = 6).

2.3. Physicochemical analysis

To assess the treatment effects on fresh samples, the sample colour was measured with a focus on the browning index development (BI) and colour changes (ΔE*). Since colour measurement is non-destructive, the same samples were followed over the storage period. In order to avoid randomness in superficial colour development, 15 specific points were selected on the surface and the colour of these samples were followed during storage (Supplementary data, Figure S4.1). The average value of colour measured at each of the 15 points was considered for calculations. Browning Index (BI) and ΔE* were evaluated as described by Lante et al. (2016) and in section 2.4 of Chapter II (equation 1), respectively. The ΔE* was calculated by the difference between an individual sample and the gold standard defined as the colour parameters of a sliced apple analysed immediately after cutting (L* = 79.81; a* = 0.90; b* = 24.46). For each storage condition, 6 different apple slices were analysed (n = 6).

2.4. Gene expression

2.4.1.RNA Extraction, purification, and quality assessment

Total RNA was isolated from each apple replicate using an adapted methodology from a CTAB-based protocol, optimizing for maximum yields concomitant with higher qualities of the extracted RNA (Gambino et al., 2008).

Firstly, 100 mg of frozen sample was ground to a powder with liquid nitrogen and transferred immediately to a 2 mL microtube with 650 mg of 1.4 mm zirconium oxide beads. While avoiding sample from thawing, a volume of 650 µL extraction buffer containing 2 % of PVP-40, 0.05 % of spermidine, 2 % β-mercaepthanol, and 1 % IGEPAL® CA-630 (NP-40) in cetyltrimethylammonium bromide extraction buffer (CTAB) was added, followed by homogenization in a bead beater using 2 cycles of 45 s at 6,200 rpm with an interval of 5 s between cycles (Precellys Evolution, Bertin, France). To promote protein degradation, 36 µL of 20 mg mL^{-1} of proteinase K was immediately added, and the mixture was gently stirred by inversion, followed by a 10-min incubation at 70 ˚C and 1,800 rpm (Thermomixer Comfort, Eppendorf, Germany). Afterwards, a centrifugation step was performed for 5 min at 13,000 g (4 ˚C). The supernatant was then collected and mixed with 530 µL of the mixture chloroform/isoamyl alcohol 24:1 (v/v) and stirred by inversion. To promote phase separation, a new centrifugation step was performed at 12,000 g for 15 min (4 ˚C). The aqueous phase was then transferred, and the RNA was precipitated with 100 % isopropyl alcohol (proportion of 1:1 to the aqueous phase) for 20 min at room temperature, followed by a second centrifugation step. The resulting pellet was washed twice with 600 µL of 75 % ethanol, stirred by inversion and centrifuged for 5 min. After the complete removal of ethanol, the RNA was re-suspended in 10 µL of DNAse/ RNAse-free water.

Next, and according to the manufacturer's instructions, pooled RNA samples were purified using the RNA Clean & Concentrator™-5 Kit from Zymo Research, including the DNAse treatment to remove traces of gDNA. In the final step, RNA was eluted with 15 µL of DNAse/ RNAse-free

water. Total RNA concentration, as well as DNA concentration for possible gDNA contamination, were determined with a fluorimeter, following the manufacturer instructions (Qubit® 2.0 Fluorometer, ThermoFisher Scientific, USA). Contamination with protein, polysaccharides, and other RNA contaminants was verified using a micro-spectrophotometer Nanodrop 2000 (Thermo Scientific, USA) by measuring the absorbance at 230 nm, 260 nm, and 280 nm and evaluating their ratios. Total RNA integrity was assessed after electrophoresis on a 1 % (w/v) agarose gel. Due to the still low RNA extraction yields obtained from the maximum biomass possible for each reaction (100 mg), five extractions of each apple replicate sample were performed and pooled, giving a final RNA amount of 666 ng in average for each pooled sample.

2.4.2.cDNA synthesis and qPCR amplification

iScript cDNA Synthesis Kit (Bio-Rad) was used for the first-strand cDNA synthesis, following the manufacturer's instructions. To this end, 150 ng of total RNA was reverse-transcribed in a total volume of 20 µL. Samples without reverse transcriptase were also amplified to assess possible interference of gDNA contamination on the RT-PCR amplification.

Next, to evaluate the effect of the seaweed extract on gene expression of browning-related enzymes (*mdPPO, mdPOD, mdPAL*), anti-oxidative enzymes (*mdSOD, mdCAT, mdDHAR*), and cell-wall related enzymes (*mdPME, mdα-Af*), the expression of those eight target genes and a reference/housekeeping gene (*mdH1*) was quantified. Oligo Explorer software (version 1.1.2, Gene Link™) was used to design primer sequences based on the available gene sequences in NCBI for this species (Table 4.1). Primer efficiency (E) was calculated according to the equation: $E= (10^{(-1/slope)} - 1) \times 100$, where the slope is obtained from the standard curve of sample serial dilution. The specificity of each primer set in producing a single and specific amplification product was assessed through melting curves analysis. To reject possible amplification resulting from gDNA contamination, as well as primer dimers formation, different controls were performed: replicate samples without the reverse transcriptase addition (-RT controls) and non-template control

replicates (NTC) (Taylor et al., 2019). For the qPCR amplification reactions, 2 µL of DNA template, 2 µL of each respective forward and reverse primer, 4 µL of DNAse/RNAse free water and 10 µL of iTAQ™ Universal SYBR® Green Supermix were added (final volume of 20 µL). The thermal cycling protocol comprised a first step of 30 s at 95 °C, followed by 60 cycles of a combined denaturation (5 s at 95 °C) and annealing (30 s at 60 °C) steps. The presence of the desired amplicon was verified by the melt curves which consisted in the measurement of fluorescence in a range from 65 to 95 °C in each increase of 0.5 °C for 5 s. Amplification reactions were performed in triplicates for all samples, using 96-well plates (Biorad, Multiplate® PCR Plates™), on a thermocycling CFX Connect™ Real-Time PCR System (Bio-Rad). The relative expression (ΔΔ CT) of the target genes was normalized by the expression of the reference gene (*mdH1*) (Storch et al., 2015), using the software CFX Connect™ Real-Time System (Biorad, USA) and following an adaptation of the equation developed by Pfaffl (2001) as: $\Delta\Delta CT = [TG E^{(CTmin\ TG - CT\ value\ TG)} / RG E^{(CT\ min\ RG - CT\ value\ RG)}]$, where E is the efficiency of each target (TG) and reference gene (RG). Despite samples were kept in storage conditions for 15 days, gene expression analysis was performed only on days 0, 5 and 10, since the molecular effects that lead to the enzymatic alterations and visual effects at day 15 are expected to occur sooner in time.

Table 4.1. Primer sequences and efficiencies of the selected genes of *Malus domestica* (*md*) for qPCR analysis.

Gene abbreviation	Accession number	Primer sequence (5'→3')	Efficiency and R^2	Reference
mdH1- Histone 1	NM_001293977	FW: CATATTTGGCAGCAGAGCAA RV: CTCGTTAGCCAACTGCATCA	E = 91 %; r^2 = 0.991	Storch et al. (2015)
mdPPO (Polyphenol oxidase)	NM_001319261	FW: CTGAGTTCCCGATAAGTCTG RV: TTCTTGCTCCTCTTCTTCTG	E = 94 %; r^2 = 0.979	In this work
mdPOD (Peroxidase)	XM_008341866.2	FW: AAGCCTATAGCCCCACCAGA RV: CTTGAAGCTACGTGGGTCGT	E = 98 %; r^2 = 0.985	Abdelhai et al. (2019)
mdSOD (Superoxide dismutase)	XM_008388606.2	FW: GCTGATCCTGATGATCTTGG RV: AACTCTTGCTCCTGCGTTC	E = 91 %; r^2 = 0.996	In this work
mdDHAR (dehydroascorbate reductase)	NM_001294110.1	FW: CCAAGGATGGAACAGAAGAA RV: AAATCAGCAGCAGAAACCAC	E = 100 %; r^2 = 0.945	In this work
mdPAL (Phenylalanine ammonialyase)	XM_008357397.3	FW: GGTGAGCCAAGTAGCAAAG RV: CAGGGATCGTCAATGTAGG	E = 97 %; r^2 = 0.995	In this work
mdα-AF (α-arabinofuranosidase)	NM_001294121.1	FW: TAGGGAATGAGGATTGTGG RV: AAGAACCGTCGCAGTTTG	E = 84 %; r^2 = 0.989	In this work
mdPME (Pectin methylesterase)	NM_001328782.1	FW: AGCTCACTAAGCGTGAGAAG RV: TACTCATGGAGATCCTCGAC	E = n.a.; r^2 = n.a.	Tanaka et al. (2018)
		FW: CATCCGAAATCTCTAATGCG RV: TGTACGAGTGGTGGTGTTTG	E = n.a.; r^2 = n.a.	In this work
mdCAT (Catalase)	XM_008350702.2	FW: AGACACCTGTCATTGTGCGT RV: CATGGATCACGTCCGGGAAT	E = n.a.; r^2 = n.a.	Abdelhai et al. (2019)
		FW: TGACTTCTTCTCCCACCATC RV: CCTTCCATGTGCCTGTAATC	E = n.a.; r^2 = n.a.	In this work

n.a.: not applicable

2.5. Enzymatic activities

The activity of four enzymes – polyphenol oxidase (PPO), peroxidase (POD), pectin methylesterase (PME), and superoxide dismutase (SOD) was determined, and frozen samples from coated and control groups were processed as described below.

To evaluate the efficacy of the seaweed extract to prevent browning development in coated and control apple slices, enzymatic activities were evaluated, namely polyphenol oxidase (PPO) and peroxidase (POD) - two enzymes which the extract appears to influence (Augusto et al., 2016) as also discussed in Chapters II and III, and be mainly responsible for the enzymatic browning in fresh-cut apples (Toivonen & Brummell, 2008). The evaluation of PPO and POD activities followed the procedure described in Chapter II (section 2.6). Briefly, frozen samples were homogenised in 50 mM sodium phosphate buffer (pH 7.0) containing polyvinylpyrrolidone (PVP) (50 g L^{-1}) and the supernatant collected for the enzyme's determination and protein quantification. For POD determination, the reaction was followed at 470 nm and catalysed by mixing the enzymatic extract with a solution containing 1 % of guaiacol and 0.3 % of hydrogen peroxide in 0.05 M of sodium phosphate buffer (pH 6.5). On the other hand, a solution of 20 mM catechol in 5 mM sodium phosphate buffer (pH 7) was used as substrate mixture for the determination of PPO, and the reaction absorbance read at 400 nm. The results of PPO and POD are expressed as U mg^{-1} protein. Protein was quantified following the Bradford methodology (Bradford, 1976).

The activity of the cell-wall related enzyme pectin methylesterase (PME) was measured following the methodology described in Chapter II (section 2.6), and results were expressed as U mg^{-1} protein. Before PME determination, frozen apple slices were used for a second extraction using an extraction buffer containing 1.5 M of NaCl and 2.5 w/v of PVP (pH 7.5) as described in Chapter II (section 2.6). The supernatant collected for the enzyme determination and protein quantification. The enzymatic reaction was measured spectrophotometrically at 610 nm, by

mixing the enzyme extract with a substrate solution with 0.01% of bromothymol blue and 5 g L^{-1} citrus pectin in 0.003 M sodium phosphate buffer (pH 7.5).

The antioxidant enzyme superoxide dismutase (SOD) activity was also evaluated. A third enzymatic extraction was carried out for SOD determination. An adapted protocol from Collazo et al. (2018), Li et al. (2019), and Wei et al. (2019) was followed. Five g of frozen apple slice was homogenised with 10mL of chilled 0.1 M sodium phosphate buffer (pH 7.5) containing 20 g L^{-1} of polyvinylpyrrolidine (PVP), 2 mM dithiothreitol (DTT), and 0.1 mM ethylenediamine tetraacetic acid (EDTA). To remove larger particles, the mixture was filtrated through a cheesecloth, followed by a 30 min centrifugation at 14,000 g (4 °C), obtaining a clear supernatant ready to be used for SOD determination. A volume of 50 µL of enzyme extract was mixed with 150 µL of a subtract mixture composed of 50 mM sodium phosphate buffer (pH 7.8) with 13 mM methionine, 75 µM NBT (nitrotetrazolium blue chloride), 10 µM EDTA and 2 µM riboflavin. The plate was incubated under fluorescent light for 10 min, and absorbance was read at 560 nm, before and after incubation. At the same time, and to discard possible interferences on the final absorbance determination, an identical plate was incubated in the dark (blank). SOD specific activity was expressed as U g^{-1} of fresh weigh (FW), where one unit (U) of the enzyme activity was defined as the photoreduction inhibition of NBT by 50 %.

All enzymatic activities were performed in six biological replicates for each storage condition (n = 6), in triplicates, using an Epoch2 Microplate reader (Biotek, USA). In all the enzymatic determinations, a control assay was performed using the homogenization buffer instead of the enzymatic extract.

2.6. Statistical analysis

To assess the seaweed extract effects on the development of superficial browning and on gene expression of the sliced apples, generalized linear models (GLMs) with logarithm link functions were used. Based on the Akaike Information Criteria (AIC) and likelihood ratio tests (LRT), the

best fitting models were chosen. For pairwise post hoc comparisons, the least significant difference (LSD) was run to test for individual differences between the factor levels. GLMs were performed in IBM SPSS Statistics version 27. CANOCO version 4.5 package was used for the Principal Component Analysis (PCA) design.

3. Results and discussion

In fresh-cut apple processing, as an immediate response to the cutting process, the antioxidant system is activated by increasing the relevant enzyme activities like superoxide dismutase (SOD) and catalase (CAT). Consequently, there is an increase in the activity of browning-related enzymes like POD and PPO, which act on the phenolic substrates that have leaked from cell vacuole to result in superficial browning (Chen et al., 2021). Changes on the visual appearance of fresh-cut apples are mainly driven by the variation in enzyme activities that are preceded by metabolic responses occurring at a cellular level which regulate gene expression and protein levels (Lemos, 2021; Simões et al., 2019). As the first alterations are observed at the sub-cellular level, the results here discussed are presented respecting the sequential events related to browning development (gene expression proceeded by the functional activity). These sequential events were then related between them and are also here discussed.

3.1. Colour development

The visual colour changes and reduction in browning are decisive factors establishing the efficacy of the seaweed extract. Browning development in fresh-cut apples can be determined by different methodologies, including spectrometric techniques and colour measurements.

In this chapter, the formation of browning compounds was determined as a function of storage time and the results, expressed as browning absorbance, browning index and colour (ΔE*), can be seen in Figures 4.1 A-C. In general, in browning absorbance results (Figure 4.1A) comparing the data on days 0 and 15 there was no significant difference between the seaweed coated and the control group ($p > 0.05$). The absorbance seems to decrease on day 5 ($p = 0.045$) but increase

after day 10 ($p = 0.002$), to reach similar values as day 0. Into the case of the seaweed extract coated group, a significant increase in absorbance was detected between days 0,10 ($p = 0.037$) and 15 ($p = 0.008$). Despite the observed increase in browning absorbance, the values for seaweed coated samples were lower than those for the control samples ($p < 0.05$). These results clearly establish the efficacy of seaweed extract coating on fresh-cut apples.

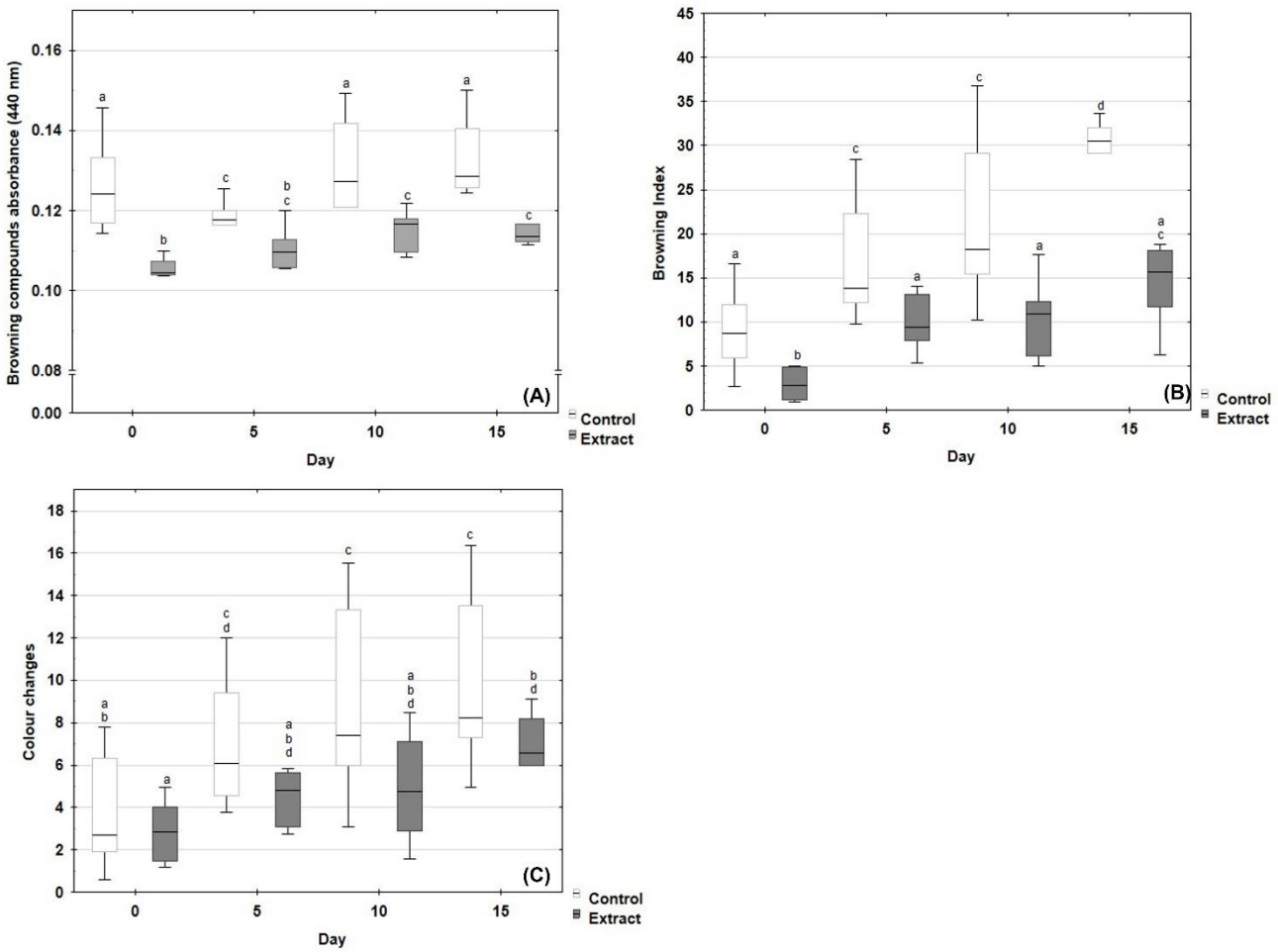

Figure 4.1. Boxplots of the **(A)** browning compounds absorbance at 440 nm (calculated by spectrophotometry), **(B)** browning index (BI, calculated by colour parameters), and **(C)** colour differences (ΔE*) between fresh-cut apples coated with seaweed extract vs control treatments. Samples were stored for 15 days at 4 ˚C, with periodic samplings on days 0, 5, 10 and 15. Boxplots with different letters represent significantly different values (ANOVA, GLM, $p < 0.05$).

The results of colorimetric measurement in terms of browning index (BI) and total colour differences (ΔE*) are presented in Figures 4.1 B-C. Significantly lower browning index values were observed in samples coated with the seaweed extract, compared to control samples (Figure 4.1 B; $p < 0.05$). As with the results of absorbance at 440 nm (Figure 4.1 A), the data on browning index also confirm the efficacy of seaweed coating treatment. The colour parameter

ΔE* increased between day 0 and 15 (control: $p < 0.001$; seaweed extract: $p = 0.042$), although the increase was much less pronounced in samples coated with the seaweed extract (ΔE^*_{15} control = 9.75 ± 4.29; seaweed extract = 6.22 ± 2.88). These changes are consistent with the results of browning index (Figure 4.1B) and browning compounds absorbance (Figure 4.1A) since the development of browning colour has a strong bearing on the final colour of the fresh-cut apples. The differences between coated and uncoated samples are more pronounced on day 10 of storage, where control samples presented significantly higher values of ΔE* (ΔE^*_{10} = 8.77 ± 1.15) than samples coated with the seaweed extract (ΔE^*_{10} = 4.93 ± 1.15) ($p = 0.019$). Similar results were obtained by Augusto et al. (2016) in fresh-cut apple slices and puree treated with a solution of *C. tomentosum* extract. In Chapter II (see section 3.1), it was also observed a reduction in the development of superficial browning in fresh-cut pears treated with a coating solution containing 0.5 % w/v of *C. tomentosum* extract. In both studies, the mechanisms underlying the reduced browning in apple and pear coated with the seaweed extract were unknown, thus, the present chapter goes beyond the anti-browning effect of this seaweed-based coating, trying to understand the mechanism of action behind these effects.

3.2. Gene expression

Three of the studied target genes, namely *mdPAL*, *mdPOD*, and *mdPPO*, were initially selected as they are believed to be implicated in the mechanisms of browning in fresh-cut apples and linked between them through phenylpropanoid pathway (Chen et al., 2021). It is through this pathway that PPO and POD substrates, the phenolic acid compounds, are produced. Phenylpropanoids are secondary metabolites that derive from phenylalanine and tyrosine amino acids. Chemically, these compounds can be divided into five sub-groups: flavonoids, monolignols, stilbenes, coumarins, and phenolic acids, with the last sub-group representing the main substrates of PPO enzyme (Chen et al., 2021; Deng & Lu, 2017). Considered the main key factor for the phenylpropanoid biosynthesis, the enzyme phenylalanine ammonialyase (PAL) plays a primary

role in the phenylpropanoid pathway, regulating indirectly the production of phenolic substrates (Chen et al., 2021; Dou et al., 2021; Liu et al., 2021a). Among phenolic compounds that can be found in apples, chlorogenic acid and procyanidin are the main phenolic substrates of PPO enzyme (Treutter, 2001). Briefly, in the presence of phenolic substrates and oxygen, the activity of PPO causes the generation of brown pigments. Firstly, the hydroxylation of monophenols to *o*-diphenols is catalysed by PPO followed by the oxidation of *o*-diphenols, resulting in the formation of *o*-quinones compounds which are responsible for the coloured brown spots in fresh-cut apples (Chen et al., 2021; Rasouli & Koushesh Saba, 2018). Although being a controversial subject, POD action in apple browning is believed to be a result of phenolic substrates oxidation, a reaction catalysed by the presence of hydrogen peroxide, which provides the free radical hydrogen essential for the enzyme activity (Chen et al., 2021; Oliveira et al., 2021).

In the present chapter, expression levels (*r*E) results of the genes encoding the mentioned browning-related enzymes PAL, POD, and PPO, after 0, 5, and 10 days of storage time, can be seen in Figures 4.2 A-C. Despite the lack of statistical differences between samples coated with the seaweed extract and control from day 0 ($p > 0.05$), it was possible to observe a different response trend in time between the control group and the seaweed extract group for these three genes. In the control treatment, expression levels tended to increase between days 0 and 5, followed by a tendency for decreased or stabilized values at 10 days of storage. On the other hand, in the samples coated with the seaweed extract it was not possible to detect any amplification signal for *mdPAL* and *mdPOD* at day 0 (Figures 4.2 A and B), which expression only started to be visible after day 5 and with a tendency to increase between days 5 and 10 of storage for both genes. This same pattern of response was observed for *mdPPO,* although in this case some low expression values could be detected at day 0 in the extract coated samples (Figure 4.2 C). For the three genes there was also an overall tendency pattern for higher expression of all of them in control samples at days 0 and 5 in comparison to the seaweed extract

coated samples, revealing a possible seaweed extract early inhibition interference on the expression of those genes. Although the non-detected *mdPAL* expression at day 0, in samples coated with the seaweed extract (Figure 4.2A), after 5 days of storage, gene expression is identical in the two sets of samples. But, at day 10, samples coated with the seaweed extract seem to present increased expression levels comparatively to control. As observed for *mdPPO*, the results suggest that *mdPAL* reached its higher average expression levels earlier in control samples (*r*E = 0.89), on day 5, while the transcript level of treated samples reached its highest detected mean expression levels on day 10 (*r*E = 0.98). Regarding *mdPOD*, similar results to those described for *mdPAL* were observed, with even more pronounced evidence of the seaweed extract influence on gene expression (Figure 4.2B). At day 5, the average transcript levels of control samples (*r*E = 1.15) were considerably higher than those of treated samples (*r*E = 0.22) ($p = 0.011$).

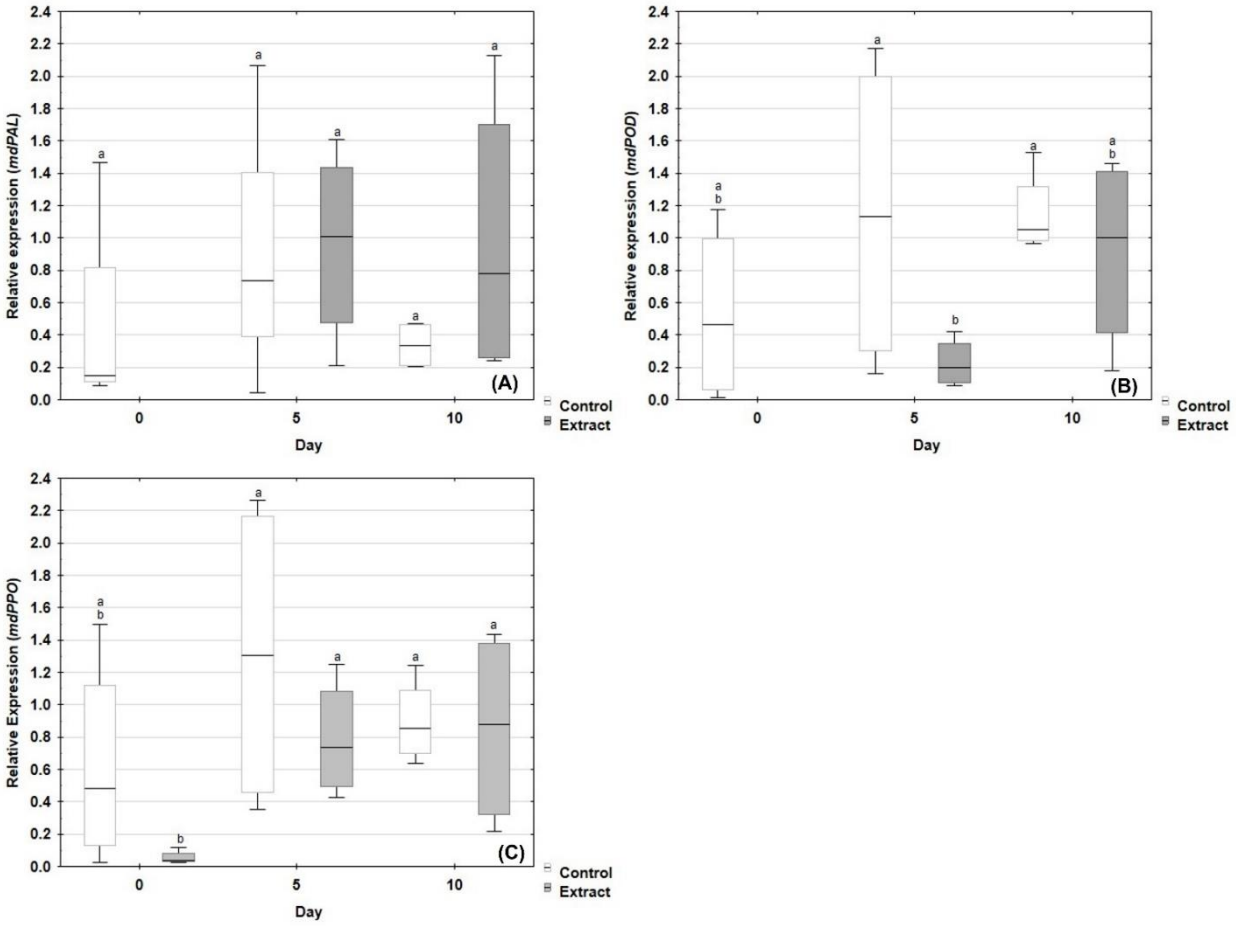

Figure 4.2. Relative expression of the browning-related genes **(A)** phenylalanine ammonialyase (*mdPAL*), **(B)** peroxidase (*mdPOD*), and **(C)** polyphenol oxidase (*mdPPO*) in fresh-cut apples coated with seaweed extract vs control treatments. Samples were stored for 10 days at 4 ˚C, with periodic samplings on days 0, 5, and 10. Boxplots with different letters represent significantly different values (ANOVA, GLM, $p < 0.05$).

In the present study, and although no statistical differences were identified between control and treated samples for *mdPPO* expression within the same time-period ($p > 0.05$), nor between control treatments through time, the extract treatment significantly inhibited *mdPPO* expression at day 0 in comparison with the following days (Figure 4.2C). As previously mentioned, it is believed that the expression of *mdPPO* is associated with browning development, and therefore the inhibition of *mdPPO* gene expression may be directly related to browning reduction in fresh-cut apples (Chen et al., 2021). After treating fresh-cut apples with hydrogen sulfide (H_2S), Chen et al. (2021) observed a positive correlation between the significant reduction of *mdPPO* expression and fresh-cut apples browning. Moreover, this inhibition was also observed in other food matrices, namely in white button mushrooms treated with the amino acid ergothioneine (Qian et al., 2021). White button mushrooms are prone to lose their original characteristics after harvesting, and when submitted to fresh-cut practices, the intensity of tissue browning increase drastically. The authors described a down-regulation in the expression of genes encoding browning-related enzymes as PPO, thereby reducing the enzymatic activity, which resulted in samples with fewer colour changes (Qian et al., 2021).

Overall, the seaweed extract addition seems to interfere with the transcription processes of *mdPPO*, *mdPOD*, and *mdPAL*, with a greater influence on *mdPAL* and *mdPOD* transcripts. This influence may lead to a delay in the transcription, resulting in lower values of gene amplification at the beginning of the storage. These results also highlight the involvement and relevance of *mdPOD* and *mdPAL* in the regulation of fresh-cut apple browning, instead of a single gene regulation like *mdPPO*. Only recently, Chen et al. (2021), Qian et al. (2021), and Liu et al. (2021a), relaunched the debate on the correlation between POD and PAL activities and browning development of fresh-cut fruits and vegetables. The results discussed in the present chapter allow to hypothesise that in the presence of the seaweed extract, the observed resistance to browning development can also be highly mediated by PAL and POD, instead of a single PPO-

browning mediation. This is also, to the best knowledge, the first report where the possible involvement of PAL on browning reduction of fresh-cut apples coated with the seaweed extract is documented and discussed, fostering pertinence of further complementary studies to further access the mechanisms underlying on fresh-cut apples browning. Additionally, in fruits and vegetables, the induction of browning-related enzymes is a fast mechanism, and with the injuries caused by cutting, this process can be almost instantaneous in fresh-cut fruits (Liu et al., 2021a). This fact can support the observed differences in gene expression at day 0, with the samples coated with the seaweed extract presenting overall lower expression levels, possibly contributing to the protection conferred by the coating in the deteriorative processes.

In fresh-cut processing, besides the changes in expression of browning-related genes (discussed above), other protective processes may be occurring, such as the detoxification of superoxide radicals that are converted into hydrogen peroxide by superoxidase dismutase (SOD). The activity of SOD during induced stress prevents the accumulation of free radicals, as reactive oxygen species (ROS), in the cell, contributing to cell protection (Rasouli & Koushesh Saba, 2018). In apples, catalase (CAT) is also involved in the scavenging of ROS avoiding oxidative damages in cells (Li et al., 2019), converting H_2O_2 into water (Abdelhai et al., 2019). Besides SOD and CAT, dehydroascorbate reductase (DHAR) is also indirectly implicated in the antioxidant mechanisms, particularly in the reduction of dehydroascorbate (DHA) into *L*-ascorbate (Davey et al., 2000; Do et al., 2016), thus turning *L*-ascorbate re-available for the antioxidant system to mitigate excessive ROS levels.

The relative expression of the selected encoding genes for the antioxidant system-related enzymes *mdSOD* and m*dDHAR* can be observed in Figures 4.3 A-B (*mdCAT* was not possible to determine as further detailed below).

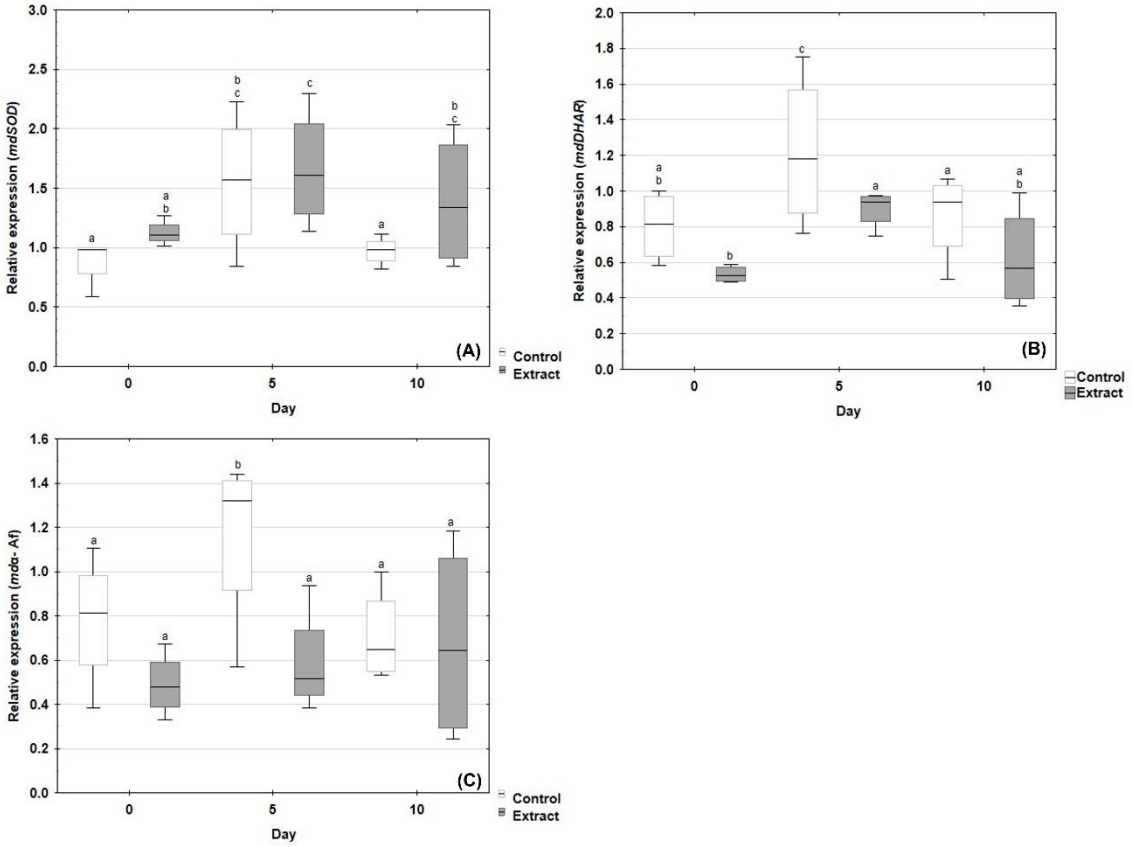

Figure 4.3. Relative expression of the browning-related genes **(A)** superoxidase dismutase (*mdSOD*), **(B)** dehydroascorbate reductase (*mdDHAR*), and **(C)** α-arabinofuranosidase (*mdα-Af*) in fresh-cut apples coated with seaweed extract vs control treatments. Samples were stored for 10 days at 4 ˚C, with periodic samplings on days 0, 5, and 10. Boxplots with different letters represent significantly different values (ANOVA, GLM, $p < 0.05$).

Considering the results of relative expression of *mdSOD* (Figure 4.3A), it is possible to observe that samples coated with the seaweed extract solution showed an overall trend for higher expression levels than control samples, through the 10 days of storage. In both control and seaweed extract coating samples, there was an increase in expression at day 5 (control: $p = 0.007$; seaweed extract: $p = 0.030$) (mean *r*E = 0.89 to 1.55 in control and mean *r*E = 1.12 to 1.66 in seaweed extract samples), followed by a significant decrease in control samples at day 10 ($p = 0.020$), which was not observed in seaweed extract samples ($p = 0.270$). In the work developed

by Liu et al. (2021a), the authors demonstrated that changes in the eggplant redox state, namely the delay of antioxidant genes transcription, could be associated with the development of fresh-cut eggplant browning. The observed differences in *mdSOD* transcript levels in the present study may thus suggest that the seaweed extract can induce the antioxidant system in fresh-cut apples, namely SOD activity, contributing to the browning resistance of samples coated with the seaweed extract (Liu et al., 2021a). Concerning *mdDHAR* relative expression (Figure 4.3B), overall, a tendency for lower expression levels in the seaweed extract group can be observed during the storage period, with a significantly lower relative expression (*r*E = 0.89) at day 5 ($p = 0.039$), when compared to control samples (*r*E = 1.22). These results suggest that the ascorbic acid recycling pathway is not activated at the same level in the seaweed extract samples as is in control samples. Although more studies are needed, increased expression of *mdDHAR* is expected to represent a protective response of the organism to ensure DHAR levels after apple slicing, which in the seaweed extract group may not be so relevant given the other protective actions in place against oxidative and deteriorating processes, as previously discussed.

Cell wall modifications involve multiple enzymes, and in fresh-cut apples, pectin methylesterase (PME) and α-arabinofuranosidase (*α*-Af) are two of the enzymes responsible for membrane integrity (Liu et al., 2021b). The study of these enzymes is important to understand their influence on browning development since, when active, these enzymes lead to cell wall degradation, promoting the contact between phenolic substrates and browning-related enzymes, stored inside the cell wall (Toivonen & Brummell, 2008). In this study (Figure 4.3C), while in the control group a sharp increase of *mdα-Af* expression was observed after 5 days of storage (average *r*E = 0.78 at day 0 to *r*E = 1.16 at day 5) ($p = 0.045$), followed by a decrease in expression at day 10 to a mean relative expression of 0.71 ($p = 0.017$), the presence of the seaweed extract in fresh-cut apples suggests an overall lower transcription of *mdα-Af.* In coated samples, no differences were observed in transcript values during the storage period ($p > 0.05$),

although it is possible to observe a slight incremental tendency in expression along the storage period, but never reaching the same maximum values as in the control group, even later in time (Figure 4.3C). Liu et al. (2021b) reported that the textural quality of fresh-cut apple during cold storage was maintained by the repression of *mdα-Af* transcript levels, which was associated with a treatment composed by 1.4 mg L^{-1} of aqueous ozone for 5 min. In the present study, the seaweed extract seems to inhibit the transcription of *mdα-Af*, contributing to the cell wall integrity maintenance.

Concerning *mdPME* and *mdCAT*, while there was an attempt to determine the expression of those genes, it was not possible to detect any amplification in both cases under the studied conditions, even with different sets of primers and performing a higher number of qPCR cycles (Table 4.1). As stated in the material and methods section, given the low yield of RNA retrieved from this type of samples, only 150 ng of total RNA were used as template for the first-strand cDNA synthesis, which may have contributed to the difficulty in detecting amplification of genes with very low transcription activities (Taylor et al., 2019).

3.3. Biochemical validation

With the fresh-cut processing, the first functional changes are observed at the biochemical level, where shifts in enzyme activities and protein levels can be directly linked to further visual effects as surface browning development. To proceed with the functional validation of effects through biochemical evaluations, and thus confirming the effect of the seaweed extract coating on the enzymatic activities of fresh-cut apples, the activities of PPO, POD, SOD, and PME enzymes were assessed (Figures 4.4 A-D).

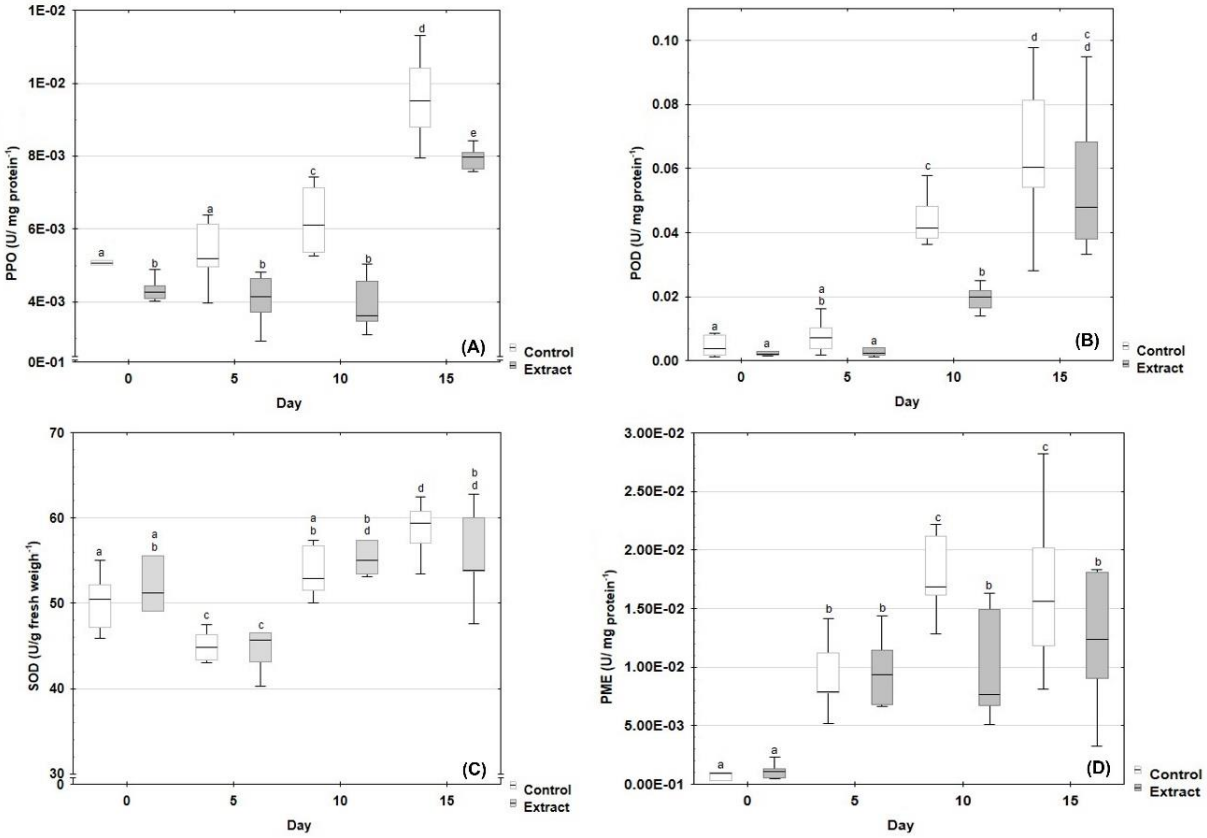

Figure 4.4. The activities of **(A)** polyphenol oxidase (PPO), **(B)** peroxidase (POD), **(C)** superoxide dismutase (SOD), and **(D)** pectin methylesterase (PME) in fresh-cut apples coated with seaweed extract vs control treatments. Samples were stored for 15 days at 4 ˚C, with periodic samplings on days 0, 5, 10, and 15. Boxplots with different letters represent significantly different values (ANOVA, GLM, $p < 0.05$).

The progress of PPO activity during the 15 days of storage period can be observed in Figure 4.4A.

Overall, during the storage, samples coated with the seaweed extract solution had a significantly lower PPO activity comparatively to control group ($p < 0.05$). In more detail, control samples presented an increment in PPO activity between 20 % and 50 % in relation to the seaweed extract group, with this difference more evidenced at day 5 ($p = 0.001$). The observed difference in PPO activity at day 5, correspond to the same time-point where a higher expression of the corresponding gene *mdPPO* was detected (Figure 4.2C), thus indicating that the increased

transcription resulted in higher translation and PPO activity. In turn, the lower activity detected in the seaweed extract group in relation to control could also be a direct effect of the overall lower *mdPPO* transcription values verified in the coated samples (Figure 4.2C). The seaweed extract effect on PPO activity observed in the present study can be corroborated by previous studies, where a reduction in PPO activity was described in fresh-cut apple (Augusto et al., 2016) and pear coated with a solution containing 0.5 % w/v of *C. tomentosum* extract, as discussed in Chapter II of this thesis.

As expected, POD activity presented a significant increase between days 0 and 15 of storage in both control and seaweed extract groups ($p < 0.05$) (Figure 4.4B). Despite the observed POD increment in both sample groups, overall samples coated with the seaweed extract present a tendency of lower values of activity over the storage period. It is at day 10 that the difference between the two set of samples is more denoted, where the enzyme activity was considerably lower in the seaweed extract group (0.0191 U mg^{-1} protein in contrast with 0.0037 U mg^{-1} protein in the seaweed extract group) ($p < 0.001$). These results strengthen those reported in previous studies conducted by Augusto et al. (2016) and presented in the second chapter of this thesis (section 3.3), documenting less POD activity in fresh-cut apples and pears coated with a seaweed extract solution and stored for 20 days and 15 days, respectively. As described for PPO, gene expression may also justify the lower values of POD activity in the seaweed extract group. In line with the observed transcription results (Figure 4.2B), the great disparity in POD values between the two sets of samples could be an effect of changes at the transcriptional level.

The effect of the seaweed extract on SOD activity was also evaluated (Figure 4.4C). To the best knowledge, this was the first report about the seaweed extract effect in SOD activity of fresh-cut apples. No differences were observed along sampling time-points between control group and samples coated with the seaweed extract ($p > 0.05$). These results contrast with those presented by Chen et al. (2021), where the authors described a higher antioxidant capacity

allegedly mediated by SOD activity, which resulted in a lower intensity of browning in fresh-cut apples. While in *mdSOD* gene expression (Figure 4.3A), differences between control and seaweed extract groups were detected, these did not affect the enzymatic level. The results suggest that at the biochemical level, the activity of SOD was not affected by adding a coating solution containing a seaweed extract. At the molecular level, the results may suggest a possible activation of the antioxidant mechanism, supported by the induction of CuZn SOD (Figure 4.3A), but this induction was not possible to verify at the enzymatic level considering that all types of SOD are present in the homogenate that is used for SOD determination.

The biochemical activity of PME was determined and results shown in Figure 4.4D. Both sampling groups presented a similar behaviour during the first 10 days of storage, including the sharp increase in PME activity of about 87 % between days 0 and 5 ($p < 0.05$). However, on day 10, control samples further increased their PME activity ($p < 0.001$) contrarily to the seaweed extract group that maintained the previous levels of activity ($p = 0.980$). The efficacy of the seaweed extract to decrease PME activity of fresh-cut pears and apples was previously evaluated in Chapters II (section 3.3) and III (section 3.2) respectively, and both studies suggested that the seaweed extract had influence in the reduction of PME activity. The results here presented may reinforce this positive influence on the activity PME, preventing the cell wall degradation and contributing to the cell integrity maintenance (Liu et al., 2021b).

3.4. Principal Component Analysis (PCA)

A Principal Component Analysis (PCA) was performed considering the results obtained for the 13 parameters through assessments of gene expression, enzymatic activities, and colour evaluations (Figure 4.5). The first and second principal components (PC1 and PC2) are represented in the X and Y axis respectively and account for 88 % of data explanation (PC1: 59.7 % and PC2: 28.3 %), representing the largest fraction of variability.

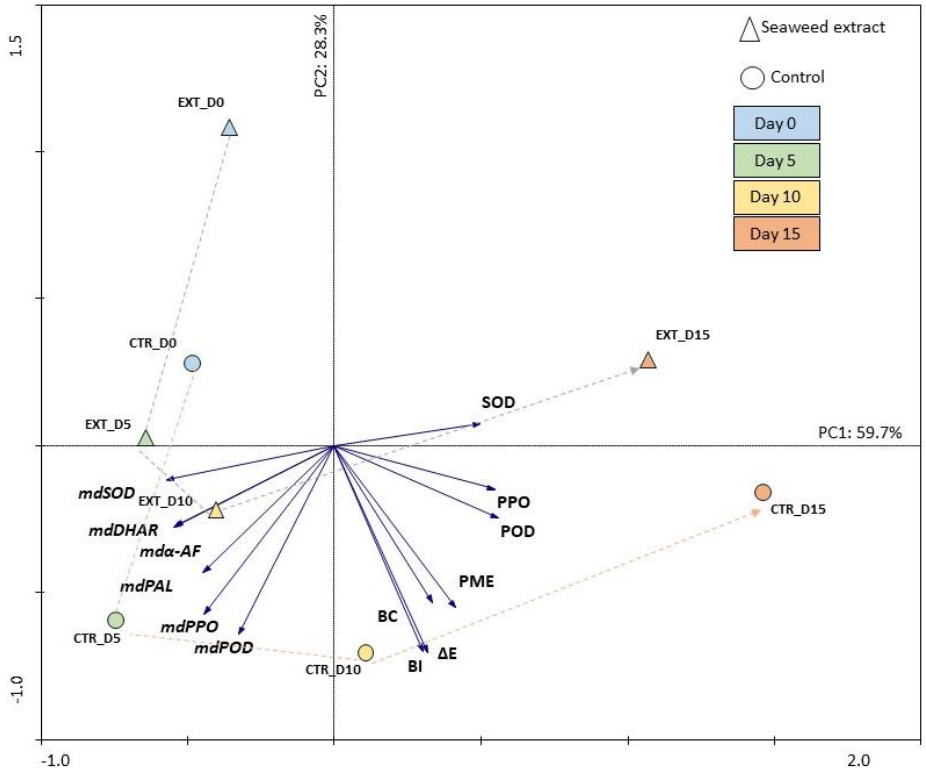

Figure 4.5. Principal component analysis (PCA) of the different responses determined in fresh-cut apples coated with seaweed extract *vs* control treatment, at the different storage times (day 0, day 5, day 10, and day 15). There were determined four latent variables, within the most representative are here represented. *mdPPO* and PPO = polyphenol oxidase gene expression and enzyme activity, respectively; *mdPOD* and POD = peroxidase gene expression and enzyme activity, respectively; *mdPAL* and PAL = phenylalanine ammonialyase gene expression and enzyme activity, respectively; *mdSOD* and SOD = superoxidase dismutase gene expression and enzyme activity, respectively; *mdDHAR* = dehydroascorbate reductase gene expression; *mdα-Af* = α-arabinofuranosidase gene expression; PME = Pectin methylesterase activity; BC = browning compounds; ΔE* = Colour changes; BI = Browning index.

The proximity of the encoding genes for PPO and POD (*mdPPO* and *mdPOD*) vectors to control samples at day 5, reinforces what was previously seen that in this storage time and group of samples, the results were mostly characterized by the increase of *mdPPO* and *mdPOD*

expression levels (Figures 4.2 B-C), with a negligible effect on seaweed extract samples group (observed by the 90° angle with *mdPPO* and *mdPOD* vectors). Only at day 10, it is possible to observe a greater influence of these genes in the seaweed extract samples, which further suggests a possible delay in gene transcription in samples coated with the seaweed extract solution, thus resulting in a lower browning rates and lower enzymatic activity, as previously reported in Figures 4.1 A-C and 4.4 A-B. This hypothesis - the delay in browning development in the seaweed extract samples group, can be strengthened by the strong proximity between the vectors of colour evaluation and browning compounds with the control group vector at day 10. Once again and considering the right angle between these parameters to the seaweed extract group vector, results suggest a lower influence of browning parameters on the coated samples results. With time, namely at day 15, the overall differences between the two sets of samples are smaller, and mostly characterized by the activity of PPO and POD. However, the lower angle between control samples and enzyme activities vectors may suggest a stronger influence of PPO and POD on the results of these groups rather than in the seaweed extract group, resulting in higher browning in non-treated samples, as observed in Figure 4.1.

4. Conclusions

This study elucidates the possible mechanisms by which a coating of seaweed extract *C. tomentosum* acts to reduce browning in fresh-cut apples. At a molecular level, the coating delayed the stimulation of the encoding genes for the main browning related enzymes namely PPO, POD and PAL. The coating effect is visible since day 0, where *mdPPO* gene expression was considerably lower in apple slices coated with the seaweed extract than in control samples. In the same period, was not possible to detect any amplification signal for *mdPAL* and *mdPOD*. Only after a period of 10 d of storage, the levels of transcripts in the seaweed extract samples group reach identical values to control samples at day 5, suggesting a delay in gene expression. The coating functionality was confirmed by the activity of PPO and POD that followed the same

trend as the gene expression results, which lead to a lower browning development in fresh-cut apples coated with the seaweed extract.

Appendix C. Supplementary data **Chapter IV** (page 155).

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CHAPTER V

The effects of *Codium tomentosum* extraction conditions influencing on anti-browning functionality

The effects of *Codium tomentosum* extraction conditions influencing on anti-browning functionality

Abstract

The application of *Codium tomentosum* extract as a coating on fresh-cut apple inhibits browning. Surface browning is a result of the oxidative processes that occur in fresh-cut fruits, and it is commonly the limiting factor determining shelf-life. In order to optimize extraction conditions for maximum functionality and understanding further the mechanisms underpinning anti-browning functionality, extracts of *C. tomentosum* were produced under different conditions and characterized. The extraction conditions were defined using a Box-Behnken Design, and the impacts of time (0 – 180 min), temperature (20 – 80 °C) and pH (3 – 10) on the extract properties were studied. Response variables giving the best-explained variances (R^2) were analysed using Response Surface Methodology. Extraction time longer than 120 min with a pH range of 6 – 10 resulted in a higher extraction yield. The extract functionality was mainly influenced by extraction temperature, suggesting that applying temperatures over 60 °C produced extracts with higher efficacy to inhibit browning. Overall, this study provides evidence that even under relatively intense extraction conditions, *C. tomentosum* has the potential to inhibit browning in fresh-cut apples.

Keywords: Seaweed extracts; *Codium tomentosum*; Extraction conditions; Anti-browning; Fresh-cut fruits

1. Introduction

Marine macroalgae or seaweeds are known for the production of relevant bioactive compounds like antimicrobial and antioxidants, mostly produced as a defence response to the extreme conditions to which they are exposed in tidal zones (Silva et al., 2020). *Codium tomentosum* is a green and edible seaweed that can be found in tidal zones of the Northeast Atlantic Ocean, from the British Isles to Azores and Cape Verde. Despite its wide distribution and biomass availability, published studies with *C. tomentosum* extracts or secondary metabolites are mainly limited to its potential for medical and biotechnological applications (Silva et al., 2020, 2021). Only recently, studies have emerged to demonstrate the exploitation of *C. tomentosum* extracts for food science applications, namely its incorporation into edible coatings and film formulations (Augusto et al., 2018; Augusto et al., 2016). Augusto et al. (2016) observed that fresh-cut 'Fuji' apples immersed in an extract of *C. tomentosum* showed lower browning rates (about 40 %), whereas apples coated with *Fucus spiralis* and *Bifurcaria bifurcata* seaweed extracts showed higher browning rates of 80 % and 60 %, respectively. These studies investigated the incorporation of the extract in alginate and chitosan edible films (Augusto et al., 2018), and noted changes in mechanical and barrier proprieties. The incorporation of the extract into films decreases solubility, water vapor permeability and elasticity of the alginate-based films. In contrast, the solubility, elasticity, and rupture strength of chitosan-based films were enhanced by extract incorporation. In both studies, as well in Chapter III, the hydro-ethanolic extract of *C. tomentosum* and the aqueous extract of *C. tomentosum* in Chapter II and IV was obtained by conventional solid-liquid extractions (SLE) which is a simple process to design, operate and scale-up (Félix et al., 2020).

Solid-liquid extraction is commonly used for the extraction of bioactive compounds from seaweeds (Cacace & Mazza, 2003; Catarino et al., 2019). The extraction rates of target components in the seaweed are mainly dependent on the characteristics of the solvent as well

as the extraction temperature and pH (Cacace & Mazza, 2003; Félix et al., 2020). In Augusto et al. (2018) and Augusto et al. (2016) and in Chapter III, *C. tomentosum* extractions were performed using a solvent composed of 75 % deionized water and 25 % ethanol (v/v) employing seaweed to solvent ratio of 6.6 % (w/v), extraction time of 6 h at room temperature or 3 h in the work detailed in Chapter III. Considering food security and cost, hydroethanolic extractions are widely accepted for the extraction of natural products for industry (Félix et al., 2020). According to the European Directive 2009/32/EC, the use of ethanol as an extraction solvent has to comply with the requirement of minimal quantities present in the final product presenting no danger to human health (Directive 2009/32/EC, 2009). Thus, despite the approved use of ethanol, the prospect of its total replacement by a "cleaner solvent" like water ought to be explored. Additionally, in preliminary studies it was observed that fresh-cut apple slices immersed in a solution of 0.5 % (w/v) of aqueous extract had similar browning rates as samples coated with the hydroethanolic extract (not published), results also corroborated by the results described in Chapter IV (see section 3.1), where the aqueous extract of *C. tomentosum* prevented the development of browning in fresh-cut apples.

The optimization of extraction parameters is critical to the production of extracts possessing maximum anti-browning functionality. Traditionally, optimization studies are designed using a one-dimensional method, where only one factor is varied keeping all other factors constant. This is a time-consuming and expensive approach (Félix et al., 2020; Liu et al., 2019; Mohammed et al., 2020). Design of Experiments (DoE) can be used to reduce the number of experiments without compromising on the quality of the result, by the simultaneous variation of all factors in the study (Mohammed et al., 2020). When working with independent factors like those that are used in extraction, a statistical tool like Response Surface Methodology (RSM) can help evaluate the effects of each variable as well as the interaction between the variables. The Box-Behnken Design (BBD) is a useful methodology to specify the experimental conditions to cover

a specified range (Ferreira et al., 2007). BBD has also been successfully used to optimize the extraction of the phenolic compounds phlorotannins from *Fucus vesiculosus* (Catarino et al., 2019), antioxidant compounds from *Grateloupia turuturu* (Félix et al., 2020) and *Ascophyllum nodosum* (Liu et al., 2019). In these studies, the effects of solvent-to-solid ratio, solvent concentration, time and temperature of extraction (Catarino et al., 2019; Félix et al., 2020; Liu et al., 2019), and pH (Félix et al., 2020) on the response variables were evaluated.

Thus, the present Chapter study builds on the earlier work Augusto et al. (2018, 2016) and on the Chapters II-IV of the present thesis, and aims to obtain an aqueous extract of *C. tomentosum* with identical proprieties as the hydroethanolic extract produced by Augusto et al. (2016) and in Chapter III and IV to reduce browning in fresh-cut apples. Furthermore, this study gives insights into the effects of time, temperature, and pH on the extraction of *C. tomentosum,* that has not been reported so far. This study provides key information needed to obtain the best performance of a marine-based anti-browning coating which can potentially be used to extend shelf-life of fresh-cut fruits.

2. Methods

2.1. Raw materials and reagents

Dried *Codium tomentosum* (particle size with an average of 1.5 mm) was obtained from a Portuguese seaweed supplier (ALGAplus, Ílhavo). 'Fuji' apples were obtained from a local supplier in Torres Vedras, Portugal (Campotec S.A.) and stored at 4 ˚C before use. All reagents used for analytical procedures were analytical grade.

2.2. Extraction Procedure

Following earlier works on the production of *Codium tomentosum* extracts (Augusto et al., 2018, 2016) and also adopted in Chapter IV (see section 2.1), the process parameters, as well as the seaweed biomass to solvent ratio, were established in this work. Six sequential steps were employed to obtain the extract in a powder form: solid-liquid extraction with a seaweed biomass

to water ratio of 6.6 % (w/v) followed by centrifugation (2,000 g, 10 min, 4 ˚C), filtration through a Buchner funnel (paper filter 20 – 25 µm) and freeze drying the filtrate at -80 ˚C. The extraction conditions investigated in this study, which are detailed in Table 5.1, include variation of time (0 – 180 min), temperature (20 – 80 °C) and pH (3 – 10). In this study, the solid to solvent ratio was kept constant due to the sweeling proprieties of the dried seaweed, with the fixed ratio was possible to achieve an adequate solvent penetration in the dried seaweed allowing to reach an equilibrium between both interfaces as detailed bellow (see section 3.1). The extractions were performed in a glass vessel, 9 cm in height and 7.5 cm in diameter. To promote mixing, a propeller (3 cm ø) was used at 1500 rpm to avoid external mass transfer effects. The extraction time excluded an initial 5-min period for the temperature and pH adjustments (with 1 N HCl or 1 M NaOH) to settle and become uniform throughout the mixture. Each extraction condition was studied in triplicate (n = 3), resulting in a total of 45 extracts. A flowchart indicating the extraction conditions and analyses performed on the extracts is given in Figure 5.1.

Table 5.1. Box-Behnken experimental design matrix, symbols, coded variables, and values for the considered independent variables (X_1: Time in min; X_2: Temperature in °C; X_3: pH). [Z] Central point.

Coded variables and variables values						
Run	***X₁***	***X₂***	***X₃***	**Time**	**Temperature**	**pH**
1	-1	-1	0	0	20	6.5
2	1	-1	0	180	20	6.5
3	-1	1	0	0	80	6.5
4	1	1	0	180	80	6.5
5	-1	0	-1	0	50	3.0
6	1	0	-1	180	50	3.0
7	-1	0	1	0	50	10.0
8	1	0	1	180	50	10.0
9	0	-1	-1	90	20	3.0
10	0	1	-1	90	80	3.0
11	0	-1	1	90	20	10.0
12	0	1	1	90	80	10.0
13[z]	0	0	0	90	50	6.5
14[z]	0	0	0	90	50	6.5
15[z]	0	0	0	90	50	6.5

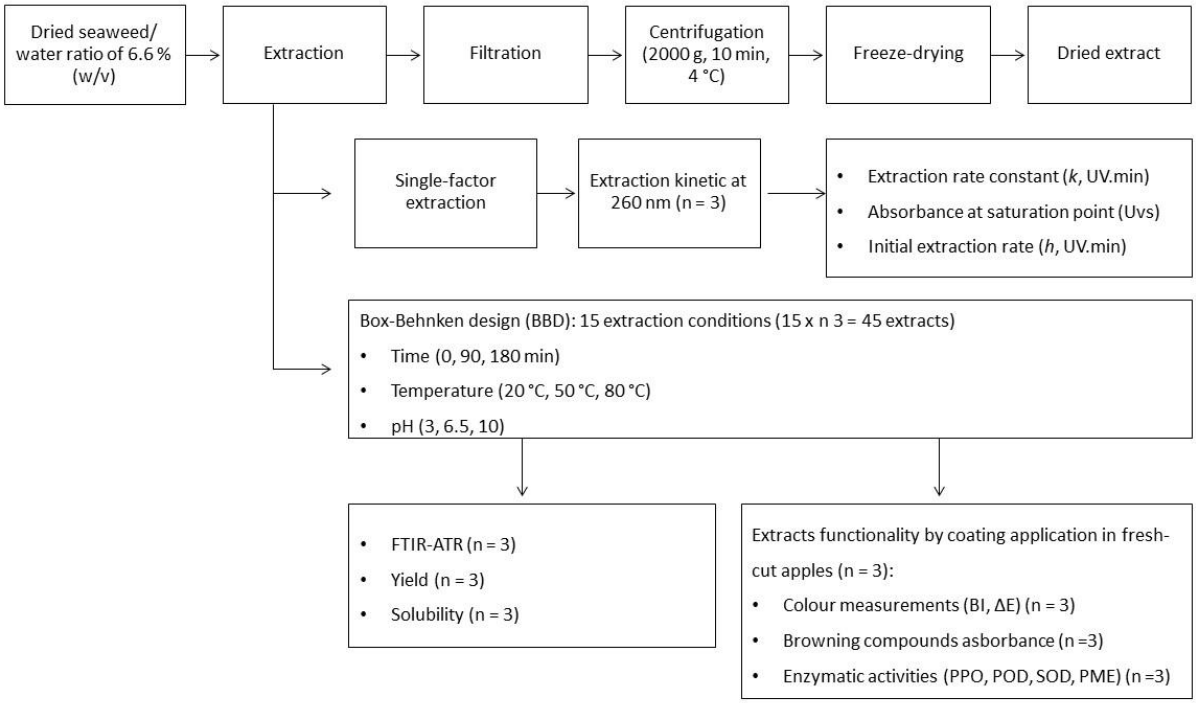

Figure 5.1. Flowchart of the experimental design.

2.3. Second order model

The extraction kinetics was modelled as a second order process (Da Porto and Natolino, 2018; Nie et al., 2021). The progress of extraction was followed by measuring absorbance at 260 nm (which gave maximum absorbance based on a wavelength scan between 200 nm and 800 nm) for 360 min. Aliquots of 200 µL were collected at 15 min intervals during the first 60 min, followed by collection at 30 min intervals until the end of the experiment. The absorbance was measured in a 96-well UV plate reader (Spectrophotometer Evolution 201, Thermo Scientific) (Venkatesan et al., 2019). The amount of liquid extract used for the analyses was kept to a minimum to avoid significant changes in the extraction volume. A total of three independent extractions were performed (n = 3).

The linearised model presented by Nie et al. (2021) (equation 1), was applied to determine the second-order extraction rate constant (*k*, UV.min), by measuring the extract absorbance at saturation (UV_s) and the initial extraction rate (*h*, UV.min) (equation 2).

$$\frac{t}{UV_t} = \frac{1}{k.UV_s{}^2} + \frac{t}{UV_s} \quad (1)$$

where, UV_t is the extract absorbance (UV) at any time *t* (min). The plots of time (min) against *t/UV* gives a gradient of 1/*UV* and an intercept of 1/*k*. UV_s^2, allowing the determination of *k* and absorbance at the saturation point. When *t* approaches 0, *h*, can be defined as (eq. 2)

$$h = k.UV_s{}^2 \quad (2)$$

2.4. Extraction yield and solubility

Extraction yield was determined based on the weights of the dried seaweed taken initially and the weight of the dried extract obtained (expressed as g of dried extract per 100 g dried seaweed, g 100 g^{-1}). The extract solubility was determined as follows Tang et al. (2019): 50 mg dried extract (*m*) was dissolved in 10 mL of deionized water, and centrifuged at 3 000 rpm for 10 min; the pellet collected was dried at 108 ˚C (m_1) and the solubility was calculated as follows:

Solubility (g 100 g^{-1}) = 100 – [(m_1 x 100) /*m*] (3)

2.5. FTIR-ATR

The Fourier transform infrared spectroscopy attenuated total reflection (FTIR-ATR) technique was used to evaluate the functional groups of the dried extracts and to detect possible differences between extraction conditions. The FTIR analysis were carried out using an Alpha-P FTIR-ATR spectrometer (Bruker Optik GmbH, Ettlingen, Germany) as described in Augusto et al. (2018). Each sample was analysed three times.

2.6. Extract functionality as anti-browning agent for fresh-cut apples

Guided by previous works, where a coating containing 0.5 % (w/v) of *C. tomensoum* extract was used to extend the shelf-life of fresh-cut apples (Augusto et al., 2016), as well by the results of Chapters II-IV, the functionality of the extracts produced was evaluated.

Fresh-cut 'Fuji' apples (*Malus domestica)* were cut into circular discs, 1.5 cm diameter, and on an average weighing 2 g. Coating solutions were prepared using the extracts produced as described in section 2.2., with a final concentration of 0.5 % (w/v). A total of 45 coating solutions were prepared (one solution from each extract replica). Maintaining a ratio of 6.6 % (w/v) of apple biomass to the coating solution, six apple discs were dipped for 5 min in the coating solutions. In the case of control samples, deionized water without any extract was used. In order to promote browning development, the coated samples were kept at room temperature, and exposed constantly to light (laboratory light intensity) for 3 hours. The functionality of the coating solutions was determined by measuring colour and recording the absorbance at 440 nm to detect browning compounds and enzymatic activities. For each of these analyses, a total of three apple disks per coating solution were analysed (n = 3).

2.6.1.Physicochemical analysis

Colour measurements were made using a colourimeter Konica Minolta CR 400 and analysed according to the CIELab system. To avoid irregularities in readings, each sample was analysed in five distinct areas, and the mean value was considered for colour calculations (n = 3). Colour variation (ΔE*) was estimated between the control and an individual sample after 3 hours incubation using the equation described by Lante et al. (2016). Superficial browning index was determined as described in Chapter II (section 2.4).

2.6.2.Browning compounds absorbance

The absorbance of browning compounds was determined at 440 nm, adapting the procedure of Rasouli & Koushesh Saba (2018) for smaller samples. Browning compounds were extracted from

1 g of apple slice and mixed with 7 mL of deionized water. After 1 hour of incubation at room temperature, the mixture was centrifuged at 1,000 g for 5 min (5810R centrifuge, Eppendorf, Germany). From the supernatant, 2.5 mL were collected and mixed with 3.75 mL of 96 % ethanol. The mixture was submitted to a second centrifugation and the supernatant was read at 440 nm in a microplate reader (Epoch2 Microplate reader, Biotek, USA). Three apple slices per treatment were evaluated (n = 3).

2.6.3.Enzymatic activities

For polyphenol oxidase (PPO) and peroxidase (POD) determination, the protocol described in Chapter IV (see section 2.5) protocol was adapted for smaller samples. Thus, 2 g of sample was homogenised with a two-fold amount of extraction buffer containing 50 g L^{-1} of polyvinylpyrrolidone (PVP) in 50 mM sodium phosphate buffer (pH 7.0), following the remaining steps as described in the Chapter IV, section 2.5. Enzymatic reactions were followed at 400 nm (for PPO) and 470 nm (for POD), and performed in a multi-well plate, leading to adjustments in reaction for a total volume of 300 µL. Enzyme activities were expressed as U mg^{-1} of protein. Protein was quantified by following Bradford methodology (Bradford, 1976). A total of three samples per treatment were used for enzyme determinations (n = 3).

For superoxidase dismutase (SOD) determination, the protocol described in Chapter IV (section 2.5) was adapted for smaller samples. To that, 10 mL of extraction buffer [10 mL of 0.1 M sodium phosphate buffer (pH 7.5) containing 20 g L^{-1} of polyvinylpyrrolidine (PVP), 2 mM dithiothreitol (DTT) and 0.1 mM ethylenediamine tetraacetic acid (EDTA)] was used to homogenise a sample with a weigh of 2 g. The enzymatic extract was centrifugated (30 min, 14,000 g, 4 °C) and the supernatant collected for enzymatic activity determination. Specific activity expressed as U g^{-1} fresh weight (FW), was assessed by absorbance at 560 nm. In a 96-well plate, 150 µL of subtract mixture prepared with 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT (nitrotetrazolium blue chloride), 10 µM EDTA and 2 µM riboflavin was added to 50 µL of

enzyme extract. After 10 min incubation under fluorescent light, the absorbance was measured, and the enzyme activity determined by the inhibition 50 % NBT photoreduction. A total of three samples per treatment were used for enzyme determinations (n = 3).

For pectin methylesterase (PME) activity determination, the methodology was adapted from previous works of Liu et al. (2016) and Delgado-Reyes et al. (2001). The extraction conditions used for SOD were replicated but using a buffer containing 1.5 M of NaCl with 2.5 % (w/v) of polyvinylpyrrolidone (PVP). The PME reaction was followed spectrophotometrically (Biotek, SynergyH1, USA) at 35 °C (610 nm, 4 min). To a volume of 50 µL of enzyme extract (pH 7.5), 15 µL of 0.01 % bromothymol blue (in 0.003 M sodium phosphate buffer, pH 7.5) and 235 µL of the substrate (5 g L^{-1} citrus pectin, pH 7.5) were added. Results were expressed as U mg^{-1} protein. A total of three samples per treatment were used for enzyme determinations (n = 3).

2.7. Experimental design and statistical analysis

A three-level-three-factor Box-Behnken design (BBD) was applied to determine the best combination of extraction variables to produce *C. tomentosum* extract with efficacy to inhibit browning in fresh-cut apples. There were considered three variables: X_1- Time (min), X_2- Temperature (°C) and X_3- pH, for which each variable was coded at levels -1, 0 and 1 (Table 5.1). The levels of these three variables were set according to single factors tests in previous works (not published). The model design includes three replicates at the central point (experiments 13^z, 14^z and 15^z), which were randomly spread within the experiments, resulting in a total of 15 runs. The use of BBD allowed a reduction of 12 experiments, which in a full factorial design would have resulted in a total of 27 experiments.

A generalized second-order polynomial model was obtained and applied in the response surface analysis and explained by equation 4 (Catarino et al., 2019):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i \neq j=1}^{k} \beta_{ij} X_i X_j \quad (4)$$

where Y is the response variables: extraction yield, extracts solubility, browning compounds absorbance, enzymatic activities, and colour parameters; β0, βi, βii and βij are the intercept, linear, quadratic and interactive coefficients of the regression, respectively; and X*i* and X*j* are the independent variables ($i \neq j$). The independent variables effect on the response variables were evaluated by the models.

Experimental design, response surface methodology and analysis of variance (ANOVA) were carried out in Minitab 20 (State College, Pennsylvania, USA) and STATISTICA (StatSoft, Inc., version 10). Coefficient of determination (R^2) and the lack-of-fit test at significant level of 5 % were used to evaluate the model adequacy. The effects of independent variables and their interactions in the response variable were visualised by response surface contour plots.

3. Results and discussion

3.1. Kinetic model

To narrow the range of selected factors to be used in the BBD experiment, a preliminary single-factor experiment was performed. The single-factor experiment with time as the independent variable (see section *2.3.*), resulted in a linear relationship between t/UV_t versus t that was fit to a second-order model (Supplementary data, Figure S5.1). The successful fit ($R^2 = 0.950$) suggests monitoring the seaweed extraction through absorbance at 260 nm as an adequate indicator of extraction kinetics. Up to now, in studies where *Codium* sp. is used, extraction variables like time and temperature are fixed variables (Augusto et al., 2018; Rodrigues et al., 2015; Silva et al., 2021, 2020). Augusto et al. (2018) fixed a six-hour extraction at room temperature, Rodrigues et al. (2015) set a 24-hour extraction at 50 °C, and Silva et al. (2021, 2020) selected an overnight extraction at room temperature. And, in Chapters II - IV of the present thesis a 3-hours extraction at room temperature was fixed. However, in none of the previous studies, the extraction kinetics were evaluated. This is the first study where the kinetics of extraction of *Codium tomentosum* is being modelled. A kinetic model was obtained to determine the kinetic parameters, such as

extraction rate constant (*k*), absorbance at saturation (UV_s) and initial extraction rate (*h*) as mentioned earlier in section 2.3 (also see Figure S5.1 A, Supplementary data).

The initial extraction rate (*h*) was 0.0594 Units of absorbance (U abs), and for every minute the extract absorbance (*k*) increased about 0.0035 U abs until reaching saturation at 4.11 U abs (UV_s). The extract absorbance increased with time, revealing a good penetration of solvent into the dried seaweed. The contact between the seaweed and the solvent induces changes in the concentration gradient leading to the diffusion of solutes from the cells to the solvent until an equilibrium is reached between both the interfaces. Nie et al. (2021) reported a similar relationship between time and fucoxanthin extraction using the brown seaweed *Sargassum fusiforme*. Usually, after attaining extraction equilibrium, especially under long-term extractions, a degradation in the compounds can be observed (Félix et al., 2020; Nie et al., 2021). To avoid degradation due to long extraction periods and observe the sole effect of time on the extracts, the absorbance at 260 nm in Figure S5.1B (Supplementary data, Figure S5.1 B), where at 180 min the absorbance reaches a stable plateau, was selected for the subsequent experiments. This results, also indicate that the external resistance as mass transfer phenomena may be neglected, since the extraction reach the stationary phase that can be observed in Figure S5.1B.

3.2. Experimental outcomes

The influence of extraction conditions using an SLE methodology of *C. tomentosum* was studied following the scheme shown in Figure 5.1. After extraction, the extracts were analysed by FTIR-ATR, water-solubility, yield, and the extracts' ability to decrease superficial browning in fresh-cut apples. Table 5.1 summarizes the processing conditions applied in the extractions, and the corresponding experimental results obtained are presented in Table S5.1 (Supplementary data, Table S5.1). The experimental results were used for the RSM model construction to understand the effect of extraction conditions on the extract proprieties. Briefly, extraction yield values

ranged between 61.51 ± 3.92 and 67.72 ± 3.56 g 100 g^{-1} of dried seaweed (Supplementary data, Table S5.1) corresponding to extraction conditions of 0 min, 50 °C, pH 3.0 and 180 min, 80 °C and pH 6.5 respectively. Extracts with an extended extraction period of 180 min, at 20 °C and pH of 6.5 presented the lower values of water solubility (63.00 ± 4.00 g 100 g^{-1}), in contrast with a solubility of 85.07 ± 5.80 g 100 g^{1} determined in the extracts produced at 80 °C and pH 6.5. In relation to the experimental assays with fresh-cut apples (described in 2.6), the observed extraction conditions with higher effectiveness to control browning were very distinct in each of the analysis. The lower browning compounds absorbance was determined in samples coated with an extract produced at 50 °C and pH 6.5 (0.095 ± 0.002). Nevertheless, extracts that resulted from experiments at 180 min, 20 °C and pH 6.5 granted lower browning index (BI) and less colour changes (ΔE*) in fresh-cut samples (BI = 36.56 ± 6.95 and ΔE* = 4.17 ± 2.83). The higher values of POD and PPO activities were determined in fresh-cut samples coated with extracts produced at 50 °C, pH 6.5 for 90 min (POD = 0.139 ± 0.024 U mg $protein^{-1}$), and 180 min at 80 °C (pH 6.5) (PPO = 0.054 ± 0.01 U mg $protein^{-1}$), respectively. High values of SOD (90.51 ± 10.15 U g^{-1} fresh weight) were observed in extracts produced at 80 °C, pH 6.5 (0 min). And, lastly, the lower values of PME were determined in fresh-cut samples coated with a solution containing extracts which resulted from an extraction with a duration of 90 min, 50 °C and pH of 6.5 (0.035 ± 0.01 U mg $protein^{-1}$).

3.3. FTIR-ATR Analysis

FTIR-ATR analysis was performed to identify the different functional groups present on the extracts and to establish possible interferences of the extraction conditions on the FTIR profile. A visual representation of the obtained FTIR spectra can be observed in Figure S5.2 (Supplementary data, Figure S5.2). FTIR spectra can be divided in three main regions: 1) 4000 – 2400 cm^{-1}, 2) 2400 – 1500 cm^{-1}, and 1500 – 400 cm^{-1}. The main peaks observed in each region are described below.

Region 1: The association of a strong and broad band around 3526 cm^{-1} with a moderate peak around 1100 cm^{-1} suggest the presence of an alcohol molecule in all samples (Coates, 2004). The medium bands observed at ~2929 cm^{-1} and 2850 cm^{-1} can be a consequence of –CH bond stretching, typical from CH_3 and CH_2 groups (Coates, 2004), suggesting a molecule with an aliphatic fragment or centre. The same functional groups were also reported on ground samples of *Codium fragile* (Agatonovic-Kustrin et al., 2020), and in hydroethanolic extracts of *C. tomentosum* (Augusto et al., 2018). However, these peaks may be caused by moisture, since the water molecule contains O-H bonds that give a stretching vibration signal in the same wavelengths (Lim et al., 2014).

Region 2: The double-bound stretching of the functional group amide (C=O band) can be observed at 1635 cm^{-1}. In the same spectra region, Fawzy (2020) observed a similar peak in dried *C. vermilara* samples and Augusto et al. (2018) in hydroethanolic extracts of *C. tomentosum*. However, it is also in this region that the unsaturated fatty acids C=O stretching occurs, suggesting the presence of fatty acids in the extract composition. In the species *C. tomentosum*, about 55 % of fatty acids are unsaturated, namely 20:5 and 22:6 species, both long-chain polyunsaturated fatty acids (PUFAs) (Costa et al., 2015).

Region 3: There were observed asymmetrical bending at 1406 – 1423 cm^{-1} and symmetrical bending at 1339 – 1374 cm^{-1}, both common in methyl groups (CH_3 and CH_2) (Lim et al., 2014). A third peak in the region 1088 cm^{-1} can be assigned to the stretching vibration of sulphoxides (S=O) from the sulphate group (SO_4) (Lim et al., 2014). The presence of these two functional groups in the seaweed extracts, strongly suggests the existence of sulphated polysaccharides in the composition of the extract (Agatonovic-Kustrin et al., 2020). These polysaccharides are components of the cell walls and are mostly linked to brown seaweeds (Agatonovic-Kustrin et al., 2020), albeit already described in green seaweeds, inclusively *Codium* species (Augusto et al., 2018; Fawzy, 2020). Further, considering the region between 1500 – 400 cm^{-1}, which

corresponds to the carbohydrate region, the high-intensity bands that can be seen in the ATR spectra, possible reveal high carbohydrate content in the seaweed extracts (Agatonovic-Kustrin et al., 2020). Although a more detailed study, including chromatographic and spectroscopic techniques, is necessary to corroborate these findings and to better describe the extracts composition. Nuclear magnetic resonance spectroscopy (NMR), a spectroscopy technique usually used for elucidation of structures of polysaccharides from seaweeds sources (Sánchez et al., 2022), can be used to distinguish between the metabolic variations of the analysed extracts, and to identify the main metabolites present in the seaweed extract which are responsible for the observed anti-browning action of the extract.

FTIR analysis thus confirmed that, within the analysed extracts, no apparent changes on the ATR spectra were observed, being possible to identify amide, methyl, and sulphate groups as the main groups present in the seaweed extracts.

3.4. Effect of extraction variables

The BBD was utilized to investigate the effect of various extract conditions, such as time, temperature, and pH in the seaweed extract functionality to prevent browning development. The raw data respecting all the experiments made under the different extraction conditions are available in Table S5.1 (Supplementary data, Table S5.1). The contour plots were investigated and the p-values from ANOVA were used to validate the importance of individual factors and the interaction between factors (Tables 5.2 – 5.4). From the analysed response variables (extraction yield, extracts solubility, browning compounds absorbance, colour parameters and enzymatic activities) the models that showed the best-explained variances were chosen (higher R^2, with lack-of-fit p-value > 0.05). From the analysis of variances, the models that showed the best fit were extraction yield (Figures 5.2 A-C, Table 5.2), browning compounds absorbance at 440 nm (BC 440 nm, Figures 5.3 A-C, Table 5.3) and peroxidase activity (Figures 5.4 A-C, Table 5.4). The analysis of variances of the remaining response variables can be consulted in Tables

S52 – 5.7 (Supplementary data, Tables S5.2 – 5.7). The experimental data were analysed by multiple regression. The response and experimental variables were related by the following regression equation:

$$\textit{Extraction yield} = 59.67 + 0.0095\ X_1 - 0.1009\ X_2 + 1.632\ X_3 - 0.000012\ X_1^2 + 0.000715\ X_2^2 - 0.1025\ X_3^2 + 0.00017\ X_1X_2 - 0.00092\ X_1X_3 + 0.00374\ X_2X_3$$

$$\textit{BC 440 nm} = 0.10096 - 0.000041\ X_1 - 0.000068\ X_2 + 0.00091\ X_3 + 0.000000\ X_1^2 - 0.000000\ X_2^2 - 0.000104\ X_3^2 - 0.000000\ X_1X_2 + 0.000006\ X_1X_3 + 0.000000\ X_2X_3$$

$$\textit{POD activity} = -0.0849 + 0.000039\ X1 + 0.00348\ X_2 + 0.0229\ X_3 - 0.000002\ X_1^2 - 0.000037\ X_2^2 - 0.001912\ X_3^2 + 0.000003\ X_1X_2 + 0.000026\ X_1X_3 - 0.000040\ X_2X_3$$

In the three response variables, a high correlation coefficient was observed ($R^2 > 77$ %) indicating that the models were successful in fitting the experimental data. From the lack-of-fit analysis, the contribution of unknown factors to the response variable is not significant ($p > 0.05$) (Tables 5.2 – 5.4). Despite the good fitness of models to the data, these models were not designed to integrate and predict extraction conditions for overall performance, being necessary to discuss simultaneous each response variable. A separate model comprising the extraction yield and extracts efficacy is needed for a more accurate variables prediction (Saravana et al., 2018).

3.4.1.Effect of time

To study the effect of time on the response variables, from the BBD resulted 3 different extraction times: 0 min, 90 min and 180 min. Time had a positive effect on the yield, suggesting that longer extraction periods allow a higher compounds recovery, as can be seen by the red coloured areas in Figures 5.2 A-B.

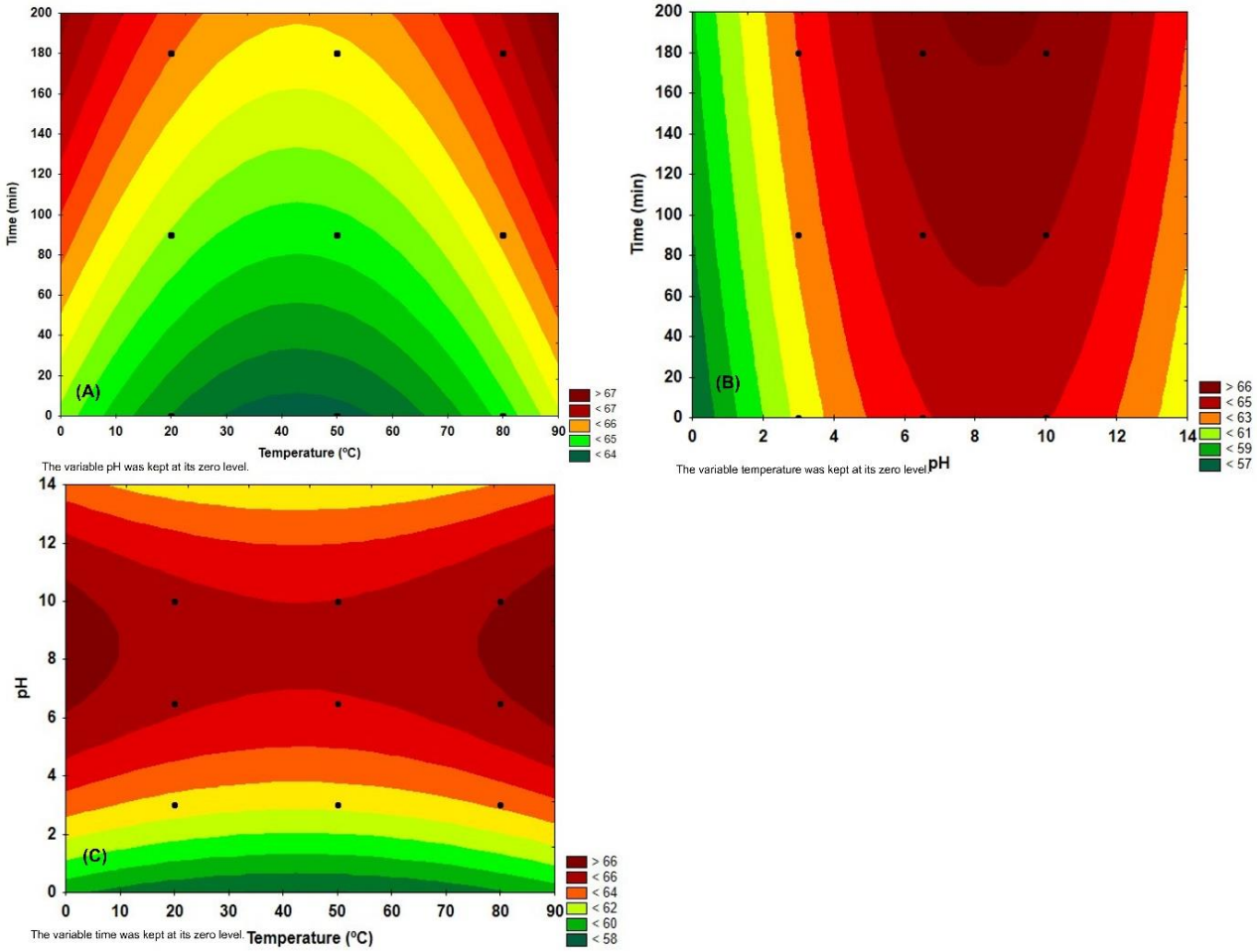

Figure 5.2. Contour plots showing combined effects of time (min), temperature (°C), and pH on the extraction yield of *Codium tomentosum*.

The relevance of time on the extraction yield is evidenced by the analysis of variance (Table 5.2), where the effect of time is significant for the model ($p < 0.05$). It is usual to observe an increase of extraction yield with time, since extended extraction periods enable solubilisation of solutes, increasing the final extracted biomass (Félix et al., 2020). Although, a compromise between long extraction periods, yield and type of extracted compounds is essential. Recurrently, the degradation of the compounds is correlated with long extraction periods. The extraction of pectin from sunflower heads showed a positive correlation between time and yield (Peng et al., 2020), however, with a higher time of extraction the degradation of pectin occurred derailing the use of extended periods, preferring lower extraction yields but high-quality pectin.

Table 5.2. Analysis of Variance (ANOVA) for the Response Surface Quadratic Model of extraction yield of *Codium tomentosum*. Variables: X_1 – time (min), X_2 – temperature (°C), X_3 – pH; *significant terms ($p < 0.05$); SS- sum of squares; Df- degree of freedom; MS- mean square.

Source	SS	Df	MS	F-value	p-value
Model	32.59	9	3.62	4.56	0.055*
X_1	6.31	1	6.31	7.95	0.037*
X_2	0.75	1	0.75	0.94	0.376
X_3	15.86	1	15.86	19.98	0.007*
X_1X_2	0.84	1	0.84	1.06	0.350
X_1X_3	0.34	1	0.34	0.43	0.542
X_2X_3	0.62	1	0.62	0.78	0.419
X_1^2	0.03	1	0.03	0.04	0.848
X_2^2	1.53	1	1.53	1.92	0.224
X_3^2	5.83	1	5.83	7.34	0.042*
Error	3.97	5	0.79		
Lack-of-fit	2.19	3	0.73	0.82	0.590
Pure error	1.78	2	0.89		
Total	36.56	14			
S model	0.891				
R^2	89.14 %				

In the case of browning compounds absorbance (Figures 5.3 A-B; Table 5.3) and peroxidase activity of coated fresh-cut apples (Figures 5.4 A-B; Table 5.4), time played a relatively weaker effect on the efficacy of the extracts to decrease peroxidase activity and browning development on samples. The low impact of time on extracts efficacy may suggest that compounds of interest that are being extracted are rapidly extracted with water at initial process stages thus independent of time. Catarino et al. (2019) observed a similar effect of time on total phlorotannin content from *Fucus vesiculosus* extractions. The authors observed an extraction

equilibrium below the 60 min, without significant variations with the extension of time on the phlorotannin's recovery. Probably, in work presented in this chapter, the extract compounds that are being extracted are mainly proteins and polysaccharides (Augusto et al., 2018) which are easily extracted with water (Félix et al., 2020), contributing largely to the recovered biomass but without impact on the extracts functionality as observed in the lack of significance of time on browning compounds absorbance and peroxidase activity ($p > 0.05$).

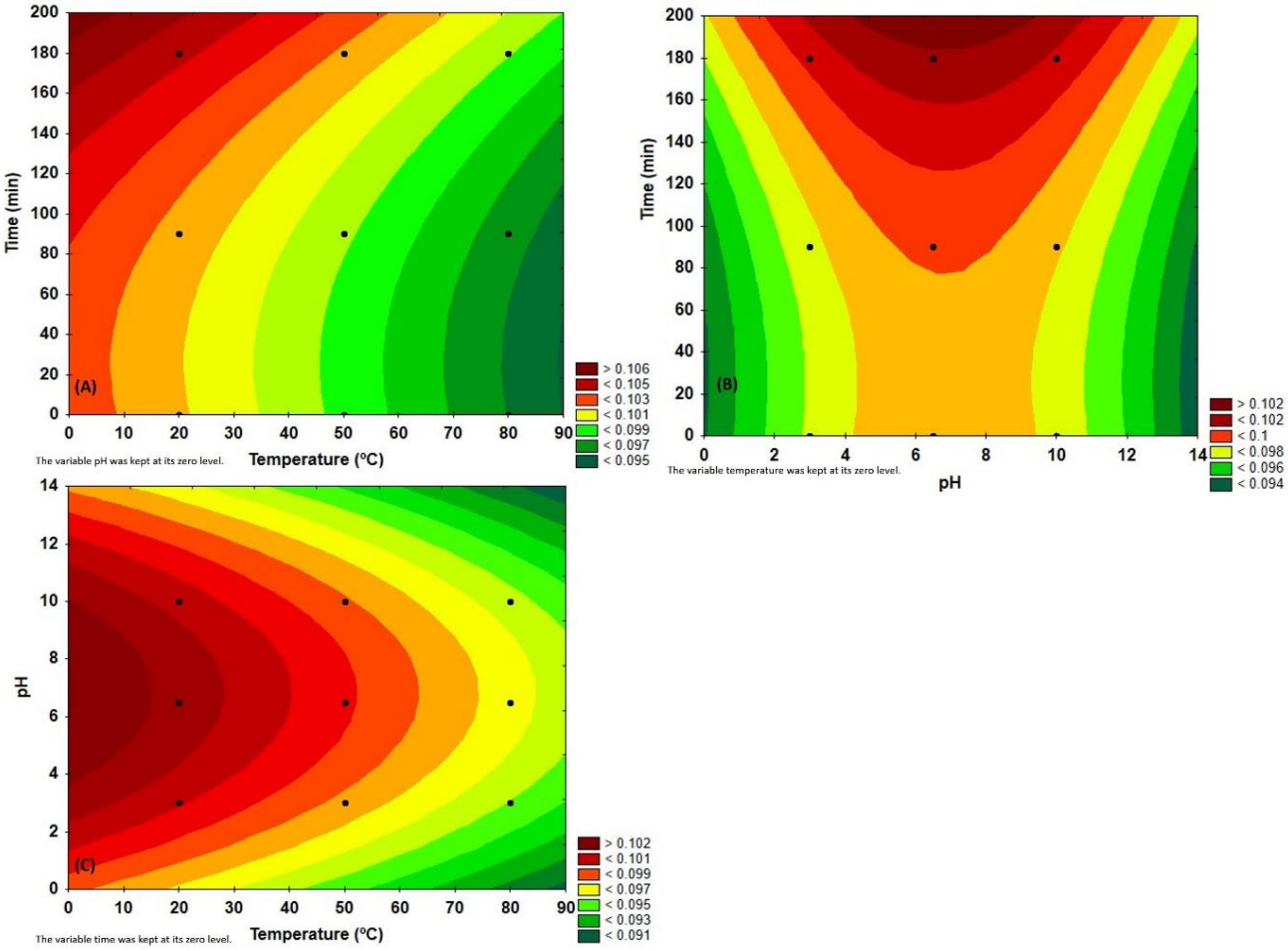

Figure 5.3. Contour plots showing combined effects of time (min), temperature (°C), and pH on browning compounds absorbance at 440 nm.

Table 5.3. Analysis of Variance (ANOVA) for the Response Surface Quadratic Model of browning compounds absorbance at 440 nm. Variables: X_1 – time (min), X_2 – temperature (°C), X_3 – pH; *significant terms ($p < 0.05$); SS- sum of squares; Df- degree of freedom; MS- mean square.

Source	SS	Df	MS	F-value	p-value
Model	0.000097	9	0.000011	1.78	0.000*
X_1	0.00002	1	0.00002	3.21	0.133
X_2	0.000054	1	0.000027	8.85	0.031*
X_3	0	1	0	0.08	0.794
X_1X_2	0	1	0	0	0.949
X_1X_3	0.000012	1	0.000012	2.01	0.215
X_2X_3	0	1	0	0	0.987
X_1^2	0.000004	1	0.000004	0.71	0.438
X_2^2	0	1	0	0.01	0.912
X_3^2	0.000006	1	0.000006	0.99	0.366
Error	0.00003	5	0.000006		
Lack-of-fit	0.000019	3	0.000006	1.15	0.497
Pure error	0.000011	2	0.000006		
Total	0.000128	14			
S model	0.0024657				
R^2	76.20 %				

3.4.2.Effect of temperature

As a result of the BBD, the effect of temperature on the extraction yield of *C. tomentosum* and in the extracts functionality was inspected at 20 °C, 50 °C and 80 °C (Figures 5.2 A, C; Figures 5.3 A, C; Figures 5.4 A, C). To avoid high energetic costs that are associated with the use of extraction temperatures below room temperature, 20 °C were chosen as the minimum temperature (Félix et al., 2020). For the same reason, and to avoid excessive solvent losses by evaporation, once at

100 °C, the mixture was near to the boiling point, 80 °C were chosen as the maximum temperature.

Contrary to expected, in the aqueous extraction of *C. tomentosum*, the temperature had a relatively lower effect on the extraction yield, without significant impact on the model ($p > 0.05$) (Table 5.2). As can be seen in Figures 5.2 A and C, with increasing temperature values, extraction yield rates are kept on the green areas of the figure, which correspond to extraction yield values lower than 65 %, never reaching values of maximum extraction yield. Usually, increasing temperature generate higher mass transfer and high solvent diffusion rates improving the recovery of biomass (Saravana et al., 2018), although in the present work this effect was not observed. In a single-factor experiment, the yield of phlorotannin extraction from *F. vesiculosus* was affected by temperature, but when integrated into a more complex experimental design which included solvent concentration and solid to liquid ratio variations, the temperature had no significant effect on phlorotannin extraction yield (Catarino et al., 2019).

Interestingly, Figures 5.3 A and C suggests an effect of temperature on the extract efficacy to decrease browning in fresh-cut apples. From the observation of the figures, lower values of browning compounds absorbance are obtained using extracts produced at higher temperatures, namely above 60 ˚C (greenish area of figures). The impact of temperature on the model is significant ($p < 0.05$; Table 5.3), revealing the importance of this variable to obtain extracts with anti-browning efficacy. Being peroxidase activity and fresh-cut apple browning related as discussed in the Chapter IV of the present thesis, a similar effect of the variable on the extract functionality to decrease peroxidase activity was expected. The temperature was also demonstrated to influence the extract ability to decrease POD activity in fresh-cut apples, but with a quadratic effect (X_2^2) on POD activity (Table 5.4, Figures 5.4 A, C). Even though the temperature had no significant influence on the yield of extraction, interestingly it influenced the extracts efficacy. The observed shifts in extracts efficacy with temperature may be justified

by changes in compounds solubility, especially organic compounds, that in normal conditions would not be extracted with water at low temperatures (Félix et al., 2020). With higher temperatures, mass transfer phenomena become facilitated increasing compounds solubility promoting their collection in the final extract. This observation also suggests that compounds which are being extracted are thermoresistant once their efficacy is not compromised by the extraction temperature. Liking type of solvent, FTIR-ATR results (in 3.2.), bibliography (Augusto et al., 2018) and temperature resistance, these results reinforce the opinion about the extracts composition and their high composition in polysaccharides, which are commonly extracted at high temperatures (Saravana et al., 2018).

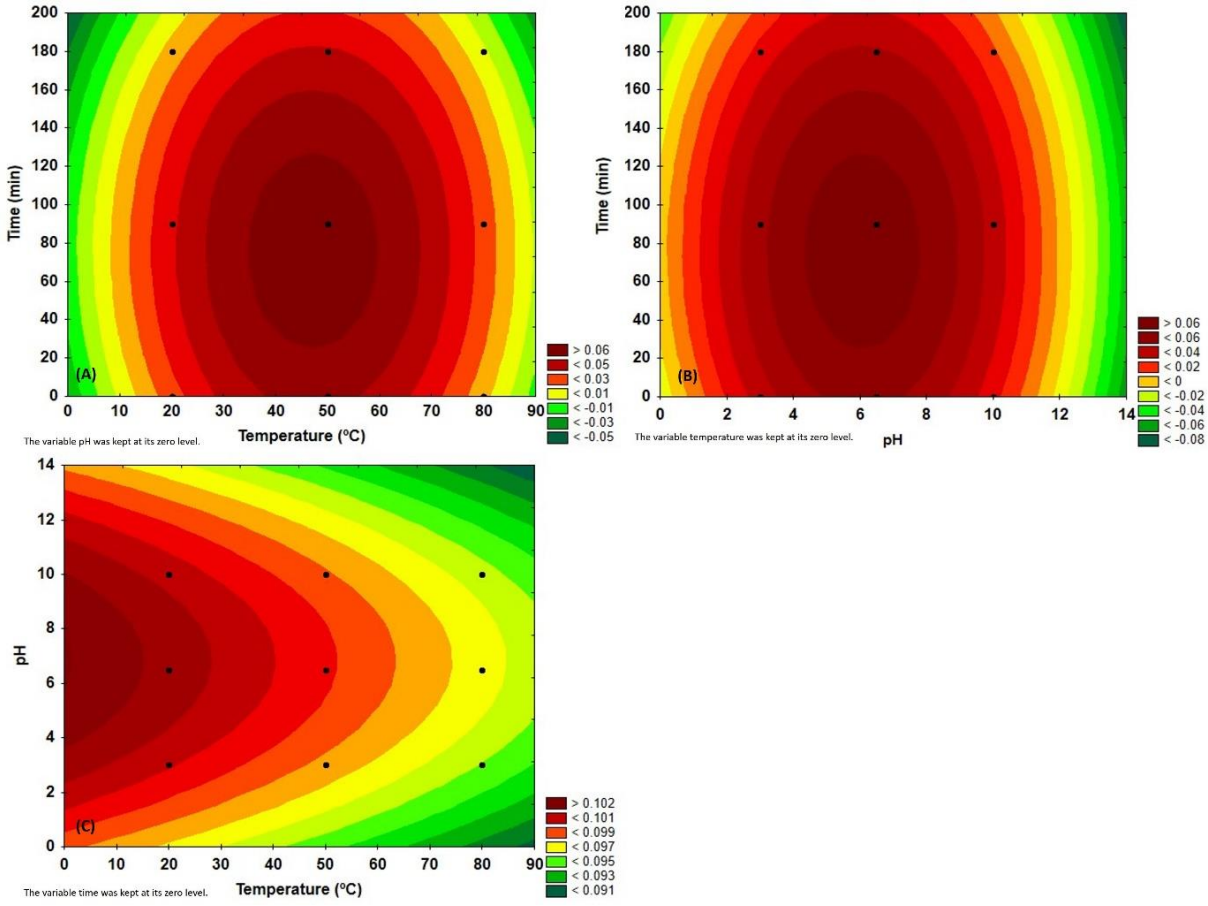

Figure 5.4. Contour plots showing combined effects of time (min), temperature (°C) and pH on peroxidase activity.

Table 5.4. Analysis of Variance (ANOVA) for the Response Surface Quadratic Model peroxidase activity. Variables: X_1 – time (min), X_2 – temperature (°C), X_3 – pH; *significant terms ($p < 0.05$); SS- sum of squares; Df- degree of freedom; MS- mean square.

Source	SS	Df	MS	F-value	p-value
Model	0.007763	9	0.000863	1.95	0.021*
X_1	0.000258	1	0.000258	0.58	0.480
X_2	0.000301	1	0.000301	0.68	0.448
X_3	0.000255	1	0.000255	0.58	0.482
X_1X_2	0.000206	1	0.000206	0.47	0.525
X_1X_3	0.000273	1	0.000273	0.62	0.468
X_2X_3	0.000071	1	0.000071	0.16	0.706
X_1^2	0.00123	1	0.00123	2.78	0.157
X_2^2	0.004025	1	0.004025	9.08	0.030*
X_3^2	0.002026	1	0.002026	4.57	0.085
Error	0.002215	5	0.000443		
Lack-of-fit	0.000875	3	0.000292	0.44	0.752
Pure error	0.00134	2	0.00067		
Total	0.009979	14			
S model	0.0210493				
R^2	77.80 %				

3.4.3.Effect of pH

Studies comprising the effect of pH on seaweed extracts are scarce, being the majority of works focused on other extraction parameters like time, temperature, solid-to-liquid ratio and solvent concentration (Félix et al., 2020). Due to the possible composition of the *C. tomentosum* extract, in the present work, pH was considered a relevant factor. The presence of polysaccharides in the extract could lead to a significant change on seaweed extract efficacy when submitted to different pH conditions.

Herein, pH was adjusted at three different levels: 3, 6.5 and 10 (Table 5.1). Considering the effect of pH on yield (Figures 5.2 B-C), which is characterized by a quadratic effect ($p < 0.05$, Table 5.2), the strongest effect was observed between pH 6 and 10, corresponding to the higher values of the extraction yield of about 67 % which can be justified by protonation and deprotonation phenomena's, increasing the yield of extraction of the seaweed *Grateloupia turuturu*. Félix et al. (2020) observed a linear increase of extraction yield with pH and temperature, suggesting that at alkalinity conditions the hydrolysis of cell wall components is more effective leading to the extraction of more compounds. The effect of pH in extracts functionality can be seen in Figures 5.3 B-C and 5.4 B-C. Although without a significant effect, the results suggest that in the pH range of 6 – 10, the extracts presented a low efficacy to prevent browning in fresh-cut apples, since the obtained values of absorbance are situated in the reddish area of the figure (Figure 5.3), which correspond to higher values of browning compounds absorbance. A similar response to pH variations was observed in peroxidase results (Figures 5.4 B-C). However, in peroxidase, the interaction between pH and time suggests a much more intensified response on the efficacy of the extract to decrease peroxidase activity. Observing Figure 5.4 B it is possible to note that, extractions performed during 90 min at 6.5 pH resulted in extracts with less capability to reduce POD activity, being these conditions the ones that allowed higher values enzymatic activity – 96 % more POD activity than the lower POD activity determined at 90 min, 80 ˚C, pH 3 (Supplementary data, Table S5.1). Despite the observed tendency, a single-factor study employing a wider range of pH, especially at alkali conditions may give a more assertive answer about the effect of pH in extracts efficacy.

4. Conclusions

The aqueous extraction of *Codium tomentosum*, a green edible seaweed with proven efficacy to reduce browning in fresh-cut apples, has been characterized regarding the effect of time, temperature, and pH on the yield of extraction, solubility, FTIR-ATR, and extracts functionality.

It was possible to demonstrate that extraction yield is mostly influenced by time and pH. Longer extraction periods and neutral to alkali pH allowed higher extracts recovery. In terms of extracts functionality, browning compounds absorbance and peroxidase activity were mainly influenced by changes in temperature. Fresh-cut apples coated with extracts which were produced above 60 °C present lower browning index, suggesting an advantage of applying extraction conditions beyond room temperature. More importantly, these findings allow definition of extraction conditions for maximum functionality without excessive costs associated with extraction optimization.

Appendix D: Supplementary data **Chapter V** (pages 156 – 164).

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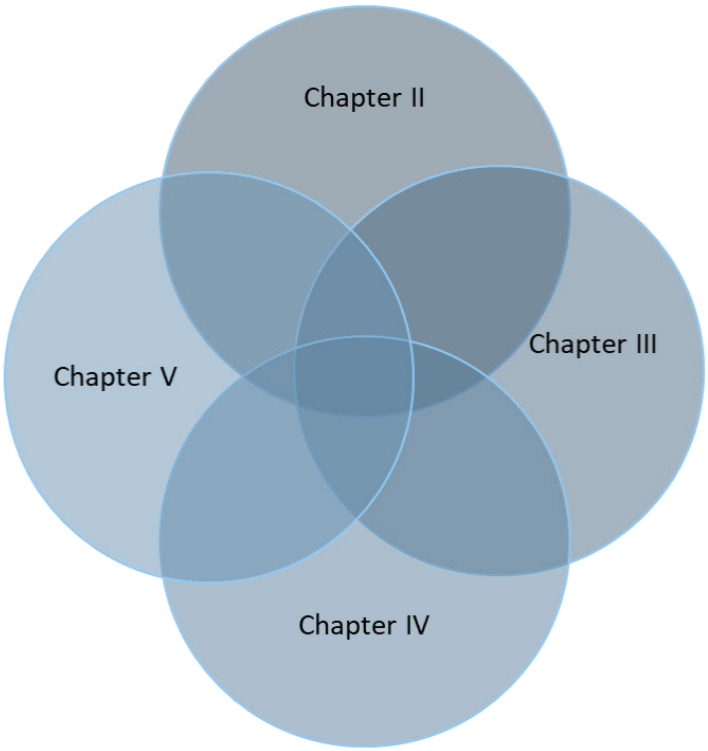

CHAPTER VI

General Conclusions and Future Perspectives

1. General Conclusions

People nowadays are more aware of the effect of their diet and lifestyle on health. The year 2021 was considered the International Year of Fruits and Vegetables by the United Nations (Bersamin et al., 2021), highlighting the importance of these products on human health. In the last decade, fresh-cut fruits and vegetables became an important way to meet consumers food requisites (Oliveira et al., 2015). Considering that one of the major requirements of modern consumers is to reduce the use of food additives, or their replacement with natural agents (Chen & Xu, 2019), the use of seaweed extracts, known for their composition in bioactive compounds, could fit the demand of the food industry to provide natural solutions to enhance the shelf-life of fresh-cut products. Thus, the overall aim of this thesis was to establish the potential of *C. tomentosum* extract to prevent browning development in fresh-cut fruits, namely in fresh-cut 'Fuji' apple and 'Rocha' pear varieties. Pears and apples were chosen for this study because these are the main fruit crops produced in the Western Region of Portugal (Almeida et al., 2017; Salta et al., 2010), and adding value to these two regional products can potentially make a significant impact on the economic development of this Portuguese Region (INE, 2021). In order to support this aim, four research chapters were developed, with the general objectives being: 1) to evaluate the efficacy of a marine-based edible coating in inhibiting superficial browning of fresh-cut 'Rocha' pear slices (Chapter II); 2) to validate the extract functionality in fresh-cut 'Fuji' apples on a pilot-scale environment (Chapter III); 3) to investigate on the mechanism of action of the seaweed extract for the prevention of browning in fresh-cut apples (Chapter IV); 4) to optimize extraction conditions for maximum functionality of the seaweed extract to prevent browning in fresh-cut apples (Chapter V).

The research presented in this dissertation contributes to the basic understanding of how *C. tomentosum* extract prevents or suppresses browning in fresh-cut fruits such as apples and pears. Considering the importance of scale-up, this thesis demonstrated for the first time, the

use of a seaweed-based natural coating applied under industrial conditions. Although the observed anti-browning efficacy was lower on a bigger scale, the reduction could be offset by the use of modified atmosphere packaging, as reported in the results of Chapter III. Here, the results showed that fresh-cut apples coated with the seaweed extract solution and packaged under ambient atmosphere developed higher rates of superficial browning when compared to the samples treated at the laboratory scale (Chapters IV and V), showing a possible limitation of the extract applicability under industrial conditions. However, when modified atmosphere packaging was used together with the seaweed extract coating, lower browning rates, as well a reduction in browning-related enzyme activities were observed in fresh-cut apples. The results of this study represent a good example of knowledge that can be transferred from academia to industry, which is an important step in the commercial exploitation of the seaweed extract.

In an earlier study (Augusto et al., 2016), and through the research presented in this thesis, a reduction in the activity of the browning-related enzymes PPO and POD was observed in fresh-cut apples coated with the seaweed extract solution. However, this reduction was only confirmed under *in vivo* conditions, which lead to the hypothesis that the extract can interfere with the processes of transcription and translation of those enzymes. From the experiments performed in Chapter IV, a reduction in the expressions of polyphenol oxidase (*mdPPO*), peroxidase (*mdPOD*) and phenylalanine ammonia-lyase (*mdPAL*) genes were observed in the first five sampling days. Since these genes are known to trigger the browning processes in fresh-cut fruits (Chen et al., 2021), the lower gene expression may explain the significant decrease in the activities of PPO and POD, which results in lower rates of browning. Additionally, the seaweed extract addition seems to have a greater influence on the activity of POD than on PPO, which can be related to the apparent reduction in the *mdPOD* expression observed at the beginning of the storage period (Chapter IV, Figure 4.2). Overall, these results suggest that the

browning development is being mediated by POD enzyme, instead of a single PPO-browning mediation.

In Chapter V, the effect of extraction variables on the seaweed extract efficacy was evaluated. Using Response Surface Methodologies, it was observed that longer extraction periods and neutral to alkaline pH allowed higher recovery of extracts. In contrast, the extract functionality is mainly affected by changes in temperature, suggesting that higher temperatures may lead to increased functionality. Considering the possible increase in the extract functionality at higher extraction temperatures and the results of FTIR, it is possible to hypothesise that the main compounds responsible for anti-browning functionality are the polysaccharides. Usually, the extraction of polysaccharides from seaweed into water is conducted at high temperatures (Saravana et al., 2018). Additionally, the presence of polysaccharides in the seaweed extract results in the maintenance of fruit firmness in the case of fresh-cut pears, as observed in Chapter II.

The seaweed extract efficacy in preventing browning was validated, showing the possibility of extending the coating application to other fresh-cut fruits, especially to products with high rates of browning development than fresh-cut pears. The seaweed extract possesses the ability to enhance the short shelf-life of fresh-cut pears from 7 days to almost 10 to 15 days. However, the seaweed extract influence on the activity of browning causing enzymes in fresh cut pears seems to be lower than in fresh-cut apple. It is possible that the presence of the seaweed extract in fresh-cut pears forms a mechanical barrier which prevents contact between the oxidative enzymes and their substrates. Finally, one can conclude that more studies are needed to understand the mechanism of action of seaweed extract.

To reduce the heterogeneity between samples collected from the sea coast, in the present thesis, *C. tomentosum* biomass produced under an Integrated Multi-Trophic Aquaculture (IMTA) system was purchased from a Portuguese seaweed supplier (ALGAplus). The use of seaweed

biomass from aquaculture had as the main objective to evaluate the seaweed extract efficacy to prevent browning development, and comparing its efficacy with the results stated in earlier studies (Augusto et al., 2016). The results obtained in this thesis confirmed that the extract obtained from an aquaculture seaweed, maintain the ability to restrict browning in fresh-cut apples; the relevant results can be observed in Chapters III-V. Thus, the use of *C. tomentosum* from aquaculture may be considered as an appealing cash crop, as it provides similar properties to prevent browning in fresh-cut fruits as the wild seaweed.

A common strategy employed to enhance the shelf-life of fresh-cut fruits is to use antimicrobial compounds in edible coatings (Sun et al., 2022). Overall, fresh-cut pears as well as fresh-cut apples coated with the seaweed extract solution (Chapters II and III respectively), showed delayed microbial growth, namely of yeasts and moulds, indicating some antimicrobial activity not yet reported for this seaweed extract. This antimicrobial activity can enhance the shelf-life, by delaying microbial spoilage.

Additionally, the growth of the Europe Market for food preservatives is largely driven by the increasing demand for foods with a longer shelf-life. The expansion of distribution channels and the growing demand for natural and organic foods without the addition of chemical compounds are some of the factors that contribute to market growth. It is estimated that from 2020 to 2025, the market for food preservatives in Europe will grow at a rate of 4.23 % (Miyazu, 2021). Within these, the segment of natural food preservatives is expected to record a robust growth when compared with synthetic preservatives (Miyazu, 2021). Given the natural composition of the coating formulation proposed in this thesis, alongside with the increased consumption of fresh-cut fruits, the scenario for the development and commercialization of this technology seems to be promising and encouraging towards a sustainable food economy.

Nowadays, the sustainability impact and social impacts of research projects are two relevant factors for their future implementation. In the present thesis, the sustainability impact may be

highlighted in terms of 1) reduction in fruit and vegetable waste and 2) replacement of a synthetic anti-browning agent with a naturally sourced and sustainable material. The key social impact will be the availability of fresh-cut fruits and vegetables which have a longer shelf-life and keeping quality for a healthier lifestyle.

In conclusion, this collective set of studies aimed at setting preliminary findings which should be taken further in order to justify the use of seaweed extracts as edible coatings in the fresh-cut industry.

2. Future Perspectives

In brief, this thesis evaluated the suitability of the seaweed extract *Codium tomentosum* to increase fresh-cut apples and pears shelf-life through the regulation of browning development. The ultimate goal would be the replacement of synthetic-based coatings used in food industry, by a natural-based coating, as it was the *C. tomentosum* extract here studied. Despite the new outcomes which resulted from this thesis, namely the seaweed extract efficacy to decrease browning in fresh-cut 'Rocha' pears (Chapter II), the scale-up process of its application in fresh-cut 'Fuji' apples (Chapter III), the possible interaction between browning regulation and the use of *C. tomentosum* extract in coating solution in fresh-cut apples (Chapter IV), and finally the extract efficacy to prevent browning when different extraction conditions are applied (Chapter V), there are still some aspects that ought to be studied in further research. Regarding the compounds responsible for the observed effect in browning reduction, the chemical characterization of the seaweed extract must be conducted to obtain relevant information on this matter. Through enrichment and fractionation of the obtained extracts, and in association with mass spectrometry techniques, it will be possible to achieve the identification of the main compounds present in the seaweed extract. From preliminary studies, it was possible to identify the presence of salt in the seaweed extract, suggesting desalination by membrane dialysis before spectrometry techniques are applied. Additionally, and following the results of FTIR-ATR

analysis, in Chapter V, it is hypothesized that polysaccharides are part of the molecules found in the seaweed extract. Based on this information, and prior to chromatographic analytical techniques, it is also suggested that a step of enzymatic digestion be included, thus obtaining molecules with lower molecular weight and easier to identify by mass spectrometry techniques.

In Chapters II and III it was observed that a reduction in the yeasts and mould growth in samples coated with the seaweed extract, suggesting that the seaweed extract may possess antifungal activities, not yet reported. Thus, a more comprehensive study of the extract bioactivities with a focus on antimicrobial properties is also suggested. Following the results of Chapter IV, where the mechanisms underpinning the seaweed extract efficacy were associated with the regulation of browning-related genes expression, the validation of these results, through full transcriptome analysis as the construction and analysis of RNA-Seq libraries, will provide a better knowledge of the overall genes and pathways being affected by the seaweed extract. Finally, studies with more fresh-cut fruits and vegetables to explore the performance of this seaweed extract to extend the product shelf-life is also important. Here, the efficacy to prevent browning in fruits and vegetables with high rates of browning, as bananas, avocados, and potatoes, may be an opportunity to expand the extract applications. Overall, the cost-effectiveness of the seaweed-based coating application needs to be studied, as well as the energy required to operate the solid-liquid system used for the seaweed extract production in Chapters II and III. To promote the industry interest and determine the extract market viability, the final price of this new coating and the economic advantages of its use to extend fresh-cut fruits shelf-life is essential for the commercialization.

As a final remark, with the seaweed extract work presented in this thesis, more comprehensive information on its functionality to increase browning resistance in fresh-cut apples and pears was achieved. This work provides a significant basal contribution for research on new natural

food preservatives, by providing encouraging results and critical considerations to be followed in the development of innovative coatings for fresh-cut fruits and vegetables.

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APPENDICES

Appendix A. Supplementary data Chapter I

Appendix B. Supplementary data Chapter II

Appendix B. Supplementary data Chapter IV

Appendix C. Supplementary data Chapter V

Appendix A. Supplementary data **Chapter I**

Table S1.1. Seaweeds commercialized in Portugal (retrieved from Portuguese Directorate-General for Natural Resources, Safety and Maritime Services (DGRM).

Type of seaweed	FAO Code	Scientific Name
Red seaweed	FYS	*Porphyra* spp.
Red seaweed	OFK	*Porphyra dioica*
Red seaweed	OFN	*Porphyra linearis*
Red seaweed	OKQ	*Pterocladiella capillacea*
Red seaweed	GEL	*Gelidium corneum*
Red seaweed	GJA	*Chondracanthus acicularis*
Red seaweed	GJE	*Chondracanthus teedei*
Red seaweed	IMS	*Chondrus crispus*
Red seaweed	RHP	*Palmaria palmata*
Red seaweed	OFH	*Porphyra umbilicalis*
Red seaweed		*Pyropia* spp.
Red seaweed	GLS	*Gracilaria* spp.
Red seaweed	GJW	*Gracilariopsis longissima*
Red seaweed	GHG	*Gigartina* spp.
Red seaweed		*Laurencia viridis*
Red seaweed	MVT	*Mastocarpus stellatus*
Red seaweed		*Osmundea pinnatifida*
Red seaweed	OKG	*Plocamium cartilagineum*
Red seaweed		*Grateloupia* spp.
Red seaweed	NLO	*Nemalion elminthoides*
Red seaweed		*Calliblepharis jubata*
Brown seaweed	ASN	*Ascophyllum nodosum*
Brown seaweed	AJC	*Alaria esculenta*
Brown seaweed	HLZ	*Himanthalia elongata*
Brown seaweed		*Bifurcaria bifurcata*
Brown seaweed	GQB	*Sargassum fusiforme*
Brown seaweed	RGV	*Sargassum vulgare*
Brown seaweed	LQX	*Saccharina latissima*
Brown seaweed		*Laminaria ochroleuca*
Brown seaweed	LAH	*Laminaria hyperborea*
Brown seaweed	UDP	*Undaria pinnatifida*
Brown seaweed	FUV	*Fucus vesiculosus*
Brown seaweed	FUU	*Fucus spiralis*
Brown seaweed	UCU	*Fucus* spp.
Brown seaweed		*Ecklonia* spp.
Brown seaweed	FUP	*Pelvetia canaliculata*
Brown seaweed	ZSY	*Saccorhiza polyschides*
Green seaweed	KJT	*Codium tomentosum, Codium* spp.
Green seaweed	EFP	*Ulva compressa*
Green seaweed	UVQ	*Ulva rigida*
Green seaweed	UVI	*Ulva intestinalis*
Green seaweed	UVU	*Ulva lactuca*

Appendix B. Supplementary data **Chapter II**

Table S2.1. Pairwise comparison matrix for significant differences among levels for firmness. Significant differences are highlighted in bold (ANOVA, GLS, p < 0.05).

Day		1		5			10			15		
	Treatment	**CS**	**SE**	**CTR**	**CS**	**SE**	**CTR**	**CS**	**SE**	**CTR**	**CS**	**SE**
1	**CTR**	**T=-2.125 P=0.034**	T=0.527 P=0.599	T=-1.311 P=0.191	T=1.956 P=0.051	T=-0.832 P=0.406	**T=-3.420 P=0.001**	**T=3.957 P=0.000**	T=1.385 P=0.167	**T=-2.117 P=0.035**	T=0.234 P=0.816	T=0.533 P=0.595
	CS		**T=2.652 P=0.008**	T=-1.956 P=0.051	T=1.459 P=0.146	**T=-2.819 P=0.005**	**T=-3.957 P=0.000**	**T=2.182 P=0.030**	**T=-2.578 P=0.010**	T=-0.234 P=0.816	T=-1.746 P=0.082	T=0.294 P=0.769
	SE			T=0.832 P=0.406	**T=2.819 P=0.005**	**T=-2.511 P=0.013**	T=-1.385 P=0.167	**T=2.578 P=0.010**	T=-1.461 P=0.145	T=-0.533 P=0.595	T=-0.294 P=0.769	T=-1.363 P=0.174
5	**CTR**				T=0.688 P=0.492	T=-0.645 P=0.519	T=-1.929 P=0.055	T=1.885 P=0.060	**T=2.153 P=0.032**	T=-0.707 P=0.480	T=-1.693 P=0.091	T=1.333 P=0.184
	CS					T=-1.368 P=0.172	T=-1.885 P=0.06	T=0.716 P=0.474	T=0.293 P=0.770	T=1.693 P=0.091	**T=-3.147 P=0.002**	**T=3.052 P=0.002**
	SE						**T=-2.153 P=0.032**	T=-0.293 P=0.770	T=1.11 P=0.268	T=-1.333 P=0.184	**T=-3.052 P=0.002**	T=1.181 P=0.239
10	**CTR**							**T=3.465 P=0.001**	**T=2.486 P=0.013**	T=1.271 P=0.205	**T=-3.647 P=0.000**	T=-0.839 P=0.402
	CS								T=-1.002 P=0.317	**T=3.647 P=0.000**	**T=-3.849 P=0.000**	**T=2.820 P=0.005**
	SE									T=0.839 P=0.402	**T=-2.82 P=0.005**	T=0.084 P=0.933
15	**CTR**										T=-1.719 P=0.087	T=1.264 P=0.207
	CS											**T=2.957 P=0.003**

Table S2.2. Pairwise comparison matrix for significant differences among levels for browning index. Significant differences are highlighted in bold (ANOVA, GLS, p < 0.05).

Day		1		5			10			15		
	Treatment	**CS**	**SE**	**CTR**	**CS**	**SE**	**CTR**	**CS**	**SE**	**CTR**	**CS**	**SE**
1	**CTR**	T=-1.262 P=0.208	T=-0.483 P=0.630	T=-0.733 P=0.464	T=0.360 P=0.719	**T=2.732 P=0.007**	T=-0.094 P=0.925	T=-0.058 P=0.954	T=0.217 P=0.829	T=1.053 P=0.293	**T=2.141 P=0.033**	T=-1.229 P=0.220
	CS		T=0.742 P=0.459	T=-0.360 P=0.719	T=-0.244 P=0.823	**T=2.376 P=0.018**	T=0.058 P=0.954	T=-0.177 P=0.859	T=0.275 P=0.784	T=-2.141 P=0.330	**T=-4.131 P=0.000**	**T=-3.370 P=0.001**
	SE			**T=-2.732 P=0.007**	**T=-2.376 P=0.018**	**T=3.107 P=0.002**	T=-0.217 P=0.829	T=-0.275 P=0.784	T=0.21 P=0.833	T=1.229 P=0.220	**T=3.37 P=0.001**	T=-0.687 P=0.493
5	**CTR**				T=-0.734 P=0.464	**T=3.412 P=0.001**	T=0.633 P=0.527	T=-0.416 P=0.678	**T=-2.259 P=0.012**	T=1.762 P=0.079	T=1.764 P=0.079	**T=-3.989 P=0.000**
	CS					**T=4.153 P=0.000**	T=0.416 P=0.678	T=0.048 P=0.962	**T=-2.529 T=0.012**	T=-1.764 P=0.079	**T=4.316 P=0.000**	**T=-5.818 P=0.000**
	SE						**T=2.529 P=0.012**	**T=2.123 P=0.035**	**T=-2.955 P=0.003**	**T=3.989 P=0.000**	**T=5.818 P=0.000**	**T=-3.911 P=0.000**
10	**CTR**							T=-1.332 P=0.184	T=-0.18 P=0.857	T=1.135 P=0.257	**T=2.188 P=0.029**	T=-1.454 P=0.147
	CS								T=1.15 P=0.251	**T=-2.188 P=0.029**	**T=4.309 P=0.000**	**T=-3.685 P=0.000**
	SE									T=1.454 P=0.147	**T=3.685 P=0.000**	T=-0.918 P=0.359
15	**CTR**										T=1.761 P=0.079	**T=-2.235 P=0.026**
	CS											**T=-4.075 P=0.000**

Table S2.1. Pairwise comparison matrix for significant differences among levels for peroxidase activity. Significant differences are highlighted in bold (ANOVA, GLS

Day		1		5			10			15		
	Treatment	**CS**	**SE**	**CTR**	**CS**	**SE**	**CTR**	**CS**	**SE**	**CTR**	**CS**	**SE**
1	**CTR**	T=-0.051 P=0.959	T=-0.453 P=0.654	**T=2.174 P=0.040**	T=-1.924 P=0.066	T=-0.439 P=0.664	T=1.746 P=0.094	T=-0.86 P=0.398	**T= 3.793P= 0.001**	T=0.028 P=0.978	T=1.111 P=0.278	T=1.306 P=0.204
	CS		T=-0.402 P=0.691	T=1.924 P=0.066	T=-0.547 P=0.589	T=1.485 P=0.151	T=0.86 P=0.398	T=0.529 P=0.601	**T= 4.653 P=0.000**	T=-1.11 P=0.278	T=1.599 P=0.123	T=0.194 P=0.847
	SE			T=0.439 P=0.664	T=-1.485 P=0.151	T=1.553 P=0.134	**T= -3.793P=0.001**	**T= -4.653P= 0.000**	**T= 7.11 P=0.000**	T=-1.306 P=0.204	T=-0.194 P=0.847	T=1.874 P=0.073
5	**CTR**				**T= -2.243P= 0.034**	T=-0.763 P=0.453	T=0.766 P=0.451	T=-0.024 P=0.981	**T= 3.805P= 0.001**	T=-0.267 P=0.792	T=1.366 P=0.184	T=1.359 P=0.187
	CS					T=1.48 P=0.152	T=0.024 P=0.981	T=0.732 P=0.471	**T=3.828P=0.001**	T=-1.366 P=0.184	T=1.666 P=0.109	T=-0.007 P=0.994
	SE						**T=-3.805P=0.001**	**T=-3.828P=0.001**	**T=6.147P=0.000**	T=-1.359 P=0.187	T=0.007 P=0.994	T=1.655 P=0.111
10	**CTR**							T=-0.892 P=0.381	**T=3.784P=0.001**	T=-0.497 P=0.623	T=1.323 P=0.198	T=0.113 P=0.911
	CS								**T=4.676 P=0.000**	T=1.323 P=0.198	T=1.373 P=0.182	T=-1.21 p=0.238
	SE									T=-0.113 P=0.911	T=1.21 P=0.238	T=-0.338 P=0.738
15	**CTR**										T=1.11 P=0.278	T=1.278 P=0.213
	CS											T=0.168 P=0.868

Table S2.2. Pairwise comparison matrix for significant differences among levels for polyphenol oxidase activity. Significant differences are highlighted in bold (ANOVA, GLS, $p < 0.05$).

Day		1		5			10			15		
	Treatment	**CS**	**SE**	**CTR**	**CS**	**SE**	**CTR**	**CS**	**SE**	**CTR**	**CS**	**SE**
1	**CTR**	T=-0.051 P=0.959	T=-0.453 P=0.654	**T=2.174 P=0.040**	T=-1.924 P=0.066	T=-0.439 P=0.664	T=1.746 P=0.094	T=-0.86 P=0.398	**T=3.793 P=0.001**	T=0.028 P=0.978	T=1.111 P=0.278	T=1.306 P=0.204
	CS		T=-0.402 P=0.691	T=1.924 P=0.066	T=-0.547 P=0.589	T=1.485 P=0.151	T=0.860 P=0.398	T=0.529 P=0.601	**T=4.653 P=0.000**	T=-1.111 P=0.278	T=1.599 P=0.123	T=0.194 P=0.847
	SE			T=0.439 P=0.664	T=-1.485 P=0.151	T=1.553 P=0.134	**T=-3.793 P=0.001**	**T=-4.653 P=0.000**	**T=7.110 P=0.000**	T=-1.306 P=0.204	T=-0.194 P=0.847	T=1.874 P=0.073
5	**CTR**				**T=-2.243 P=0.034**	T=-0.763 P=0.453	T=0.766 P=0.451	T=-0.024 P=0.981	**T=3.805 P=0.001**	T=-0.267 P=0.792	T=1.366 P=0.184	T=1.359 P=0.187
	CS					T=1.480 P=0.152	T=0.024 P=0.981	T=0.732 P=0.471	**T=3.828 P=0.001**	T=-1.366 P=0.184	T=1.666 P=0.109	T=-0.007 P=0.994
	SE						**T=-3.805 P=0.001**	**T=-3.828 P=0.001**	**T=6.147 P=0.000**	T=-1.359 P=0.187	T=0.007 P=0.994	T=1.655 P=0.111
10	**CTR**							T=-0.892 P=0.381	**T=3.784 P=0.001**	T=-0.497 P=0.623	T=1.323 P=0.198	T=0.113 P=0.911
	CS								**T=4.676 P=0.000**	T=-1.323 P=0.198	T=1.373 P=0.601	T=-1.21 P=0.238
	SE										T=1.21 P=0.238	T=-0.338 P=0.738
15	**CTR**										T=1.11 P=0.278	T=1.278 P=0.213
	CS											T=0.168 P=0.868

Table S2.3. Pairwise comparison matrix for significant differences among levels for pectin methylesterase Activity. Significant differences are highlighted in bold (ANOVA, GLS, p< 0.05).

Day		1		5			10			15		
	Treatment	**CS**	**SE**	**CTR**	**CS**	**SE**	**CTR**	**CS**	**SE**	**CTR**	**CS**	**SE**
1	**CTR**	**T=4.914 P=0.000**	T=1.152 P=0.261	**T=2.58 P=0.016**	**T=-4.072 P=0.000**	T=-1.552 P=0.134	**T=2.367 P=0.026**	T=0.505 P=0.618	T=-1.236 P=0.228	**T=4.563 P=0.000**	**T=-2.658 P=0.014**	T=0.327 P=0.747
	CS		**T=-3.762 P=0.001**	**T=4.072 P=0.000**	**T=-3.179 P=0.004**	**T=2.520 P=0.019**	T=-0.505 P=0.618	**T=3.081 P=0.005**	T=-1.741 P=0.095	**T=2.658 P=0.014**	T=0.805 P=0.429	**T=2.984 P=0.006**
	SE			T=1.552 P=0.134	**T=-2.52 P=0.019**	T=0.385 P=0.703	T=1.236 P=0.228	T=1.741 P=0.095	T=0.619 P=0.542	T=-0.327 P=0.747	**T=-2.984 P=0.006**	**T=5.026 P=0.000**
5	**CTR**				T=-1.871 P=0.074	T=-1.128 P=0.271	T=1.002 P=0.326	**T=2.412 P=0.024**	T=-0.426 P=0.674	**T=3.434 P=0.002**	T=-1.038 P=0.310	T=0.896 P=0.379
	CS					T=0.744 P=0.464	**T=-2.412 P=0.024**	**T=4.412 P=0.000**	**T=-2.837 P=0.009**	T=1.038 P=0.310	T=1.966 P=0.061	T=1.934 P=0.065
	SE						T=0.426 P=0.674	**T=2.837 P=0.009**	T=0.400 P=0.693	T=-0.896 P=0.379	T=-1.934 P=0.065	**T=4.701 P=0.000**
10	**CTR**							T=1.800 P=0.084	T=-0.977 P=0.338	**T=2.228 P=0.036**	**T=-2.474 P=0.021**	T=1.037 P=0.310
	CS								**T=-2.778 P=0.010**	**T=2.474 P=0.021**	T=-1.27 P=0.216	**T=3.511 P=0.002**
	SE									T=-1.037 P=0.310	**T=-3.511 P=0.002**	**T=3.695 P=0.001**
15	**CTR**										T=-1.739 P=0.095	T=0.56 P=0.580
	CS											**T=2.3 P=0.030**

Appendix C. Supplementary data **Chapter IV**

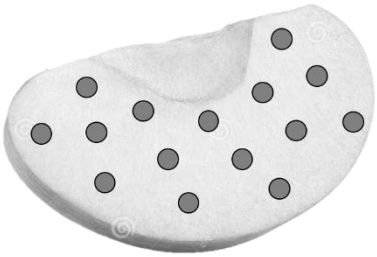

Figure S4. 1. Measuring points of colour parameters of apple slices.

Appendix D. Supplementary data **Chapter V**

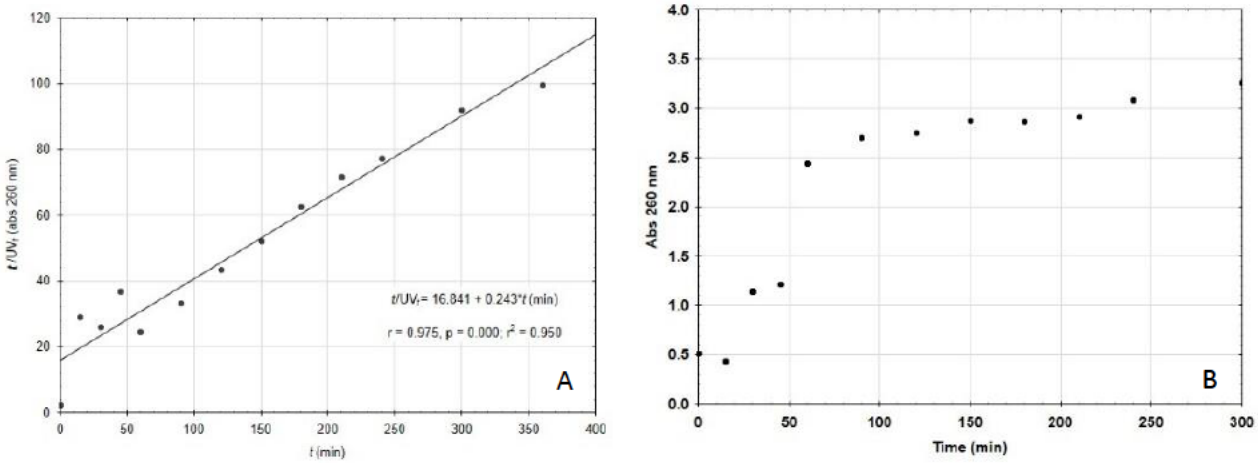

Figure S5.1. Linear relationship between t/Uvt and t in a single-factor experiment with time as a variable **(A)**. Effect of extraction time (in min) on the extracts absorbance at 260 nm **(B)**.

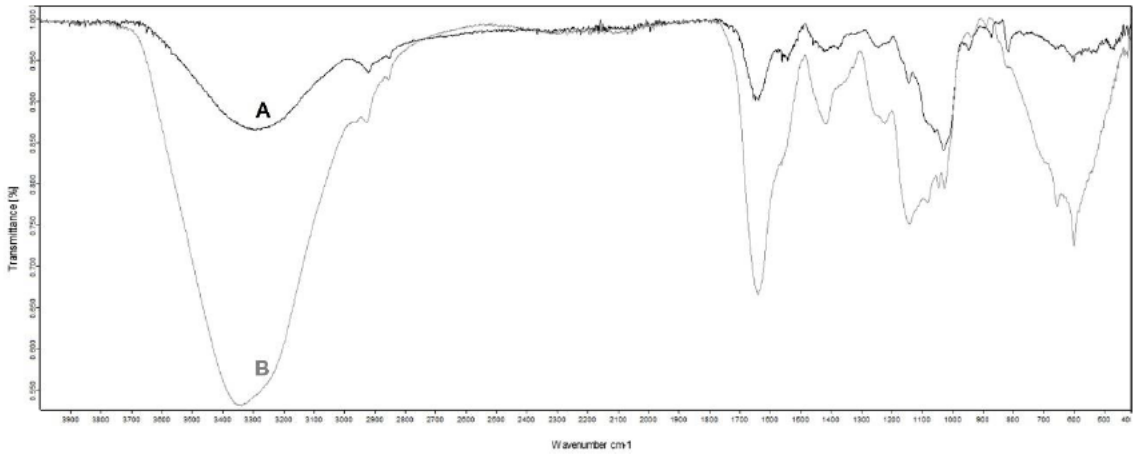

Figure S5.2. FTIR spectrum representation of dried *Codium tomentosum* (**A**, black line) and seaweed extracts (**B**, grey line).

Table S5.1. Raw experimental data on yield of the produced extracts produced and extract efficacy to prevent browning in fresh-cut apples in the Box-Behnken design performed of *Codium tomentosum* extractions. Extraction Yield: g $100g^{-1}$ dried seaweed; Solubility: g $100g^{-1}$ extract; BC 400nm: browning compounds absorbance at 440 nm; PPO, POD and PME: U mg $protein^{-1}$; SOD: U g^{-1} fresh weight.

Extraction Run	Variables			Experimental parameters								
	Time (min)	Temperature (°C)	pH	Yield	Solubility	BC 440 nm	BI	ΔE*	PPO	POD	SOD	PME
1	0	20	6.5	64.06±0.59	84.00±3.60	0.103±0.009	45.04±9.85	6.68±3.66	0.033±0.01	0.044±0.019	74.88±4.40	0.065±0.01
2	180	20	6.5	65.52±0.56	63.00±4.00	0.105±0.007	36.56±6.95	4.17±2.83	0.037±0.00	0.010±0.009	84.08±1.86	0.086±0.01
3	0	80	6.5	64.42±1.24	85.07±5.80	0.096±0.001	37.57±4.05	4.76±1.58	0.021±0.00	0.004±0.002	90.51±10.15	0.087±0.01
4	180	80	6.5	67.72±3.56	68.20±3.94	0.097±0.003	38.73±3.52	4.42±1.61	0.054±0.01	0.010±0.006	68.68±13.68	0.131±0.04
5	0	50	3.0	61.51±3.92	78.33±1.03	0.098±0.002	58.19±7.69	11.56±2.69	0.032±0.00	0.055±0.021	74.08±2.05	0.091±0.02
6	180	50	3.0	63.27± 3.64	75.90±4.90	0.100±0.004	59.50±8.09	12.43±2.53	0.027±0.01	0.024±0.007	73.16±4.19	0.085±0.01
7	0	50	10.0	64.38±0.51	77.87±2.39	0.095±0.002	46.03±3.36	7.48±1.16	0.020±0.01	0.007±0.002	65.74±3.20	0.107±0.01
8	180	50	10.0	64.97±1.37	78.80±5.20	0.103±0.008	39.62±4.49	5.03±1.28	0.024±0.00	0.009±0.002	54.49±4.39	0.101±0.05
9	90	20	3.0	63.01 ± 0.82	72.60 ± 4.00	0.099±0.005	50.53±4.74	9.84±2.25	0.017 ±0.00	0.005±0.001	38.75±5.17	0.107±0.01

10	90	80	3.0	62.17 ± 1.63	79.87 ± 3.72	0.096±0.001	38.67±6.02	5.47±1.63	0.017 ±0.00	0.003±0.001	42.85±9.03	0.118±0.01
11	90	20	10.0	65.58 ± 0.47	74.40 ± 1.80	0.100±0.007	44.41±5.29	6.77±1.89	0.032 ±0.01	0.022±0.012	38.04±3.84	0.103±0.00
12	90	80	10.0	66.30 ± 0.37	70.93 ± 4.55	0.097±0.003	48.68±2.99	8.64±1.20	0.009 ±0.00	0.004±0.002	33.76±10.21	0.043±0.01
13*z*	90	50	6.5	64.35 ± 1.11	79.00 ± 1.40	0.097±0.003	50.32±4.91	9.08±1.73	0.020 ±0.00	0.072±0.032	37.11±7.46	0.035±0.01
14*z*	90	50	6.5	64.32 ± 1.58	76.40 ± 4.04	0.101±0.001	50.99±5.39	9.74±1.89	0.019 ±0.00	0.050±0.014	31.65±5.58	0.042±0.00
15*z*	90	50	6.5	65.97 ± 0.60	81.40 ± 3.17	0.100±0.002	42.41±6.60	6.57±2.45	0.028 ± 0.01	0.139±0.024	40.69±6.47	0.044±0.00

Table S5.2. Analysis of Variance (ANOVA) for the Response Surface Quadratic Model of solubility. Variables: X_1 – Time (min), X_2 – temperature (°C), X_3 – pH; *significant terms ($p < 0.05$); SS- sum of squares; Df- degree of freedom; MS- mean square

Source	SS	Df	MS	F-value	p-value
Model	408.68	9	45.41	1.25	0.423
X_1	229.69	1	229.69	6.32	0.054*
X_2	83.64	1	83.64	2.3	0.19
X_3	24.27	1	24.27	0.67	0.451
X_1X_2	0.03	1	0.03	0.00	0.979
X_1X_3	1.44	1	1.44	0.04	0.85
X_2X_3	1.52	1	1.52	0.04	0.846
X_1^2	18.05	1	18.05	0.50	0.512
X_2^2	46.82	1	46.82	1.29	0.308
X_3^2	3.61	1	3.61	0.10	0.765
Error	181.66	5	36.33		
Lack-of-fit	146.47	3	48.82	2.78	0.276
Pure error	35.19	2	17.59		
Total	590.33	14			
S model	6.03				
R^2	69.23 %				
Equation:	Solubility = 70.5 - 0.021 X_1 + 0.544 X_2 - 1.42 X_3 - 0.000273 X_1^2 - 0.00396 X_2^2+ 0.081 X_3^2- 0.00003 X_1X_2 + 0.00190 X_1X_3 - 0.0059 X_2X_3				

Table S5.3. Analysis of Variance (ANOVA) for the Response Surface Quadratic Model of browning index. Variables: X_1 – Time (min), X_2 – temperature (°C), X_3 – pH; *significant terms ($p < 0.05$); SS- sum of squares; Df- degree of freedom; MS- mean square.

Source	SS	Df	MS	F-value	p-value
Model	517.09	9	57.45	1.38	0.378
X_1	19.28	1	19.28	0.46	0.526
X_2	20.80	1	20.80	0.5	0.511
X_3	99.03	1	99.03	2.38	0.184
X_1X_2	23.17	1	23.17	0.56	0.489
X_1X_3	14.89	1	14.89	0.36	0.576
X_2X_3	64.95	1	64.95	1.56	0.267
X_1^2	9.261	1	9.26	0.22	0.657
X_2^2	173.03	1	173.03	4.16	0.097
X_3^2	75.26	1	75.26	1.81	0.236
Error	208.08	5	41.61		
Lack-of-fit	162.55	3	54.18	2.38	0.31
Pure error	45.53	2	22.76		
Total	725.18	14			
S model	6.45				
R^2	71.31 %				
Equation	$BI = 66.6 + 0.013\ X_1 + 0.377\ X_2 - 7.16\ X_3 - 0.000196\ X_1^2 - 0.00761\ X_2^2 + 0.369\ X_3^2 + 0.00089\ X_1X_2 - 0.0061\ X_1X_3 + 0.0384\ X_2X_3$				

Table S5.4. Analysis of Variance (ANOVA) for the Response Surface Quadratic Model of colour changes (ΔE*). Variables: X_1 – Time (min), X_2 – temperature (°C), X_3 – pH; *significant terms ($p < 0.05$); SS- sum of squares; Df- degree of freedom; MS- mean square.

Source	SS	Df	MS	F-value	p-value
Model	72.89	9	8.099	1.79	0.271
X_1	2.452	1	2.452	0.54	0.495
X_2	2.178	1	2.178	0.48	0.519
X_3	16.196	1	16.196	3.58	0.117
X_1X_2	1.175	1	1.175	0.26	0.632
X_1X_3	2.768	1	2.768	0.61	0.47
X_2X_3	9.777	1	9.777	2.16	0.202
X_1^2	3.721	1	3.721	0.82	0.406
X_2^2	22.18	1	22.18	4.9	0.078
X_3^2	10.279	1	10.279	2.27	0.192
Error	22.645	5	4.529		
Lack-of-fit	17.031	3	5.677	2.02	0.348
Pure error	5.614	2	2.807		
Total	95.536	14			
S model	2.12815				
R^2	76.30 %				
Equation	$\Delta E^* = 14.67 + 0.0233\ X_1 + 0.140\ X_2 - 2.68\ X_3 - 0.000124\ X_1^2 - 0.00272\ X_2^2 + 0.1362\ X_3^2 + 0.000201\ X_1X_2 - 0.00264\ X_1X_3 + 0.0149\ X_2X_3$				

Table S5.5. Analysis of Variance (ANOVA) for the Response Surface Quadratic Model of polyphenol oxidase activity (PPO). Variables: X_1 – Time (min), X_2 – temperature (°C), X_3 – pH; *significant terms ($p < 0.05$); SS- sum of squares; Df- degree of freedom; MS- mean square.

Source	SS	Df	MS	F-value	p-value
Model	0.00123	9	0.000137	1.82	0.264
X_1	0.00017	1	0.000167	2.23	0.196
X_2	0.00004	1	0.000043	0.58	0.482
X_3	0.00001	1	0.000008	0.11	0.751
X_1X_2	0.00021	1	0.00021	2.8	0.155
X_1X_3	0.00002	1	0.00002	0.26	0.63
X_2X_3	0.00012	1	0.000123	1.64	0.256
X_1^2	0.00039	1	0.000394	5.26	0.07*
X_2^2	0.00005	1	0.000045	0.61	0.472
X_3^2	0.00018	1	0.000178	2.38	0.184
Error	0.00038	5	0.000075		
Lack-of-fit	0.00033	3	0.000108	4.33	0.193
Pure error	0.00005	2	0.000025		
Total	0.00160	14			
S model	0.00866				
R^2	76.62 %				
Equation	$PPO = 0.0186 - 0.000359\ X_1 - 0.000366\ X_2 + 0.00909\ X_3 + 0.000001\ X_1^2 + 0.000004\ X_2^2 - 0.000568\ X_3^2 + 0.000003\ X_1X_2 + 0.000007\ X_1X_3 - 0.000053\ X_2X_3$				

Table S5.6. Analysis of Variance (ANOVA) for the Response Surface Quadratic Model of superoxide dismutase (SOD). Variables: X_1 – Time (min), X_2 – temperature (°C), X_3 – pH; *significant terms ($p < 0.05$); SS- sum of squares; Df- degree of freedom; MS- mean square.

Source	SS	Df	MS	F-value	p-value
Model	5603.55	9	622.62	39.7	0.00*
X_1	76.83	1	76.83	4.9	0.078
X_2	0.00	1	0.00	0.00	0.997
X_3	169.3	1	169.3	10.8	0.022*
X_1X_2	240.67	1	240.67	15.35	0.011*
X_1X_3	26.66	1	26.66	1.7	0.249
X_2X_3	17.58	1	17.58	1.12	0.338
X_1^2	4728.4	1	4728.4	301.5	0.00*
X_2^2	195.03	1	195.03	12.44	0.017*
X_3^2	107.8	1	107.8	6.87	0.047*
Error	78.42	5	15.68		
Lack-of-fit	37.01	3	12.34	0.6	0.676
Pure error	41.41	2	20.7		
Total	5681.96	14			
S model	3.9602				
R^2	98.62 %				
Equation	$SOD = 61.2 - 0.6327\ X_1 - 0.419\ X_2 + 6.16\ X_3 + 0.004418\ X_1^2 + 0.00808\ X_2^2 - 0.441\ X_3^2 - 0.002873\ X_1X_2 - 0.00820\ X_1X_3 - 0.0200\ X_2X_3$				

Table S5.7. Analysis of Variance (ANOVA) for the Response Surface Quadratic Model of pectin methylesterase (PME). Variables: X_1 – Time (min), X_2 – temperature (°C), X_3 – pH; *significant terms ($p < 0.05$); SS- sum of squares; Df- degree of freedom; MS- mean square.

Source	SS	Df	MS	F-value	p-value
Model	0.00888	9	0.000987	1.26	0.421
X_1	0.000347	1	0.000347	0.44	0.536
X_2	0.00004	1	0.00004	0.05	0.831
X_3	0.000278	1	0.000278	0.35	0.578
X_1X_2	0.000125	1	0.000125	0.16	0.707
X_1X_3	0.000	1	0.000	0.000	0.994
X_2X_3	0.001253	1	0.001253	1.59	0.262
X_1^2	0.002807	1	0.002807	3.57	0.117
X_2^2	0.002199	1	0.002199	2.8	0.155
X_3^2	0.002877	1	0.002877	3.66	0.114
Error	0.00393	5	0.000786		
Lack-of-fit	0.003889	3	0.001296	64.14	0.015*
Pure error	0.00004	2	0.00002		
Total	0.012809	14			
S model	0.028034				
R^2	69.32 %				
Equation	$PME = 0.1874 - 0.000645\ X_1 - 0.00173\ X_2 - 0.0229\ X_3 + 0.000003\ X_1^2 + 0.000027\ X_2^2 + 0.00228\ X_3^2 + 0.000002\ X_1X_2 + 0.000000\ X_1X_3 - 0.000169\ X_2X_3$				