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Bachelor degree in Biology

AlgaeCoat: Development of edible coatings from marine-based extracts

A Market Strategy

Dissertation to obtain the Master degree in Biotechnology

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AlgaeCoat: Desenvolvimento de revestimentos comestíveis a partir de extratos de origem marinha

Uma Estratégia para o Mercado

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Every day, new ideas sprout from creators' minds, driving society towards a world that is gradually changing.

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Abstract

The present work aims to study a go-to-market strategy and validate the pilot-scale functionality of AlgaeCoat – a marine extract with anti-browning activity – used as a post-harvest treatment in minimally processed ‘Cripps Pink’ apple (MP apple) in order to extend its shelf-life, while preserving its freshness and visual appeal.

Therefore, this entirely natural formulation – composed uniquely by *Codium tomentosum* and distilled water – could replace synthetic additives (widely used in Food Industry) potentially harmful for consumer’s health.

Previous research projects published by MARE-IPLeiria team (Augusto, Simões, Pedrosa, & Silva, 2016) validated the efficacy of this solution at laboratorial level, demonstrating an inhibitory effect of the extract over polyphenoloxidase (PPO) and peroxidase (POD) enzymes, resulting in the reduction of apple’s browning index (BI) and also in the preservation of apple’s mechanical properties (texture and firmness) during a 20 days’ storage, at 4 ± 2°C. However, after scale-up, the results revealed some limitations, indicating a need for the optimization of pilot-scale extraction and application conditions.

In 2016, Europe accounted for the major share (28.1%) in the global Food Preservatives market, registering a predicted value of US$ 850 million (≈ €708 million). It’s expected that the European Food Preservatives Market continues to follow a positive tendency, achieving US$ 950 million (≈ €792 million) by 2021, with a Compound Annual Growth Rate (CAGR) of 2.25% between 2016 and 2021.

Additionally, the demand for natural, organic foods, free of synthetic and complex ingredients, as well as for ready-to-eat products, has been increasing and it’s expected to stimulate the development of new, natural additives, revolutionizing Food Additives market.

Keywords: *C. tomentosum*; Natural additive; Minimally Processed Products; Enzymatic Browning; Scale-up; Food Preservative

Resumo

O presente trabalho teve como principais objetivos estudar uma estratégia de mercado e validar à escala piloto, a funcionalidade do AlgaeCoat – um extrato marinho com propriedades anti-escurecimento – usado como um tratamento pós-colheita em maçã 'Cripps Pink' minimamente processada (maçã MP), a fim de aumentar a sua validade, preservando a sua frescura e aparência.

Com efeito, esta formulação totalmente natural – composta unicamente por *Codium tomentosum* e por água destilada – poderá vir a substituir a aplicação de aditivos sintéticos (amplamente usados na Indústria Alimentar) potencialmente nocivos para a saúde dos consumidores.

Trabalhos de investigação previamente publicados pela equipa do MARE-IPLeiria (Augusto, Simões, Pedrosa, & Silva, 2016) validaram a eficácia desta solução a nível laboratorial, demonstrando um efeito inibitório do extrato sobre as enzimas polifenoloxidase (PPO) e peroxidase (POD), resultando na diminuição do índice de escurecimento (BI) das fatias de maçã e também na manutenção das suas propriedades mecânicas (textura e firmeza) ao longo de 20 dias de armazenamento, a 4 ± 2°C. Contudo, após o aumento de escala, os resultados revelaram algumas limitações, indicando uma necessidade de otimização das condições de extração e aplicação na escala piloto.

Em 2016, a Europa alcançou a maior quota de mercado (28,1%) relativamente ao mercado global dos Conservantes Alimentares, registando um valor previsto de US$ 850 milhões (≈ €708 milhões). Espera-se que o mercado Europeu dos Conservantes Alimentares continue a seguir uma tendência positiva, atingindo os US$ 950 milhões (≈ €792 milhões) até 2021, com uma taxa de crescimento anual composta (CAGR) estimada de 2,25%, entre 2016 e 2021.

Acrescenta-se ainda que a procura de produtos naturais, feitos com alimentos orgânicos, livres de ingredientes sintéticos complexos, bem como de produtos prontos-a-consumir, tem vindo a aumentar e espera-se que venha a estimular o desenvolvimento de novos aditivos naturais, revolucionando o mercado dos aditivos alimentares.

Palavras-Chave: *C. tomentosum*; Aditivo Natural; Produtos Minimamente Processados; Escurecimento Enzimático; Aumento de Escala; Conservante Alimentar

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List of Acronyms

AA Ascorbic Acid
B2B Business-to-Business
B2C Business-to-Consumer
BI Browning Index
CAGR Compound Annual Growth Rate
CFIA Canadian Food Inspection Agency
CT *C. tomentosum*
CTE *C. tomentosum* Extract
ECT Edible Coating
EFSA European Food Safety Authority
FAO Food and Agriculture Organization
FDA Food and Drug Administration
GMO Genetically Modified Organism
GRAS Generally Recognized As Safe
IMTA Integrated Multi-Trophic Aquaculture
MA Modified Atmosphere
MAP Modified Atmosphere Packaging
MD Market Dimension
MPP Minimally Processed Products
PDO Protected Designation of Origin
PGI Protected Geographical Indication
PME Pectin Methylesterase
POD Peroxidase
PPA Plasma Processed Air
PPO Polyphenoloxidase
PUFA Polyunsaturated Fatty Acids
RNS Reactive Nitrogen Species
ROS Reactive Oxygen Species
SCF Scientific Committee on Food
SSC Soluble Solids Concentration
TRL Technology Readiness Level
TV Technology Viability
USDA US Department of Agriculture
WHO World Health Organization

Introduction

In the last years, the awareness towards the need to adopt healthier consumption habits fostered the demand for fresh food and minimally processed products (MPP), particularly fruit and vegetables, rich in valuable nutrients (micronutrients and fibres) (Ragaert, Verbeke, Devlieghere, & Debevere, 2004; Bansal, Siddiqui, & Rahman, 2015).

Fruits and vegetables have a protective function, preventing the development of several types of cancer, as well as other chronic and degenerative diseases, thus being essential to keep a strong immune system. For those reasons, WHO (World Health Organization) recommends the consumption of about 400 g of fruit and vegetables per day (Ragaert, Verbeke, Devlieghere, & Debevere, 2004; Ahern, et al., 2013).

Minimally processed products are fresh, ready-to-eat products, very similar to the initial feedstock given they're only constituted by the edible parts of the produce. After undergoing cutting and packaging, the metabolism of MPP tissues increases and a stress response is induced. As a consequence, the respiration rate rises, leading to product's deterioration and rotting. When tissues start to decompose, opportunistic microorganisms colonize them, accelerating the deterioration process and making the product unable for consumption (Watada & Qi, Quality control of minimally-processed vegetables, 1999; Watada & Qi, Quality of fresh-cut produce, 1999). Therefore, appropriate measures have to be implemented to preserve MPP freshness and consequently deliver high quality products to the final customer.

The application of synthetic additives seems to be the easiest and the most effective way to achieve this purpose, turning MPP much more attractive and contaminant free. Recent studies show that synthetic additives (frequently used in Food Industry) are being progressively banned from the GRAS list ("Generally Recognized as Safe"), due to the evolution of risk analysis tools used to reassess product's toxicity. Consequently, the new regulation guidelines – that impact preservation methodologies – reduced the available strategies for MPP stabilization.

Additionally, a recent study published by the Royal DSM Company (2013) – a multinational that supports scientific investigation, with application in Health, Nutrition and Materials fields – revealed that urban consumers, who used to buy pre-prepared foods were generally seeking fresh and natural flavours that tasted as good as the real home meals. This consumer survey showed that more than a half (55%) believed that a "fresh or natural" flavour was what made the food they had bought tastes delicious. Besides flavour, also cost, convenience ("easy to use and prepare") and taste ("very good taste") were among the attributes mostly valorised by customers, leading them to purchase MPP, while the major concerns comprised the preservation of food's nutritional value and safety (Institute of Food Technologists, 2013; Bansal, Siddiqui, & Rahman, 2015; Nielsen, 2016).

Another report, developed by the Nielsen Company (US) in 2016, reveals that one of the top ingredients mostly avoided by consumers are artificial preservatives, due to their long-term health impact.

There's then an increasing search for new, natural-based additives capable of substituting synthetic additives, responding simultaneously to consumers' request for fresh, safe and more convenient foods.

The preference for natural foods, made with simple ingredients, meets the concept of "Clean Label", defined by Go Clean Label™ as a "consumer driven movement, demanding a return to 'real food' and transparency through authenticity".

The present work aims to study a go-to-market strategy and validate the pilot-scale functionality of a marine extract (with anti-browning activity) used as a post-harvest treatment in minimally processed apples – AlgaeCoat. The idea relies on the development of an original natural formulation, for a safer preservation of MP fruit, allowing people to consume these kind of products, in a more convenient way, with minimum preparation time.

Besides keeping fruit's nutritional value, this ecological and sustainable solution will contribute to extend produce shelf-life, consequently reducing food waste. It's proven that extended date labels lead people to keep their products for longer periods. Contrastingly, short date labels lead to an increase in food waste and consequently to higher pollution levels (Broad Leib, Ferro, Nielsen, Nosek, & Qu, 2013).

At a laboratorial scale, previous results demonstrated that AlgaeCoat was able to reduce the activity of Polyphenoloxidase (PPO), Peroxidase (POD) and its metabolic pathways, contributing to a reduction of tissue browning (oxidation) and softening.

The first chapters will begin with a brief literature review about the evolution of algae market around the world and about the properties and current applications of *Codium tomentosum* (CT). Then, after the brainstorm of possible applications, a "go-to-market strategy" for the selected application will be defined. However, before exploring the technology, the importance of apple in the context of this work will be explained, as well as MP apple production and deterioration (browning) processes. After a brief literature review about MP apple´s metabolism, the experimental work and the main results obtained – colour evaluation, enzymatic activity and microbiological analysis – will be exposed and discussed.

In the second half of this thesis, the market analysis for AlgaeCoat will be explored, evaluating the potential market dimension, competition and market trends. Business model, commercialization strategies and the SWOT analysis will be also suggested.

In the end, the thesis concludes with a reflection about the next steps of this project.

CHAPTER 2

Codium tomentosum: An Overview

2.1 Marine Algae – A Valuable Resource

For hundreds of years, production and consumption of marine algae have been fundamental activities of the oriental culture. These ancestral practises began in oriental countries, spreading later to other countries. As Beetul, et al. (2016), affirms: "Their use in the human society dates back to 500 B. C. in China and later across the globe; they are still being used for similar purposes and more today.".

In oriental culture, ancestral knowledge has been preserved. As an example, seaweeds have been applied in many natural products and formulations and used for different purposes, in very distinct areas, such as Medicine, Food Industry and Handicraft.

Marine algae may be considered a key ingredient in some traditional recipes, being frequently introduced on daily meals. Nutritional and medicinal properties linked to seaweed intake have been identified, particularly in oriental countries, such as China, Philippines or Korea, where the production of these marine organisms is massive and where the technology in this field is developing rapidly (McHugh, 2003).

In alternative medicine, algae and their extracts are used to prepare alternative formulations to alleviate symptoms and even cure some diseases. People usually find this kind of treatments when they look for a less aggressive treatment, without synthetic drugs.

On the other hand, in Handicraft, some pigments may be extracted from seaweeds and then be used in tissue coloration or in traditional paintings, very typical of Chinese and Japanese ceramic and porcelain.

In Europe, the commercial interest in these marine organisms, dates back to the 17^{th} century in France and Norway, since they could be used for glass and soap production (netalgae - EU Project, 2012). Nowadays they are classified as a highly valuable resource (with high commercial and nutritional value), due to the development of applications in Cosmetics and Dietetics (that will be analysed further), as well as in Biofuel Production.

Recent findings suggest marine algae application as animal and fish feed, biofertilizer, bioplastics production, fluorophores, food colourants and phytoremediation (Beetul, et al., 2016).

The present project will focus on the utilization of *Codium tomentosum* – a marine green alga, found across the Portuguese coast (Guiry, 2016) – to produce a formulation able to preserve fresh-cut products, like fruits and vegetables. Next, a brief review of this alga is presented, focusing on its distribution and properties.

2.2 *Codium tomentosum* Alga

Codium tomentosum (Chlorophyta) is a species that can be produced and collected in Portugal's territorial sea, serving as food with exclusive properties, beneficial to human health.

There are numerous applications in which this alga can be used, from several cosmetic products (repair and restoration moisturizers, hydration serums, bath and shower creams, eye and anti-aging creams, masks, lip balms, among other (Wang, Chen, Huynh, & Chang, 2015)), until nutraceutical products with excellent properties, namely: antioxidant (Valentão, 2009), antibacterial (Ibtissam, et al., 2009), antifungal (Aparanji, Atika, Venu, & Rajan, 2013), anticoagulant (Wang, Wang, Wu, & Liu, 2014), anti-genotoxic (Celikler, Vatan, Yildiz, & Bilaloglu, 2009), anti-arthritis (Aparanji, Atika, Venu, & Rajan, 2013).

C. tomentosum belongs to a group of organisms that grow in intertidal marine zones, where they are subjected to repeated immersion and emersion, leading them to experience different kinds of stress. The exposure to stressful environmental conditions induces the formation of free radicals and other oxidising agents, like ROS (Reactive Oxygen Species) and RNS (Reactive Nitrogen Species). As an adaptation, seaweeds developed defence mechanisms against oxidation, involving, for example, the production large amounts of antioxidant compounds such as proteins, phenolic compounds (flavonoids and coumarins), tocopherols, alkaloids, chlorophyll derivatives, amino acids and amines, carotenoids, ascorbic acid, glutathione and uric acid (Valentão, 2009).

According to Ibtissam et al. (2009) and Lowy (2003), *C. tomentosum* presented an intense antimicrobial activity against *Staphylococcus aureus*, one of the most resistant pathogenic agents, usually found in hospital units, responsible for several kinds of infections. Additionally, the application of different seaweed extracts (constituted by *C. tomentosum*) revealed an antibacterial activity against *Streptococcus* sp., *Bacillus subtilis* and *Proteus vulgaris*, besides an antifungal activity against *Trichophyton mentagrophytes*, *Candida albicans* (pathogenic fungus), *Aspergillus niger* and *Aspergillus flavus* (which produces aflatoxins, with a potential health risk) (Aparanji, Atika, Venu, & Rajan, 2013; Nidhina, Bhavya, Bhaskar, Muthukumar, & Murthy, 2016).

Marine algae are also known for their high content of sulphated polysaccharides (SPs). SPs have been intensively explored as supplements to keep a healthy diet and also as medical products (new medicines) with great potential. Likewise, *Codium* sp. revealed to produce a specific type of SPs (sulphated arabinogalactans) with anticoagulant capacity. The anticoagulant activity related to this specific type of SPs (similar to heparin) is universally recognized and was recently investigated as a potential and novel heparin substitute in order to achieve a safer prevention and even a safer cure for cardiovascular and cerebrovascular diseases (Wang, Wang, Wu, & Liu, 2014).

Additionally, Celikler, Vatan, Yildiz, & Bilaloglu (2009) verified an anti-genotoxic activity associated to the application of a *C. tomentosum* ethanolic extract. After extract's application on human lymphocyte cultures (previously exposed to different genotoxic agents), a reduction of the damages induced in DNA was observed, indicating a potential protective activity explained by the presence of antioxidant molecules as polyphenols, chlorophyll compounds, vitamins, proteins and/or SPs.

Another study demonstrated a suppression of an autoimmune response related to Rheumatoid Arthritis (RA), after an intraperitoneal administration of a *C. tomentosum* ethanolic extract to mice previously injected with type II collagen, to induce arthritis (Aparanji, Atika, Venu, & Rajan, 2013). Once autoimmune response is supressed, articulation cells do not suffer auto-degradation, removing therefore pain symptoms characteristic of this disease (cartilage is maintained, protecting articulation bones).

Recent studies suggest the application of this alga in Food Industry. Augusto, Simões, Pedrosa, & Silva (2016) proposed the application of a seaweed extract as a post-harvest treatment for MP 'Fuji' apple. The immersion of apple wedges in an aqueous solution containing *C. tomentosum* extract (CTE), resulted in a significant reduction of wedges' browning index (BI) and also in a reduced enzymatic activity of PPO (polyphenoloxidase) and POD (peroxidase), sustaining the utilization of CTE as novel solution to preserve MP products.

Considering the vast properties and applications of *C. tomentosum* (CT) – in Chapter 3 – a diverse set of possible applications (new and pre-existing concepts) are proposed and evaluated according with different criteria, ending with the selection and the definition of a specific go-to-market strategy for the best idea.

CHAPTER 3

Brainstorming of Applications

3.1 Brainstorming and Evaluation of Possible Applications for CT Alga

In the previous chapter, it was seen that *Codium tomentosum* (CT) is a simple and versatile organism with excellent properties that can be used in many different ways and sectors.

In this chapter, a brainstorming of possible applications – divided into 4 distinct sectors (food, pharmaceutical, cosmetics and industry) and summarized in Tables 3.1, 3.2, 3.3 e 3.4 (shown in the next section) – is exposed.

In order to judge the potential market value of each application, two sets of technology and market criteria (or filters) were chosen. Technology criteria (innovation, technology viability and technology readiness level) are related to the product *per se*, while market criteria (market dimension, competition and regulation) act as surrounding factors, external to the product, that need to be overcome to properly introduce the technology in the market.

Each criterion was evaluated with a score from 1 to 3, as explained below.

- Innovation: What's the novelty of the application? Is it possible find similar products in the market? **Score**: 1 – Low (no innovation); 2 – Moderate (the application shows some novelty, comparing to the existing ones); 3 – High (highly innovative application);
- Technology Viability (TV): Is it possible to bring the specific application to the market? **Score**: 1 – Low (there are no scientific and concrete evidences of the possibility of developing the specific application); 2 – Moderate (there are some scientific evidences that prove that it's possible to develop the specific application, but additional tests are still necessary); 3 – High (there are scientific and concrete evidences proving there is a possibility to develop the specific application);
- Technology Readiness Level (TRL[1]): If it's possible to bring the specific application to the market, which is the current phase/stage of development of the technology needed to introduce the application in the market? **Score**: 1 – Not Developed (the technology is not developed yet – technology readiness level 1 or 2); 2 – Partially Developed (the technology needed is still being developed – technology readiness level 3 to 6); 3 – Developed (the technology has already been tested in the market – technology readiness level 7 to 9);
- Market dimension (MD): Number of potential customers with the need for the specific application. **Score**: 1 – Low (small number of potential customers); 2 – Moderate (medium number of potential customers); 3 – High (large number of potential customers in the market);
- Competition: Considering a given sector, how many companies produce the same product (direct competition), similar products (indirect competition), or might easily include the

[1] According to the European Commission TRL (based on a scale from 1 to 9) identify the readiness level of the technology associated with the project as well as the planned progression during the course of project execution (European Commission Decision C, 22 July 2014).

proposed application in their production line? **Score**: 1 – High (there are too many companies competing directly and indirectly against the proposed application); 2 – Moderate (there are some companies competing directly and indirectly against the proposed application); 3 – Low (there are few or none competing products against the proposed application and this competition is mainly indirect);

- Regulation: Is it a highly regulated sector according with the legal terms imposed by EFSA (European Food Safety Authority)? **Score**: 1 – Highly regulated sector; 2 – Medium regulation (there are some tests to which the technology has to be submitted and protocols to obey, in order to reach the final consumer); 3 – Weak (there are not many tests to which the technology has to be submitted or protocols to obey, in order to reach the final consumer).

3.2 Description of the CT applications and potential market value

A. Food Sector

Possible Applications of CT in food sector are:

- Fresh Algae, Dehydrated Alga: Being an edible alga, CT can be easily introduced in daily meals under different ways (mostly in Europe, since in oriental countries this is already a common practice).
- Fruit Preservative (whole fruit, fresh-cut fruit, fruit puree), Juice Preservative, Meat Preservative: Natural preservatives will allow the substitution of synthetic additives, improving consumers' health.
- Canned Products: In Portugal, adding algae or algae extracts to canned fish products is becoming a common practice. The application of CT in canned food will preserve product's freshness and improve its flavour.
- Canned Sausages Preservative: The addition of a CT extract could help to substitute the use of nitrites (potentially carcinogenic molecules), to preserve this kind of products.
- Natural Pigments/Dyes: The extraction of natural pigments and dyes from algae have been explored for a few decades, being commonly used in Food Industry.
- Coloured Apple (Kids): When dissolved in water, natural pigments extracted from CT would result in coloured dipping solution. After being immersed, apples would assume solution's colour[2].
- Gelatine, Healthy Candies: Texture is the critical point that differentiates animal and vegetable-based gelatines. It would be beneficial to improve vegetable gelatine formulation consistency (texture).

[2] The actual contract with a fast food global brand (McDonald's®) may help to develop this application.

- Chocolate Cream: When heated, chocolate melts. When cooled, it gets solid. A possible way of give it a fluid texture, even when cooled, could involve the addition of a CT extract. This product would serve as a topping to be added on ice creams, fruit, toasts, etc.
- Aroma Preservative: Due to the large application of natural aromas in Food Industry, CT extracts (rich in vitamins and antioxidants) could be applied in order to preserve natural aromas, producing stronger flavours and consequently more valuable products.
- Aromatized Olive Oil, Aromatized Salt[3]: The addition of algae to olive oil and to salt bottles contributes to a better preservation of the products, keeping all its properties (given that algae have strong antioxidants). Since algae also have an equilibrate salt content, smaller amounts of salt and different flavours would be added to home meals.
- Butter, Cooking Oil: Algae are a good source of lipids, namely, polyunsaturated fatty acids (PUFAs), that may be used to produce healthier butter and oils.
- Protein Drinks/Shakes/Powders: Nowadays, protein drinks have an increasing market tendency, being commonly commercialized as powders, liquid drinks or shakes. Due to its high protein content, algae may be considered a good source to produce this kind of drinks.
- Infant Formula: Powdered milk (containing algae compounds) is already being commercialized by some companies. Long-chain PUFAs, such as docosahexaenoic acid (DHA) and arachidonic acid (ARA), ω-3 and ω-6 respectively, are commonly included in infant formulas, showing several benefits to baby's health.
- Animal and Fish Feed: An ancient application that offers a good source of nutrients, due to the excellent properties of algae.

[3] These ideas were already launched in the market by some companies. Several are present in Gourmet sections of supermarkets and classified as excellence products.

Table 3.1: Applications included in Food Sector

Food Sector							
	Technology Criteria			Market Criteria			
Application	Innovation	TV	TRL	MD	Competitors	Regulation	TOTAL
Fresh Alga	1	3	3	3	1	2	13
Dehydrated Alga	1	3	3	3	1	2	13
Fruit Preservative (whole fruit, fresh-cut fruit, fruit puree)	**3**	**3**	**2**	**3**	**2**	**2**	**15**
Juice Preservative	**3**	**3**	**2**	**3**	**2**	**2**	**15**
Meat Preservative	3	3	1	3	2	2	14
Canned Products	2	3	3	3	2	1	14
Canned Sausages Preservative	3	2	1	3	1	1	11
Natural Pigments/Dyes	1	3	3	3	1	2	13
Coloured Apple (Kids)	3	3	2	2	2	2	14
Gelatine	1	3	3	3	2	2	14
Healthy Candies	2	3	3	2	1	2	13
Chocolate Cream	2	3	2	3	1	2	13
Aroma Preservative	3	1	1	3	2	2	12
Aromatized Olive Oil	2	3	3	3	1	2	14
Aromatized Salt	2	3	3	3	1	2	14
Butter	3	3	1	3	1	2	13
Cooking Oil	2	3	3	3	1	2	14
Protein Drinks	2	3	3	1	1	2	12
Shakes, Powders	2	3	3	1	1	2	12
Infant Milk	2	3	2	1	2	1	11
Animal and Fish Feed	1	3	3	2	1	3	13

Notes: Each application was evaluated (from 1 to 3), according to technology and market filters. In the end of each row, the final column (Total) shows the sum of scores attributed after the application of each filter. The selected applications (in bold) received the highest score.
Score: Innovation: 1-Low, 2-Moderate, 3-High; Technology Viability: 1-Low, 2-Moderate, 3-High; TRL: 1-Not Developed, 2-Partially Developed, 3-Developed; Market Dimension: 1-Low, 2-Moderate, 3-High; Competition: 1-High, 2-Moderate, 3-Low; Regulation: 1-Very Tight, 2-Moderately Tight, 3-Weak.

Table 3.1 shows a comparison between different applications, included in Food sector, using technology and market filters. According to Table 3.1 the most interesting applications in food sector are: fruit and juice preservative, since these applications received a total score of 15.

B. Pharmaceutical Sector

Possible Applications of CT in pharmaceutical sector are:

- Food Supplement, Dietary Supplement, Weight Loss Supplement: As previously referred, algae are a very nutritive source (with a low caloric content and with an anti-liquid retention capacity), containing high amounts of protein and PUFAs, besides a high content of vitamins, antioxidants and antimicrobial compounds.
- Cardiovascular Supplement: Among the PUFA present in these organisms, ω-3 and ω-6 are beneficial for cardiovascular health, regulating cardiac beating and enhancing cardiovascular system.

- Sunscreen: Algae usually produce and release antioxidant compounds that protect them against violent ultraviolet (UV) lightning. The protective capacity of these compounds could be explored to develop natural based sunscreens (substituting the actual formulations with metal particles that are a potential risk for consumers' health).
- Antimicrobial *Aloe vera* Gel, Wound Care Solution, Disinfectant: The combined action of both species (*Aloe vera* and *C. tomentosum*) would result in an improved formulation, used to hydrate the skin, as a smoothing (to irritated skin, for example), disinfectant and/or as a cicatrizing agent. Moreover, given algae capacity to form films, a novel wound care solution could function as chemical and physical barrier, also suitable to treat burnings (from minor to severe burns).
- Mouthwash Solution, Teeth Whitening: Algae are already used in some toothpastes. Due to its antimicrobial power, CT could be included in teeth whitening products, preventing the development of dental caries, that affect many people around the world, promoting a better oral care.
- Antiarthritis Gel: A study carried by Aparanji, Atika, Venu, & Rajan (2013) demonstrated the antiarthritic effect of the CT extract. In this way, a gel formulation could be developed to combat pain and other symptoms linked to Rheumatoid Arthritis.
- Medicines: Since investigation is permanently evolving, new properties (with medical application) were and continue to be found. Anticancer, nematicidal (to combat internal parasitic infections) and anticoagulant properties were already identified in solutions containing CT.
- Deodorant, Foot Deodorant (antifungal): The scientific evidences of CT antibacterial and antifungal properties support the development of natural deodorant solutions. As an antifungal agent, the development of a foot deodorant could help to prevent the appearance of athlete's foot, especially in sports complexes.

Table 3.2: Applications included in Pharmaceutical Sector

Pharmaceutical Sector							
	Technology Criteria			Market Criteria			
Application	**Innovation**	**TV**	**TRL**	**MD**	**Competitors**	**Regulation**	**TOTAL**
Food Supplement	1	3	3	3	1	1	12
Dietary Supplement	1	3	3	3	1	1	12
Weight Loss Supplement	2	3	3	2	1	1	12
Cardiovascular Supplement	**2**	**3**	**3**	**3**	**1**	**1**	**13**
Sunscreen	3	2	1	3	2	1	12
Antimicrobial *Aloe vera* Gel	2	2	2	3	1	1	11
Wound Care Solution	**3**	**3**	**2**	**3**	**1**	**1**	**13**
Disinfectant	2	2	1	3	1	1	10
Mouthwash Solution	2	2	2	3	1	1	11
Teeth Whitening	3	2	1	2	1	1	10
Antiarthritis Gel	2	3	2	1	1	1	10
Medicines	**3**	**2**	**1**	**3**	**3**	**1**	**13**
Deodorant	3	3	2	2	1	1	12
Foot Deodorant (antifungal)	2	3	2	2	1	1	11

Notes: Each application was evaluated (from 1 to 3), according to technology and market filters. In the end of each row, the final column (Total) shows the sum of scores attributed after the application of each filter. The selected applications (in bold) received the highest score.

Score: Innovation: 1-Low, 2-Moderate, 3-High; Technology Viability: 1-Low, 2-Moderate, 3-High; TRL: 1-Not Developed, 2-Partially Developed, 3-Developed; Market Dimension: 1-Low, 2-Moderate, 3-High; Competition: 1-High, 2-Moderate, 3-Low; Regulation: 1-Very Tight, 2-Moderately Tight, 3-Weak.

Table 3.2 shows a comparison between different applications, included in Pharmaceutical sector, using technology and market filters. According to Table 3.2 the most interesting applications in pharmaceutical sector are: cardiovascular supplement, wound care solution and medicines, since all these applications received a total score of 13.

C. Cosmetics Sector

Possible Applications of CT in cosmetics sector are:

- Anti-Aging Cream, Eye Cream, Day/Night Cream, Hydration Serum, Lip Balm: The strong antioxidant and hydrating power of CT extracts allows a quick and efficient action as an anti-aging or, when applied in particular areas, as an eye or lip contour cream, nourishing and eliminating visible wrinkles. Serums are high concentrated formulas that have an even quicker and more efficient action. Several algae-based cosmetics are currently available in the market.
- Shower Gel/Cream, Body Lotion, Hand Soap: CT may be a key ingredient for the production of shower gel/cream (shower creams became very popular in the last years), with a nourishing effect. Nowadays, it's common to find these products in the market.
- Shampoo (regular, dry, etc.), Treatment Shampoo: The vitamin and mineral content of CT could be used to develop shampoos able to strengthen and hydrate the hair. Specific

formulas, appropriate to treat hair falling and weak and easy brake hair, could be developed as well.

- Hydrating Mud/Butter, Massage Oils, Masks, Exfoliating Products: Several algae-based products can be applied in people's skin, having a hydrating, nourishing and smoothing effect.
- Makeup Products, Makeup Remover: Gathering pigment extraction and the hydration and protective properties of algae-based products, it's possible to produce safer and natural makeup solutions. These formulas may help to prevent skin dehydration. Likewise, a makeup remover can also be proposed, refreshing, hydrating and protecting the skin simultaneously.
- Thalassotherapy: Very popular in Europe, this therapy is quite common in Spas. It's based on the healing properties of sea water and also of algae. The goal is to relieve pain and heal several kinds of damage (bone and muscle).

Table 3.3 shows a comparison between different applications, included in Cosmetics sector, using technology and market filters. According to Table 3.3 the most interesting applications in cosmetics sector are: shower gel/shower cream, body lotion, hand soap, shampoo and thalassotherapy, since all these applications received a total score of 13.

Table 3.3: Applications included in Cosmetics Sector

Cosmetics Sector							
	Technology Criteria			Market Criteria			
Application	**Innovation**	**TV**	**TRL**	**MD**	**Competitors**	**Regulation**	**TOTAL**
Anti-Aging Cream	1	3	3	1	1	1	10
Eye Cream	1	3	3	2	1	1	11
Day/Night Cream	1	3	3	2	1	1	11
Hydration Serum	1	3	3	2	1	1	11
Lip Balm	1	3	3	2	1	1	11
Shower Gel/Cream	**1**	**3**	**3**	**3**	**1**	**2**	**13**
Body Lotion	**1**	**3**	**3**	**3**	**1**	**2**	**13**
Hand Soap	**1**	**3**	**3**	**3**	**1**	**2**	**13**
Shampoo (regular, dry, etc.)	**1**	**3**	**3**	**3**	**1**	**2**	**13**
Treatment Shampoo	1	2	2	1	1	1	8
Hydrating Mud/Butter	1	3	3	1	1	1	10
Massage Oils	1	3	3	1	1	2	11
Masks	1	3	3	1	1	1	10
Exfoliating Products	1	3	3	1	1	2	11
Makeup Products	1	2	1	1	1	1	7
Makeup Remover	1	3	2	1	1	1	9
Thalassotherapy	**1**	**3**	**3**	**3**	**1**	**2**	**13**

Notes: Each application was evaluated (from 1 to 3), according to technology and market filters. In the end of each row, the final column (Total) shows the sum of scores attributed after the application of each filter. The selected applications (in bold) received the highest score.
Score: Innovation: 1-Low, 2-Moderate, 3-High; Technology Viability: 1-Low, 2-Moderate, 3-High; TRL: 1-Not Developed, 2-Partially Developed, 3-Developed; Market Dimension: 1-Low, 2-Moderate, 3-High; Competition: 1-High, 2-Moderate, 3-Low; Regulation: 1-Very Tight, 2-Moderately Tight, 3-Weak.

D. Industrial Sector

Possible Applications of CT in Industrial sector are:

- Biofuel: An intensely studied area over the last decade. In order to become a valuable product/resource it's still necessary to reduce production costs. Biofuel results from the oil extraction form natural resources (like microalgae). Some studies already demonstrated the use of CT for this purpose.
- Bioplastics: A largely explored industry nowadays. It will serve to substitute plastic produced from fuel. Algae may serve as a raw material to produce biodegradable and more sustainable products.
- Fertilizers: It's maybe the most ancient application of algae. These organisms are rich in nutritive compounds, being used to fertilize large agricultural fields.
- Paper: Paper production begins with cellulose extraction from vegetable matter. Having faster reproduction cycles, green algae may be a suitable raw material for this industry.
- Textile: Fibre production, as well as pigment extraction make these organisms a raw material for this industry[4].
- Aquarium Filters, Filters: It's already known that algae can filter and depurate water. In this way, it would be interesting to develop an organic filter, creating a confined ecosystem (mimicking the natural environment) more appropriate for the survival of animals present in aquariums. Also by changing the matrix thickness, filters could be adapted for many distinct applications.
- Glass: Another ancient application. The ashes obtained from the burning of dry algae were used to produce glass. It would be interesting to rehabilitate this industry.
- Paints: Utilization of natural pigments to produce paints.
- Detergents: Due to the high lipid content and antimicrobial power of CT, natural based detergents could be produced.
- Bioremediation: Algae accumulate large amounts of heavy metals, contributing to purify the surrounding environment.
- Pollution Indicator: Algae, like many other vegetable species, react differently to slight changes in the air, light or water quality. Thus, they can be used to register environmental changes, allowing an early and controlled prevention of the environmental damage.

[4] Seacell® is an algae-based fibre produced by a German company that promises to revolutionize textile industry.

Table 3.4 shows a comparison between different applications, included in Industrial sector, using technology and market filters. According to Table 3.4 the most interesting applications in industrial sector are: bioplastics, glass, detergents, bioremediation and pollution indicator, since all these applications received a total score of 14.

Table 3.4: Applications included in Industrial Sector

Industrial Sector							
	Technology Criteria			Market Criteria			
Application	Innovation	TV	TRL	MD	Competitors	Regulation	TOTAL
Biofuel	1	2	3	3	1	3	13
Bioplastics	**2**	**3**	**2**	**3**	**1**	**3**	**14**
Fertilizers	1	3	3	2	1	3	13
Paper	2	2	2	2	1	3	12
Textile	2	2	2	3	1	3	13
Aquarium Filters	3	3	2	1	1	3	13
Filters	3	1	1	1	1	2	9
Glass	**1**	**3**	**3**	**3**	**1**	**3**	**14**
Paints	3	1	2	2	1	3	12
Detergents	**2**	**3**	**2**	**3**	**1**	**3**	**14**
Bioremediation	**1**	**3**	**3**	**2**	**2**	**3**	**14**
Pollution Indicator	**2**	**3**	**3**	**1**	**2**	**3**	**14**

Notes: Each application was evaluated (from 1 to 3), according to technology and market filters. In the end of each row, the final column (Total) shows the sum of scores attributed after the application of each filter. The selected applications (in bold) received the highest score.
Score: Innovation: 1-Low, 2-Moderate, 3-High; Technology Viability: 1-Low, 2-Moderate, 3-High; TRL: 1-Not Developed, 2-Partially Developed, 3-Developed; Market Dimension: 1-Low, 2-Moderate, 3-High; Competition: 1-High, 2-Moderate, 3-Low; Regulation: 1-Very Tight, 2-Moderately Tight, 3-Weak.

3.3 The Selected Application (Go-To-Market Strategy)

Table 3.5 summarizes the highlighted applications in each sector. Comparing the selected applications, it's possible to observe that the formulation of a CT extract to preserve fruit and/or juices seems to be the best applications for this alga (having a final score of 15, as previously stated), deserving a deeper study of a go-to-market strategy.

The natural additive will provide a safer and extended preservation of fresh products, promoting fruit and vegetables consumption, ultimately helping consumers to maintain a healthier diet. Thus, it's a suitable product for a large part of the population (high market dimension, scoring 3 points).

Its classified as a highly innovative technology (scoring 3 points) due to the fact of being already protected by a patent (original product).

Scientific articles (published by MARE-IPLeiria investigation team) support the viability and efficacy of this additive (TV, with a score of 3 points). The patent also demonstrates the development of a prototype obtained in a laboratorial scale, although it needs additional tests to become a scale-up prototype (TRL, with a score of 2 points), adapted to an industrial production line, through which the final product will be obtained and then launched in the market.

Table 3.5: Highlighted Applications by Sector

		Technology Criteria			Market Criteria			
Sector	**Application**	**Innovation**	**TV**	**TRL**	**MD**	**Competitors**	**Regulation**	**TOTAL**
Food	**Fruit Preservative (whole fruit, fresh-cut fruit, fruit puree)**	**3**	**3**	**2**	**3**	**2**	**2**	**15**
	Juice Preservative	**3**	**3**	**2**	**3**	**2**	**2**	**15**
Pharmaceutical	Cardiovascular Supplement	2	3	3	3	1	1	13
	Wound Care Solution	3	3	2	3	1	1	13
	Medicines	3	2	1	3	3	1	13
Cosmetics	Shower Gel/Cream	1	3	3	3	1	2	13
	Body Lotion	1	3	3	3	1	2	13
	Hand Soap	1	3	3	3	1	2	13
	Shampoo (regular, dry, etc.)	1	3	3	3	1	2	13
	Thalassotherapy	1	3	3	3	1	2	13
Industrial	Bioplastics	2	3	2	3	1	3	14
	Glass	1	3	3	3	1	3	14
	Detergents	2	3	2	3	1	3	14
	Bioremediation	1	3	3	2	2	3	14
	Pollution Indicator	2	3	3	1	2	3	14

Notes: Each application was evaluated (from 1 to 3), according to technology and market filters. In the end of each row, the final column (Total) shows the sum of scores attributed after the application of each filter. The selected applications (in bold) received the highest score.
Score: Innovation: 1-Low, 2-Moderate, 3-High; Technology Viability: 1-Low, 2-Moderate, 3-High; TRL: 1-Not Developed, 2-Partially Developed, 3-Developed; Market Dimension: 1-Low, 2-Moderate, 3-High; Competition: 1-High, 2-Moderate, 3-Low; Regulation: 1-Very Tight, 2-Moderately Tight, 3-Weak.

The number of synthetic additives currently used in Food Industry to preserve fruit (citric acid, ascorbic acid, NatureSeal® or Food Freshly® for example) and the new formulations that are being developed to substitute the aforementioned solutions (indirect and direct competitors, respectively), make the marketing of CT additive a difficult process. Besides, it is a relatively simple solution, susceptible of being copied/adapted by other companies. Thus, competition is classified as moderate (2 points). Nevertheless, it's a good classification, comparing with the remaining applications, many of which have several direct competitors.

Being a food additive, ingested by consumers, it has to be assured that there are no risks associated with its consumption. In Europe, the authorities responsible for food safety are the Scientific Committee on Food (SCF) and/or the European Food Safety Authority (EFSA). In order to integrate the product in the European Union List of Food Additives, SCF and EFSA have to evaluate and classify the additive as safe.

On the other side, in the Pharmaceutical sector, products have to pass through a very strict regulation, including several tests/stages before being introduced in the market (that generally takes 20 years). So, considering that in the Pharmaceutical sector regulation procedures are more complex, in Food and Cosmetics sectors regulation was classified as moderately tight (scoring 2 points).

As earlier explained, one of the purposes of this project – also supported by the results obtained in this brainstorming of possible applications for the CT alga – was the development of a natural additive (AlgaeCoat), to preserve fresh-cut fruit, particularly apple. In the future is expected to apply this solution over other fruits as well (whole or minimally processed). It's interesting to add that this formulation was also successfully applied over custard apples (*Annona* sp.) and pears, like 'Rocha' pear (*Pyrus communis* L.).

A detailed study of technology functionality and pilot-scale validation will be exposed in the next chapter (Chapter 4), including the protocols and experiments followed, as well as the main results achieved.

CHAPTER 4

Technology: AlgaeCoat

4.1 How to obtain an Extract from CT Alga?

In the previous chapter several applications for the CT alga were suggested. Between the numerous ideas, the most interesting one was the formulation of a natural additive towards the preservation of fresh-cut fruit.

Due to the partnership established between IPLeiria (Instituto Politécnico de Leiria) and Campotec IN – a company that produces minimally processed fruit and vegetables – the investigation project resulted in the development of AlgaeCoat – an edible coating extracted from CT alga to preserve fresh-cut apple. In a lab scale, this goal was successfully accomplished, resulting in the submission of a patent (PT 107369 B) of national invention.

The following step – scale-up – was the basis for this thesis project, which will be explored further in this chapter.

To sustain the aim of this work, it's necessary to understand first which are the main causes that contribute to MP fruit deterioration. It's also necessary to understand the main metabolic pathways involved in MP fruit softening and browning, as well as the role that specific enzymes like POD, PPO and PME (Pectin Methylesterase) may play in those pathways. It's crucial to know and understand the role of this biological factors, to avoid undesired side effects, like aroma loss, sugar loss, etc.

In the next sections the reasons by which apple was the fruit selected to test the efficacy of the CT extract will be explained.

In section 4.4, a detailed description of the technology is provided, and in the final sections, laboratorial work will be explained, as well as the discussion of the main results obtained.

4.2 Why Apple?

As written in the previous section, the partnership between MARE-IPLeiria and Campotec IN led to the application of AlgaeCoat in fresh-cut apples. Since Campotec IN is the main supplier of MP apples to one of the most famous fast-food chains in Portugal (McDonald's®) and to the leading Portuguese airline (TAP), the need to preserve the freshness and to extend the shelf-life of this highly perishable fruit were the guidelines for the development of this solution.

Apples are fleshy fruits, with a firm texture and an acid flavour which determine its uniqueness. These features change progressively during maturation stages. As climacteric fruits, apples maintain a high metabolic activity, leading to great biochemical transformations during ripening, and consequently to a very quick deterioration, if the appropriate post-harvest treatment is not applied (Sacilik & Elicin, 2006).

Apple's firm and crunchy texture, as well as its reduced size and all the benefits associated, make this one of the most appreciated and consumed fruits in whole world – about 10.1 Kg *per capita* in 2013 (FAOSTAT, 2017). Apples also have a tremendous importance in the national diet. According to Instituto Nacional de Estatística (INE, 2017), in 2016, an average of 29.8 Kg of apple *per capita* was consumed in Portugal.

According to Instituto Nacional de Saúde Doutor Ricardo Jorge (INSA), the edible part of an apple matches 90% of the whole fruit and the daily portion recommended for an adult is one unit with approximately 160 g (INSA, 2017).

Apple's daily intake helps to prevent and combat several chronic diseases, reducing cholesterol levels in the blood. This is also an ideal food supply for people that want to lose weight, allowing them to stay healthy at the same time. Its texture and high fibre content contribute to a sensation of satiety (Córdova, 2006; Augusto, Desenvolvimento de revestimentos comestíveis para produtos de IV gama, 2013; Instituto Nacional de Estatística, I.P., 2016).

Given all the features and properties of apples, it's crucial to create the perfect conditions to produce them with the maximum quality, so consumers can eat and enjoy the taste and benefits offered by this particular fruit.

It was precisely to take advantage of product's quality that the European Commission (EC) created the PGI (Protected Geographical Indication) and PDO (Protected Designation of Origin) quality logos.

PGI includes the areas known to have the perfect conditions for the development of products with exclusive and distinctive features (like taste and texture), that make them very appreciated around the world. Products certified with this logo are produced according to the local know-how, by traditional techniques (DGADR, 2014).

In Portugal there are four regions/locations indicated as PGI (IGP, or *Indicação Geográfica Protegida*), recognized by the EU and by the Portuguese Agriculture Ministry (DGADR – Direção Geral de Agricultura e Desenvolvimento Rural), where apples and apple products can be produced with high quality: Western Region ("Maçã de Alcobaça"); Northern Region ("Maçã da Beira Alta"); Central Region ("Maçã da Cova da Beira") and Portalegre ("Maçã de Portalegre").

In Portugal there are also other varieties of apple recognized as PDO (or DOP, *Denominação de Origem Protegida*), namely "Maçã Bravo de Esmolfe" and "Maçã Riscadinha de Palmela".

Jorge Soares, Executive Administrator in Campotec S.A., affirms that apple production is following an intensive process of modernization and reorganization, in order to promote fruits' quality and food safety. In account of that, producers must promote their regional products and/or develop new products to meet consumers' needs.

4.3 Minimally Processed Apple

4.3.1 Production Process – Minimal Processing

As stated in the Book of Specifications published by Direção-Geral de Agricultura e Desenvolvimento Rural (DGADR, 2014), there is a set of rules to prepare minimally processed apples, before being introduced in the market.

The production process of MP apples includes several consecutive steps (schematically represented in Fig. 4.1), namely, washing; disinfection; cutting, peeling or slicing and finally packaging (storing the final product under refrigeration, according to legal provisions, without compromising fresh-like attributes and nutritional value of the produce).

Nonetheless, before packaging, apple slices can be immersed in aqueous solutions containing natural preservatives or edible coatings, to extend product's shelf-life and to maintain its essential features (texture and flavour).

Before cutting, fruit must be intact to go along to the washing process, to prevent changes in fruit characteristics (fruit's freshness). Additionally, any kind of cutting operation must be done only by physical methods. To reduce fruit oxidation, cutting, packaging and transport operations have to be performed under refrigeration.

Apples must be stowed in packages, or in a specific film, that act as a barrier, able to control gas diffusion. To further prevent product's degradation, a modified atmosphere with low oxygen levels may be introduced in the packages.

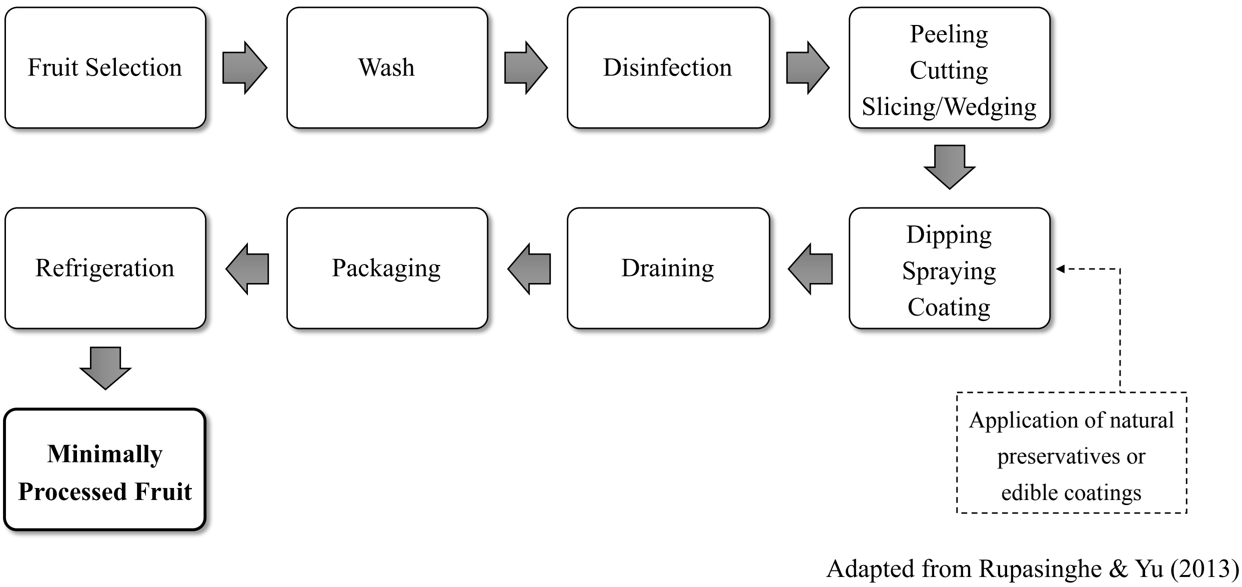


Figure 4.1: Major Processes for fresh-cut processing (revised from Rupasinghe & Yu (2013))

4.3.2 Browning Process

In order to explain the need beyond the development of the proposed technology – a natural edible coating composed by a seaweed extract – in this section, metabolic pathways related to MP apple browning will be approached.

According to Lee, Park, Lee, & Choi (2003), minimally processed products, commonly known as fresh-cut produce (fruits and vegetables), are exposed to a greater and faster deterioration, as a consequence of their production process. The deterioration of fresh-cut produce is associated with many factors, namely: water loss; softening (linked to PME activity); microbial contamination; increased respiration and ethylene production and finally cut-surface browning (linked to PPO and POD activity).

Tissue injury caused by slicing and chopping triggers a defence response characterized by an increasing in the respiration rate, that will, consequently, determine faster deterioration process. For this reason, MPP shelf-life is much more limited than the uncut produce shelf-life (Lee, Park, Lee, & Choi, 2003; Rocha & Morais, 2003).

Along with the deterioration process, tissue browning is also a factor that should be considered by producers and processors, since products' aesthetics is a quality feature immediately perceived and evaluated by consumers, determining their choice (Lee, Park, Lee, & Choi, 2003). Moreover, browning also affects the organoleptic properties and nutritional quality of fresh products (Rocha & Morais, 2003).

Since the lower shelf-life is mainly associated with mechanical damages (wounding), there's a need for a product that can interferes in produce's defence response, controlling the respiration rate and the enzymatic browning.

Tissue browning is a visible consequence of cellular decompartmentalization that occur after a mechanical damage, allowing the contact between PPO and polyphenolic compounds.

Mechanical Damages

As stated above, mechanical damages lead to tissue browning as the loss of cellular compartmentalization enhances enzyme/substrate contact.

Montero, Santos, & Bender (2016) observed that when the intensity of mechanical injuries increased, electrolyte loss also increased, pointing to a high permeability of the cellular membrane on the impacted areas.

The increase in membrane permeability is frequently associated with changes in membrane fluidity, affecting consequently cellular compartmentalization and tissue disorganization. Decompartmentalization, in turn, leads to changes in cellular pH and in enzyme and/or substrate concentrations, affecting subsequently the enzymatic activity and other metabolic events (ie.: the activity of the receptors, starch breakdown, etc.).

Stress damages (ie.: chilling injuries, drought, senescence or overexposure to O_2 and CO_2 atmospheres) also weaken cell membranes, leading to enzymatic browning.

The lack of cell stability can also occur as an outcome of lipid peroxidation or free radicals' upsurges. Lipid peroxidation due to ROS production (under freezing and similar stress conditions) is considered the major cause of membrane damage in plant tissues, being an indication of injuries and electrolyte loss. Evidences show that electrolyte leakage is also connected to fruit quality (Mditshwa, et al., 2016; Montero, Santos, & Bender, 2016).

Phytomelanins' Production and Accumulation

When fruits' tissue is injured, the action of polyphenoloxidase (PPO) and peroxidase (POD), located in thylakoid membranes and in cell walls, respectively, allows the production and accumulation of phytomelanins, a brown pigment visible in fruits' superficies (Taiz & Zeiger, 2002; Chisari, Barbagallo, & Spagna, 2007).

PPO belongs to a group of copper-containing enzymes (Cu^{2+}) that catalyse the hydroxylation of monophenols to *o*-diphenols, as well as the oxidation of *o*-diphenols to the respective *o*-quinones in the presence of oxygen (O_2) (Chisari, Barbagallo, & Spagna, 2007; Araji, et al., 2014).

The enzymatic reaction between PPO and the phenolic compounds (stored in cell vacuole), after cell damage (ie.: cut/mechanical damage), triggers the reactions described above (Araji, et al., 2014).

In plants, PPOs are commonly encoded by multigene families. According to Araji et al. (2014) responses to stress varies between species and within PPO gene families. Some authors affirm that within a single plant species, different members of the PPO gene family may be expressed in different or in the same tissues, at different developmental stages, exhibiting distinctive patterns of expression. Moreover, PPO have different roles in processes like pigment biosynthesis, senescence, wound healing and defence against pathogens.

On the other hand, POD is an iron-containing enzyme, having multiple molecular forms and being present in many fruits and vegetables. Its catalytic properties are influenced by variety and/or cultivar and also by growth and physiological stages (Neves & Lourenço, 1998).

In fruits and vegetables POD reveals quality deterioration through flavour loss and several biodegradation reactions. During processing and preservation, POD also contributes to the enzymatic browning, since diphenols may function as reducing substrates in the reaction (Chisari, Barbagallo, & Spagna, 2007; Jang & Moon, 2011).

Additionally, POD participates in primary and secondary metabolism processes, like regulation of cell elongation, cross-linking between cell wall polysaccharides, synthesis of lignin and suberin polymers (defence), cross-linking catalysis between ferulic acid substituents of pectin, wound healing, phenol oxidation and defence against pathogens (Lagrimini, 1991; Chisari, Barbagallo, & Spagna, 2007).

Precipitation of proteins by polyphenols leads to the development of an astringent flavour and consequently to a decrease in palatability. Polyphenols also contribute to a reduced digestibility through protein denaturation and cross-links with carbohydrates (Lagrimini, 1991).

4.3.3 Flavour and Texture – Quality Factors

Flavour is a key quality attribute of fresh products, being especially perceptible when fruit and vegetables are consumed raw. Besides flavour, apple quality is also determined by features like the visual appeal, firmness and certainly by the absence of any physiological or pathological damages.

Quality is nowadays perceived by consumers as a powerful criterion for fruit acceptance or rejection, being primarily determined by the interactions between the pectic substances and pectinases (such as PME) coexisting in fruits and vegetables. Pectinases are complex macromolecules that catalyse numerous reactions, namely the degradation of pectin (a major component of plant cell wall), affecting the mechanical properties of fruit's tissues. Like other enzymes, PME activity is modulated by the pH and temperature, through which is possible to control the enzymatic interactions during food storage and processing (Espino-Díaz, Sepúlveda, González-Aguilar, & Olivas, 2016; Lv, Zhao, & Ning, 2017).

Flavour and texture also rely on the contents of water (that may achieve 85% fresh weight); micronutrients (like potassium); sugars (mainly fructose, sucrose and glucose, easily assimilated by human organism) and fibres (cellulose, hemicellulose and pectin, responsible for water retention) in apples composition (Córdova, 2006; Augusto, Desenvolvimento de revestimentos comestíveis para produtos de IV gama, 2013).

Apples' protein content is not significant (protein content rounds 1% fresh weight), despite the crucial role of enzymes in the regulation of fruits' metabolism (Augusto, Desenvolvimento de revestimentos comestíveis para produtos de IV gama, 2013).

Moreover, apples have a low lipid content. Lipids are mainly present in the protective cell layers and in the epidermal cells (Córdova, 2006; Augusto, Desenvolvimento de revestimentos comestíveis para produtos de IV gama, 2013).

Malic acid and citric acid are among the most important organic acids, in spite of being present in very small amounts when compared with the other components. Malic acid, together with sugars, esters and aldehydes, contributes to apples' distinctive flavour (Córdova, 2006; Rupasinghe & Yu, 2013; Augusto, Desenvolvimento de revestimentos comestíveis para produtos de IV gama, 2013).

From other point of view, it's possible to think that flavour results from the combination between both taste and aroma.

Apples' taste is determined by the concentration of sugars and organic acids, while aroma is a complex mixture of several volatile compounds, whose composition is specific and unique for each species or even for each variety/cultivar (Espino-Díaz, Sepúlveda, González-Aguilar, & Olivas, 2016).

Volatiles' production increases during the climacteric period, in which ethylene production induces a series of physical and chemical changes in the expression of specific genes and, consequently, in the activity of specific enzymes (encoded by those genes) (Espino-Díaz, Sepúlveda, González-Aguilar, & Olivas, 2016).

4.4 Technology Description – Intellectual Property

Patent of invention (PT 107369 A, also published as PT 107369 B):

- Title: "Revestimento de origem marinha para aplicação em produtos minimamente processados ou de quarta gama e respectivo método de produção";
- Filing date (16/12/2013);
- Current status (patent granted in 10/03/2016);
- International Patent Classification (IPC): A23B7/00; A23L3/00.

Description

The Description from the patent PT 107369 B is discussed next:

The present invention describes a marine-based edible coating (ECT) and/or film suitable for the preservation of minimally processed products (MPP), such as fresh-cut fruits and vegetables, with the purpose of extending produce shelf-life.

As described in the patent, this formulation can be applied as an immersion solution, prepared from the dissolution of a *C. tomentosum* concentrate (that functions as the active ingredient) in distilled water, or else, applied as an edible film, with a 0.03 mm to 0.06 mm thick, constituted by distilled water, 0.5% CT concentrate and 1% alginate and/or chitosan.

Edible coatings form a protective layer in the superficies of the fresh produce, acting as a semipermeable barrier against gas diffusion, namely, oxygen (O_2), carbon dioxide (CO_2) and water (H_2O), that accelerate the ripening process, leading to subsequent food deterioration. Additionally, edible coatings may have the ability to retain aromatic volatile compounds (naturally produced by fruits and vegetables), responsible for the characteristic aroma and flavour of some fruits and vegetables. However, there are no evidences, yet, of the presence of this capacity in the current formulation.

Accordingly, the main purpose of an edible coating is to achieve an extended shelf-life, without compromising product's quality and consumer's health.

An increased shelf-life is accomplished due to extract's capacity of inhibiting the enzymatic activity of Polyphenoloxidase (PPO) and Peroxidase (POD), responsible for tissue browning (oxidation). PPO and POD inhibition results in the retardation of cell walls' degradation and other metabolic pathways involved in fruits and vegetables' ripening (decreasing oxidation and respiration rates), thus maintaining product's texture and firmness (mechanical properties). This inhibitory capacity results in the retention of product's visual appeal during storage, at room temperature or under refrigeration (between 2ºC and 4ºC).

Besides the antioxidant power, CT extract also has an antimicrobial activity, protecting fruit's tissues against the microbial spoilage, that accelerates the deterioration (tissue softening).

The application of CT extract (AlgaeCoat) in minimally processed 'Fuji' apples (MP apples) reduced significantly slices' browning index (BI), and also the variation of mass loss percentage (preventing softening), water content, soluble solids concentration (SSC) and pH (retaining sugar content and acidity, which define apples' flavour). After the treatment with AlgaeCoat, apples retained their visual appeal for approximately 20 days.

The results obtained were corroborated by a significant decrease in PPO and POD enzymatic activity, registered after the treatment with the protective solution, as previously referred.

The film was also submitted to quality tests that confirmed its low solubility (not allowing an easy dissolution of the film); great perforation resistance and elasticity (allowing a strong physical protection of food matrixes, even under mechanical aggressions, during handling); low water content equilibrium, even when stored in areas with different water activity values (a_w); and the presence of antibacterial properties against apple's flora.

Claims

Claims from the patent PT 107369 B are transcribed and discussed next:

1. "Edible coating obtained from a marine source, consisting of a marine, edible macroalga concentrate (*Codium tomentosum* concentrate), distilled water and alginate and/or chitosan, to be applied over minimally processed products.
2. Edible coating, according to claim 1, characterized by a concentration of 0.5% (w/v) of *Codium tomentosum* in distilled water and 1% of alginate and/or chitosan.
3. Edible coating, according to claim 1, characterized by having a thickness between 0.03 mm and 0.06 mm.
4. Edible coatings' production method, according to claims 1 and 2, characterized by being constituted by the following steps:
    a. Washing of the edible seaweed *Codium tomentosum*, lyophilisation, packaging and storage at – 80°C;
    b. Obtainment of the concentrates from the seaweed previously lyophilised, using a polar solvent;

c. Weighing of 2g of dry biomass, addition of distilled water and ethanol in a proportion of 3:1 (v/v);
d. Agitation of the obtained solution for 6 hours, protected from the light, at room temperature;
e. Centrifugation at 2000g, for 10 min., at 4°C, and collection of the supernatant;
f. Filtration, followed by vacuum evaporation of the supernatant, to remove ethanol, at 30°C, proceeded by lyophilisation to remove water;
g. Incorporation of the resulting concentrate in polymeric coatings of alginate and/or chitosan.

5. Utilization of the edible coating, according to claims 1, 2 and 3, characterized by being applied in minimally processed products".

To sum up, regarding the description and claims exposed in the patent of invention displayed above, it's possible to confirm the natural composition of AlgaeCoat that, unlike many other solutions, doesn't have synthetic ingredients (it's a 100% natural solution), meeting the concept of "clean label[5]".

Also in this patent, it's explained that is possible to create a film just by adding natural polysaccharides (alginate and/or chitosan) to the underlying formulation. However, the addition of these ingredients raises the final cost of the additive.

It's interesting to add that this is a "green" technology (environmentally friendly), not only due to its natural base, but also to its contribution to reduce food waste[6], given fruits/vegetables' high degradation rate (between 4 and 7 days).

Besides sustainability, it can be stated this is also a safe technology, being an alternative solution to GMOs (Genetically Modified Organisms) – whose acceptance by the community (both consumers and food safety entities) is controversial – and also to synthetic additives (such as citric and ascorbic acid, produced by genetic recombination), largely used in Food Industry, representing a risk for human health when consumed for long periods and/or in large amounts. For that reason, risk reassessment of some synthetic additives has resulted in the progressive elimination and reduction of its maximum permitted levels by the SCF and/or EFSA (European Comission Food Safety, 2016).

Moreover, besides all the qualities mentioned above, this technology may add nutritional value to the one that is naturally owned by fresh products (like fruits and vegetables). Nevertheless,

[5] Clean label foods are natural and organic foods, free of synthetic additives or artificial and complex ingredients, such as artificial flavours, dyes, preservatives, etc. (Go Clean Label™, 2018)

[6] According to Broad Leib (2013), date labels are one of the main factors leading to food waste. This happens because these labels transmit the wrong idea (misinterpreted by the consumer) that the product, after the expiry date, is no longer fresh or viable, representing a risk for his/her own health.

since AlgaeCoat is just a superficial treatment, the amount of alga present in the formulation may not be enough to produce effects on the human organism. Despite the low amount of alga, given the nutritional and medicinal properties linked to seaweed intake, consumers can develop a positive perception about AlgaeCoat additive.

According to several studies, seaweed consumption prevents the appearance and development of several diseases, exhibiting visible benefits such as anticoagulant, anti-proliferative, antiviral and antimicrobial properties, among other (Santos, et al., 2015). Their high nutritional value is represented by a low lipid content (though, poly-unsaturated fatty acids are good for human health), high protein content (through which it's possible to substitute meat and fish consumption need), as well as high polysaccharide, mineral and vitamin content (Augusto, Simões, Pedrosa, & Silva, 2016). Algae are also a source of antioxidants ("anti-aging" compounds), very important for managing a younger and healthy organism.

Given all the reasons exposed above, it's reasonable to think that this technology shall integrate the GRAS product list ("Generally Recognized as Safe"), assuring the quality, safety and stability of MPP.

4.5 Materials and Methods

4.5.1 Materials

4.5.1.1 Apple

Apples (*Malus x domestica* Borkh., cv. Cripps Pink) were directly obtained from Campotec S.A., a local producer in Torres Vedras (Lisboa, Portugal). Apples were stored under refrigeration (4 ± 2 °C) until use.

To validate the results obtained (after additive's application), apples were sliced, treated and packaged in Campotec S.A. facilities, reproducing the regular production chain (outlined by the company).

4.5.1.2 Seaweed (*C. tomentosum*)

The marine green macroalgae (*Codium tomentosum*), used to prepare the natural additive, was purchased from Algaplus Lda., placed in Ílhavo (Aveiro, Portugal), already dried and in a green, pulverized state.

4.5.2 Methods

4.5.2.1 Additive's Preparation (pilot-scale)

Through a solid-liquid extraction (Pilodist SL 5 Solid-liquid Extractor, Meckenheim/Bonn, Germany), using water and ethanol (in a ratio of 3:1), a *Codium tomentosum* extract (CTE) was obtained. For this purpose, the dried algae concentrate was weighted and dropped into the filter[7], then placed in the extraction vessel (Fig. 4.2). Then, water and ethanol were added into the bath vessel.

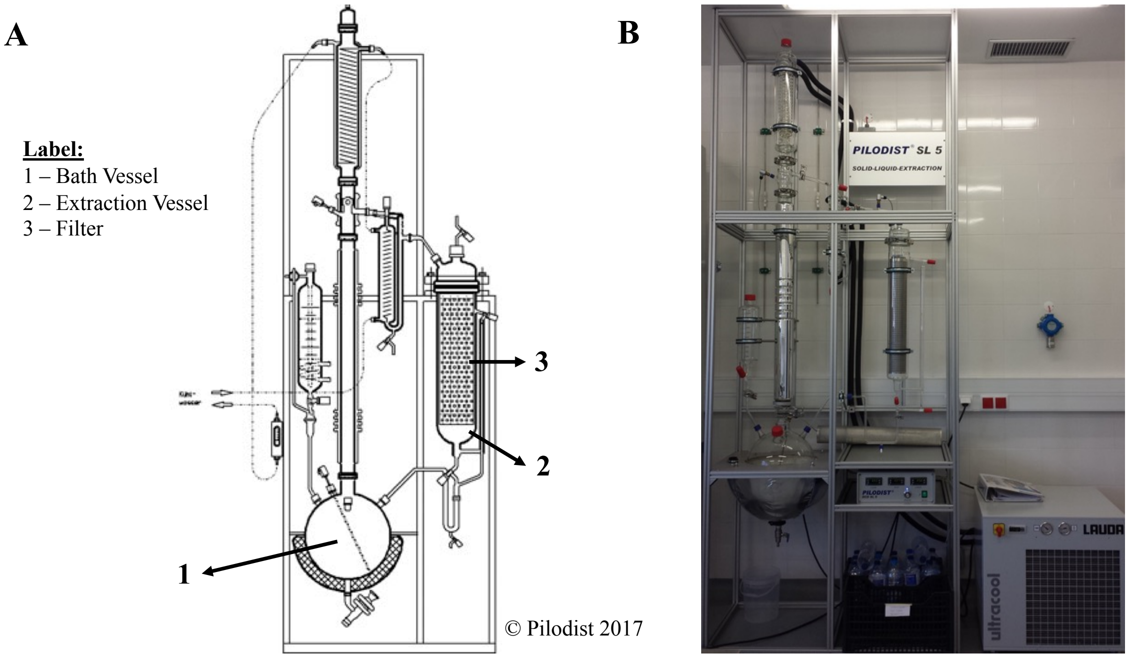


Figure 4.2: Solid-Liquid Extractor PILODIST® SL 5. Schematic representation of the equipment (A) and photographic register of the equipment in the lab (B)

Figure 4.3 (A) displays a scheme of the entire production process. The extraction process began a few seconds after the ebullition of the extraction solvent (water:ethanol). The process ended when 1 L of CTE was attained and 3 hours later it was collected and stored under refrigeration.

In the next step, ethanol was removed in a vacuum-evaporator IKA RV 10 Control (VWR, Pennsylvania, USA), under standard conditions of velocity, pressure and temperature (75 rpm, 95 mbar e 38 °C, respectively).

[7] This filter was washed with tap water and dried inside an incubator before the addition of the alga.

After ethanol removal, CTE was frozen at −80 °C, to be freeze dried later on. During lyophilisation, water was removed to obtain the final product (natural additive) – a solid dry powder ready to be used (as displayed in Fig. 4.3 (B)). Therefore, to prepare the treatment solution, the additive was dissolved in water, as described next.

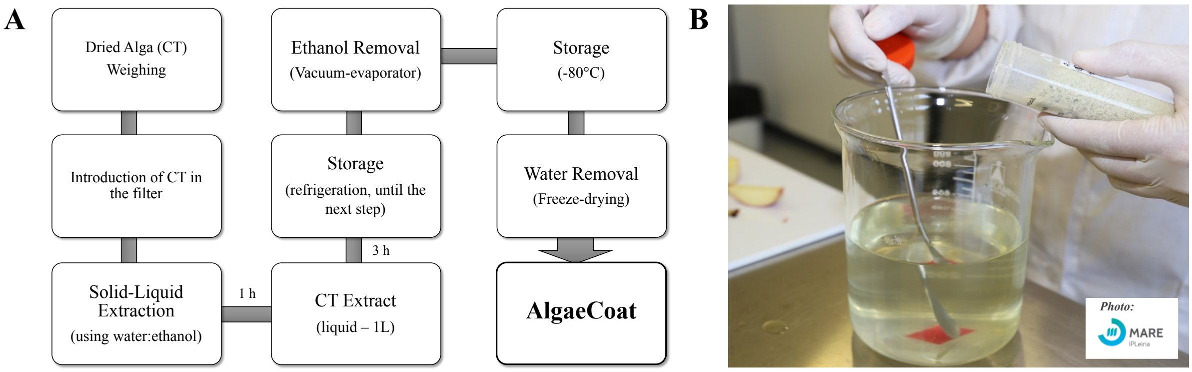


Figure 4.3: Scheme of AlgaeCoat production process (A) and photographic register of the additive (B). Photo ©MARE-IPL

4.5.2.2 Apple Treatment

In Campotec S.A. facilities, apples were uniformly sliced in a suitable cutting machine.

After cutting, 6 Kg of apple slices were collected and immersed for approximately 90 seconds in a container (with a 5 L capacity), where treatment solutions were previously prepared: (A, D) Control Solution (containing only water), (B, E) Commercial Solution (containing water and a commercial additive) and (C, F) *C. tomentosum* Extract Solution (containing water and 0.5% of freeze dried seaweed). Treatments are summarized in Table 4.1.

Table 4.1: Treatments and Packaging conditions applied to the apple slices prepared in Campotec S. A. facilities

Treatments		**Composition**			
Package Headspace	**Solutions**		O_2	CO_2	N_2
Modified Atmosphere (MAP)	(A) Control Solution	Water (only)	8%	22%	70%
	(B) Commercial Solution	Commercial additive + Water			
	(C) CT Extract Solution	*C. tomentosum* (0.5% (w/v)) + Water			
Ambient O_2 Atmosphere (AIR)	(D) Control Solution	Water (only)	22%	1%	77%
	(E) Commercial Solution	Commercial additive + Water			
	(F) CT Extract Solution	*C. tomentosum* (0.5% (w/v)) + Water			

Notes: Commercial Solution is the treatment solution used by the company to preserve apple slices. Since this was a real time experiment, this solution was not prepared and the slices from the production line were directly packaged (in some bags, it was injected air, while in the others a modified atmosphere was introduced).

To assure the complete immersion of the slices, those were manually dipped and agitated in the respective treatment solution. Given the small dimensions of the immersion container, apple wedges weren't immersed all at the same time (slices were divided in several portions and immersed one at a time).

After the treatment, slices were introduced in a packaging machine, where some parameters like quantity of apple per package and gas levels (O_2, CO_2 and N_2) were defined. In some packages it was introduced a Modified Atmosphere (with low levels of O_2), in order to see the combined action of the treatment solution and gas levels.

Bags were stored under refrigeration conditions (at a temperature of 4°C ± 2 °C) until use.

The assays were carried for 30 days and the analyses were performed every 5 days. In day 0 the analyses were directly performed in the raw material (apple without treatment).

4.5.2.3 Physicochemical Analysis

Colour Assessment

For this analysis, 5 slices per treatment were randomly selected. The colour of apple slices was assessed at room temperature (around 23°C). Each sample was measured in two different locations (values reported as the mean value of 2 measurements in each side). The equipment, Konica Minolta CR-400 Colorimeter (Minolta, INC., Tokyo, Japan), was calibrated with a standard white ceramic plate prior to the measurements (Palou, López-Malo, Barbosa-Cánovas, Welti-Chanes, & Swanson, 1999).

Colorimetric parameters[8] (L, a* and b*) were analysed using CIELAB System software. These parameters, were used to calculate the browning index (BI), as shown in Equations 1 and 2:

$$BI = [100\,(x - 0.31)]/\,0.172 \qquad \text{(Equation 1)}$$

where:

$$x = (a + 1.75L)/[5.645L + (a - 3.012b)] \qquad \text{(Equation 2)}$$

BI represents the true origin/nature of the browning phenomenon ("purity of brown colour") in food products with sugar (like fruits), in which enzymatic and non-enzymatic browning take place (Palou, López-Malo, Barbosa-Cánovas, Welti-Chanes, & Swanson, 1999; Perez-Gago, Serra, Alonso, Mateos, & Río, 2005).

[8] L* indicates lightness, a* is the red/green coordinate, and b* is the yellow/blue coordinate.

To assess (visually) the appearance of dark stains in the apples through the entire experiment (in each day), slices (about 4 slices/treatment) were photographed under similar light conditions.

Enzymatic Extraction

Enzyme extraction was carried out according to Jang & Moon (2011), Sulaiman et al. (2015) and Augusto, Simões, Pedrosa, & Silva (2016) with minor modifications.

Apple wedges were first frozen at −80 °C in order to minimize the enzymatic activity changes and preserve their characteristics. Apples were cut (chopped) until a final amount of 5 g was attained (each enzyme extract was obtained from three apples, to reduce variability between sample results) and then homogenized (Ystral®, Dottingen, Germany) with 10 mL of chilled 0.05 M sodium phosphate buffer (pH 7.0) containing polyvinylpolypyrrolidone (PVP, 50 g L^{-1}), protected from light. Enzymatic extractions for PME analysis were a little different, since the extraction solution was constituted by sodium chloride (NaCl, 1.5 M), containing PVP (2.5 g L^{-1}).

Additionally, homogenized samples were filtered through gauze pads and centrifuged at 14.000× g for 30 min. at 4 °C, using Eppendorf 5810 R Centrifuge (Hamburg, Germany).

Finally, supernatant solutions (enzymatic extract) were divided into microtubes (1 mL needed to determine PPO enzymatic activity, 1 mL to determine POD enzymatic activity an 0.5 mL to determine total protein content of each sample). For PME, the supernatant was divided between 2 microtubes (1 mL for each tube). All samples were frozen at −80 °C, until further analysis (Enzymatic Activity and Total Protein content, explored next).

Enzymatic Activity and Total Protein Quantification

Following the protocols presented in Augusto, Simões, Pedrosa, & Silva (2016), Bravo & Osorio (2017), Rodríguez-Verástegui et al. (2016) and Liu et al. (2016) with some modifications, enzymatic activity, as well as total protein content were determined.

To determine the activity of each enzyme (and total protein content), the respective standard-curves were defined, in quadruplicate (each solution of the standard-curve filled 4 different pits) and all samples were analysed in triplicate, through microplate reader Synergy™ H1 Hybrid Multi-Mode Reader (BioTek, Winooski, USA) and Gen5™ Data Analysis software.

a) Peroxidase (POD) Activity Assay

According to Rodríguez-Verástegui et al. (2016) and Augusto, Simões, Pedrosa, & Silva (2016), POD activity was determined spectrophotometrically at 470 nm (for 10 minutes), using guayacol as the substrate and hydrogen peroxide (H_2O_2) as the hydrogen donor.

The substrate mixture contained 11.8 µL of 1% guayacol, 16 µL of hydrogen peroxide (0.3%) and 40 mL of sodium phosphate buffer (0.05 M, pH 6.5). In the microplate, 271.2 µL of substrate mixture and 28.8 µL of enzyme extract (or control solution, constituted by sodium phosphate buffer and water) were added to start the enzymatic reaction.

Enzymatic activity was defined by the slope of absorbance vs. time with one unit of activity defined as a change in absorbance of 0.001 min^{-1} per milligram of protein per gram of apple fresh weight (U mg^{-1} protein g^{-1} FW).

b) Polyphenoloxidase (PPO) Activity Assay

PPO activity was measured spectrophotometrically at 400 nm, following the protocols presented by Bravo & Osorio (2017) and Augusto, Simões, Pedrosa, & Silva (2016).

The substrate mixture contained 0.066 g of cathecol (20 mM), as substrate, dissolved in 30 mL of sodium phosphate buffer (0.005 M, pH 7.0). In the microplate, 288 µL of substrate mixture and 12 µL PPO extract (or control solution, constituted by sodium phosphate buffer and water) were added to start the enzymatic reaction.

Enzymatic activity was defined by the slope of absorbance vs. time with one unit of activity defined as a change in absorbance of 0.001 min^{-1} per milligram of protein per gram of apple fresh weight (U mg^{-1} protein g^{-1} FW).

c) Pectin Methylesterase (PME) Activity Assay

According to the protocol followed by Delgado-Reyes, Fernández-Romero, & Castro (2001) and Liu et al. (2016), before determining PME activity, the pH of the enzymatic extract[9] was adjusted to a value of 7.5, using a NaOH (1 M) solution. Additionally, pectin (5 g L^{-1}) and bromothymol blue indicator were incubated at 35 °C, before the readings.

The reaction mixture, constituted by 50 µL of PME extract, 235 µL of pectin (citrus, 5 g L^{-1}, pH 7.5) and by 15 µL of indicator (bromothymol blue, 0.01%, pH 7.5), was added in the microplate and as a reference (control), it was only used the substrate mixture that consisted of pectin and indicator (without the enzyme).

Enzymatic activity was obtained spectrophotometrically at 610 nm, for 4 minutes, at 35 °C. According to Delgado-Reyes, Fernández-Romero, & Castro (2001), PME activity was monitored through the colour decrease of bromothymol blue.

Enzymatic activity was defined by the slope of absorbance vs. time with one unit of activity defined as a change in absorbance of 0.001 min^{-1} per milligram of protein per gram of apple fresh weight (U mg^{-1} protein g^{-1} FW).

[9] PME is an extremely sensitive enzyme, and for this reason all solutions (pectin, bromothymol blue indicator and enzymatic extract) shall be adjusted to an optimum pH of 7.5. PME is active at an optimum temperature of 35 °C.

d) Total Protein Quantification (Bradford method)

Total protein concentration was determined by the Bradford method (Bradford, 1976), having been defined a standard curve from an initial solution (stock solution) of Bovine Serum Albumin, (BSA 0.5 mg mL^{-1}), in Milli-Q water.

The reaction mixture, composed by 210 µL of Bradford reagent and 30 µL of sample (or of a standard-curve solution) was dispensed into a microplate, mixing well, and then it was incubated at room temperature, for 30 minutes.

The absorbance was measured at 592 nm, in the microplate reader, and the total protein was quantified using the standard-curve formula.

Microbiological Analysis

For the microbiological analysis, adapting the protocol followed Moreira, Roura, & Ponce (2011) and Olivas, Mattinson, & Barbosa-Canovas (2007), 25 g of sliced apple (1 bag/treatment) were homogenized in 250 mL diluent (Buffered Peptone Water), both added in a BagFilter®. All samples were diluted and homogenized with a Stomacher (Classic Panoramic Masticator, IUL Instruments, Barcelona, Spain).

Then, 1 mL was removed from the BagFilter® to perform serial dilutions (1:7) of each homogenized sample.

For total microorganisms and *Entrerobacteriaceae*, in each plate, 1 mL of each dilution was pour-plated in duplicate. On the other hand, for moulds and yeasts, only 0.1 mL of each dilution was spread-plated (also in duplicate).

Enumeration and differentiation of microorganisms were performed according to ISO 4833-1:2013, ISO 21528-2: 2004 and ISO 21527-1: 2008, respectively, by using the following culture media and culture conditions: total microorganisms on Plate Count Agar (PCA) incubated at 30°C ± 1°C, for 72 h ± 3 h. *Enterobacteriaceae* in Violet Red Bile Glucose Agar medium (VRBGA), incubated at 37°C for 24 h ± 2 h. Moulds and yeasts were counted in Dichloran-rose Bengal Chloramphenicol Agar medium (DRBCA), incubated at 25°C ± 1°C for 5 d.

Microbial counts were performed by duplicate, in each day of experimentation (Moreira, Roura, & Ponce, 2011).

4.5.2.4 Statistical Analysis

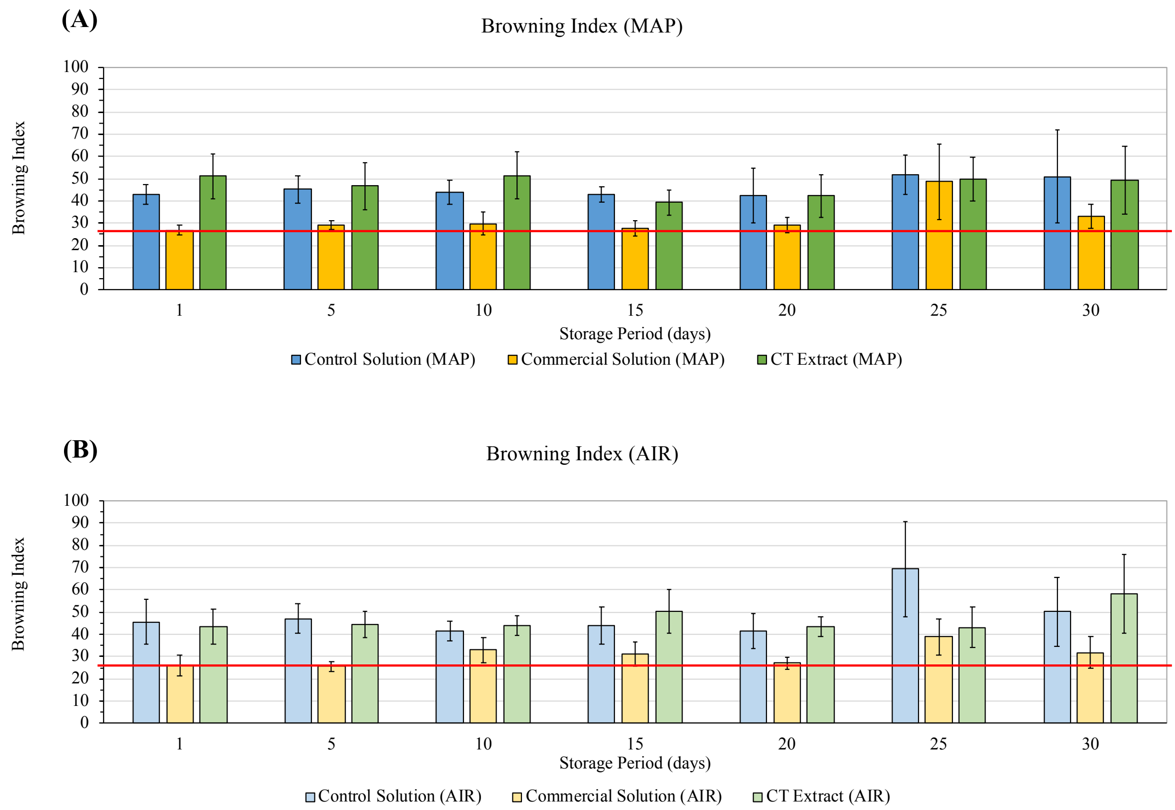
All data were analysed using the Microsoft Excel (2016) Analysis ToolPak statistical software, through Descriptive Statistics and ANOVA: Single Factor tools. Analysis of variance (ANOVA) was applied, with a level of significance of 0.05, to identify significant differences between groups.

4.6 Results and Discussion

4.6.1 Colour Evaluation

As previously referred, BI is commonly used as an indicator of browning in food products that contain sugar (Palou, López-Malo, Barbosa-Cánovas, Welti-Chanes, & Swanson, 1999; Perez-Gago, Serra, Alonso, Mateos, & Río, 2005).

Looking at Figure 4.4 it's possible to observe that, for all days tested, samples treated with the commercial solution resulted in a significantly lower BI than samples treated with the control and CTE solutions. Comparing BI values registered after the treatment with the commercial solution and the initial browning state of the raw material, no significant differences are noticed.



Notes: The red threshold indicates the raw material BI (mean value).

Figure 4.4: Evaluation of Browning Index during storage (30 days) under MAP packaging (A) and AIR packaging (B) conditions

Under packaging with modified atmosphere (Fig. 4.4 (A)), in the first 10 days CTE dipping solution samples presented higher BI than the control solution, but from day 15 onwards, comparatively to the control solution, CTE registered slightly lower BI levels, despite not being significantly different. However, from this day, the variation relatively to the initial browning state (red threshold) seems to be lower that the variation registered in days 1 and 10.

Unexpectedly, in day 25, samples treated with the commercial solution achieved its highest BI value (Fig. 4.4 (A)). These odd results can be explained by the challenging sampling of apple surface for colour measurement (as browning occurs heterogeneously throughout the fruits' surface).

On the other hand – under packaging with air (Fig. 4.4 (B)) – a different effect was observed for the CTE solution. In the first 5 days, samples immersed in the CTE presented slightly lower BI values than samples dipped in the control solution, indicating a better action as an anti-browning agent. The same was observed in day 25.

The overall results also indicate, apparently, a better performance of CTE solution under AIR packaging conditions.

In day 15, the refrigeration at 5°C was interrupted during approximately 12 hours, due to an electric problem. Given this situation, results from this day onwards may not express the normal evolution of the BI. This situation may also explain the unexpected results formerly mentioned.

A closer look at CIELAB coordinates (L*, a* and b*) helps to better understand how slices' colour changed during storage.

To assess ΔE values (total colour difference or colour variation), additional formulas had to be applied (Equations 3 and 4, below):

$$E = \sqrt{(\Delta a^2 + \Delta b^2 + \Delta L^2)} \qquad \text{(Equation 3)}$$

$$\Delta E_{(0-20)} = E_{20} - E_{raw\ material} \qquad \text{(Equation 4)}$$

ΔE measures the total colour difference between samples (in this case, between day 20 samples and raw material samples), while ΔL measures lightness variation between samples. ΔL negative values mean that samples became darker during the period considered.

In Figure 4.5 it's perceptible that the commercial solution applied in Campotec S.A displayed significantly better results than both control (water) and CT extract solutions, though under MAP conditions (Fig. 4.5 (A) and 4.5 (C)) the CTE had demonstrated better results than the control solution. Under AIR packaging, total colour difference (ΔE) of apple slices immersed in the CTE was slightly higher than ΔE of slices treated with the control solution.

Figure 4.5 (B) and 4.5 (D) indicate that under AIR packaging the difference between the commercial and CTE solutions was slightly shorter, comparatively to the situation observed in MAP condition (Fig. 4.5 (A) and 4.5 (C)). This might suggest that the commercial solution works better when slices are packaged with MA.

Lightness variation (ΔL) results (Fig. 4.5 (C) and 4.5 (D)) reveal that all samples became darker through time, although slices treated with the commercial solution had remained lighter than slices treated with the other solutions. In both packaging conditions, immersion in the control solution led to an intense browning of apple wedges (becoming the darkest ones), so that the CTE solution prevented somehow apple's darkening – though at a lower extent than what was initially expected.

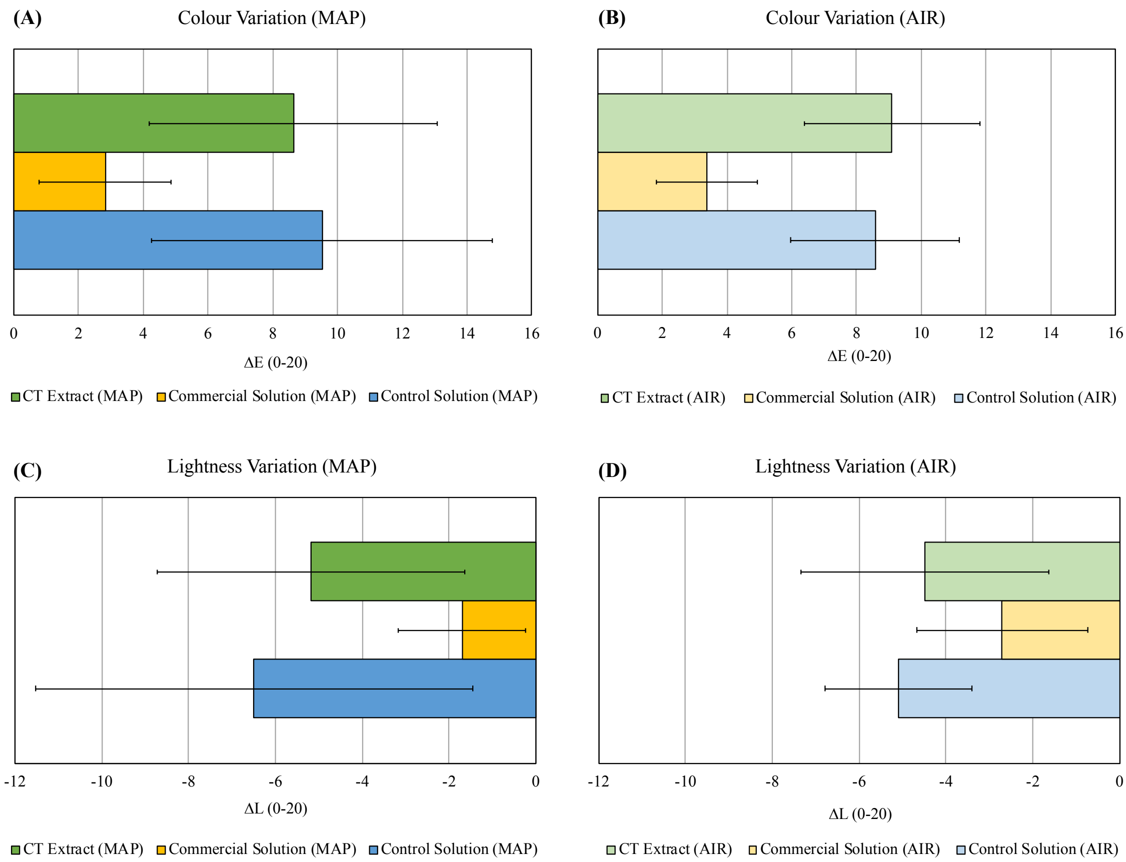


Figure 4.5: Evaluation of Colour Variation (ΔE) and Lightness Variation (ΔL) between day 0 and day 20, under MAP packaging (A, C) and AIR packaging (B, D) conditions

Photographic registers allow an easier visual assessment of colour changes in apple slices during storage. Figure 4.6 only displays a comparison between slices photographed in days 1 and 20 (more registers are annexed in Appendix A).

These images corroborate the previous results since it's clear that wedges became visually darker when dipped in the control solution (water). It's also evident that the commercial and CTE solutions had much better performances than control solution, as expected.

Moreover, registers show that wedges treated with the commercial solution or with the CT extract are very similar, despite the slightly darker look of the wedges immersed in the last solution.

Finally, comparing packaging conditions, MAP seems to favour slices' darkening. Further analyses have to be performed to clarify these results.

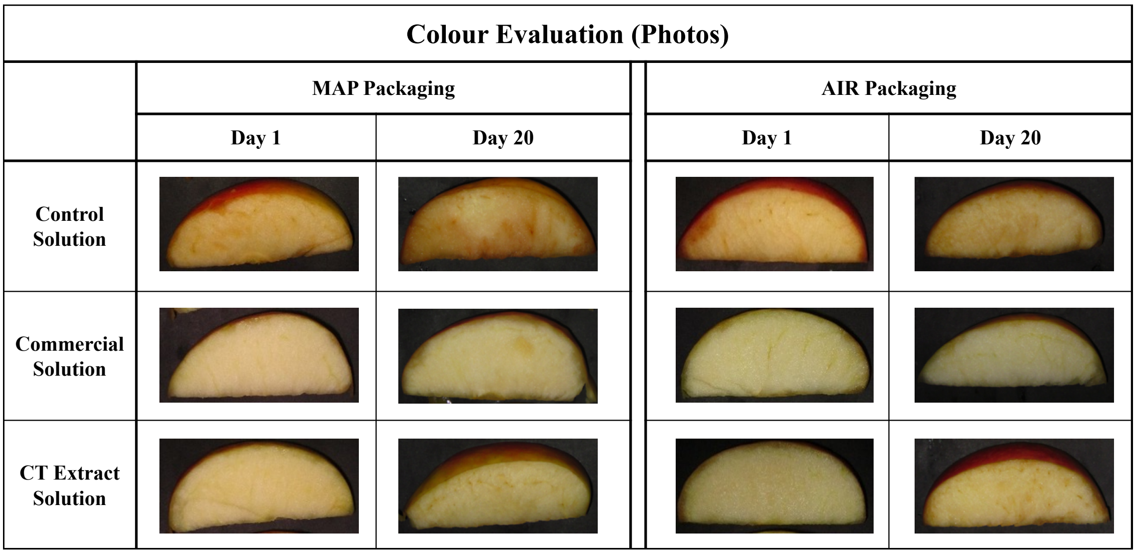


Figure 4.6: Visual Assessment of colour changes occurred in apple slices during the storage, between day 1 and day 20, under MAP packaging (photos on the left) and AIR packaging conditions (photos on the right)

4.6.2 Enzymatic Activity

Specific enzyme activity (or simply, specific activity) is an important measure of enzyme purity. It can be obtained dividing enzyme activity (U mL^{-1}) by the total protein concentration (mg). In this assay, one unit of enzyme activity was defined as a change in absorbance of 0.001 min^{-1} per milligram of protein per gram of apple fresh weight (U mg^{-1} protein g^{-1} FW).

Regarding enzymatic activity assays (Fig. 4.7, 4.8 and 4.9), registers revealed that in the first 15 days, in both atmospheres (MAP and AIR), POD and PPO generally presented a lower specific activity after slices' immersion in the control solution (water). This suggests that somehow during storage, POD and PPO were more easily activated in both commercial and CTE solutions (even though, in most of the days, activation had been stronger in the commercial solution).

POD specific activity results (Fig. 4.7 (A) and 4.7 (B)) indicate a good performance of the CT extract, especially in apples packaged with air, in which the situation is more stable. It's interesting to note that in both packaging conditions (MAP and AIR), CTE was more efficient than the commercial solution currently used in Campotec S.A. (with the exception of day 25, under MAP packaging), blocking enzymatic activity.

These results contrast with the browning index results, that indicated a better performance of the commercial solution, with lower ΔE and ΔL. This may suggest that apple browning was not caused by enzymatic reactions (non-enzymatic browning).

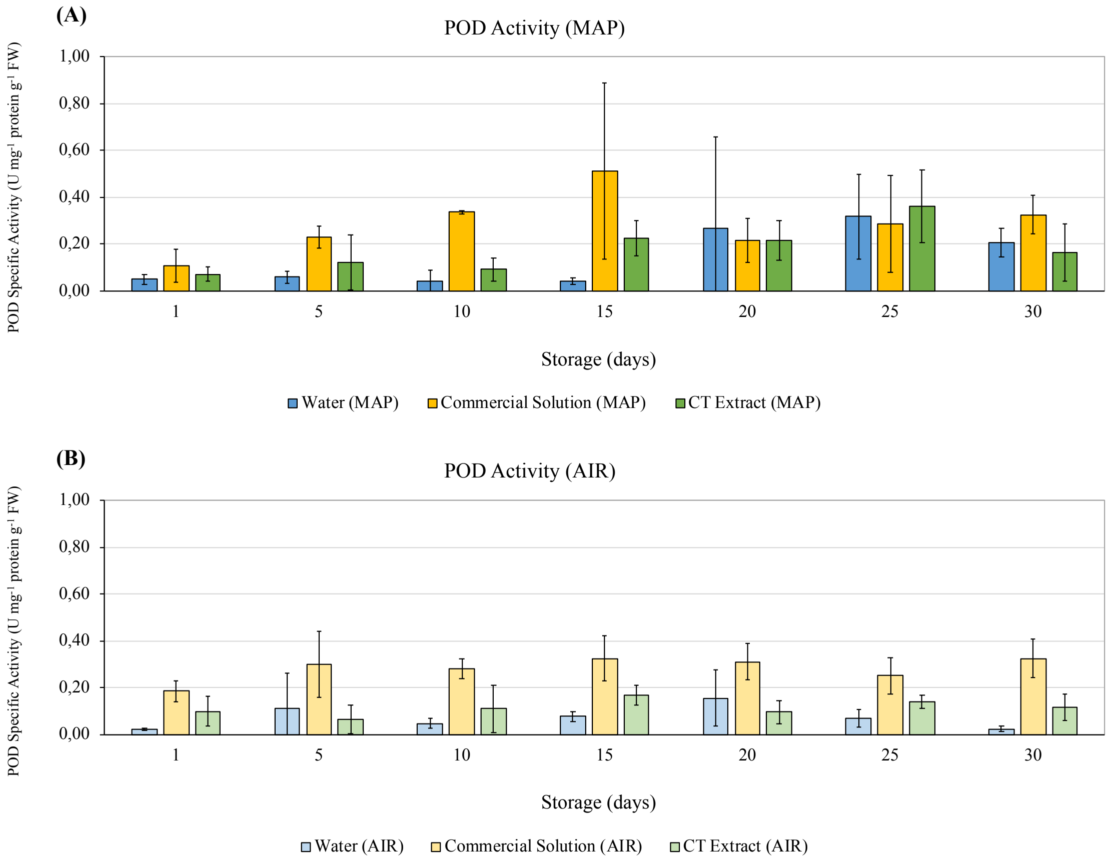


Figure 4.7: Evaluation of POD enzymatic activity during the storage period (30 days) under MAP packaging (A) and AIR packaging (B) conditions

In Fig. 4.8 (A), PPO specific activity in slices dipped in the control solution, contrastingly with the activity observed slices treated with both commercial and CTE solutions, increases exponentially from day 20 onwards. Moreover, it's interesting to note that in commercial and CTE solutions, the values of specific activity are not significantly different, demonstrating similar performances while blocking the enzymatic activity.

Again, it's possible to see that packaging with air (Fig. 4.8 (B)) contributed to slightly lower specific activity in apple slices treated with the CT extract. Also in this condition the difference between slices dipped in the control solution and slices dipped in CTE solution is shorter than in MAP condition.

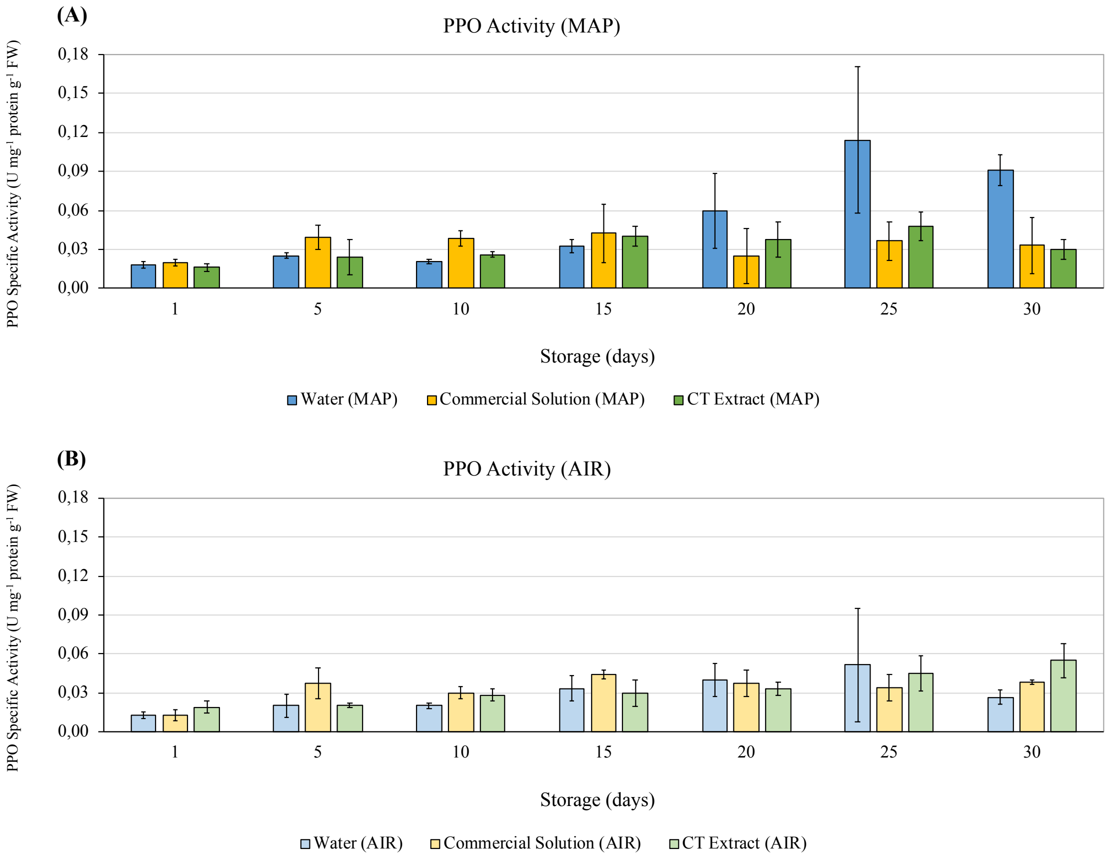


Figure 4.8: Evaluation of PPO enzymatic activity during the storage period (30 days) under MAP packaging (A) and AIR packaging (B) conditions

PME assay revealed that in the first day of experiment (Fig. 4.9 (A) and 4.9 (B)), specific activity in slices treated with CTE was lower than in slices treated with the control and commercial solutions. However, between days 10 and 20, PME increased significantly its activity in slices treated with the CT extract in both atmospheres, and, from day 20 until the end of the assay, PME experienced again a decrease in its specific activity, getting close to the initial values.

The strange results observed in days 15 and 20 may be related to the electric problem that interupted the refrigeration at 5°C. As early stated in this work, PME is an extremely sensitive enzyme, being activated at an optimum temperature of 35°C, which may have promoted a certain volatility of the results.

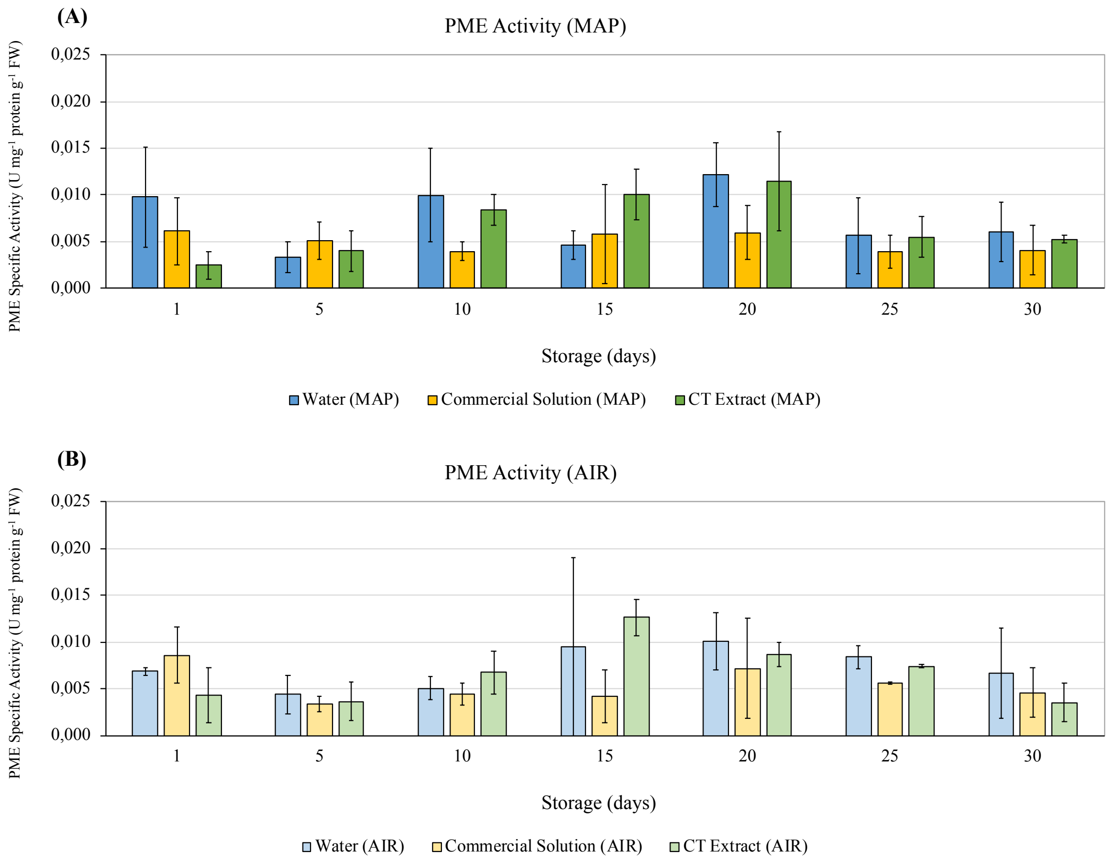


Figure 4.9: Evaluation of PME enzymatic activity during the storage period (30 days) under MAP packaging (A) and AIR packaging (B) conditions

Knowing PME is the enzyme responsible for tissue softening (a lower enzymatic activity would contribute to a decrease in tissue deterioration), it can be stated that excluding the period between days 10 and 20, CTE theoretically exhibited a good capacity to preserve slices' texture, indicating that the proposed technology shows some potential that needs to be further explored.

A research work performed by Gwanpua et al. (2014) revealed a higher activity of PME in 'Jonagold' apples stored under air conditions than in apples stored under modified atmosphere. However, the authors also reported a strong and negative correlation between enzymatic activity and firmness. Contrastingly, under air, the high enzyme activities were positively correlated with the very high rates of ethylene production, reaching a peak when fruit was placed under ambient shelf conditions. In the present work, results do not clarify which packaging condition promotes a higher PME activity.

Looking carefully at the data presented (Fig. 4.7, 4.8 and 4.9), it's possible to see that CTE has a slight tendency to be more efficient under packaging with ambient O_2 atmosphere (AIR packaging) than under packaging with modified atmosphere (MAP packaging). Similar results were analysed by Castro, Barrett, Jobling, & Mitcham (2008), Johnston, Hewett, & Hertog (2002) and Barrett, Lee, & Liu (1991).

After studying the biochemical factors associated with the mechanism of CO_2-induced flesh browning susceptibility in 'Pink Lady' apples, Castro, Barrett, Jobling, & Mitcham (2008) suggest that apples' browning can be caused by the stress induced by high levels of CO_2. Apparently, modified atmospheres (MA) rich in CO_2 trigger the accumulation of H_2O_2 (that act as a signal for the activation of stress-response and defence pathways), leading to elevated oxidative levels in cells present in apple's susceptible tissues. In these areas, cell membranes are very damaged, allowing enzymes and substrates react and form brown pigments. Additionally, in this oxidative environment, ascorbic acid (AA) is oxidized, allowing the expression of flesh browning (FB) symptoms (further aggravated by low O_2 concentrations). Finally, the extreme stress conditions drive to cellular death.

Also softening is influenced by the levels of O_2 and CO_2 during storage. While some authors suggest that progressive increases in CO_2 concentration from 0 to 50% slowed softening in several cultivars, others found that atmospheres composed by CO_2 and concentrations greater than 1-1.5% O_2 reduced softening more efficiently. However, if fruit enters in the rapid phase of softening (irreversible process), it's very difficult to control the loss of firmness, even under packaging with MA (Johnston, Hewett, & Hertog, 2002).

Barrett, Lee, & Liu (1991) studied the effect of modified atmosphere packaging on PPO activity, observing that MAP with high levels of CO_2 led to a slight decrease in PPO activity, but when stored under varying high CO_2 conditions, fruit developed an initial browning, darkening fast after 30 min. of exposure to air.

If future analyses support this theory, MAP packaging – followed by a large part of fruit producers – may be removed along with the underlying costs. Besides, it's known that MAP may alter products' features, like flavour and texture. Thus, removing the need of introducing gas mixtures in the packages, producers can offer fresher products (such as fresh-cut fruit) to consumers.

Enzymatic assays do not corroborate the highest efficiency of the commercial solution over CT extract and control (water) solutions. It was already mentioned that PPO plays a crucial role in tissue browning. The reaction between this enzyme and oxygen molecule triggers hydroxylation and oxidation reactions that end with phytomelanins' production and accumulation in fruit's tissue. So, considering this fact, it would be expected that a lower activation of PPO would contribute to prevent tissue browning.

However, crossing colour evaluation and enzymatic activity data, the reverse situation was observed.

In most of the days, apples treated with the commercial solution demonstrated higher POD and PPO activities and, simultaneously, lower BI levels, as well as colour (ΔE) and lightness (ΔL) variation during storage (slices immersed in the commercial solution remained similar to raw material slices, despite the activation of POD and PPO). Contrastingly, slices immersed in water (control solution) became the darkest (Fig. 4.6).

In spite of these apparently strange results, other authors, like Castro, Barrett, Jobling, & Mitcham (2008), also didn't find a direct correlation between browning and PPO activity.

Notwithstanding, Billaud, Roux, Brun-Mérimee, Maraschin, & Nicolas (2003) proposed that also sugar concentration may contribute to these results. Authors observed that by raising the concentration of fructose and glucose, enzymatic browning was inhibited by 47% and 33%, respectively. As suggested by Pitotti, Nicoli, Sensidoni, & Lerici (1990) the inhibiting effect of sugars may be related to their capacity to reduce water activity, causing an increase in the reaction medium viscosity and/or a change in the oxygen solubility.

4.6.3 Microbiological Analysis

Minimally processed fruits are perishable products, very susceptible to microbial contamination. Fruits have a natural microflora associated due to their content in water, nutrients and sugar, used by microorganisms to survive and disperse. Even though, some of them are acquired during harvest, processing and handling until they reach the final consumer (Graça, et al., 2015).

In order to assure food safety, a set of hygiene standard procedures defined in the *Codex Alimentarius* – a food code respected worldwide – include some guidelines, control parameters and legal boundaries that must be obeyed. This is a global reference for consumers, food producers and processors, national food control agencies and the international food trade (WHO/FAO, 2006).

In the European Union, EFSA established some microbiological criteria, which define the acceptability of a product or process, based on the absence or presence of certain microorganisms (number or amount of toxins/metabolites) per unit (mass, volume, area, etc.) (Commission of the European Communities, 2005). There are three levels admitted to classify the microbiological quality of foodstuff: Satisfactory (good microbiological quality – $\text{cfu g}^{-1} < m$), Borderline (when the product's microbiological content is between the upper limit of acceptability and the limit of unacceptable risk – $m < \text{cfu g}^{-1} < M$) and Unsatisfactory (the product's microbiological content compromises the human organism, representing an unacceptable risk – $M < \text{cfu g}^{-1}$) (Hong Kong Centre for Food Safety, 2014).

Guideline limits summarized in Table 4.2 can be specifically applied to ready-to-eat foods included in the "extended shelf-life food products requiring refrigeration" category.

Table 4.2: Guidelines for Microbiological Safety

	Microbiological Guideline Limits (cfu g^{-1})	
Indicators	**m**	**M**
Aerobic Colony Counts (ACC)	10^6	10^8
***Enterobacteriaceae* [a]**	NA	NA
Moulds and Yeasts		
Moulds	10^2	10^3
Yeasts	10^2	10^5
Sources: FSAI (2016); CFSHK (2014) and Santos et. al (2005)		
Notes: [a]According to the European and international regulation, *Enterobacteriaceae* guideline limits do not apply to fresh fruit, because these foods can naturally contain high levels of *Enterobacteriaceae* as part of their normal micro-flora (FSAI, 2016). **NA – Not Applied**		

Moreover, the results obtained in these laboratorial assays were compared with the results obtained by Graça et al. (2015) – that studied the microbial quality of MP apples commercialized in Portugal – and by Abadias et al. (2008), since little data exists on this matter in the Portuguese commerce.

As seen previously, three types of microorganisms (parameters) were assessed: total microorganisms (or ACC), *Enterobacteriaceae* and moulds and yeasts.

Aerobic Colony Counts

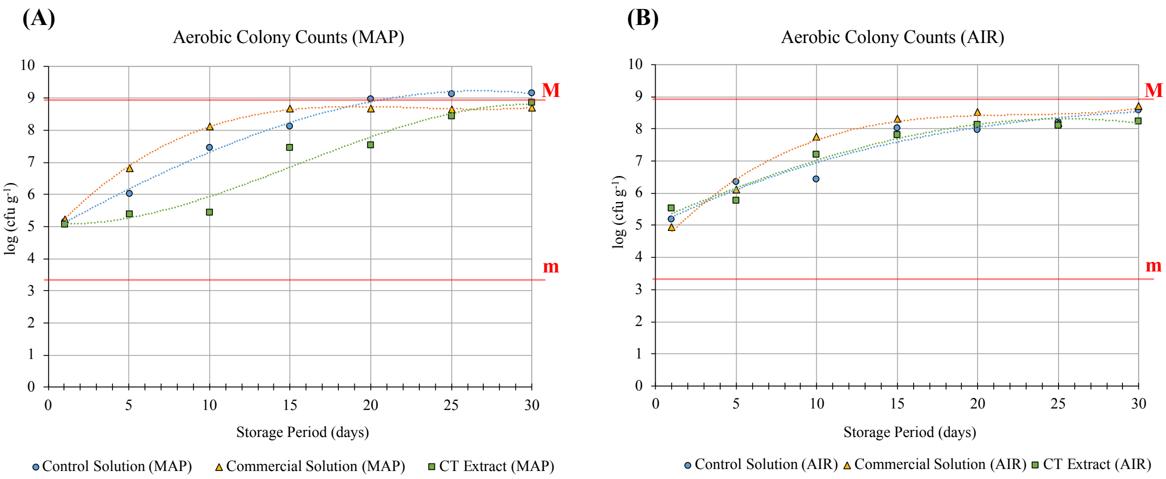
Total microorganisms grown at 30°C embrace aerobe mesophylls, that serve as good Quality Indicator Organisms – recognised as Aerobic Colony Count (ACC).

ACCs provide useful information about the general quality of the product, usually affected by storage and handling (Hong Kong Centre for Food Safety, 2014).

Comparing the results obtained in this assay (Figure 4.10) with the reference values obtained by Graça et al. (2015) ($m = 3.3$ and $M = 8.9$, expressed in log (cfu g^{-1})) it's possible to see that, with the exception of samples tested between days 20 and 30 immersed in the control solution and packaged with MA, all values were below 8.9 log (cfu g^{-1}). Moreover, the maximum numbers registered by Abadias et al. (2008) ($M = 7.1$ log (cfu g^{-1})) are generally crossed after day 5, except samples immesed in the CTE and packaged with MA (see Appendix B, Table B.1).

Results indicate a better performance of the CT extract. Fig. 4.10 (A) shows that until day 25, less aerobic colonies were grown in the apple slices treated with the CT extract and that the maximum limit ($M = 8.9 \log (\text{cfu g}^{-1})$) was crossed only after day 25.

According to the microbiological criteria presented by the Food Safety Authority of Ireland (FSAI) in Table 4.2, until day 10 (MAP), samples treated with the CTE are classified as Satisfactory, thus having a good microbiological quality.



Notes: The red thresholds represent the maximum (M) and minimum (m) microbiological reference limits according to Graça et al. (2015)

Figure 4.10: Evolution of ACC microbiological counts during storage (30 days) in apples treated with the control (●), commercial (▲) and CTE (■) solutions, under MAP packaging (A) and under AIR packaging (B)

In Fig. 4.10 (B) it's possible to see that all treatments exhibited similar performances. Considering, again, the guideline microbiological limits summarized in Table 4.2, until day 15, samples treated with the CTE are classified as Borderline, remaining constant until the end of the assay.

These are acceptable results considering the fact that fresh products, such as fresh-cut apple, have a natural micro-flora associated. However, samples treated with the CTE have to be submitted to additional tests, to better understand the composition of apple's micro-flora and if there are microorganisms deserving a special attention, like, for example faecal coliforms or pathogens, that compromise product's consumption.

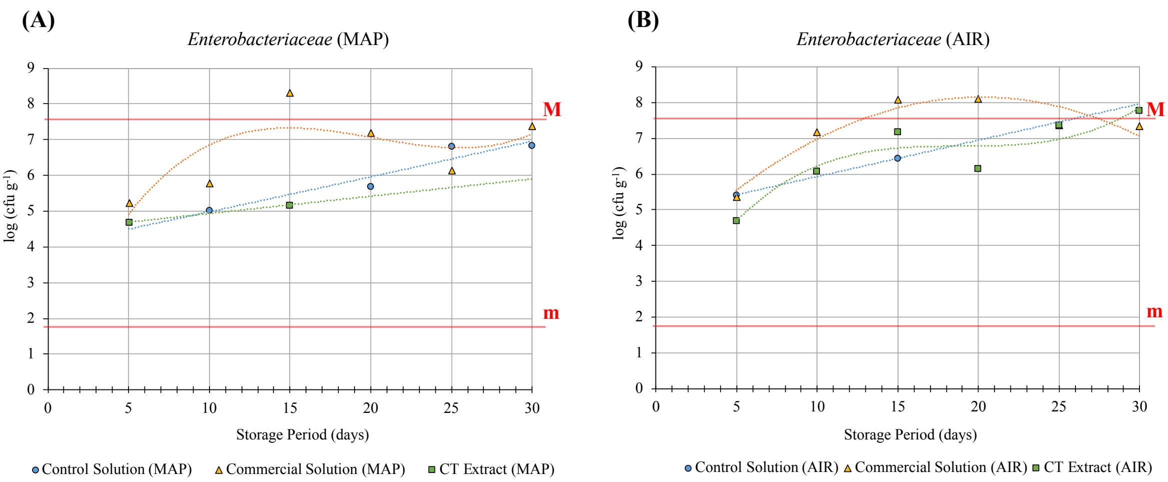
Enterobacteriaceae

Enterobacteriaceae and particularly *E. coli* serve as good Hygiene Indicator Organisms, since their presence results from a lack of cleanliness in product's handling and improper storage. *Enterobacteriaceae* family comprises the group of coliform bacteria (or total coliforms), where faecal coliforms (like *E. coli*) are included. The presence of these microorganisms in foodstuff may indicate direct or indirect faecal contamination.

In terms of European regulation – (EC) No 2073/2005 – *E. coli* is the only reference for "Vegetables, fruits and products thereof" category. To assure consumers' safety, the detection of *E. coli* cannot overcome the maximum limit of 10^3 cfu g^{-1}. All tests shall be performed during the manufacturing process (Commission of the European Communities, 2005).

However, in this microbiological analysis, the purpose was not to identify the presence of specific *Enterobacteriaceae* strains or species, like *E. coli*. The results obtained only reveal the general microbiological content of *Enterobacteriaceae* in apple slices. As the EC do not establish microbiological guidelines for this broad group of microorganisms, some recent studies performed by Graça, et al. (2015), about fresh-cut apple microbial quality were used to establish some reference values. These reference levels refer to total coliforms counts.

Comparing the obtained results with the reference values ($m = 1.8$ and $M = 7.6$, expressed in log (cfu g^{-1})) it's possible to witness a better performance of the CTE relatively to the commercial solution (Figure 4.11). Values obtained for the control solution are very close to the values obtained for the CTE. Though, there are few values to compare (Table B.2., Appendix B).



Notes: The red thresholds represent the maximum (M) and minimum (m) microbiological reference limits according to Graça et al. (2015)

Figure 4.11: Evolution of *Enterobacteriaceae* microbiological counts during storage (30 days) in apples treated with the control (●), commercial (▲) and CTE (■) solutions, under MAP packaging (A) and under AIR packaging (B)

Looking at the reference values, samples from each day are generally within the range reported by Graça et al. (2015). In day 5, values are very close to 4.8 log (cfu g^{-1}), reported by Abadias et al. (2008) – that represent the maximum numbers of *Enterobacteriaceae* obtained by the authors.

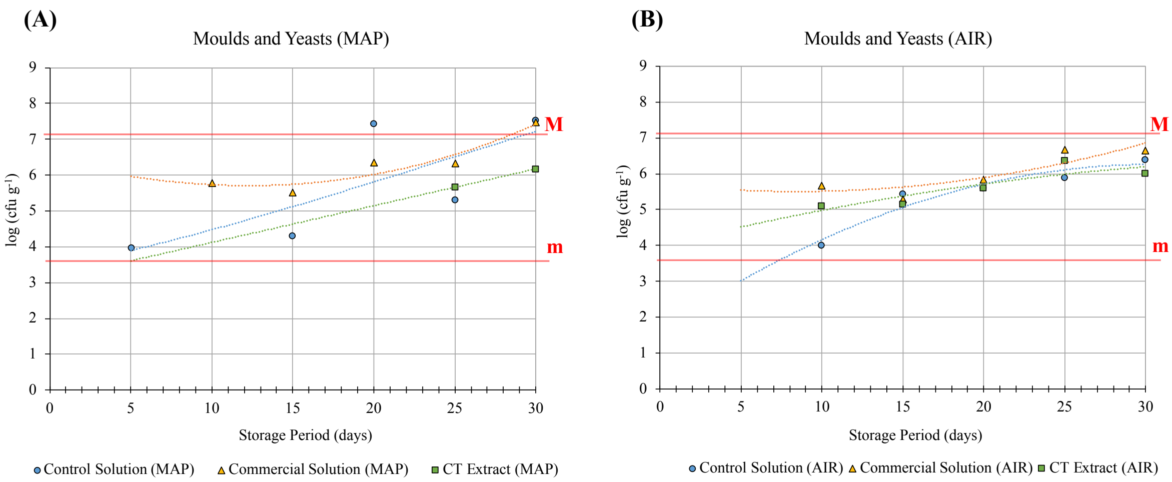
It’s also possible to see that CTE allowed an extended preservation, only crossing the reference value ($M = 7.6 \log\ (\text{cfu g}^{-1})$) in day 30 (AIR), but still very close from the upper limit.

Some inconsistencies are noted from day 15 onwards since some values started to decrease. Numbers were expected to increase, because microbial content usually rises during storage.

In the first days of storage, counts were not statistically valid (out of the 30 to 300 colonies range).

Moulds and Yeasts

Moulds and yeasts (or fungi, in general) are responsible for food deterioration and decomposition (spoilage by changing colour, producing gas, off-flavours and souring (Graça, et al., 2015)). These organisms can pose a risk for the human organism, since they produce toxic metabolites, known as mycotoxins, involved in some allergic reactions and infections (Tournas, Stack, Mislivec, Koch, & Bandler, 2001). For those reasons, their presence must be confined and properly controlled.



<u>**Notes:**</u> The red thresholds represent the maximum (M) and minimum (m) microbiological reference limits according to Graça et al. (2015)

Figure 4.12: Evolution of moulds and yeasts counts during storage (30 days) in apples treated with the control (●), commercial (▲) and CTE (■) solutions, under MAP packaging (A) and under AIR packaging (B)

Moulds and yeasts counts also reveal a good performance of the CTE (Fig. 4.12). Under AIR packaging, CTE and control solutions resulted in very similar values, but conversely, under MAP packaging counts registered for the control solution were generally higher than those registered for the CTE solution, crossing the reference values ($m = 3.6$ and $M = 7.1$, expressed in $\log\ (\text{cfu g}^{-1})$) presented by Graça et al. (2015) in days 20 and 30 (see Table B.3., Appendix B).

Most of the values are higher than those obtained by Abadias et al. (2008) – $m = 1.7 \log(\text{cfu g}^{-1})$ and $M = 4.9 \log(\text{cfu g}^{-1})$.

According to INSA, the maximum limits are 10^5 cfu g^{-1} and 10^3 cfu g^{-1}, for yeasts and moulds respectively (Santos, Correia, Cunha, Saraiva, & Novais, 2005). In either packaging conditions, these limits were crossed, in spite of the counts in samples dipped in the CTE solution being very close to 5 log (cfu g^{-1}), the maximum limit accepted for the presence of yeasts.

Nevertheless, the values are within the ranges established in other studies, revealing again a good extended preservation of slices treated with the CT extract.

Like in *Enterobacteriaceae*, in the first days of storage, counts were not statistically valid (out of the 10 to 150 colonies range).

In the future, it would be interesting to identify the main foodborne pathogens indicated in the "food safety criteria" established by the European Commission, in order to validate the microbiological safety of the final product (apple slices treated with the CT extract).

These results demonstrated that the CTE prevented the contamination and spoilage caused by some microorganisms, especially when compared with apples immersed in the commercial solution, after their best before period (10-15 days at refrigeration temperatures, in accordance with Graça et al. (2015)).

For all groups of microorganisms, it's possible to witness an increase during storage, which is consistent with the natural deterioration of apple slices during storage. The breakdown of cell walls in apple tissues allows the colonization by opportunistic organisms responsible for fruit deterioration.

The action of tyrosinases exuded from the fungal spores germinating on the cut surfaces of the slices triggers some biochemical changes that will ultimately lead to flesh browning. This natural phenomenon – known as secondary browning – suggest a different cause for apple browning. According to the authors, between 1 and 3 weeks in storage, dark stains begin to appear in fresh-cut fruit, being influenced by the handling temperature of the product (Toivonen & Brummell, 2008).

Secondary browning can also explain some of the results observed.

In the next chapter a market study for AlgaeCoat will be discussed.

CHAPTER 5

Technology Transfer: Market Analysis

5.1 Potential Market Dimension

In this chapter, the addressable market of AlgaeCoat additive will be studied: which customers are interested in the product, which business-to-business (B2B) and/or business-to-consumer (B2C) customers are willing to pay for this product; what's the market size of the extract and which are the most interesting countries to commercialise it. Also, the strategic triangle (segmentation, targeting and positioning); competition and market trends will be discussed.

This study will be focused in the apple market, since the laboratorial and scale-up experiments were carried in this fruit. Nevertheless, as early mentioned, in a near future, it's expected to apply the additive to preserve other type of fruits as well, in order to postpone their maturation.

AlgaeCoat customers include primarily fruit processors (that sell fresh-cut fruit, particularly, sliced or minimally processed apple), and in a smaller scale, catering companies, hotels, restaurants and cafes. It's important to stress that this is a growing market due to the increase in consciousness about health issues.

Thus, it is interesting to look at apple production data in Europe, where the target market of AlgaeCoat is predominantly concentrated. These data (summarized in Table 5.1), show the global apple production and the quantity of apples produced in Europe (in tonnes) between 2014 and 2016, and whether these were used for Fresh Consumption (directly sold to consumers) or for Processing (to produce juices, jams, pies or to be sold as MP apples).

Table 5.1: Total Apple Production (tonnes) between 2014 and 2016 and its main destinies (Fresh Consumption and Processing)

	Total Apple Production (tonnes)			Apples for Fresh Consumption (%)			Apples for Processing (%)		
Year	**2014**	**2015**	**2016**	**2014**	**2015**	**2016**	**2014**	**2015**	**2016**
World	**84 630 275**	**:**	**:**	**:**	**:**	**:**	**:**	**:**	**:**
EU-28	**12 893 900**	**12 764 120**	**12 568 460**	**:**	**80%**	**:**	**:**	**20%**	**:**
Poland	3 195 300	3 168 800	3 604 270	60%	69%	56%	40%	31%	44%
Italy	2 473 610	2 487 950	2 455 620	:	100%	100%	:	0%	0%
France	1 847 550	1 968 190	1 819 760	75%	73%	74%	25%	27%	26%
Germany	1 115 900	973 460	1 032 910	69%	71%	69%	28%	28%	29%
Spain	620 820	598 210	621 160	88%	86%	87%	12%	14%	13%
Portugal	**273 720**	**324 990**	**241 610**	**93%**	**93%**	**93%**	**7%**	**7%**	**7%**

Source: Eurostat (2018) and FAOSTAT (2018)

Notes: In this table, global and the top 5 EU apple producers are listed, as well as Portugal. Additionally, the table also shows the percentage of apple that is directly sold to consumers and the percentage of apples destined to processing, in order to produce juices, jams, pies, and also to be sold as MP apple. Dots (:) represent the data not available.

In the referred table, it can be seen that Poland is the largest producer inside the European Union (EU), followed by Italy, France, Germany and Spain. According to Cicco (2016), about one third (33.9%) of all EU apple orchards were situated in Poland, where a quarter of all EU apple production was harvested.

In Portugal, the favourable development of apple trees and the abundant sprout observed in 2015 contributed to the increase of apple production, but contrastingly, in November 2016, INE confirmed the negative predictions of a decrease[10] of 26% in the production.

A report published by the European Commission's Directorate-General for Agriculture and Rural Development (2017) indicates that in the season of 2016/2017, apple production in the EU was affected by the climate conditions in April and September 2016, registering an amount near 12.6 million tonnes. Though, apple harvest remained the 4th largest of the last decade.

Looking at apple main destinies (Table 5.1), it's possible to see that in Germany, France and Poland about 30% of total apple production went to processing due to the lower quality of the apples produced in those countries. In 2015, the median price of dessert apples[11] was near €44.15 per 100 Kg, while in Portugal the median price was €57.59 (and €64.04/100 Kg in 2016) (Eurostat, 2016).

Table 5.2, shows an estimation of the amount of MP apple (in tonnes) produced per country, including Portugal.

Table 5.2: Potential Market Dimension (2014-2016)

	Total Apple Production (tonnes)			MP Apple* (tonnes)		
Year	**2014**	**2015**	**2016**	**2014**	**2015**	**2016**
World	**84 630 275**	**:**	**:**	**21 024,6**	**:**	**:**
EU-28	**12 893 900**	**12 764 120**	**12 568 460**	**3 203,2**	**2 721,8**	**3 797,4**
Poland	3 195 300	3 168 800	3 604 270	793,8	675,7	1 089,0
Italy	2 473 610	2 487 950	2 455 620	614,5	530,5	741,9
France	1 847 550	1 968 190	1 819 760	459,0	419,7	549,8
Germany	1 115 900	973 460	1 032 910	277,2	207,6	312,1
Spain	620 820	598 210	621 160	154,2	127,6	187,7
Portugal	**273 720**	**324 990**	**241 610**	**68,0**	**69,3**	**73,0**

Source: Eurostat (2018) and FAOSTAT (2018)

Notes: **(*)** In the literature there are no data about MP apple production. Thus, it was assumed that the percentage of MP apple annually produced in Portugal was similar to the percentage of MP apple annually produced in each country. It was also assumed that Campotec® is the current market leader of MP apple production in Portugal with a market share larger than 50%. Therefore, an estimated percentage of the MP apple produced in Portugal was assessed in each year, between 2014 and 2016. This percentage was applied to other EU countries and also to the total apple production in the EU (EU-28) and to the global apple production, in order to estimate the quantity (tonnes) of MP apple produced in each country. The assumptions made in order to estimate MP apple production in each country have limitations, since the number of companies that produce apple and MP apple, as well as, consumer preferences differ from country to country. These projections only consider the apple production in each country, whose data are available in Eurostat database. Dots (:) represent the data not available.

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[10] The main cause for this drop, relied on the adverse climate conditions experienced during the vegetative dormancy period of apple and pear orchards. Adverse conditions included a mild winter – that didn't allow flowers' formation – intense rain periods and low temperature in the blossom phase, compromising pollination and fruits' development (Instituto Nacional de Estatística, I.P., 2016).

[11] According to the Regulation 1337/2011 " 'dessert apple tree' (…) means apple tree plantations (…) except those specifically intended for industrial processing.".

Due to the lack of data regarding the production of minimally processed apples and given the importance of this data to predict the potential market size for AlgaeCoat, in Table 5.2 MP apple production in Portugal and in the other countries had to be estimated as explained in table footnotes.

Assuming that Campotec IN is the market leader of MP apple production in Portugal with a market share larger than 50%[12] (the amount of MP apple annually produced by Campotec IN is shown in Appendix C) and that the percentage of MP apple annually produced in Portugal was similar to the percentage of MP apple annually produced in each country, it was possible to assess the annual production of MP apple per country (in tonnes) according to the following equations (Equations 5 to 7):

$$\boldsymbol{MP\ Apple\ Produced\ in\ Portugal}\ (\%) = \frac{MP\ apple\ produced\ by\ Campotec®\ (tonnes)}{Total\ Apple\ Production\ in\ Portugal\ (tonnes)} \times 100 \quad \text{(Equation 5)}$$

where,

$$MP\ Apple\ Produced\ in\ Portugal\ (\%) \approx \boldsymbol{MP\ Apple\ Produced\ in\ each\ country}\ (\%) \quad \text{(Equation 6)}$$

$$MP\ Apple\ Produced\ in\ each\ country\ (tonnes) = \boldsymbol{MP\ Apple\ Produced\ in\ each\ country}\ (\%) \times Total\ Apple\ Production\ in\ each\ country\ (tonnes) \quad \text{(Equation 7)}$$

The assumptions made in order to estimate MP apple production in each country have limitations, since the number of companies that produce apple and MP apple, as well as, consumer preferences differ from country to country. These projections only consider the apple production in each country, whose data are available in Eurostat database. Nevertheless, this is a better option than not to show any numbers about the market dimension for AlgaeCoat, given that with no available data it's hard to establish the real market size for the additive.

5.2 Competition Analysis – Performance Map

There are several techniques used in Food Industry to preserve fresh-cut fruits and vegetables, particularly MP apple. These techniques may be divided into three different groups: biopreservation, chemical-based preservation and physical-based preservation (Ma, Zhang, Bhandari, & Gao, 2017).

Biopreservation group comprises more traditional solutions, based on the antimicrobial potential of natural microorganisms, used to extend and enhance food shelf-life and safety.

[12] Campotec IN is the main supplier of MP apple in Portugal, distributing the product to fast food restaurants, supermarkets and air companies, respectively McDonalds®, Pingo Doce®, Lidl®, Intermarché Group, and TAP.

Chemical-based preservatives includes organic or synthetic additives commonly used in Food Industry.

On the other side, physical-based preservation group is composed by highly complex technologies used to change the physical parameters (temperature, humidity, pressure and gas composition) of the environment that surrounds food products, requiring therefore large amounts of energy. These treatments are usually more effective when combined with synthetic additives or with biopreservatives, thus being applied to guarantee an extended preservation.

In the B2B market, fruit processing companies are developing new formulations to extend the shelf-life of MPP. However, it's interesting to note that currently these companies continue to choose synthetic additives to preserve MP apple, because it's generally the cheapest and more efficient option. In this way, it's expected to integrate AlgaeCoat in the biopreservation group, using CT alga as the natural raw material to combat the lack of natural alternatives and then compete against chemical-based solutions.

The Performance Map below (Table 5.3) shows the analysis of some these competitors, comparing them with AlgaeCoat. The performance of each product was evaluated, considering three possible classifications: positive (green), intermediate (yellow) or negative (red).

- **AlgaeCoat:**

In Table 5.3 it's possible to observe that AlgaeCoat is an efficient solution, with a biological source, that doesn't change fruit's flavour or texture.

Despite the slight decrease in treatment's efficacy observed after the scale-up, AlgaeCoat was already validated at a laboratorial scale, as explained in Chapter 4.

Flavour and texture preservation during storage was supported by laboratorial results and also by a sensorial analysis – Triangle Test[13]. Moreover, as early explained, products obtained from a natural, biological source are generally safer for consumption. Although this additive hadn't been evaluated by EFSA yet, it's estimated to be approved soon after its submission.

Regarding scalability, since AlgaeCoat is obtained by a simple solid-liquid extraction process, scale-up production can be easily achieved.

Due to product's novelty and to the fact of still being developed (not currently available in the market), AlgaeCoat doesn't have full trust of companies yet. Nevertheless, in the future, once it gets into the market, trust inside Food Industry is expected to increase.

[13] To test the influence of AlgaeCoat in MP apple's flavour, slices with and without treatment were disposed under the same light conditions, and then, tasted individually. In the end of triangle test, no differences between samples were noticed.

Table 5.3: Performance Map

Competitors / Attributes	Biopreservation			Chemical-based Preservation			Physical-based Preservation		
	AlgaeCoat	Edipeel™	Arctic® Apples	Food Freshly® O Plus BIO-3	NatureSeal®	Ascorbic Acid (AA)	UV-A	Modified Atmosphere Packaging (MAP)	Plasma Processed Air (PPA)
Efficacy	☺	☺	☺	☺	☺	😐	☺	😐	☹
Biological Source	☺	☺	☺	☹	☹	☹	☹	☹	☹
Flavour/Texture	☺	☺	☺	☺	☺	😐	☺	☹	😐
Safety (EFSA)	☺	☺	☹	😐	😐	😐	😐	☺	☺
Scalability/Complexity	☺	😐	☹	☺	☺	☺	☹	😐	☹
Trust (Brand)	😐	😐	☹	☺	☺	☺	😐	☺	😐
Market Price	**NA**	**NA**	**NA**	**NA**	**32.00 €/Kg**	**9.40 €/Kg**	**NA**	**NA**	**NA**

NA – Not Available

Notes: Efficacy: this attribute measures treatment's success in preventing the enzymatic browning of apple slices and the preservation of apple's freshness for extended periods; Biological Source: this attribute evaluates the biological and natural character of the treatment, based on its source; Flavour/Texture: this attribute measures the quality of fresh-cut apple after treatment application; Safety: this attribute accounts for the risks that treatments may pose to consumers' health, according to EFSA regulation; Scalability/Complexity: this attribute evaluates the capacity and the difficulty of product's production and application (industrial scale); Trust: this attribute defines how the product is perceived in food industry and by consumers; Market Price: this attribute compares market prices between treatments.

- **Edipeel™:**

Edipeel™ is a new technology developed and patented (patent number: US 2017/0049119 A1) by Apeel Sciences™. This is a post-harvest treatment, functioning as an edible coating made of edible oils (lipids and glycerolipids), extracted from the peels, seeds and pulp of fruits and vegetables. It's undetectable on produce and the results support product's efficacy (Apeel Sciences™, 2017).

Like AlgaeCoat, Edipeel™ is applied as a dipping solution, forming a thin layer on fruit's surface, protecting it against biotic and abiotic stresses, such as, microbial spoilage (bacteria, viruses, fungi or pests), water stress and tissue oxidation, preventing water evaporation and gas diffusion, respectively. Additionally, this solution offers mechanical stability and prevents photodegradation, with no need of refrigeration. Since this product is a completely natural solution, allergen-free, already approved by the FDA (GRAS), it's safe for consumption, being used to substitute synthetic additives. Despite the reduced production cost, it has a more complex production process, requiring a fully equipped laboratory, as well as a specialized staff.

Finally, Apeel Sciences™ (2017) announces that this product will only be available in 2018, to select commercial partners (there are no references to products' price yet).

- **Arctic® Apples:**

Arctic® Apples is the trademark created and patented (patent number: US 2014/0041079 A1) by the Okanagan Specialty Fruits™ (OSF) company to designate the genetically modified (GM) apples, in which the expression of PPO gene is reduced, blocking enzymatic browning. Therefore, apples can keep their initial colour and flavour (even when cut and sliced), promoting its consumption and reducing food waste.

In spite of being genetically manipulated, Arctic® Apples derive from a biological source. In North America, they have been rigorously reviewed by reputable regulatory teams at the USDA, FDA, CFIA and Health Canada, having been approved for a safe consumption. In Europe, regulation about GM organisms (GMO) is still very strict and EFSA has to evaluate product's safety before its commercialization in the EU.

Regarding convenience, Arctic® Apples have a highly complex and slow production process, given that genome editing and GM crops harvesting demand high technological procedures and a highly qualified staff. These products are not well accepted by consumers, existing many movements and campaigns against GMO consumption.

OSF affirms that Artic® Apples will be cheaper than the regular MP apples, because it's not necessary to add flavourings or other preservatives. However, for the reasons explained above, production costs may increase the final price. Artic® Apples are available in select cities around the Midwestern United States.

- **Food Freshly® – O Plus BIO-3:**

O Plus BIO-3 is the most recent solution developed by Food Freshly® North America to preserve fresh-cut produce (over 21 days) (Food Freshly®, 2017). It's protected by a patent (patent number: EP 1301084 A1) and like AlgaeCoat and NatureSeal®, is a convenient dipping solution, easy to apply, being currently used in Food Industry as post-harvest treatment.

The mixture approved worldwide is mainly composed by vitamins, organic acids and minerals, including ascorbic acid, among other, thus being GMO free, free of chlorine, sulphite, sodium or other allergens. The amount of each component is adapted to the product that is being preserved, preventing enzymatic browning and microbiological spoilage. Thus, O Plus BIO-3 can be accurately dosed during production, avoiding over usage and reducing labour costs.

It's not an expensive treatment since the amount of product needed to treat 1Kg of fresh fruit is lower than in most of its competitors, resulting in the addition of €0.04 to €0.10 per kilogram of final product.

- **NatureSeal®:**

NatureSeal® is the worldwide leader in fresh-cut produce preservation, developed and patented (patent number: US 2011/0111103 A1) by NatureSeal Inc.

It's a shelf-life extension technology, sulphite and allergen free, mainly composed by a mixture of vitamins and minerals, including ascorbic acid, among other compounds, being approved by the FDA.

Like AlgaeCoat, this formula is dissolved in water to prepare a dipping solution for the treatment of fresh-cut apple, preserving its texture, flavour and appearance, at low but above-freezing temperatures. Additionally, it doesn't require packaging with modified atmosphere. When applied in fresh-cut apples (results assessed over 21 days), this solution showed better results than ascorbic acid (vitamin C) and citric acid solutions (Rupasinghe, Murr, DeELL, & Odumeru, 2005; Rößle, Gormley, & Butler, 2009; NatureSeal®, 2017).

NatureSeal® is available in the markets, being sold at €32.00/Kg of product, considered by many producers/processors as an expensive treatment.

- **Ascorbic Acid (AA):**

Commonly known as vitamin C, AA is an organic acid with good antioxidant properties that can be used as a reducing agent, to enhance food quality and to improve food stability. Additionally, it helps to preserve the aroma, colour and nutrient content of the produce (Martin-Diana, Rico, Henehan, Frias, & Barat, 2007).

Gil, Gorny, & Kader (1998) observed that solutions containing AA reduced the surface browning and increased the shelf-life of fresh-cut products, but only for a short-period of time (tissue softening and mould growth were observed in longer treatments).

Ascorbic acid was re-evaluated by EFSA in 2015, that confirmed the safety of this additive.

It's largely and easily obtained and applied, being explored by Food Industry for many years (highly trustable). The current price for AA is approximately €9.40/Kg.

- **UV-A LED:**

The application of UV-A light (390 nm) treatment using a LED illuminator decreased the colour change of fresh-cut apples. As UV-A light irradiation is less powerful than the UV-C, browning can be effectively controlled, without compromising the organoleptic properties, neither the nutritional quality of fresh-cut fruits. Moreover, UV LED technology may be an eco-friendly alternative that helps to save energy, reducing the environmental impact (Lante, Tinello, & Nicoletto, 2016).

It's known that UV radiation affects the human skin (when directly absorbed), having some risks associated. For this reason, products have to be treated in specific and specialized facilities, turning it an expensive and less convenient process. Despite the efficacy of UV light, customers may not rely in the product.

- **Modified Atmosphere Packaging (MAP):**

Successful applications of modified atmosphere packaging (MAP) with low O_2 and high CO_2 levels for MPP preservation have been extensively reported in the literature. According to Ghidelli & Pérez-Gago (2016) MAP contributes to a reduction in the respiration rate, ethylene biosynthesis, water loss and phenolic oxidation, contributing to MPP preservation during storage.

However, the exposure of fresh-cut produce to extremely low and excessively high levels of O_2 and CO_2, respectively, may trigger anaerobic respiration and fermentation, with the consequent production of undesirable metabolites or even the development of physiological disorders. Therefore, the range of O_2 and CO_2 in the package must be defined for each product and handling/processing conditions (e.g. processed form, package format, storage conditions, etc.).

This is a safe process largely used in food industry for many years, but it generally works as a secondary treatment (as part of a combined treatment), to assure an extended preservation. It requires large and expensive machines, that must be present in the company.

- **Plasma Processed Air (PPA):**

In their research, Bußler, Ehlbeck, & Schlüter (2017) used a microwave-driven discharge to generate plasma processed air (PPA), also known as "cold plasma". This treatment reduced the microbiological spoilage and also the activity of PPO and POD, through a change in their secondary structure. Nevertheless, after applying PPA treatment, it was also observed that the colour of apple tissues was significantly affected. The authors believe that plasma-oxidative degradation and the polymerization of the components present in apple flesh contributed to increase tissue browning.

The fact of being a non-thermal approach, operated under atmospheric pressure, make this a cheaper and suitable treatment for heat-sensitive foods, like food and vegetables, substituting the addition of anti-browning compounds.

Despite the clear need to optimize treatment parameters, this technology promises to innovate the minimal processing industry for fruit and vegetables, possibly as a pre-drying procedure, reducing food waste and providing food quality and safety through an improved sustainable strategy.

The complexity and novelty of this process affect the trust among consumers and Food Industry. In spite of being a cheaper treatment, it requires specialized machinery and staff, increasing the final costs.

5.2.1 Leading Company – NatureSeal®

Considering the Performance Map (Fig. 5.3), NatureSeal® is considered the most efficient solution in the market (the main competitor) to treat fresh-cut produce, acting as an anti-browning solution. NatureSeal® is a patented chemical-based formulation – its main disadvantage, when compared with AlgaeCoat – being classified as a GRAS ingredient. However, due to its elevated price, fruit processors usually prefer to use cheaper solutions, such as ascorbic acid and modified atmospheres.

5.3 Market Trends

Since AlgaeCoat is a natural additive that will be used to preserve and extend the shelf-life of fresh MPP (anti-microbial and anti-browning agent), the following market trends show the projections of the market value in each sector – Food Preservatives and Fresh-cut Fruits and Vegetables Market – as well as the growth of each market in the next years.

Coherent Market Insights (2017) define food preservatives as "substances added or sprayed in food to restrict the growth of fungi, bacteria, and other harmful microorganisms", increasing the shelf-life and maintaining an optimal quality of food.

5.3.1 Food Preservatives Market

According to Coherent Market Insights (2017), the global Food Preservatives market size was estimated at US$ 2 230 million in 2016 (≈ €1 859 million[14]) and is predictable to exceed a revenue of US$ 3 000 million (≈ €2 501 million) by 2025, growing at a Compound Annual Growth Rate[15]

[14] Conversions based on daily reference rates published by Banco de Portugal and the European Central Bank.

[15] Compound Annual Growth Rate (CAGR) measures the rate of return for an investment assuming that every year the investment grows at a constant rate.

(CAGR) of 3.35% between 2016 and 2025. Moreover, it's expected that, during the same period, the global anti-oxidants market grows with a CAGR of 3.32%, stimulating the growth of this market.

In Europe, Food Preservatives Market was projected to reach US$ 850 million (≈ €708 million) in 2016, and US$ 950 million (≈ €792 million) by 2021, with a CAGR of 2.25% between 2016 and 2021. Moreover, in 2016, Europe (followed by North America and Asia Pacific) held a share of 28.1% in terms of value, accounting for the major share in the global Food Preservatives Market. The trend is estimated to remain the same between 2017 and 2025 (Market Data Forecast, 2016; Coherent Market Insights, 2017).

The need for functional ingredients with emulsification, flavour enhancing and shelf-life extension properties, as well as consumer's demand for safer, convenient and more natural foods ("clean label" foods), such as frozen and ready-to-eat foods, mainly among corporate employees and students will drive positively to the growth of this industry (Hexa Research, Inc., 2014; Grand View Research, 2015; Grand View Research, Inc., 2016; Market Data Forecast, Inc., 2016).

Global Market Insights report (2016) indicates that Germany, France and the United Kingdom (UK) are among the most demanding countries in Europe in terms of Food Additives.

BASF Corporation; Cargill Inc.; Celanese Corporation are the major players in the European Food Preservatives Market, while Univar Inc.; Hawkins Watts Ltd.; DSM N.V.; Danisco A/S; Tate & Lyle PLC; Brenntag AG; Kemin Industries Inc.; AkzoNobel N.V. are among the major players in the global market (Market Data Forecast, 2016; Coherent Market Insights, 2017).

Natural Preservatives Market

Statistics provided by Coherent Market Insights in 2016, showed that natural food preservatives dominated Global Food Preservatives market. Moreover, according to Grand View Research report (2015), the overall demand for natural additives is expected to increase in the next years, especially in Europe.

Population growth and the high prevalence of foodborne diseases and disorders will drive the market growth and the demand for healthy food preservatives (Coherent Market Insights, 2017).

5.3.2 Fresh-cut Fruits and Vegetables Market

Fruits and vegetables constitute the largest segment in the global Fresh Food market, generating almost 60% of the overall revenue. Inside this segment, fresh-cut fruit (fruit-salads, snack fruit, etc.) is a growing market, although it remains too small (Business Wire, 2017; Fresh Plaza, 2016).

A cooperative study between the University of Foggia and the University of Massachusetts showed that the consumption of fresh-cut fruit and vegetables in Europe has been following an increasing tendency, in spite of the gradual decrease in its production from 10% to 3% between 2005 and 2010. As a consequence of this decrease, in 2010 fresh-cut fruit achieved a market-share of 1%

regarding the total volume of fruit sold in Europe (Baselice, Colantuoni, Lass, Gianluca, & Stasi, 2014).

This study also demonstrated that the UK, followed by Italy, dominated the fresh-cut market sales. Also in Germany and Spain, the market is growing faster than in other European countries (like the Netherlands), since this kind of products still represent a novelty to the consumers.

The promotion of fruit and vegetable consumption by international organizations like WHO and FAO, along with the awareness about the health benefits related with the consumption of fresh produce and with the increasing of vegetarianism, will drive the growth of this market in the next years (Technavio, 2017; Business Wire, 2017).

Analysing the market trends, it's possible to understand that Food Preservatives, and Natural Preservatives in particular, are following a positive tendency, in spite of growing slowly in the next years. Given the natural composition of AlgaeCoat and its anti-oxidant capacity, this seems to be a favourable scenario for the development and commercialization of this technology, especially in Europe, where the MPP market and the demand for healthier products is increasing too.

CHAPTER 6

Technology Transfer: Marketing Plan

6.1 Segmentation

As early stated, AlgaeCoat market is an organizational market (B2B) since it's composed by companies that will purchase the additive in order to produce and preserve MP fruit. However, it can be sold to consumers market[16] (B2C) too.

Organizational market is characterized by a small number of customers (more geographically concentrated), who often buy large volumes of goods ("purchase in bulk"). For those reasons, the financial effort needed is much more reduced, since the investment in marketing is less demanding than for the B2C market.

The criteria used to segment the organizational or B2B market are:

a) Sector of Activity: Inside Food Industry, companies that sell and prepare fruit.
b) Geographic Location: Companies situated in regions with the proper climate conditions and optimal trade policies to easily produce and commercialize large amounts of fruit with the desired features (taste, texture and nutritional content).
c) Innovation Degree: Companies willing to develop and/or introduce some innovations to improve its productivity and offer.

6.2 Target Market

Given that AlgaeCoat is a product with exclusive properties, intended to add a 'clean label' status to MP fruit, it's interesting to consider a **niche marketing**, offering the product to a single market segment. This strategy will allow Campotec IN to focus on a particular segment and adjust the marketing mix to fulfil the needs of that specific segment. Despite the lower dimension of the target market, the company has the possibility to explore deeply the selected segment.

The descriptions below show that the most interesting target market for AlgaeCoat additive are companies in the EU dedicated to the processing of large amounts of apple, willing to invest in new technologies in order to offer products with a higher quality (taste, firmness, etc.).

Sector of Activity:

In B2B market, the apple processors and also the food service companies such as catering companies, hotels, restaurants and cafes (HORECA channel) are among the potential customers in terms of activity sector.

Processors could introduce MP apple in vending-machines, widely spreading fruit consumption. Currently it's possible to find vending-machines in many public places (like subway and train stations), companies, universities, and even along the streets (like Grab&Go® stores, for example).

[16] The fact of being a ready-to-eat product, will allow consumers to promptly consume the recommended daily portion of fruit. Since this is a new product, the B2C market would require large investments, in order to be well known to the public.

In restaurants, large quantities of fresh fruit and fresh fruit-salads are prepared every day. In schools, instead of the unhealthy desserts usually offered, it would be beneficial to give children some fresh-cut, ready-to-eat fruit. With AlgaeCoat, fruit can be sliced a week before being consumed, saving time and avoiding troubles in the busy school kitchens. In addition, the substitution of sugary desserts by healthy fruit snacks will certainly help to fight infant obesity, a serious problem, that is affecting many children of the modern society.

Geographic Location:

Due to the climate conditions and soil properties, apples produced in Europe generally have better quality, being exported all over the world. According to FAOSTAT (2017), the European continent is the largest global exporter of fresh and processed apple (respectively, 49% and 46% of the global exports in 2013).

In terms of geographic location, the proper conditions along with the free trade and transport policies, make EU countries the primarily target market for AlgaeCoat.

The **EU Trade Policy** settles in a single market that allows the free circulation of people, goods, services and capital within the EU borders. Trade relations across the world (outside the borders) are also managed by the European Union, the biggest trading partner for 59 countries and therefore considered the largest economy in the world (The EU explained: Trade, 2016). Moreover, in order to ensure cheaper, safer, faster and more transparent cross-border payments and pricing, the European Commission created the single euro payments area (SEPA), that supports the **EU Transport Policy**, based on the free movement of individuals, services and goods (Treaty of Rome, 1957).

Innovation Degree

Small and medium-sized enterprises (SME's) dedicated to fruit processing are probably the mostly interested parties, willing to invest in new and improved technologies, given the necessity for an extended preservation of large amounts of fresh-cut fruit. In these companies, several tonnes of MPP are produced, packaged and sent every day to the delivery channels, before reaching the final consumer. In case of exportation, distance and delivery time are also aspects to consider.

Traditional companies usually do not have enough capacity to apply the natural additive, selling fresh products directly to the final customer. These small producers tend to simplify their businesses and, sometimes, are not willing to pay for food additives.

So, considering the selected segment, the main potential customers **in Portugal** are, for example Nuvi Fruits®, Easy Fruits&Salads®, Catefru® (Sogenave S.A.), Frulact® and, **in Europe**, Crop's NV® (Belgium), Comiver® (Spain), Scandic Food A/S (Denmark), among many members of the European Association of Fruit and Vegetable Processing Industries (PROFEL).

6.3 Positioning

Positioning is related with product's concept (identification) and consumer's perception of the product in relation with its competitors (differentiation): how is perceived and remembered and what senses or feelings it might trigger. Real positioning (identification) is a more objective look, a direct definition of the product and all its features, while psychological positioning (differentiation) is a more subjective approach, demonstrating consumers/customers' point of view about the symbolic value of the product.

For the B2B market, real and psychological positioning are defined and analysed next:

Real Positioning

AlgaeCoat is an innovative, natural formulation used to preserve MP apple properties (colour, flavour, nutritional content), that will be used to substitute synthetic additives (potentially harmful for consumers' health). This marine extract is obtained from a seaweed – *C. tomentosum* – with several benefits[17], due to its content in polyunsaturated fatty acids (PUFA), vitamins and antioxidants. Besides, it's a new solution already protected by patent, that will make a difference in the market.

Psychological Positioning

AlgaeCoat is a new "clean label" product (natural and simple), safer for consumer's health, that will add value to fresh-cut apple. Moreover, as mentioned in Chapter 4, given the nutritional and medicinal properties linked to seaweed intake, consumers can develop a positive perception about AlgaeCoat additive.

6.4 Marketing Mix

Product

Seaweed extract with anti-browning and anti-microbial activity, used as a post-harvest treatment for minimally processed products, in order to extend shelf-life, while preserving its freshness and visual appeal. It's obtained through a solid-liquid extraction, resulting in a dry powder (after being freeze-dried) ready to be dissolved in water to prepare an immersion solution.

AlgaeCoat aims to be a "clean-label" brand associated to convenience, allowing a healthier and safer preservation of fresh-cut fruit and vegetables.

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[17] Several studies demonstrated that *C. tomentosum* exhibits good properties, functioning as an antioxidant (Valentão, 2009), antibacterial (Ibtissam, et al., 2009), antifungal (Aparanji, Atika, Venu, & Rajan, 2013), anticoagulant (Wang, Wang, Wu, & Liu, 2014), anti-genotoxic (Celikler, Vatan, Yildiz, & Bilaloglu, 2009), anti-arthritis (Aparanji, Atika, Venu, & Rajan, 2013).

Price

As indicated in the Performance Map (Chapter 5), it was not possible yet to define the final price for this natural additive because it's still being tested (not ready to use in an industrial scale). Once achieved the pilot-scale validation, it will be easier to predict a market value, based on the actual cost of production.

Place

In Portugal, the distribution of AlgaeCoat can be made directly by Campotec IN or by an external distributor.

Internationally, there can be used exclusive distribution agreements, in which the supplier chooses a single distributor to work in a particular market/geographic region. In these agreements, the supplier usually claims something in exchange, like, minimum purchase commitments or other performance-related obligations.

Promotion

To promote the product, it will be interesting to participate in trade fairs, meetings, conferences, workshops and similar events, establishing contact with potential partners. It would be also interesting to send free samples, allowing customers to test the product in their own facilities and make a purchase decision.

6.5 SWOT Analysis: Introducing AlgaeCoat into the Market

The SWOT analysis – presented in Table 6.1. – suggests some strategies that can be followed by Campotec IN in order to introduce AlgaeCoat in the Portuguese and EU markets, taking advantage of product's properties (strengths) and external opportunities to overcome or minimize the weaknesses and threats that may compromise the viability of the project. This framework will help to evaluate the competitive position of AlgaeCoat in the market.

a) Strengths

Throughout this thesis it was seen that AlgaeCoat project aims to develop a natural and sustainable technology that prevents the flesh browning and softening of fresh-cut products, hopefully substituting the application of synthetic additives. This solution is protected by patent (national invention patent – PT 107369 B), filled in December 16th 2013.

As seen in Chapter 2, *Codium tomentosum* has several nutritional and medical properties, and, like other algae, is a very simple organism, with fast reproduction and growth cycles. The primordial structures and the new techniques/tools provided by the evolution of scientific knowledge enable the manipulation of these organisms. Thus, specific targets such as specific biomolecules or metabolic pathways become achievable.

b) Weaknesses

Independently of the commercial strategy chosen by Campotec IN, this project is still an ongoing investigation. Therefore, project's industrial implementation (after pilot-scale validation) will involve large investments not only in infrastructures, machines and research and development (R&D), but also in the production process itself (raw material, water, energy, heat loss, drying process).

Another weakness is the raw material dependency. CT is a living organism that needs to grow and reproduce.

AlgaeCoat has a simple production process, which makes it an easily reproducible project. Moreover, results acquired in the present work (Chapter 4) continue to demonstrate some lack of stability of the final product (pilot-scale production is not validated by the results obtained in this experiment). However, in the future it's expected to obtain a completely stable formula.

Finally, given that an international patent was not applied at the time (the national patent was filled 5 years ago), it will be very difficult to protect the formulation and its production process outside Portugal.

c) Opportunities

Market growth, global health concern, the increasing knowledge and the advanced technology, as well as investing programmes and regulation constitute the main opportunities to successfully introduce AlgaeCoat in the market.

The growing market of apples, food preservatives and algae represent good prospects for the success of this project (Eurostat, 2017; FAOSTAT, 2017).

Global health concern is one of the most defended causes worldwide. According to Food Aid Foundation and to World Food Programme, about one in nine people on earth (FAOSTAT predicts approximately 815 million people in 2016) "do not have enough food to lead a healthy active life". On the other hand, according to the World Health Organization, in 2016 more than 1 900 million adults were overweight, including over 650 million obese. Additionally, over 340 million children and adolescents (aged between 5-19) and 41 million children (under the age of 5) were overweight or obese.

Nowadays, the information flux between national and international universities has been crucial to access global knowledge and also to get answers promptly. In Europe, the Erasmus + Programme (created by the European Commission) has driven many students to learn and bring new ideas to their countries. This has also made possible to develop novel and advanced technologies, that in turn provide tools that help scientific investigation to proceed. The development of artificial intelligence and advanced software is allowing scientists to transfer their own lab-scale projects to the industrial level in a more easy and faster manner.

Moreover, many investment programmes and initiatives are supporting the Blue Biotechnology sector (estimated to reach over €6 000 million by 2025).

Regulation (controlled by EFSA in the EU and by Food and Drug Administration in the US) is becoming very strict and recent studies show that synthetic additives are being progressively banned from the GRAS list due to the evolution of risk analysis tools used to reassess product's toxicity. Therefore, this is probably the most difficult and challenging phase before the commercialization of the extract.

d) ***Threats***

Competition and the ancestral knowledge of the algae market by oriental countries constitute some of the threats. In China, Indonesia and Korea, the algae market is particularly well-known and well-established. Contrastingly, in western countries, it's still a relatively new industry. For this reason, oriental countries like China can easily develop and improve a similar or competitive project.

Additionally, in Chapter 5, several competing products/techniques were analysed and compared with AlgaeCoat. Nevertheless, synthetic additives combined with MAP are still the first choice for MPP producers, given the low cost and high effectiveness of this method.

Strategies

The combination between opportunities and strengths will certainly foster the development of this natural additive, expanding it to new markets in Europe and even beyond the EU borders.

The nutraceutical properties of *C. tomentosum* can also be used to develop new products and to build strong marketing campaigns, promoting the high quality of the product and its application to preserve fruit snacks, fruit-salads, etc., given that B2B customers are becoming very interested in knowing all the details about the products they will apply.

Taking advantage of the investment programmes, like Horizon 2020, it would be interesting to finance project's research and development (R&D), creating new pipelines, through which the increasing knowledge and the information flux, as well as the development of advanced technologies might also contribute to enhance extract's production and to develop new and improved formulations, for example, through the addition of new algae (micro and/or macroalgae), different polysaccharides or other ingredients capable of stabilize the final formula.

Since it's expected to extend produce shelf-life, AlgaeCoat may ultimately contribute to boost the Portuguese economy through the exportation of national fresh-cut fruits, particularly "Maçã de Alcobaça" and "Pêra Rocha", internationally recognized by their distinctive, high standard quality.

To overcome project weaknesses, opportunities must be explored.

In Europe's Bioeconomy Strategy, €1 200 million from the European Maritime and Fisheries Fund were addressed to the sustainable development of EU aquaculture and between 2016 and 2017, the Horizon 2020 invested about €46 million in research on aquaculture management, spatial planning and diseases (EC, 2017). Together with the increasing knowledge and the advanced technology, these investment programmes could certainly help to develop new pilot-scale tests to validate extract's industrial production and to implement an Integrated Multi-Trophic Aquaculture system (IMTA), in which the CT alga and other marine species (of finfish, shellfish and marine plants) can naturally grow together, increasing system productivity and project self-sustainability, consequently reducing raw material cost.

Portugal geographical position, climate conditions and wide maritime area are propitious to develop an IMTA system, like the project Fish n' Greens, created by Aquaponics Iberia® (a Portuguese start-up).

Cooperative work between institutions (for example through BLUEBIO ALLIANCE©, an organization that promotes the contact between the scientific community and companies) could also support product's development and transfer to the market.

To optimize extract's production and reduce costs, heat loss, among other requirements have to be well studied to be minimized. Also, the adjustment in the proportion of the ingredients (alga, water and ethanol) used in the extraction process, as well as the choice of a cheaper drying process (discussed in the Business Model, in the next Chapter) are some of the modifications that could help to reduce costs and stabilize the final formula.

To avoid threats and take advantage of the strengths, two strategies can be considered: use less common species and use species that can be classified as GRAS. By working with uncommon/rare species (unexplored in oriental countries), project's idea (intellectual property, etc.) can be more easily protected.

It's also essential to include AlgaeCoat in the European List of Additives. If the alga species is not considered GRAS yet, it's crucial to prove its safety for consumption (through laboratorial analyses, toxicity data, documents that attest its consumption as food/ingredient, used in traditional recipes, etc.). Otherwise, the product will be rejected, and the project can no longer proceed. In Malaysia, India, Indonesia, Thailand, Japan and Hawaii, *C. tomentosum* (edible seaweed) is commonly consumed raw, in salads and it can also be used to prepare soups (Leite, 2017).

To overcome weaknesses, it would also be interesting to establish partnerships with other companies to reduce competition and some production costs (the drying process can be outsourced, for example).

Table 6.1: SWOT Analysis - AlgaeCoat introduction into Portuguese and EU markets

	Strengths	**Weaknesses**
	• **Nutritional Value (vitamins, proteins, antioxidants, etc.)** • **Natural Product** • **Sustainable (environmentally friendly)** • **Reduction of Fruit Browning and Softening (fruit preservation)** • **National Invention Patent (granted)**	• **Great Investment Need (Infrastructures, R&D)** • **Raw Material Dependency** • **Technology Easily Reproducible** • **Product Stability (pilot-scale production not validated)** • **Extraction Costs (Heat loss, Energy, Water, etc.)** • **International Patent Not Applied**
Opportunities		
• **Growing Market** • **Global Health Concern** • **Increasing Knowledge (Universities, Institutes)** • **Advanced Technology** • **Investment Programmes (Horizon 2020 Programme)** • **Regulation (EFSA, FDA, etc.)**	+ Expand the product to new markets + Develop different products (taking advantage of *C. tomentosum* properties) + Promote product quality and properties (Marketing) + Project submission to Horizon 2020 Programme, or similar Programmes + Invest in R&D to create new pipelines	+ Invest in new pilot-scale tests + IMTA Implementation + Cooperative work with Universities and Institutes (BLUEBIO ALLIANCE©) + Production Optimization (cost reduction)
Threats		
• **Oriental Countries (ancestral knowledge of algae market)** • **Competition**	+ Work with less common alga species (unexplored in oriental countries) + Use GRAS alga species (or test them to enter on the GRAS list)	+ Partnerships (outsource production, etc.)

CHAPTER 7

Technology Transfer: Business Strategy for Commercialization of AlgaeCoat Extract

As previously referred AlgaeCoat is being developed through a partnership between MARE-IPLeiria and Campotec IN.

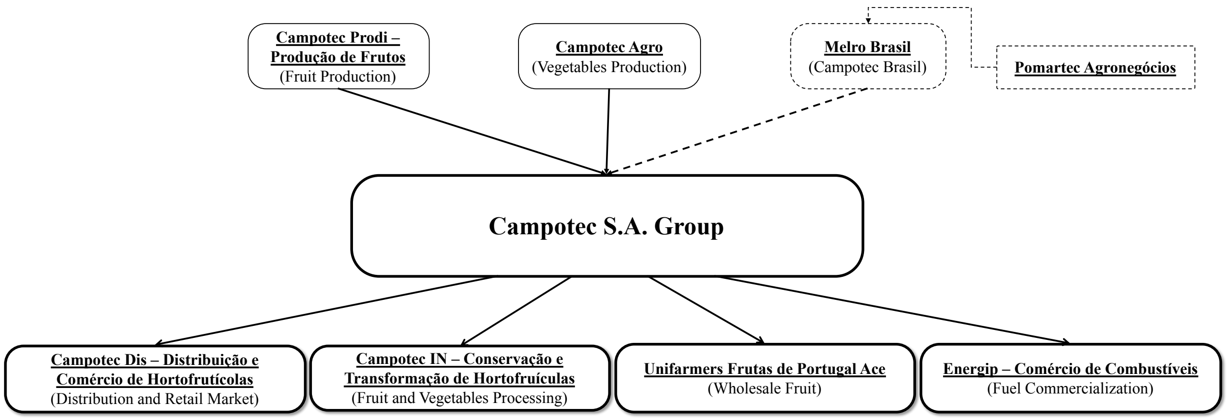
Before analysing which are the best strategies to commercialize AlgaeCoat, it's important to understand Campotec S.A. structure, how is the business organized, which are the current target markets, etc. In the next chapters, after a brief presentation of Campotec S.A., the business model and some commercial strategies will be suggested and explored.

7.1 Campotec S.A. History

Campotec S.A. was founded in 1994, being subsequently recognized as an Association of Fruit and Vegetable Growers. Its core business integrates the purchase and commercialization (sale and distribution) of fruit (*Pomoideae* and *Prunoideae*), potatoes and pre-package agricultural produce (MP products) (Campotec®, 2017).

Assuming an ambitious vision, the company aims to achieve the highest quality production (with food safety as a priority), supported by an advanced technical "know-how" based on scientific research and on the comprehensive knowledge of the latest production techniques (Campotec®, 2017).

In 2008, Campotec S.A. group decided to embrace a restructuring project, constituting a set of smaller and specialized companies (spin-offs) organized across the value-chain (Fig. 7.1). This restructure allowed Campotec S.A. to make a business association with Pomartec Agronegócios Ltda, entering in the international market, particularly in Brazil, as Melro Brasil Ltda (Campotec®, 2017).

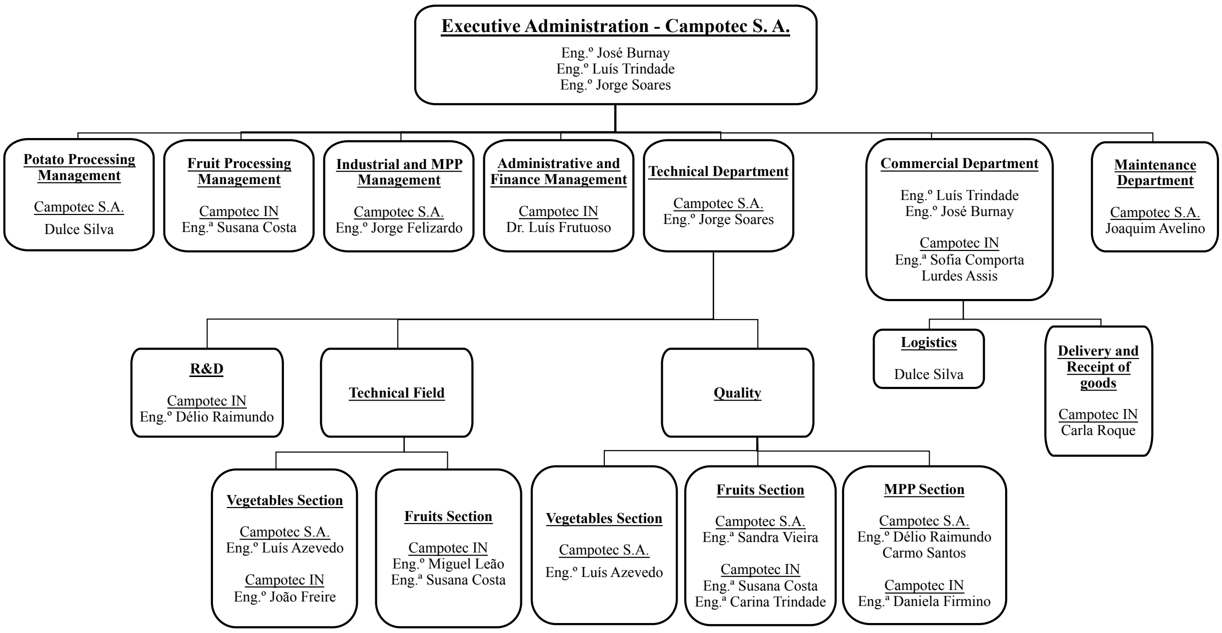


Notes: Business organization in Brazil (---) Adapted from Campotec® (2014)

Figure 7.1: Organization of Campotec S.A. Group (after being restructured in 2008)

Also in the internationalization strategy, Campotec S.A. participates in a partnership with other five Portuguese companies – Ferreira da Silva®, Frutoeste®, Ecofrutas®, Granfer® and Lusopera® – to create Unifarmers ACE, the largest fruit company in Portugal.

Campotec S.A. is currently composed by 800 shareholders, more than 150 employees and 15 high level technicians, distributed by several departments, including administration; commercial department; technical department; quality and food safety department; R&D department and maintenance department. Company's organogram is represented in Figure 7.2., below.



Adapted from Campotec® (2014)

Figure 7.2: Campotec S.A. Organogram

To pursue the new tendencies of the fresh food sector, in 2000, Campotec S.A. decided to produce minimally processed products (in the form of pre-packaged fruits and vegetables). Then, as a consequence of the restructuring plan, in 2008 Campotec IN was created with the purpose of develop and innovate the agricultural and food business.

In its facilities, large and automated systems are currently used to preserve, package and store the fresh produce, guaranteeing food safety and quality (considered priority policies). For this reason, the company received a BRC certificate, a leading global brand developed by the British Retail Consortium (BRC) that assures consumer protection. Also, due to the success in this particular business, between 2012 and 2016, Campotec IN was successively distinguished by the Portuguese Agency for Competitiveness and Innovation (IAPMEI) as a "PME Líder" ("SME Leader"), a status that recognizes some Small and Medium-sized Enterprises (*Pequenas e Médias Empresas*) as important contributors to the national economy.

Among the MP products available in the national markets are: fresh-cut salads and apple slices, as well as pre-prepared mixtures for soups, detox juices and vegetables (to stir-fry), showed in Figure 7.3.

Photos © Campotec (2017)

Figure 7.3: Range of minimally processed products offered by Campotec IN

7.2 Business Model: AlgaeCoat

The Business Model Canvas displayed in Figure 7.4 was developed assuming Campotec IN position. Although the B2B market had been classified as the main target market in the previous chapters, this Business Model also includes the B2C market, given that AlgaeCoat can be sold directly in the consumers market too.

This Business Model represents a possible strategy for the introduction of the natural additive in the market, focusing on specific aspects and key activities that need to be developed in order to organize product's commercialization.

The **value proposition** of AlgaeCoat is:

A new, easy and simple solution, capable of acting as an anti-browning agent of fresh-cut produce (fruits and vegetables). Moreover, it's an original and natural solution, already protected by patent, safer for human consumption, that will help to substitute the synthetic additives currently used in Food Industry, that generally represent a risk for consumer's health. Finally, by acting as a protective layer (blocking gas diffusion and inhibiting enzyme activity), this technology will deliver an increased shelf-life for fresh-cut products, helping customers to preserve them for longer periods, ultimately reducing food waste, reported as one of the biggest problems of the modern society.

A **customer relationship** based on trust (associated with product's safety) and satisfaction (linked to product's efficiency), will reach B2B customers truly concerned about consumers' needs. Through an always informed and updated society, interested in products' origin and in food ingredients, it's advantageous to apply a natural-based formulation.

Additionally, with this solution, fruit processing companies (like Campotec IN) may offer convenience to the consumers that frequently eat fruits and vegetables. Some of these products are difficult to peel and cut, given their large volume and/or hard skin (i.e.: pineapple or coconut), existing therefore the need to pre-prepare them for consumption. In this way, final consumers can promptly eat them outside home with no trouble.

Regarding **customer segments**, there are two possible approaches:

B2B Market

Following a B2B strategy, the target market is composed by fruit producers and processing companies (primarily, to MPPs industry) or to Food Service, situated in the EU, willing to invest in technology (open to innovation).

To reach this market segment, some interesting **channels** may be considered. For instance, Campotec IN could participate in trade fairs, conferences and/or workshops to show product's functionality and establish contact with potential partners, to whom free samples could be sent.

Investigation centres, suppliers, external distributor, as well as other fruit producers and processors would constitute some of the **key partners** in this project, being essential to reach final consumers in the best way possible. Partnership with other companies could result in the development of new products, like the ones presented in the Brainstorming of Applications exposed in Chapter 3.

Since these products are frequently designed and conceived through a cooperative work with universities and research centres, some of the **key activities** would include the launch and promotion of incentive programmes (at least, annually), where scientists/investigators and company's team could exchange some ideas (Frulact®, another fruit processing company, is implementing, successfully, these programmes every year). Incentive programmes are a good strategy to establish and consolidate the relationship between the company and the community (consumers, professionals and students). Another key activity could be the creation of a spin-off (discussed in the next chapter).

To produce AlgaeCoat there are indispensable **key resources** such as the raw material (*Codium tomentosum*), proper equipment and specialized machines (needed to extract AlgaeCoat) and finally, hire highly qualified staff (to assure the production and accelerate company's activity).

Revenue streams are going to be the result of the amount of additive sold and product's price. It's important to note again that AlgaeCoat is a product with an added value, and for this reason it's expected to be more expensive than the current solutions. Nevertheless, competitive pricing will be followed to set a value adjusted to this product.

Being a value and cost driven business (focused on value creation and cost minimization), the **cost structure** will be mostly based in the purchase of the raw material and in the extraction process.

C. tomentosum is a biological source and the active ingredient of this solution. In Portugal, just a few companies can produce and sell algae and, therefore, the unit price is relatively high (€55.00/Kg). In the extraction process, the drying process (lyophilisation) is also a very expensive technique (€30.00/L). However, since these are both economies of scale – where the fixed costs per unit decrease as production increases – at an industrial-scale, production cost is expected to decrease.

Another option that would help to reduce production costs is the substitution of lyophilisation (or freeze drying) by a cheaper drying technology. Some examples are refractance window dehydration (RWD) – suitable to convert liquid foods into powder – or high electric field drying (HEF) – a convective drying technique able to remove product's moisture, while retaining heat-sensitive components (such as ascorbic acid) (Moses, Norton, Alagusundaram, & Tiwari, 2014). This study refers that HEF is a promising method for drying seafood and that both technologies offer high energy efficiency and a lower drying time.

B2C Market

On the other hand, in the second approach (B2C strategy), **customer segment** would include customers with higher incomes, more willing to pay for costlier products on the behalf of their quality and high standard features. AlgaeCoat could be sold to the final consumer as a liquid solution (similar to Amukina®), easy to apply at home, over apple slices, or fresh fruit-salads, keeping their freshness and preserving them for longer periods. Following this approach, several **channels** may be considered to reach customer segments.

One of the most effective ways to aware consumers for a new product is the advertisement through TV commercials. Advertise the product through the television would be a **key activity** with a strong impact on people's minds, leading them to better understand product's concept and application. In Portugal, many companies choose this channel to display their products, specially companies that sell natural products like calcium, omega-3 and vitamin supplements. Nevertheless, this is a very expensive option.

Another way to get B2C target market is to establish contact through the social media (Facebook®, Twitter®, Instagram®, among others). In Campotec IN website, a contacts platform it's already available. Through that platform people can contact directly AlgaeCoat producer and suggest their own ideas (this is also important for the B2B customers, who can easily contact Campotec IN).

Concerning distribution **channels,** in the B2C market AlgaeCoat could be delivered through retail stores, for example supermarkets.

Given that in the B2C market, the financial effort need is much more demanding than for the B2B market, the **cost structure** would have to include large investments in the Marketing campaign.

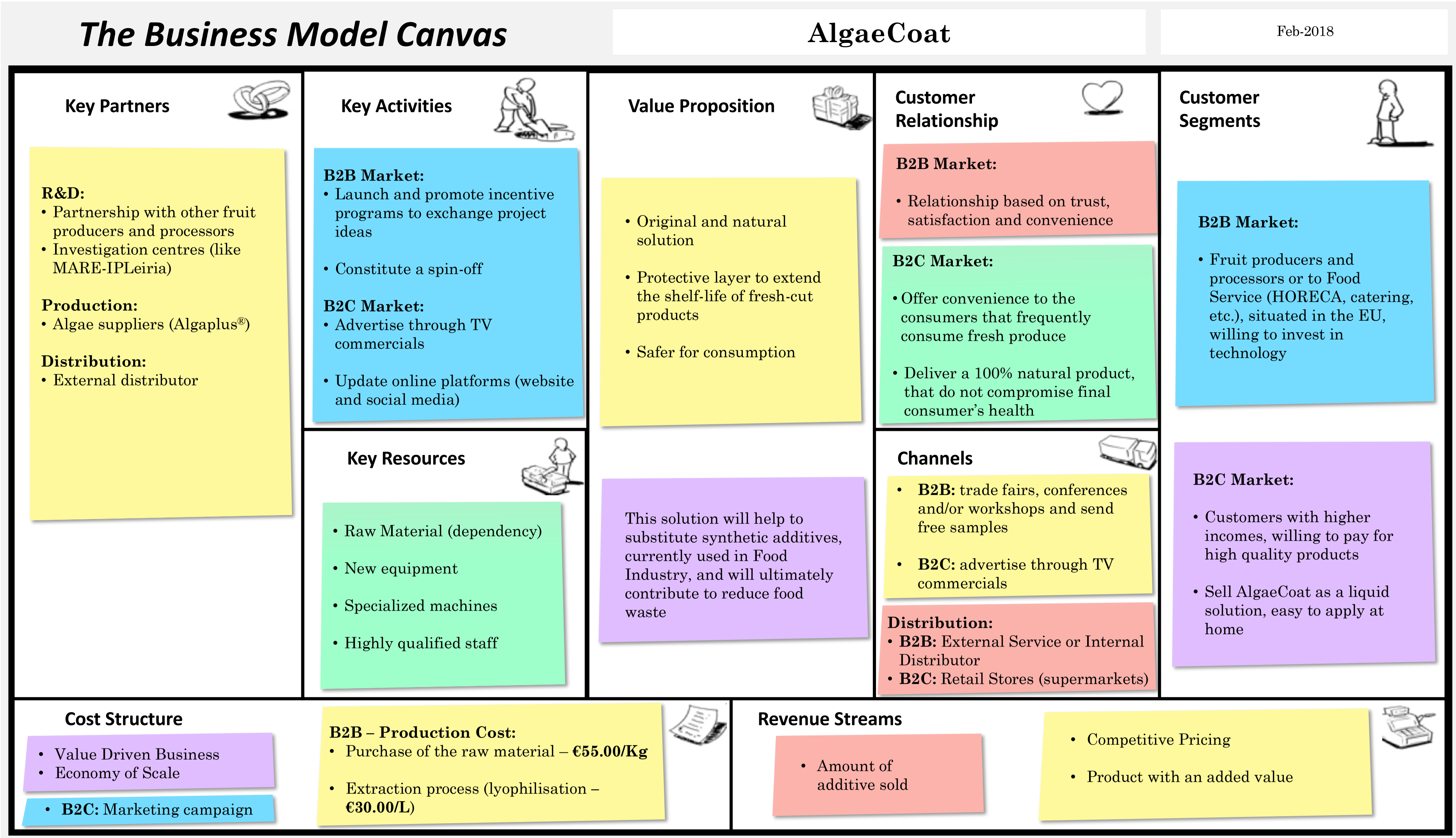


Figure 7.4: Business Model Canvas of AlgaeCoat

CHAPTER 8

Technology Transfer: Business Plan

8.1 Company strategy: core business, outsourcing, spin-off or patent licensing?

After analysing market growth, competitors and market trends, it's necessary to evaluate which strategies can be followed by Campotec IN to commercialise AlgaeCoat. Depending on company's current needs and future perspectives, there are four available strategies: integrate AlgaeCoat production in the core business; outsource AlgaeCoat production; create a spin-off or patent licensing.

a) ***Integrate AlgaeCoat production in the core business***

This strategy allows to control the entire business process, from the production, until product's application and delivery. This is probably its major advantage. The final product (fresh-cut apple preserved with AlgaeCoat) is directly sent to the distribution channel, reaching promptly the final customers.

Another advantage is the protection of the intellectual property and all the information concerning the project (production process, ingredients, etc.).

Nonetheless, there are some disadvantages to consider. First, costs related to AlgaeCoat production, namely, raw material, machines/equipment, energy, water, salaries (highly qualified staff), etc., would be entirely supported by the company. The creation of a single department, focused on the key-activities linked to the extract production would also raise costs.

Finally, new buildings and warehouses would probably be necessary to place the equipment/machinery and also to store the extract, as well as the raw materials, in the appropriate conditions.

b) ***Outsource AlgaeCoat production***

Outsourcing, as the name implies, is the production out of the source. Companies trust the production to a third party, with whom establishes a commercial relationship (agreements, etc.). This is a business practice that companies usually use to reduce costs and to delegate some functions that doesn't fit in their core businesses. Therefore, companies gain time (increasing productivity) and become more efficient, focusing on the usual tasks (key-activities).

As previously reported, this is probably the most economic choice, given that Campotec IN would just have to buy the final product – AlgaeCoat extract – to a third party (that would become the extract supplier). Additionally, to hire qualified staff and buy machines or raw materials required to produce the marine extract, wouldn't be needed.

However, there are some disadvantages associated with this strategy. For instance, all the profits related with product's commercialization, as well as brand authenticity are lost when part of the business is attributed to a third party.

Another disadvantage is the amount of time spent in the agreements and in the discussion of contracts' legal terms (that occasionally offer some limitations). Furthermore, there is sometimes lack of communication between the outsourced provider and companies, delaying project's conclusion. Finally, there is a risk of copy. Expose the formulation to a third party may guarantee the access to confidential information by other companies.

c) Create a Spin-off

A spin-off is an independent company created from a larger company (parent company) to explore a business autonomously. So, this strategy allows companies to focus on a particular product/service, that otherwise would have little or no growth.

This is probably the most interesting strategy for Campotec IN, since it combines some of the advantages offered by the previous strategies.

A spin-off would allow to control the entire business process, having the advantage of being an independent company, completely focused on food additives market, particularly on food preservatives market, managing its own business plan (costs, profits, human resources, etc.).

The biggest disadvantages of this strategy are the initial investment (even higher that in the first option) and time needed to build a whole new company. Therefore, AlgaeCoat production; hire a qualified and multidisciplinary team; protect the intellectual property and raise/acquire new buildings to place the machinery and store company goods, would be entirely supported by the spin-off (assisted, in turn, by the parent company whenever necessary).

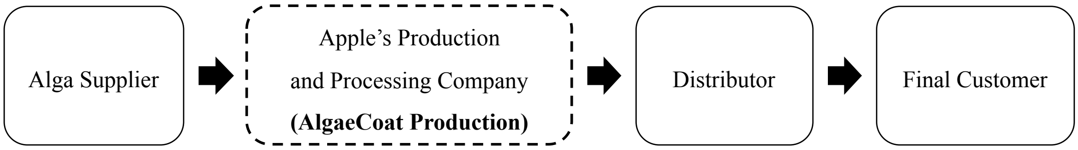
d) Patent Licensing

The last strategy, and also the less interesting one, is to establish a licence agreement and grant patent licence to other interested companies in return for a fee. Following this strategy Campotec IN would lose the total business control.

8.1.1 Value Chain

After understanding the fundaments of each commercial strategy, it's interesting to analyse how would the product be delivered to the final customer. Therefore, value chains are respectively represented in Figures 8.1, 8.2 and 8.3:

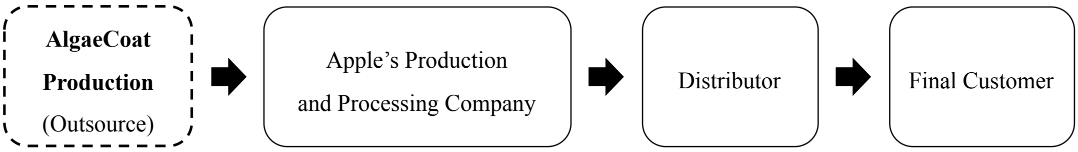
- If Campotec IN decides to produce AlgaeCoat, the value chain (Fig. 8.1) would begin with the alga supplier selling the raw material to produce the natural additive. Then, the treatment would be directly applied over MP apples in Campotec IN facilities. The final product (MP apples preserved with AlgaeCoat) would be delivered to the final customer through the distribution channel.



Notes: The dotted line (---) represents where AlgaeCoat production takes place in the value chain

Figure 8.1: Schematic representation of the Value Chain assuming that AlgaeCoat is produced by Campotec IN

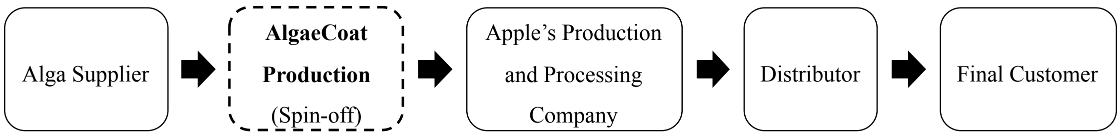
- If the strategy goes through the outsourcing of AlgaeCoat production (Fig. 8.2), the value chain would begin with the production of additive by an external company, which would consequently function as a supplier. The solution would be then applied over MP apple in Campotec IN facilities and the final product sent to the distribution channel, reaching the final customer.



Notes: The dotted line (---) represents where AlgaeCoat production takes place in the value chain

Figure 8.2: Schematic representation of the Value Chain assuming the outsourcing of AlgaeCoat production

- If Campotec IN chooses to create a spin-off, the company would have two separate activities in the value chain (Fig. 8.3): one to produce and process apple and the other to produce AlgaeCoat (depending on alga suppliers). The spin-off would, therefore, act as the additive supplier (internally). In Campotec IN facilities, the extract would be used to preserve MP apples, and the final product would be launched to the distribution channel, to be finally delivered to the final customer.



Notes: The dotted line (---) represents where AlgaeCoat production takes place in the value chain

Figure 8.3: Schematic representation of the Value Chain assuming that AlgaeCoat is produced by a spin-off created by Campotec IN

- If Campotec IN decides to grant the licence, the value chain is inexistent.

8.1.1.1 Suppliers

Suppliers include companies that produce algae, particularly the CT alga – the raw material of this formulation. In Portugal, there are few companies that produce and sell this kind of feedstock. Algaplus® stands-out by the knowledge and growing activity in this sector.

There is also a need to find suppliers that can provide a service concerning the drying process of the marine extract (currently, lyophilisation). Some Universities, like Faculdade de Ciências da Universidade de Lisboa, are offering a freeze-drying service, but not at an industrial scale.

8.1.1.2 Distributors

As referred in Marketing Mix (Chapter 6), distribution in Portugal can be done directly by Campotec IN or by an external distributor. Internationally, exclusive distribution agreements may be an option to distribute the product in a particular market/geographic region.

8.1.1.3 Raw Material

As *Codium tomentosum* is the raw material of this product, there is a clear dependence on alga production (reproduction) cycles. CT is a perennial alga, meaning it has matching reproduction and growing peaks. These peaks normally occur between the end of Summer and the beginning of Fall, when hot temperatures allow a faster growth and development of the alga (Pereira, 2014).

Algae Market

Data shown by Food and Agriculture Organization (FAO) indicate that in 2015, the world production of aquatic plants, mostly seaweeds[18], reached 30.5 million tonnes, of which 96% (29.4 million tonnes) was harvested from aquaculture (FAO, 2017). Nowadays, seaweeds production is mainly directed to human consumption and also to food additives and fertilizers industries, though in lower amounts.

In 2014, seaweed aquaculture accounted for 49% of the global mariculture production, reaching a value of US$ 5.7 billion (approximately €4 863 million) (Cottier-Cook, et al., 2016; FAO, 2017).

According to Cottier-Cook et al. (2016), in the future, FAO is expecting a continuous growth of seaweeds industry throughout the world. This will raise new challenges to both producers and the environment.

China, Indonesia, Philippine Islands, Democratic Republic of Korea and Japan are among the largest producers worldwide, having a huge expression nowadays. In 2015, China was the largest

[18] According to FAO (2017), the global cultivated seaweed production represents about 96% to 99% of the global production of aquatic plants.

producer, achieving approximately 47% (≈13.9 million tonnes) of the global seaweed production. Indonesia conquered the second position, registering a production of approximately 11.3 million tonnes (38% of the global seaweed production) (Cottier-Cook, et al., 2016; FAO, 2017).

In Europe, numbers are far behind. Comparing the global algae market and the algae market in Europe (Fig. 8.4) it's possible to notice a significant discrepancy between the amount of seaweeds produced. As referred above, it's clear that China, alone, supported almost a half of the global seaweed production in 2015.

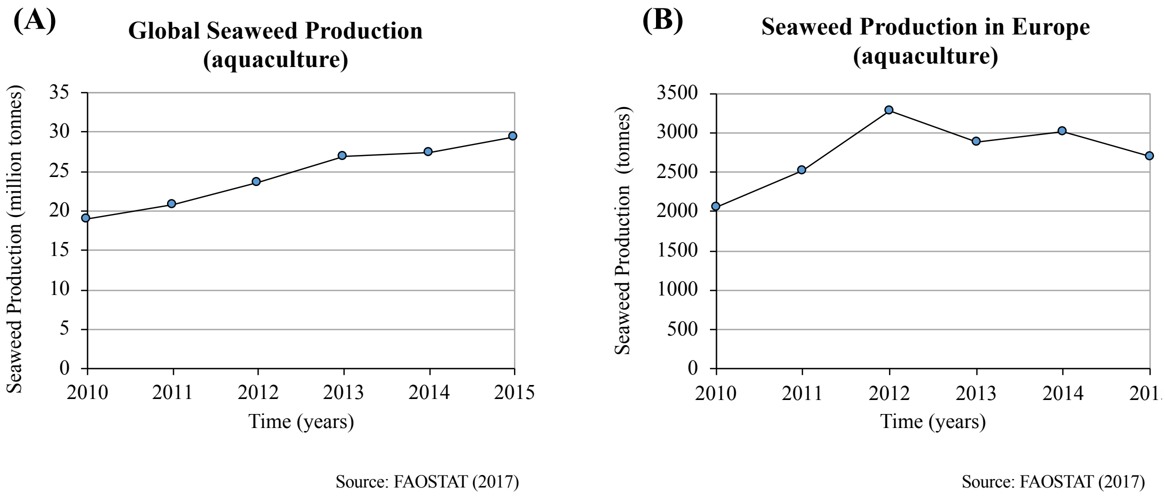


Figure 8.4: Global seaweed production (A) and seaweed production in Europe (B) harvested from aquaculture

However, despite the significant differences, it's possible to observe that Europe is looking for an increase in this sector. Until 2012, evidences show a positive tendency (≈3 273 tonnes, valued in US$ 4.4 million, or approximately €3.7 million) (FAO, 2017).

In the last years, seaweed production in Europe registered a slight decrease, reaching an amount of ≈2 709 tonnes in 2015, valued in US$ 4.2 million (near €3.5 million) (FAO, 2017).

Nevertheless, the European Commission has shown that some efforts are being made to reduce the discrepancy in those numbers.

8.1.1.4 Partnership Opportunities

The partnership between Campotec IN, IAPMEI, INOVA + (two consulting agencies for competitiveness and innovation) and MARE-IPLeiria can be profitable in every direction, not only because Campotec IN is interested in producing and commercializing AlgaeCoat, but also because it will certainly promote innovation in Food Industry.

Other interesting partners are algae producers, like Algaplus®, as well as international distributors, that may deliver the additive.

Another partnership suggestion is Aquaponics Iberia® (already established with MARE-IPLeiria) that develops aquaculture systems to produce fish and vegetables.

8.1.1.5 Stakeholders

As explained above, MARE-IPLeiria and Campotec S.A. are the main interested parties in the commercialization of AlgaeCoat. The two entities detain a shared patent of the marine extract (PT 107369 B). IAPMEI and INOVA + sustained project's innovation and provided the financial support needed.

In the B2B market, it was already seen that apple processors will constitute AlgaeCoat target market. Other stakeholders to consider are Nuvi Fruits®, Easy Fruits&Salads® and Catefru® (Sogenave®) that besides Campotec IN, also expressed some interest in this product.

Additionally, regulation agents (EFSA and SCF) are also important entities to consider, given their role in the evaluation and inclusion of the proposed formulation in the List of European Additives.

Agencies concerned about public health, like World Health Organization (WHO) and Direção Geral de Saúde (DGS), in Portugal, can also be considered stakeholders in this project.

Conclusion and Future Work

AlgaeCoat – the value proposition presented throughout this thesis – is a new post-harvest treatment that aims to improve consumers' daily-lives.

Nutrition is becoming an important issue for the current society, not only to fight overweight and hunger, but also to prevent other health problems, such as cancer, food allergies, vitamin deficiency, among other. For those reasons, people are becoming more concerned about what they eat, about food properties, food origin and usually prefer to buy natural products ("clean-label" demand). However, despite the preference for fresh products completely free of additives, in many EU countries several ready-to-consume meals are displayed in supermarkets' shelves.

The application of AlgaeCoat will allow B2B customers to produce "clean label" products (MPP) – highly demanded by the current society (B2C market) – allowing customers to prepare their own healthy, ready-to-eat snacks, providing a more convenient way to consume fresh fruit outside home. By attributing a "clean label" status to their fresh products, companies can establish a trademark, winning the trust and respect by their customers. Not only products, but also brands will benefit with AlgaeCoat.

Moreover, it's expected that the application of the new additive over fresh products can extend foods' shelf-life, allowing its exportation to the most affected countries. In this way, besides hunger and obesity, this technology will also be used to fight food waste witnessed worldwide. FAO affirms that, every year, roughly one third of the food produced in the world for human consumption (1300 million tonnes) gets lost or wasted, and that fruit and vegetables (plus roots and tubers) represent 40% to 50% of these losses. So, there is a clearly urgent need to reduce these numbers.

Notwithstanding, the purpose of this thesis project was the validation of the extract (CTE) functionality at pilot-scale (scale-up). It's important to note that scale-up procedures, normally help to find the main limitations of the production process before going to the industrial level, where problems in the production chain must be avoided (or, at least, be thought), in order to be instantly solved.

At a pilot-scale, results revealed some limitations of CTE, particularly concerning apples' browning index and visual look. These are the first quality indicators evaluated by consumers, leading them to accept or reject a determined piece of fruit.

Additionally, results indicated that CTE had a slight tendency to be more efficient under packaging with air, than with modified air (MAP packaging). Comparing samples from both packaging conditions, MAP seemed to favour the browning of apple slices treated with AlgaeCoat. If future analyses continue to sustain the results obtained, packaging with modified atmosphere (largely used by fruit processors) may be removed, along with the underlying costs. Furthermore, this offers the possibility to deliver fresher MP fruit, since MAP sometimes induces the modification of quality features, like flavour and texture.

Microbiological analysis revealed that AlgaeCoat prevented the contamination and spoilage caused by some microorganisms.

It's still important to note that these laboratorial results are far from being the initially desired. Knowing that the application of the treatment solutions in Campotec S.A. facilities didn't work exactly as planned in the experimental design, the inconsistencies verified can be understood.

After a careful analysis of the situation, there are some adjustments that must be done to optimize extract's production and to minimize costs.

One of the most interesting and important aspects of the production process is the agglomeration of biomass in the extraction vessel. Previous works demonstrated that smaller particles contribute to higher solute transfer rates, increasing therefore extraction's efficiency. However, smaller particles also suffer higher agglomeration (Bucić-Kojić, Planinić, Tomas, Bilić, & Velić, 2007; Chanioti, Liadakis, & Tzia, 2014). An attempt to solve this problem would be the introduction of an agitation system, which would create a turbulent diffusion and increase the transfer rates of material from the surface of the particles to the bulk of the solution. According with Chanioti, Liadakis, & Tzia (2014) given the time spent in the extraction and in solvent's evaporation and given the low recovery of the solute obtained, this type of flow is rarely used on an industrial scale.

In order to overcome the problems imposed by solid-liquid extraction, it would be interesting to test other methods of extraction, like Supercritical Fluids Extraction (SFE) or Pressurized Liquid Extraction (PLE, which includes Subcritical Water Extraction, SWE), usually used to obtain polyphenolic compounds. These procedures represent more cost-effective solutions that are being implemented at an industrial scale.

SFE, for example, doesn't require solvents in the solute phase, neither high temperatures, besides being low energy demanding (Garcia-Salas, Morales-Soto, Segura-Carretero, & Fernández-Gutiérrez, 2010). On the other hand, PLE is the most effective method used to extract polyphenols from solid samples, having a low solvent consumption and short extraction times, despite the requirement for high temperature and pressure conditions (Garcia-Salas, Morales-Soto, Segura-Carretero, & Fernández-Gutiérrez, 2010). Subcritical Water, in turn, has also shown good results, being the most environmental-friendly technique. It's however important to note that the extremely high temperature of the water also triggers other chemical reactions like caramelization and the Maillard reaction, which may influence the antioxidant activity of the extract (the stability of phenolic compounds is affected by temperature, polyphenolic structure, pressure, sample characteristics, etc.) (Ibañez, Herrero, Mendiola, & Hayes, 2012; Castro-Puyana, Herrero, Mendiola, & Ibáñez, 2013).

Another adjustment to consider is to increase apple's immersion time in the treatment solution, in larger containers, introducing mechanical agitation to assure the complete coating of wedges' superficies.

Regarding production costs, although the current extraction process is not highly energy demanding, heat loss, among other requirements, have to be well studied, in order to be minimized. Also, the regulation of the relative proportion of the ingredients (alga, water and ethanol) used in the extraction process, as well as the determination of the number of solution renovations necessary to treat a certain amount (Kg) of apples, are some of the modifications that could help to evaluate and minimize these costs. Recent works carried by MARE team demonstrated the possibility to remove ethanol, suppressing a step in the production process.

Implement an IMTA system to produce raw material and choose a different drying process (suitable, cheaper and low energy demanding) to substitute extract's lyophilisation would also help to reduce the cost of the final product.

From an economical point of view, after a brief market analysis, it was possible to conclude that the ideal target market for this natural solution included fruit processors in the EU, able to afford investments in new technologies and products with the purpose of offering to their customers high quality products.

It was also seen that Portugal, like other EU State Members, is currently experiencing a favourable environment for business development. Moreover, the strategy followed by the European Commission to support a strong Bioeconomy ('Blue Economy'), investing in a more sustainable growth will undoubtedly contribute to the development of projects that, as AlgaeCoat, explore bio-resources. It's expected that the synergy between investment and quality drive business success.

In spite of the big market potential, the lack of data about the precise amount of MP apples annually produced in each country made difficult the intention to establish its real market size. In Portugal, the MPP market is still a novelty (not well established yet), being nowadays mainly represented by MP vegetables, such as pre-prepared salads and fresh-cut preparations for soups. Only recently, fresh-cut fruits were introduced in the main supermarkets. Nevertheless, the availability of MP fruit is expected to increase, given the convenience offered by these products. According to Ragaert et al. (2004), "sales of minimally processed vegetables and packaged fruits are rapidly increasing thanks to their image of convenience and healthiness".

Finally, after exploring some commercial strategies, it was concluded that a spin-off creation seemed to be the best strategy, since it allows to separate two different businesses (fruit and vegetables processing and AlgaeCoat production), helping the company to focus in each issue. Besides, spin-off companies generally worth more as independent entities, than as parts of larger businesses.

To sum up, evidences corroborate the potential application of AlgaeCoat additive as a natural food preservative. Future work must then be oriented towards the improvement of the final formula (increasing its stability) and towards the adaptation the production process to the industrial scale. Moreover, there is also the need to introduce the solution in the European list of Food Additives (currently in progress).

Once validated product's functionality (after scale-up), it will be much easier to present the product to potential customers, promoting it through workshops, conferences and other events and guaranteeing business success.

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Appendices

Appendix A

Colour Evaluation (Photos)			
MAP Packaging			
	Control Solution	**Commercial Solution**	**CT Extract Solution**
Day 1			
Day 15			
Day 20			
Day 30			

Figure A.1: Visual Assessment of colour changes occurred in apple slices during storage (30 days), under MAP packaging

Colour Evaluation (Photos)			
AIR Packaging			
	Control Solution	**Commercial Solution**	**CT Extract Solution**
Day 1			
Day 15			
Day 20			
Day 30			

Figure A.2: Visual Assessment of colour changes occurred in apple slices during storage (30 days), under AIR packaging

Appendix B

Table B.1: Aerobic Colony Counts during storage (30 days) and Reference Values (log (cfu g^{-1}))

	Aerobic Colony Counts (MAP) (log (cfu g^{-1}))			Aerobic Colony Counts (AIR) (log (cfu g^{-1}))			Reference Values (log (cfu g^{-1}))	
Day	**Control Solution**	**Commercial Solution**	**CT Extract**	**Control Solution**	**Commercial Solution**	**CT Extract**	**Graça et al. (2015)**	**Abadias et al. (2008)**
0	-	-	-	-	-	-	3,3 - 8,9	2,0 - 7,1
1	5,16	5,24	5,09	5,19	4,95	5,54		
5	6,05	6,83	5,39	6,38	6,13	5,77		
10	7,46	8,13	5,45	6,43	7,76	7,20		
15	8,13	8,69	7,47	8,03	8,31	7,83		
20	8,98*	8,70	7,56	7,99	8,53	8,14		
25	9,13*	8,67	8,44	8,23	8,20	8,13		
30	9,16*	8,71	8,87	8,58	8,74	8,26		

*The highlighted values represent the samples that exceeded the reference values obtained by Graça et al. (2015)
The blank spaces (-) represent the counts that remained below the 30 to 300 colonies range (counts not statistically valid)

Table B.2: *Enterobacteriaceae* counts during storage (30 days) and Reference Values (log (cfu g^{-1}))

	Enterobacteriaceae (MAP) (log (cfu g^{-1}))			*Enterobacteriaceae* (AIR) (log (cfu g^{-1}))			Reference Values (log (cfu g^{-1}))	
Day	**Control Solution**	**Commercial Solution**	**CT Extract**	**Control Solution**	**Commercial Solution**	**CT Extract**	**Graça et al. (2015) [a]**	**Abadias et al. (2008) [b]**
0	-	-	-	-	-	-	1,8 - 7,6	4,8
1	-	-	-	-	-	-		
5	-	5,24	4,69	5,42	5,37	4,71		
10	5,03	5,79	-	-	7,17	6,09		
15	-	8,32*	5,18	6,44	8,09*	7,18		
20	5,70	7,21	-	-	8,11*	6,16		
25	6,81	6,14	-	-	7,38	7,37		
30	6,83	7,40	-	-	7,34	7,77*		

*The highlighted values represent the samples that exceeded the reference values obtained by Graça et al. (2015)
The blank spaces (-) represent the counts that remained below the 30 to 300 colonies range (counts not statistically valid)
[a] Values obtained for total coliforms
[b] Values obtained for *Enterobacteriaceae*

Table B.3: Moulds and yeasts counts during storage (30 days) and Reference Values (log (cfu g^{-1}))

	Moulds and Yeasts (MAP) (log (cfu g^{-1}))			Moulds and Yeasts (AIR) (log (cfu g^{-1}))			Reference Values (log (cfu g^{-1}))	
Day	**Control Solution**	**Commercial Solution**	**CT Extract**	**Control Solution**	**Commercial Solution**	**CT Extract**	**Graça et al. (2015)**	**Abadias et al. (2008)**
0	-	-	-	-	-	-	3,6 - 7,1	1,7 - 4,9
1	-	-	-	-	-	-		
5	3,98	-	-	-	-	-		
10	-	5,79	-	4,00	5,69	5,53		
15	4,31	5,52	-	5,43	5,30	5,15		
20	7,43*	6,36	-	5,60	5,85	5,60		
25	5,30	6,34	5,66	6,28	6,69	6,38		
30	7,54*	7,48*	6,18	6,39	6,67	6,24		

*The highlighted values represent the samples that exceeded the reference values obtained by Graça et al. (2015)
The blank spaces (-) represent the counts that remained below the 10 to 150 colonies range (counts not statistically valid)

Appendix C

1. Qual o preço atual da maçã (€/Kg)? E da maçã minimamente processada?	2. Qual foi a evolução das vendas (de maçã inteira e minimamente processada) nos últimos anos?	3. Qual a percentagem representada pela maçã MP?	4. Que quantidade de maçã acaba por se estragar antes de chegar ao cliente final?	5. A fruta comercializada a nível nacional e a nível internacional tem tratamentos distintos?
Consultar Tabela C.1.	Consultar Tabela C.1.	Em 2016, a Maçã MP representou cerca de 1% sobre as vendas da IV Gama.	Os resíduos têm 2 destinos: Indústria ou Alimentação Animal. Para a alimentação animal, esta matéria não é valorizada, representando um valor muito residual.	Não. A questão do tratamento não tem a ver com a distância, nem com o tipo de transporte. Tem apenas a ver com o período que passa entre a colheita e o consumo/processamento da fruta. • Se a fruta for para consumo imediato, não é aplicado qualquer tratamento. • Se, pelo contrário, a fruta colhida em Setembro, for apenas consumida em Fevereiro/Março, é necessário aplicar uma solução de tratamento (longa conservação).

6. Quais são as soluções usadas atualmente para tratar os problemas de oxidação associados ao corte da maçã?	7. Estes tratamentos têm encarecido o produto de alguma forma? Qual o custo dos tratamentos atuais?	8. A aplicação de uma formulação natural conferiria vantagem ao produto final?	9. O aditivo natural é importante para os Processadores, para os Produtores ou para ambos? Tem conhecimento de outras empresas dedicadas ao processamento de maçã?	10. Tendo em conta que atualmente existe necessidade constante de inovação, considera que a aplicação desta formulação pode marcar a diferença no mercado?
Ácido Ascórbico e NatureSeal®.	Sim. É preparada uma solução de 5Kg, onde podem ser tratadas quantidades variáveis de maçã (0,2 a 0,5Kg de maçã), com várias renovações de solução. O NatureSeal® é bastante mais eficaz, dado que não é necessário fazer tantas renovações de solução, embora seja um tratamento significativamente mais caro. Consultar Tabela C.2.	Sim. A Campotec® pretende produzir e vender o extrato, caso este seja validado.	Para ambos, mas caso se opte por aplicar o extrato apenas em produtos MP (maçã MP), torna-se um pouco mais interessante para os processadores. Não tenho conhecimento de mais nenhuma empresa que comercialize maçã MP em Portugal.	Sim.

Tabela C.1: Produção de Maçã e Evolução das Vendas

Ano	Produto	Produção (Kg)	Vendas (€)	Preços Médios (€/Kg)
2014	Maçã	2 360 000	1 500 000,00 €	0,64 €
	Maçã MP	68 000	269 000,00 €	3,96 €
2015	Maçã	3 760 000	3 100 000,00 €	0,82 €
	Maçã MP	69 300	285 000,00 €	4,11 €
2016	Maçã	4 350 000	4 000 000,00 €	0,92 €
	Maçã MP	73 000	311 000,00 €	4,26 €

Tabela C.2: Custo das Soluções de Tratamento

Produto	Soluções de Tratamento (antioxidantes)	Preço (€/Kg)	Preço Médio (€/Kg maçã)
Maçã MP	Ácido Ascórbico (AA)	9,40 €	0,01 €
	NatureSeal®	32,00 €	0,04 €

Figure C.1: Interview to Eng. Délio Raimundo (Quality Department of Campotec IN)

Appendix D

1. Qual foi a evolução das vendas nos últimos anos?	**2. Existem outros países para os quais exportem fruta MP?**	**3. Quais são as soluções usadas atualmente para tratar os problemas de oxidação associados ao corte dos frutos?**	**4. Estes tratamentos têm encarecido o produto de alguma forma? Qual o custo dos tratamentos atuais?**	**5. Após a aplicação do tratamento, quanto tempo demora a fruta a escurecer?**	**6. Está satisfeito com os tratamentos atuais?**
3.000.000,00 €/ano A nível de produtos minimamente processados, o coco seco é líder de vendas, sendo o produto com maior potencial de venda e com mais sucesso.	Não.	Ascorbato.	O preço deste aditivo é 21,90€/Kg, o que representa um custo de cerca de 4.500,00 €/mês, contando com a manutenção e renovação da solução, que envolve a utilização de vários litros de água.	Entre 7 a 8 dias.	Sim, embora seja preferível optar por uma solução natural (‘clean label’).

7. O que mais valoriza numa solução para evitar o escurecimento da maçã?	**8. Caso se comprove o sucesso deste novo aditivo/solução, apostaria no produto?**	**9. Qual o preço máximo que estão dispostos a pagar pelo novo aditivo?**	**10. Para além da maçã e da manga, que outras frutas gostaria de ver preservadas?**
O preço e a eficácia. A empresa está a procurar reduzir custos a nível do tratamento das várias frutas MP, estando a testar várias soluções antioxidantes. Até ao momento estas soluções têm apresentando baixa eficácia (preservação entre 4 a 7 dias).	Sim, penso que é uma oportunidade para melhorar a qualidade dos nossos produtos e de oferecer aos nossos clientes um produto ainda mais saudável	Preço competitivo. Entre 7,00€ e 7,50€, caso a nova solução tenha a capacidade de preservar a fruta no mínimo entre 7 a 9 dias, sem manifestação de oxidações nas frutas (principalmente manga e maçã). Convém que após a aplicação do aditivo, todas as frutas tenham o mesmo prazo de validade. Caso contrário, a entrada destes produtos no mercado será bastante dificultada ou até mesmo rejeitada. A solução também não deverá conferir aroma, cor ou sabor atípico.	• Banana, devido à perda de textura e aos problemas de oxidação. • Abacaxi, devido à sua rápida maturação. • Coco

Figure D.1: Interview to Eng. Daniel Marques (Plant Manager in Nuvi Fruits S.A.)

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(54) Epígrafe: **REVESTIMENTO DE ORIGEM MARINHA PARA APLICAÇÃO EM PRODUTOS MINIMAMENTE PROCESSADOS OU DE QUARTA GAMA E RESPETIVO MÉTODO DE PRODUÇÃO**

(57) Resumo:
A PRESENTE INVENÇÃO É UM REVESTIMENTO E/OU FILME EDÍVEL DE ORIGEM MARINHA QUE SE DESTINA À APLICAÇÃO E CONSERVAÇÃO DE PRODUTOS MINIMAMENTE PROCESSADOS OU DE QUARTA GAMA, COMO POR EXEMPLO FRUTAS E VEGETAIS FATIADOS E REFERE-SE A UM REVESTIMENTO E/OU FILME EDÍVEL DE ORIGEM MARINHA PARA APLICAÇÃO EM PRODUTOS HORTOFRUTÍCOLAS DE QUARTA GAMA, CARACTERIZADO POR SER CONSTITUÍDO POR UMA SOLUÇÃO CONTENDO ÁGUA, CONCENTRADO DA MACROALGA EDÍVEL MARINHA CODIUM TOMENTOSUM TENDO INCORPORADO POLISSACÁRIDO DE ORIGEM MARINHA PARA MANIPULAÇÃO DAS PROPRIEDADES FÍSICAS DO REVESTIMENTO E/OU FILME. A PRESENTE INVENÇÃO TEM COMO PRINCIPAL OBJETIVO AUMENTAR O TEMPO DE PRATELEIRA DE PRODUTOS MINIMAMENTE PROCESSADOS OU DE QUARTA GAMA RECORRENDO A ALTERNATIVAS BIOLÓGICAS ATRAVÉS DA APLICAÇÃO DE UMA SOLUÇÃO DE REVESTIMENTO OU FILME EDÍVEL FORMULADA POR CONCENTRADO DA MACROALGA CODIUM TOMENTOSUM COMO INGREDIENTE ATIVO, ÁGUA E POLISSACÁRIDO DE ORIGEM MARINHA PARA MELHORIA DAS PROPRIEDADES FÍSICAS DO REVESTIMENTO.

RESUMO

"Revestimento de origem marinha para aplicação em produtos minimamente processados ou de quarta gama e respetivo método de produção"

A presente invenção é um revestimento e/ou filme edível de origem marinha que se destina à aplicação e conservação de produtos minimamente processados ou de quarta gama, como por exemplo frutas e vegetais fatiados e refere-se a um revestimento e/ou filme edível de origem marinha para aplicação em produtos hortofrutícolas de quarta gama, caracterizado por ser constituído por uma solução contendo água, concentrado da macroalga edível marinha *Codium tomentosum* tendo incorporado polissacárido de origem marinha para manipulação das propriedades físicas do revestimento e/ou filme. A presente invenção tem como principal objetivo aumentar o tempo de prateleira de produtos minimamente processados ou de quarta gama recorrendo a alternativas biológicas através da aplicação de uma solução de revestimento ou filme edível formulada por concentrado da macroalga *Codium tomentosum* como ingrediente ativo, água e polissacárido de origem marinha para melhoria das propriedades físicas do revestimento.

DESCRIÇÃO

"Revestimento de origem marinha para aplicação em produtos minimamente processados ou de quarta gama e respetivo método de produção"

Domínio técnico e âmbito da invenção

O âmbito da presente invenção insere-se na área dos revestimentos para aplicações em produtos minimamente processados ou de quarta gama. Acrescenta-se que ao nível da classificação internacional de patentes enquadra-se na A23G9.

A presente invenção é um revestimento e/ou filme edível de origem marinha que se destina à aplicação e conservação de produtos minimamente processados ou de quarta gama (4ªgama), como por exemplo frutas e vegetais fatiados e refere-se a um revestimento e/ou filme edível de origem marinha para aplicação em produtos hortofrutícolas de quarta gama, caracterizado por ser constituído por uma solução contendo água e concentrado da macroalga edível marinha *Codium tomentosum*. A presente invenção tem como principal objetivo aumentar o tempo de prateleira de produtos minimamente processados ou de quarta gama recorrendo a alternativas biológicas através da aplicação de uma solução de revestimento ou filme edível formulada por concentrado da macroalga *Codium tomentosum* como ingrediente ativo e água destilada. O composto ativo presente na formulação da presente invenção, concentrado de *Codium tomentosum*, permite aumentar o tempo de prateleira dos produtos acima mencionados uma vez que: i) possui a capacidade de inibir as enzimas polifenol oxidase e peroxidase, responsáveis pelo escurecimento enzimático com consequente degradação dos tecidos e diminuição do tempo de prateleira de produtos hortofrutícolas de 4ª gama, ii) como demonstrado em ensaios de aplicação em maçã fatiada de 4ª gama e puré de maçã contribui para a manutenção de um maior apelo visual dos produtos ao longo do armazenamento a temperatura ambiente e de refrigeração.

Estado da técnica

Nos países da União Europeia o consumo de produtos hortofrutícolas ainda se encontra abaixo do recomendado pelos especialistas (é recomendada uma média de 400g *per capita*). Os hortofrutícolas são componentes importantes de uma alimentação saudável pois possuem baixa densidade energética e são fonte de micronutrientes, fibras e outros componentes com propriedades funcionais. Graças a todos os seus componentes são produtos muito procurados e consumidos. Assim a necessidade do desenvolvimento de novos produtos que promovam o consumo de frutas e vegetais é essencial. A indústria alimentar apostou nos produtos de 4ª gama ou Produtos Minimamente Processados (PMP), que apresentam como principal característica a combinação de frescura e conveniência na preparação, embalagem e comercialização de vegetais ou outros produtos tradicionalmente frescos como produtos minimamente transformados prontos a consumir. Os PMP são descritos como produtos que sofreram apenas operações unitárias simples, onde as células e os tecidos da matéria-prima permanecem vivos com qualidade idêntica à dos produtos que lhes deram origem. São produtos prontos a consumir, onde o produto final é utilizado na sua totalidade havendo uma ausência de subprodutos ao nível do consumidor.

A manutenção da qualidade de produtos frescos cortados baseia-se na tecnologia de barreiras, tirando benefício do efeito sinergético entre vários fatores individuais. A adequada seleção das barreiras, tanto em número como em intensidade e sequência de aplicação, apresenta-se como o futuro do processamento mínimo de produtos hortofrutícolas. Neste âmbito surgiram os revestimentos e filmes edíveis.

Os filmes e revestimentos alimentares constituem uma tecnologia amiga do ambiente que permite aumentar a qualidade, segurança, estabilidade e propriedades mecânicas dos alimentos, promovendo uma barreira semipermeável ao vapor de água, oxigénio e dióxido de carbono, retenção dos compostos voláteis do aroma entre o alimento e a atmosfera circundante e veículo para transporte de aditivos alimentares.

As maçãs são um dos frutos mais consumidos por todo o mundo. De acordo com a FAOSTAT, em 2009, foram consumidos uma média de 28,1 kg de maçã *per capita* em Portugal, mostrando a sua importância na dieta Portuguesa.

A presente invenção diz respeito a um revestimento de origem marinha para aplicação em produtos minimamente processados ou de 4ª gama, e perspetiva aumentar o seu tempo de prateleira recorrendo a alternativas biológicas.

Em pesquisa ao estado da técnica da invenção, foram identificados os seguintes documentos de patente, dos quais destacamos a sua distinção com a presente invenção:

(1) Investigadores de Peniche estão a inventar novos negócios com base nas riquezas do mar in Região de Leiria de 28.02.2013 - A presente invenção difere de (1) na medida em que este apresenta biofilmes formulados por quitosano e extratos de algas com atividade antioxidante permitindo a prevenção na oxidação da maçã. Na presente invenção recorre-se a um revestimento alimentar formulado por concentrado de uma alga específica *Codium tomentosum* diluído na razão de 0,5% (m/v) em água destilada e que tem como principal ação a inibição das enzimas polifenol oxidase e peroxidase;

(2) Valentão P. et al, *Codium tomentosum* and *Placamium cartilagineum*: Chemistry and antioxidante potential in Food Chemistry 119 (2010) 1359-1368, 09.2009 - A presente invenção difere de (2) na medida em que a seleção da alga *Codium tomentosum* na presente invenção foi baseada também na sua atividade especifica na inibição das enzimas polifenol oxidade e peroxidase e na sua ação anti escurecimento *in vivo*;

(3) Acevedo O. Et el. Food Hydrocolloid Edible Films and Coatings, 14.04.2009 (Introdução, pontos 2 e 4) – a presente invenção difere de (3) uma vez que neste documento referem que utilizam soluções de água-álcool seguida de secagem para a preparação de filmes hidrocoloides, pelo contrário na presente invenção recorre-se a água e etanol para a obtenção do concentrado de macroalga. Na presente invenção para obtenção dos filmes utilizam-se soluções de alginato ou de quitosano que são posteriormente sujeitas a secagem. Apesar de ter sido testada a solução com quitosano esta

não demonstrou a máxima eficácia pelo que a presente invenção refere-se a um revestimento formulado por 0,5% de concentrado da alga *Codium tomentosum* em água destilada;

(4) CN 20121526585 - A presente invenção difere de (4) na composição, uma vez que embora em (4) serem utilizados compostos de origem marinha, não é referida a utilização de concentrado de nenhuma alga específica. Na presente invenção a funcionalidade, inibição do escurecimento da fruta, é alcançada através da aplicação específica de concentrado de *Codium tomentosum* diluído em água destilada. Em D4 são utilizados polissarídeos de algas, polímeros que podem ser extraídos da maioria das algas e são amplamente utilizados na indústria alimentar como espessantes, sendo provavelmente essa a sua função em D4: fornecer ao revestimento as propriedades físicas para formação de uma pelicula insolúvel na superfície da fruta. A formulação do revestimento da presente invenção poderá compreender apenas 0,5% de concentrado da alga *Codium tomentosum* em água destilada, não inclui a carboximetil quitosano, lisozima de origem marinha nem polissacarídeos de algas. A água é utilizada de modo a permitir a aplicação do revestimento através da imersão do produto alimentar no revestimento.

Acrescenta-se para efeito de compreensão da invenção que um concentrado pode definir-se como um produto de onde foi retirada a parte/fração aquosa, resultando numa fase sólida.

Descrição da invenção

Para a concretização da presente invenção e da potencial utilização de diferentes concentrados de organismos marinhos como ingredientes ativos na formulação de revestimentos comestíveis, foi analisada a caraterização da qualidade da maçã processada ao longo de 20 dias de armazenamento. A caraterização da qualidade da maçã processada baseou-se em alterações de cor, atributos texturais (firmeza e crocância), teor de humidade, pH, teor de sólidos solúveis e na atividade enzimática de polifenol peroxidase (POD) e polifenol oxidase (PPO).

Após seleção do concentrado de organismo marinho com maior potencial para a aplicação em causa, concentrado da alga *Codium tomentosum*, este foi incorporado em filmes edíveis poliméricos de alginato e quitosano. Os filmes comestíveis foram caraterizados em termos de solubilidade, transparência, propriedades mecânicas (testes de tensão/perfuração e de rutura dos filmes), permeabilidade ao vapor de água, isotérmicas de sorção e propriedades antimicrobianas.

Para a seleção dos revestimentos a utilizar foram recolhidas na costa de Peniche, Portugal, as algas *Bifurcaria bifurcata*, *Fucus spiralis*, *Codium tomentosum* e *Codium vermilara*, as quais foram lavadas em laboratório a fim de remover organismos invertebrados e outros detritos presentes nas amostras. Foi utilizada a seguinte nomenclatura para as algas recolhidas: *Fucus spiralis*: MN 1; *Bifurcaria bifurcata*: MN 2; *Codium tomentosum*: Q1 e *Codium vermilara*: Q2.

Após a lavagem, as algas foram liofilizadas, embaladas e armazenadas a -80°C. Para a preparação dos concentrados das diferentes algas adaptou-se a seguinte metodologia: os concentrados foram obtidos a partir da alga anteriormente liofilizada e com recurso a um solvente polar. Foram pesados 2g de biomassa seca e adicionados 22,5ml de água e 7,5 ml de etanol (proporção de 3:1).

A solução obtida ficou em agitação durante 6h, protegida da luz e à temperatura ambiente. De seguida realizou-se uma centrifugação (2000g; 10min; 4°C) e o sobrenadante foi recolhido. O sobrenadante foi filtrado utilizando um filtro n° 4 da whatman, de modo a reter as partículas suspensas. O filtrado foi assim sujeito a evaporação a vácuo para remoção do etanol, a 30°C, prosseguido de liofilização para remoção da água, resultando assim num concentrado em pó.

Foram preparadas soluções com uma concentração de 0,5% (m/V) de concentrado de alga, em água destilada, 0,5% (m/V) ácido cítrico (AC) em água destilada e um controlo com água destilada. A aplicação das soluções nas fatias de mação foi realizada pela imersão das fatias de maçã, com uma duração de 2 minutos, nas várias soluções anteriormente formuladas. Após a aplicação do revestimento, as fatias de maçã revestidas sofreram uma secagem (30 minutos, protegidas da luz) de modo a retirar o excesso de solução de revestimento. Por fim as fatias de maçã foram armazenadas a 4±2°C durante 20 dias, com amostragens periódicas (figura 1).

Primeiramente foi realizado um teste de cor a maçãs revestidas, durante 90min, e a puré de maçã, durante 10 min, armazenados à temperatura ambiente e sem proteção da luz.

Maçãs revestidas foram homogeneizadas com recurso a um homogeneizador formando um puré. Foram registadas as alterações de cor recorrendo a um colorímetro. As leituras foram realizadas com um ângulo de 90°, em cada amostra em diferentes locais. As medições de cor das maçãs revestidas e armazenadas a 4°C foram realizadas periodicamente, e a cor na superfície de corte foi avaliada recorrendo a um colorímetro.

Tanto para as fatias de maçã mantidas à temperatura ambiente, como para o puré de maçã e para as maçãs armazenadas em refrigeração foi avaliado o índice de escurecimento das maçãs revestidas. O índice de escurecimento é definido como a pureza da cor castanha, que é geralmente utilizada como um indicador do grau de escurecimento em produtos alimentares.

A firmeza e o atributo crocante das amostras foram medidos com um texturómetro e foram efetuados vários ensaios por amostra em diferentes locais de cada fatia.

Os atributos texturais avaliados foram a firmeza definida como a área total da curva de compressão (N.s) correspondente à firmeza da amostra, e o atributo crocante relacionado com o número de picos positivos da curva força *vs* tempo.

O peso das fatias de maçã foi determinado durante o armazenamento. A percentagem de peso perdido foi calculada medindo o peso segundo a seguinte equação:

$$\%\ \text{Peso perdido} = \frac{\text{Massa inicial} - \text{Massa final}}{\text{Massa inicial}} \times 100 \quad (1)$$

O teor de humidade das amostras de maçã foi avaliado com recurso a um analisador de humidade automático, a uma temperatura de 130°C, e para a avaliação do pH das amostras seguiu-se a norma portuguesa NP EN 1132:1996.

O teor de sólidos solúveis foi determinado de acordo com a norma portuguesa EN 12143:1999. Os resultados apresentados estão expressos em g sacarose/100 g produto.

A atividade das enzimas polifenol oxidase (POD) e polifenol peroxidase (PPO) foram seguidas espectrofotometricamente a 25°C. A atividade da enzima POD foi seguida a 470nm usando guaiacol como substrato e peróxido de hidrogénio (H_2O_2) como dador de hidrogénio. Para o ensaio da atividade da PPO, foi utilizado o catecol como substrato e a atividade foi avaliada a 400nm. Ambas as reações foram seguidas durante 120s, com intervalos de 20 s. A atividade enzimática foi determinada pelo declive da reta resultante do acompanhamento da evolução da absorvância durante 120s. Os resultados obtidos foram expressos em abs.s^{-1}.

Para a preparação de filmes de alginato e quitosano enriquecidos com concentrado etanólico de *Codium tomentosum*, foram testadas 4 diferentes formulações de filmes comestíveis (tabela I).

Controlo 1	1% Alginato de sódio + 1% glicerol
AC	1% Alginato de sódio + 0,5% concentrado Q 1 + 1% glicerol
Controlo 2	1% Quitosano + 0,1% Tween 80
QC	1% Quitosano + 0,5% concentrado Q 1 + 0,1% Tween 80

Tabela I- Tipos de soluções formadoras de filmes formuladas.

Na determinação de solubilidade dos filmes formulados foi delineado o seguinte protocolo. Os filmes foram secos a 105°C durante 24h, após a secagem o peso seco inicial foi determinado (Wi), de seguida os filmes secos foram imersos em 30ml de água destilada em suave agitação por 24h à temperatura de 21°C (±2°C). As soluções resultantes foram filtradas com um filtro whatman 4.

Os filtros que continham a fração insolúvel foram secos a 105°C durante 24h e no final foram pesados, de modo a obter o peso seco final (Wf), correspondente à matéria insolúvel. A solubilidade do filme (SF%) foi calculada segundo a equação:

$$SF(\%) = \frac{W_i - W_f}{W_i} \times 100 \qquad (2)$$

Onde, Wi corresponde ao peso inicial do filme seco e Wf é o peso da matéria insolúvel presente no resíduo seco do filme.

A espessura dos filmes obtidos foi determinada com recurso a um micrómetro digital de precisão de 0,01mm, e a transparência dos filmes foi medida colocando amostras retangulares dos filmes com uma área de 4,5cm^2 diretamente dentro de uma célula do espectrofotómetro. A transparência (T) foi calculada de acordo com a equação:

$$T = \frac{A_{550}}{x} \qquad (3)$$

Onde A550 corresponde à leitura da absorvância da amostra a 550nm e o x corresponde à espessura do filme (mm). De acordo com esta equação, um maior valor de T poderá indicar um menor grau de transparência.

Para avaliar a resistência à perfuração um pedaço de filme com $4cm^2$ foi colocado sobre uma plataforma para o teste com um orifício de diâmetro 1cm. O filme foi perfurado com uma sonda cilíndrica de 2mm de diâmetro. A força de perfuração foi definida como a força máxima medida ao longo do teste, ou seja a força máxima que a sonda precisa fazer para a perfuração do filme. A partir dos valores da distância que a sonda percorreu até romper o filme foi possível calcular a taxa de deformação (%) dos filmes através da seguinte equação 4):

$$\text{Taxa de deformação } (\%) = \frac{L - Li}{Li} \times 100 \qquad (4)$$

Onde L alongamento na perfuração (mm); Li distância inicial (mm).

A transferência de vapor de água através dos filmes formulados foi medida de acordo com a ASTM E-96-95. Foram utilizados recipientes de plástico, onde se depositou água destilada (HR 100%; 2800 Pa) até 2cm do filme que se encontrava no topo do recipiente, com uma área de filme exposta de $6cm^2$. Este recipiente foi acondicionado dentro de um dissecador com uma solução saturada de NaCl (HR 75,7%; 2119,6 Pa). O declive da perda de peso dos recipientes *vs* tempo de ensaio foi obtido por uma regressão linear.

A medição da permeabilidade ao vapor de água dos filmes foi determinada segundo a equação:

$$WVP = \frac{WVTR \times L}{\Delta P} \qquad (5)$$

Onde WVP corresponde à permeabilidade ao vapor de água ($kg.m/s.m^2.Pa$); WVTR taxa de transferência de vapor de água determinada pelo declive da reta a dividir pela área de filme exposto; L é a espessura média dos filmes; e ΔP corresponde à variação de pressão parcial entre as duas fases do filme.

Os filmes foram expostos a várias humidades relativas proporcionadas por diferentes soluções saturadas correspondendo a diferentes atividades de água (aw). As soluções utilizadas foram MgCl2 (RH 33%; aw 0,34), KCl (RH 44%; aw 0,88), $Mg(NO_3)_2$ (RH 50%; aw 0,59), NaCl (RH 75%; aw 0,76) e KNO_3 (RH 93%; aw 0,93).

A microflora nativa da maçã foi preparada com 10g de matéria-prima macerada em 90ml de *Mueller-Hinton Broth* (MHB) (pH 7,4 ± 0,1) com o auxílio de um *stomache*r de seguida foi inoculado 18± 2 h a 37°C.

Para o método de difusão em disco, a microflora da maçã preparada anteriormente foi inoculada na superfície de placas (ø 90mm) com *Mueller-Hinton Agar* (MHA) (pH 7,4 ± 0,2). Cinco discos de papel de filtro estéreis impregnados com as soluções formadoras de filme e solução de extrato Q1 foram colocados na superfície de cada uma das placas com o auxílio de uma pinça estéril. Foi também utilizado um disco de antibiótico comercial, a Amoxicilina (10µg). As placas foram incubadas aerobicamente a 37°C, 24h. O diâmetro da zona de inibição foi medido após as 24h de incubação. A sensibilidade às diferentes soluções utilizadas foi classificada pelo diâmetro dos halos de inibição como: diâmetros inferiores a 8mm, não sensível; diâmetros entre 9-14mm, sensível;

diâmetros entre 15-19mm, muito sensível; diâmetros superiores a 20mm, extremamente sensíveis.

Com o intuito de avaliar o potencial de utilização dos concentrados de algas produzidos na formulação de revestimentos comestíveis para produtos de 4ª gama foram efetuados dois testes preliminares.

O índice de escurecimento nas fatias de maçã foi determinado (figura 2). As amostras tratadas com as soluções de revestimento com os concentrados de algas apresentaram um índice de escurecimento significativamente inferior ao controlo (água) ($p<0,05$, ANOVA, teste *Dunnett*). Pode-se afirmar que a aplicação das soluções de revestimento enriquecidas com os concentrados de algas traz benefícios para a conservação de maçã minimamente processada relativamente a este atributo de qualidade, bastante valorizado pelo consumidor. Os resultados obtidos no índice de escurecimento são assim corroborados pois as fatias de maçã revestidas com soluções enriquecidas com concentrados de alga apresentaram, após 90 minutos, menores alterações visuais do que o controlo e o ácido cítrico. Foi observada uma redução significativa do índice de escurecimento do puré de maçã nas amostras tratadas com os concentrados de origem marinha relativamente à amostra de controlo ($p<0,05$, ANOVA, teste *Dunnett*). (figura 3)

As amostras tratadas com as soluções de revestimento com concentrado de alga proporcionaram algum tipo de proteção ao puré de maçã não permitindo uma extensa oxidação das amostras.

Os resultados obtidos no índice de escurecimento são corroborados pois os purés provenientes de fatias de maçã revestidas com soluções com concentrado de algas apresentaram menores alterações visuais do que as tratadas com controlo e ácido cítrico. Após a aplicação das soluções de revestimento e a da avaliação do seu efeito em maças fatiadas e em homogeneizado de maçã, pode-se concluir que os compostos testados têm potencial de aplicação na formulação de revestimentos comestíveis para produtos de 4ª gama.

Para avaliação da funcionalidade destes num produto produzido e armazenado em condições semelhantes às dos produtos análogos comercializados foi investigado o efeito da aplicação das soluções de revestimento em maçãs minimamente processadas, armazenadas a 4°C, protegidas da luz durante 20 dias de armazenamento.

A avaliação da qualidade da maçã Fuji revestida com as soluções de revestimento utilizadas no presente estudo foi baseada em análises efetuadas na literatura, de forma a contemplar a evolução de parâmetros físicos e químicos significativos para a qualidade do fruto.

	% Perda de massa	Variação Teor de humidade	Variação °Brix (g/100g)	Variação pH
Controlo	0,73±0,42	-3,04 ± 0,39	2,8 ± 0,06	-0,03 ± 0,01
AC	1,64±0,98	0,22* ± 1,06	0,4* ± 0,17	0,26* ± 0,03
MN1	0,87±0,65	0,73* ± 1,32	0,6* ± 0,23	0,07* ± 0,01
MN2	1,07±0,37	-0,99±1,36	0,9* ± 0,06	0,15* ± 0,05
Q1	1,41±1,63	0,50* ± 0,81	-0,5* ± 0,12	0,06* ± 0,02
Q2	1,20±1,24	-0,54* ± 0,27	0,2* ± 0,12	-0,09* ± 0,04

Tabela II- Diferenças entre dia 0 e dia 20 do ensaio. Os valores correspondem à média ± desvio padrão (*n*=3) (*Diferenças estatisticamente significativas em relação ao controlo (p<0,05, ANOVA, teste *Dunnett*)

Não foram encontradas diferenças estatisticamente significativas entre as amostras submetidas aos diferentes tratamentos na percentagem de peso perdido entre o dia 0 e o dia 20 dos ensaios (p< 0,05) (tabela II). Em relação à variação do teor de humidade entre os dias 0 e 20 de ensaio foi possível verificar que as fatias de maçã sem tratamento apresentaram uma perda de humidade significativamente superior (p <0,05) às restantes amostras (tabela II). As restantes amostras apresentaram menores perdas de humidade uma vez que com a aplicação de um revestimento em alimentos frescos, como é o caso de maçã fatiada, a transferência de vapor de água diminui, reduzindo a oxidação e as taxas de respiração, prolongando o tempo de vida do produto final.

Os açúcares presentes nas fatias de maçã ou o teor em sólidos solúveis (TSS) (°Brix) foram determinados ao longo do ensaio, permitindo obter a variação de TSS entre os dias 0 e 20 de ensaio (tabela II). O controlo foi a amostra que apresentou o maior aumento de TSS (p <0,05), enquanto nas restantes amostras os valores de TSS não variaram significativamente entre o início e o fim do ensaio (p>0,05).

As variações do teor de sólidos solúveis podem ser atribuídas à quebra dos hidratos de carbono (amido) em açúcar durante o processo de armazenamento. Curiosamente a amostra tratada com a solução de revestimento Q1 teve um decréscimo nos valores de TSS, poderá ser justificável graças à elevada quantidade de hidratos de carbono presentes no concentrado da alga utilizada também observado num estudo da alga Q1, onde encontraram 20,47 ± 0,5% de hidratos de carbono.

Quanto às variações de pH (tabela II) das amostras entre os dias 0 e 20 do ensaio, foram encontradas diferenças entre o controlo e os restantes tratamentos (*p* <0,05). Na amostra controlo observou-se uma diminuição dos valores de pH enquanto nos outros tratamentos ocorreu uma ligeira subida dos mesmos, exceto nas amostras Q2.

Em PMP dá-se uma deterioração bastante rápida do produto o que pode ser prevenido pela aplicação de um revestimento. Os resultados do atributo crocante não estão representados graficamente uma vez que não foram encontradas diferenças significativas no atributo crocante das fatias de maçã estudadas ao longo do ensaio.

Firmeza (N.s)	Dia0	Dia 20
Controlo	11,26±1,02	18,13*±3,16
Ácido Cítrico	11,35±2,39	19,24*±2,41
MN1	11,13±1,07	19,88*±2,02
MN2	10,79±1,47	14,95±5,56
Q1	11,24±0,96	21,56*±3,34
Q2	10,66±0,93	19,28*±8,33

Tabela III- Efeito das soluções de revestimento na firmeza (N.s). Os valores correspondem às médias ± desvio padrão (*n*=3). Diferenças estatisticamente significativas (*p*< 0,05, ANOVA, teste de LSD) em relação ao * dia 0.

Da análise da tabela III pode-se constatar que se deu um aumento da firmeza das amostras entre o dia 0 e o dia 20 do ensaio. Este aumento pode ser justificado pela temperatura de armazenamento. A utilização dos revestimentos em estudo pode ser mais vantajosa do que alguns revestimentos estudados anteriormente. Em maçãs revestidas com gel de aloé vera, com quitosano e cloreto de cálcio e pulalano enriquecido com agentes anti escurecimento e antibacterianos não foi observada uma manutenção da firmeza da amostra ao longo do armazenamento através da aplicação dos tratamentos. Da análise dos resultados pode-se afirmar que a utilização do revestimento Q1 induz um aumento de firmeza das fatias de maçã ($p < 0,05$, ANOVA, teste Dunnet) comparativamente com o controlo e as restantes soluções de revestimento em estudo.

As amostras que apresentaram um maior índice de escurecimento comparativamente com o controlo foram as revestidas com AC e MN1 ($p < 0,05$) (figura 4). Este resultado demonstra que a utilização de água, de ácido cítrico e MN2 não é eficaz na inibição da oxidação dos tecidos da maçã durante o seu armazenamento.

Os valores mais baixos de índice de escurecimento foram os dos tratamentos com Q1 e Q2 com 41,8% e 45,5% respetivamente. Os valores de índice de escurecimento do Q1 são significativamente inferiores ao controlo (41,81%) ($p<0,05$, ANOVA, teste de LSD), permitindo ainda escolher a solução de revestimento Q1 como aquela que impede de forma mais eficaz o escurecimento da maça, embora ainda com mecanismos desconhecidos. Os resultados obtidos com o cálculo do índice de escurecimento, foram confirmados visualmente, onde as amostras tratadas com a solução de ácido cítrico e MN1são as que escureceram mais enquanto as amostras com Q1 foram as que mantiveram a aparência durante os 20 dias de ensaio.

Das soluções de revestimento utilizadas a que mais inibiu a atividade enzimática foi a Q1, com diferenças estatisticamente significativas aos dias 0, 4, 8, 16 e 20 entre Q1 e o controlo (figura 5). A menor atividade na polifenol oxidase (PPO) pode ser interpretada como a inibição do escurecimento enzimático, porque a atividade da PPO é geralmente considerada como o primeiro passo para o escurecimento enzimático.

As alterações na atividade da polifenol oxidase (POD) ao longo dos dias do ensaio estão representadas na figura 6. Também foram detetadas diferenças estatisticamente significativas (p< 0.05, ANOVA, teste de *Dunnett*) nos diferentes dias de ensaio nos revestimentos utilizados. As amostras tratadas com Q1 e Q2 foram as que apresentaram uma menor evolução da atividade da POD podendo indicar que a aplicação das soluções de revestimento Q1 e Q2 resultam numa inibição da atividade enzimática da maçã processada.

Com base nos resultados obtidos com a aplicação das soluções de revestimento com concentrado de algas em maçã minimamente processada foi possível escolher o concentrado com maior potencial de aplicação na formulação de revestimentos comestíveis Q1. As amostras revestidas com o concentrado Q1 apresentaram um índice de escurecimento reduzido, como verificado instrumentalmente e corroborado pela análise da aparência das amostras. A aplicação deste concentrado inibiu significativamente a atividade das enzimas oxidativas de PPO e POD.

Após a escolha do concentrado Q1, foram realizados testes a filmes de alginato e quitosano enriquecidos com Q1.

A avaliação das propriedades de filmes edíveis quando aplicados nos produtos é muito complexa pelo que a sua avaliação foi feita sem estar aplicado no filme. O facto de estudar os filmes e os resultados serem transponíveis para matrizes alimentares suporta esta decisão.

Para a avaliação de propriedades físicas dos filmes de alginato a 1% e quitosano a 1% enriquecidos com 0,5% de solução concentrado de alga Q1 foram determinadas as seguintes características: solubilidade em água, espessura, transparência, tensão de perfuração, taxa de deformação, permeabilidade ao vapor de água, isotérmicas de absorção a 21 ± 2°C e capacidade antibacteriana.

A solubilidade de filmes edíveis em água é um indicador da resistência do revestimento à água. Uma maior solubilidade poderá indicar uma menor resistência do filme, ou seja na presença de água o filme poderá dissolver-se perdendo o seu efeito protetor à superfície do alimento. A solubilidade é ainda um fator importante que determina a biodegradabilidade dos filmes quando utilizados como cobertura de alimentos.

A figura 7 mostra a solubilidade em água dos filmes formulados. Os filmes formulados com quitosano apresentam uma menor solubilidade do que os filmes formulados com alginato ($p< 0,05$, 2 way ANOVA, teste LSD).

O alginato tratando-se de um hidrocolóide terá sempre uma grande solubilidade em água. A solubilidade do quitosano em água será mais baixa pois o quitosano é um polissacarídeo apenas solúvel em soluções com pH ácido.

Verificou-se que a adição do concentrado da alga Q1 resulta numa diminuição estatisticamente significativa ($p < 0,05$, 2 way ANOVA, teste LSD) da solubilidade dos filmes de alginato e num aumento significativo da solubilidade dos filmes de quitosano. Os resultados obtidos correspondem a uma mistura entre os polímeros utilizados e o concentrado da alga, o que pode resultar em interações entre os compostos utilizados, causadas por exemplo por forças electroestáticas e ligações de hidrogénio. Estas interações poderão justificar as alterações divergentes de solubilidade causadas pela adição de concentrado de alga ao alginato e ao quitosano.

A espessura dos filmes produzidos variou entre 0,03mm e 0,06mm, sendo que a espessura maior corresponde ao filme Q e o de menor ao filme de AC. Os filmes de quitosano apresentaram-se flexíveis e fáceis de remover da placa, pelo contrário os filmes de alginato apresentaram-se suaves, pegajosos e encolhendo facilmente quando removidos das placas, depois de expostos, por um curto período de tempo, ao ambiente de laboratório (75% humidade relativa).

As propriedades óticas são essenciais para definir a funcionalidade dos filmes e/ou revestimentos a serem aplicados em alimentos, uma vez que afetam a aparência do produto revestido, o que é um importante fator de qualidade. Tal como foi referido na secção dos métodos, um maior valor de T (equação 3) revela um filme com um menor grau de transparência, mas com uma elevada absorvância de luz.

De acordo com os resultados obtidos e representados na tabela III, o filme que apresentou uma menor transparência foi o filme AC (p< 0,05, 2 way ANOVA, teste LSD). Ao apresentar o valor mais elevado de T, significa que o filme AC é um filme que absorve a luz o que poderá ser vantajoso aquando da sua possível aplicação em alimentos, pois estudos indicam que ao absorverem a luz estes filmes poderão proporcionar excelentes barreiras à oxidação induzida pela luz envolvente. A adição do concentrado de alga ao alginato poderá trazer assim benefícios para a proteção dos alimentos à luz. No entanto, será necessário assegurar que a sua aplicação não compromete o apelo visual dos produtos. A aparência do produto deverá manter-se inalterada para que continue a ser apelativo para o consumidor.

A adição do concentrado de alga ao quitosano provoca uma diminuição dos valores de transparência, quando comparados com os filmes Q (p< 0,05, 2 way ANOVA, teste LSD).

Filme	Espessura (mm)	Transparência (T)
A	0,05±0,02	2,4±0,1
AC	0,03*±0,01	13,1*±0,4
Q	0,05$^{\#}$±0,01	3,1*$^{\#}$±0,3
QC	0,06*$^{\#t}$±0,01	2,5*$^{\#t}$±0,1

Tabela III- Espessura e transparência dos filmes alginato (A), alginato e concentrado (AC), quitosano (Q) e quitosano e concentrado (QC). Os valores correspondem à média ± desvio padrão (espessura n=15; transparência n=3). Diferenças estatisticamente significativas em relação * ao alginato # ao alginato com concentrado; *t* ao quitosano (p< 0,05, 2 way ANOVA, teste LSD).

Uma resistência mecânica adequada é geralmente necessária para um filme de modo a suportar o esforço externo de manter a sua integridade durante o transporte e manuseamento necessários à distribuição e venda do produto, bem como as propriedades de barreira durante a aplicação em alimentos. A rutura do filme irá originar zonas de fragilidade no alimento. Estas zonas ficam desprotegidas do efeito protetor dos filmes, o que irá prejudicar a qualidade do produto e reduzir o tempo de prateleira do mesmo.

Os filmes de alginato (A e AC) apresentaram uma tensão de perfuração significativamente menor comparativamente com os filmes de quitosano (Q e QE) (p< 0,05, 2 way ANOVA, teste LSD). Apesar de o polímero apresentar um elevado potencial para o desenvolvimento de filmes edíveis biodegradáveis tem, no entanto, baixa resistência e elasticidade.

Os filmes Q e QC apresentam valores de tensão de perfuração superiores (p< 0,05, ANOVA, teste LSD) o que indica que é necessária a aplicação de uma maior força para romper este tipo de filmes. O quitosano é um polímero bastante resistente, tornando-se um ótimo filme a ser aplicado em alimentos devido às suas propriedades de resistência mecânica e protetoras. Ao apresentarem uma elevada resistência à perfuração, este tipo de filmes poderá ter uma durabilidade superior comparativamente aos de alginato.

Quanto à taxa de deformação entre filmes de A e AC, os filmes de AC apresentam uma menor taxa de deformação comparativamente aos de A (p< 0,05, ANOVA, teste LSD). A adição do concentrado ao alginato poderá ter um efeito estabilizante nas ligações intermoleculares do alginato, proporcionando uma maior capacidade de manter a sua forma original.

Da análise dos resultados da taxa de deformação nos filmes Q e QC (figura 8), estes apresentam diferenças significativas entre si ($p< 0,05$, ANOVA, teste LSD). Ao contrário do efeito que o concentrado de alga terá nos filmes de AC, a taxa de deformação nos filmes QC aumenta. Tal poderá ter acontecido pela facilidade que os componentes do concentrado de alga terão em ser inseridos entre as cadeias do quitosano, produzindo um efeito de *cross-linker*, que diminuirá a resistência mecânica dos filmes, aumentando a sua aptidão para se deformarem.

O filme de QC aparenta ser o mais resistente pelo que a sua aplicação em matrizes alimentares poderá permitir uma proteção mais eficaz do alimento. A adição do concentrado contribui ainda para a maior elasticidade dos filmes, quando comparados com os filmes de Q. Esta propriedade é importante, pois durante as agressões físicas do exterior os filmes de quitosano são resistentes, apresentando no entanto alguma elasticidade auxiliando na proteção de matrizes alimentares.

As propriedades barreira ao vapor de água dos diferentes filmes estão representadas na figura 9. As condições de humidade relativa utilizadas durante as medições de permeabilidade (gradiente de humidade relativa de 100:75) foram estabelecidas de modo a reproduzir a ação dos revestimentos em produtos frescos e armazenados até 10°C. A média de espessura dos filmes foi a indicada na tabela IV. Apenas foram encontradas diferenças estatisticamente significativas entre os filmes AC e os restantes filmes estudados (p< 0,05, ANOVA, teste LSD).

Os filmes de AC foram os que apresentaram menor permeabilidade ao vapor de água, exibindo assim uma boa barreira ao vapor de água em relação aos outros filmes estudados. A adição do concentrado ao alginato trará assim vantagens relativamente à capacidade de redução de transferência de vapor de água entre o alimento e o meio ambiente, o que poderá contribuir para uma maior eficiência do filme em manter as características organoléticas típicas do produto, como por exemplo a suculência. Nas maçãs revestidas com a solução Q1 deu-se uma baixa perda de humidade, levando a crer que as trocas de vapor de água com o meio ambiente foram baixas. Daí o concentrado de alga Q1, utilizado na formulação do filme AC, de alguma forma estará a fornecer uma barreira extra ao vapor de água impedindo as trocas gasosas.

Uma consequência da elevada solubilidade dos filmes AC será a grande absorção de água do meio para o filme, também visível nas curvas de sorção (figura 10). A retenção de água na estrutura do filme poderá justificar a menor permeabilidade dos filmes. A água que estava dentro do sistema utilizado para a avaliação do WVP (e que representa um alimento fresco) ao ser transferida do sistema pelo filme, fica retida no filme permitindo manter os níveis de água no sistema/matriz alimentar.

A análise das isotérmicas de sorção poderá fornecer informação importante para a avaliação da funcionalidade de filmes comestíveis, pois permitem a medição da estabilidade dos filmes em relação à humidade relativa de um ambiente, determinando o seu tempo de armazenamento e a sua funcionalidade em geral.

A figura 10 mostra as isotérmicas de sorção obtidas a 21±2°C dos filmes A, AC, Q e QC. Os filmes em estudo tinham os seguintes teores de humidade iniciais: A: 24,86%; AC: 28,29%; Q: 9,57%; e QC: 10,06%, tendo sido atingido o equilíbrio após 144 horas de exposição aos diferentes meios. Os filmes A e AC foram os que apresentaram uma maior absorção de humidade quando expostos a diferentes condições ambientais. Os filmes de alginato ao absorverem a água presente no meio, caso esta absorção seja excessiva (provável em humidades relativas mais altas), poderão levar à solubilização do filme, o que provoca falhas na estrutura do filme e consequentemente compromete a proteção física do produto onde esteja inserido. Pela observação do gráfico, os filmes que apresentaram um comportamento mais interessante relativamente à funcionalidade requerida para os filmes foram os Q e QC. Os filmes Q foram os que apresentaram menores alterações de humidade quando expostos aos diferentes ambientes, o que indicará que absorvem um menor teor de água.

A utilização dos filmes de Q e QC, será vantajosa quando aplicada a matrizes alimentares pois absorve menos água, fator essencial na proteção de PMP frescos. A menor absorção de água diminui os riscos de falhas físicas nos filmes e permitirá igualmente a manutenção de condições à superfície do alimento que contribuirão para uma inibição da colonização por microrganismos devido à baixa disponibilidade de água.

O estudo da sensibilidade da microflora às diferentes soluções formadoras de filmes pelo método de difusão em disco está representado na tabela IV.

A microflora da maçã apenas apresentou sensibilidade às soluções formadoras de filme Q e QC e ao controlo ampicilina (10µg). A atividade antimicrobiana do quitosano já é bastante estudada, sendo a capacidade antibacteriana do quitosano contra uma grande variedade de organismos uma das características mais conhecidas deste composto. A adição do concentrado da alga Q1 ao quitosano de algum modo estará a reduzir a sua atividade antibacteriana visto que o halo de inibição foi mais pequeno do que no filme de quitosano. As soluções formadoras de filmes de A e AC não apresentaram qualquer atividade contra a microflora da maçã. Quanto à atividade antibacteriana do concentrado da alga Q1 não existe bibliografia que permita comparar os resultados com trabalhos anteriores.

Amostra	Halo de inibição(cm)	Sensibilidade microflora
A	0 ±0,0	Não sensível
Ac	0 ±0,0	Não sensível
Q	1 ±0,0	Sensível
QC	0,9 ±0,0	Sensível
Concentrado	0±0,0	Não sensível
Ampicilina (10µg)	2±0,0	Extremamente sensível

Tabela IV- Sensibilidade microflora de maçã Fuji às soluções formadoras de filmes alginato (A), alginato e concentrado (AC), quitosano (Q) e quitosano e concentrado

(QC). Os valores correspondem à média ± desvio padrão (n=3).

Conclusões

A presente invenção diz respeito a um revestimento de origem marinha para aplicação em produtos minimamente processados ou de 4ª gama. Foi testada em maçãs Fuji minimamente processadas, para quatro tipos de concentrados etanólicos de algas edíveis, um controlo com água destilada e um com ácido cítrico (antioxidante amplamente utilizado na indústria alimentar).

Num primeiro ensaio, os revestimentos foram aplicados em maçã fatiada e maçã homogeneizada. No segundo ensaio com uma duração total de 20 dias, os revestimentos foram aplicados em maçãs de 4ªgama embaladas, armazenadas a 4°C, protegidas da luz, nos quais eram realizadas análises de 4 em 4 dias. Aquando da aplicação das soluções de revestimento em fatias e homogeneizado de maçã, expostos à temperatura ambiente e sem proteção da luz, as amostras tratadas com a solução de revestimento Q1 apresentaram um índice de escurecimento menor (IE de 41,81) comparativamente com as amostras submetidas aos restantes tratamentos. Das análises físico-químicas realizadas ao longo do armazenamento refrigerado da maçã fatiada (% perda de massa; variação teor de humidade, TSS, pH; firmeza; índice de escurecimento) as amostras de maçã revestidas com a solução de revestimento Q1 foram as que apresentaram menores alterações. Também a nível visual foram as que apresentaram menores alterações entre os dias 0 e 20. A aplicação da solução Q1 demonstrou igualmente ter um efeito significativo na redução da atividade enzimática de PPO e POD.

Face aos resultados obtidos a solução Q1 foi selecionada para incorporação em filmes comestíveis poliméricos e investigação das propriedades físicas e antibacterianas dos mesmos. Foram testados filmes de 1% de alginato e 1% de quitosano suplementados com 0,5% de concentrado da alga Q1. Os filmes foram caraterizados em termos de solubilidade; espessura; transparência; características mecânicas, permeabilidade ao vapor de água, isotérmicas de sorção e características antimicrobianas de modo a averiguar qual seria a melhor formulação para os filmes edíveis, e posterior aplicação como revestimento alimentar.

Dos testes efetuados, os filmes com melhores características para aplicação em alimentos de 4ª gama será o QE. As características que poderão permitir a escolha destes filmes serão: a baixa solubilidade não permitindo a dissolução fácil do filme; elevada resistência à perfuração e elevada elasticidade permitindo uma proteção física das matrizes alimentares mesmo quando sujeitos a agressões mecânicas no manuseamento, um teor de humidade de equilíbrio mais baixo quando expostos a ambientes com diferentes valores de atividade da água e a presença propriedades antibacterianas relativamente à flora bacteriana típica da maçã.

Descrição das figuras

Figura 1- Fluxograma do processo de produção das amostras de maçã fatiada resultante do presente estudo.

Figura 2 - Índice escurecimento das amostras (maçã fatiada) após 90 minutos de exposição à temperatura ambiente (21 ± 2°C), sem proteção da luz. Valores correspondem à média ± desvio padrão ($n=3$). * Diferenças estatisticamente significativas em relação ao controlo ($p<0,05$, ANOVA, teste *Dunnett*).

Figura 3 - Índice de escurecimento de homogeneizado de maçã exposto após 10 minutos de exposição à temperatura ambiente (21 ± 2°C), sem proteção da luz. Valores correspondem à média ± desvio padrão ($n=3$). * Diferenças estatisticamente significativas em relação ao controlo ($p<0,05$, ANOVA, teste *Dunnett*).

Figura 4 - Índice de escurecimento das maçãs ao dia 20 do ensaio. Os valores correspondem à média ± desvio padrão ($n=9$). * Diferenças estatisticamente significativas em relação ao controlo ($p<0,05$, ANOVA, teste *Dunnett*).

Figura 5 - Efeitos das soluções de revestimento AC, MN1, MN2, Q1 e Q2 na atividade enzimática da enzima polifenol oxidase de maçãs tratatas, entre os dias 0 e 20 de ensaio, a 4°C. Os valores correspondem às médias ± desvio padrão ($n=3$). Diferenças estatisticamente significativas em relação ao controlo ($p< 0,05$, ANOVA, teste de *Dunnett*): * dia 0; # dia 4; *t* dia 8; £ dia 16; § dia 20;

Figura 6 - Efeito das soluções de revestimento na atividade enzimática da enzima peroxidase de maçãs tratatas, entre os dias 0 e 20 de ensaio, a 4°C. Os valores correspondem às médias ± desvio padrão ($n=3$). Diferenças estatisticamente significativas em relação ao controlo (p< 0,05, ANOVA, teste de Dunnett): * dia 0; # dia 4; t dia 8; & dia 12; £ dia 16; § dia 20;

Figura 7 - Solubilidade filmes de 1% alginato (A), 1% alginato com 0.5% concentrado (AC), 1% quitosano (Q) e quitosano com concentrado (QC). Os valores correspondem às médias ± desvio padrão (n=3). Diferenças estatisticamente significativas em relação * ao alginato; # alginato com concentrado; *t* quitosano (p< 0,05, 2 way ANOVA, teste LSD);

Figura 8 - Relação entre tensão de perfuração (Tp) (N/mm^2) e taxa de deformação (%) dos filmes (n=9). Diferenças estatisticamente significativas em relação ao *alginato # alginato com concentrado *t* quitosano (p< 0,05, ANOVA, teste LSD);

Figura 9 - Permeabilidade ao vapor de água (WVP) de filmes de alginato (A), alginato e concentrado (AC), quitosano (Q) e quitosano e concentrado (QC). Os valores correspondem à média ± desvio padrão (n=3). * Significam diferenças estatisticamente significativas quando comparadas com o filme AE (p< 0,05, ANOVA, teste LSD);

Figura 10 - Isotérmica de sorção a (21 ± 2°C) dos filmes de alginato (A), alginato e concentrado (AC), quitosano (Q) e quitosano e concentrado (QC) em ambientes com diferentes atividades de água.

Leiria, 11 de fevereiro de 2016

REIVINDICAÇÕES

1. Revestimento de origem marinha para aplicação em produtos minimamente processados ou de quarta gama caracterizado por consistir em um concentrado da macroalga edível marinha *Codium tomentosum* em água destilada e alginato e/ou quitosano.

2. Revestimento de acordo com a reivindicação 1 caracterizado pela concentração do concentrado da macroalga edível marinha *Codium tomentosum* em água destilada ser de 0,5% (m/V) e 1% de alginato e/ou quitosano.

3. Revestimento de acordo com a reivindicação 1 caracterizado por apresentar uma espessura entre 0,03mm e 0,06mm.

4. Método de produção de revestimentos de acordo com as reivindicações 1 e 2 caracterizado por ser constituído pelas seguintes etapas:
   a) lavagem da macroalga edível marinha *Codium tomentosum*, sua liofilização, embalagem e armazenamento a -80°C;
   b) obtenção dos concentrados a partir da alga anteriormente liofilizada e com recurso a um solvente polar;
   c) pesagem 2g de biomassa seca, adição de água destilada e de etanol numa proporção de 3v:1v;
   d) agitação da solução obtida durante 6h, protegida da luz e à temperatura ambiente;
   e) centrifugação a 2000g, 10min a 4°C, e recolha do sobrenadante;
   f) filtragem do sobrenadante e sua evaporação a vácuo para remoção do etanol, a 30°C, prosseguido de liofilização para remoção da água;

g) Incorporação do concentrado resultante em revestimentos poliméricos de alginato e/ou quitosano.

5. Utilização do revestimento de origem marinha de acordo com as reivindicações 1, 2 e 3, caracterizada por ser para aplicação em produtos minimamente processados ou de quarta gama.

Leiria, 11 de fevereiro de 2016

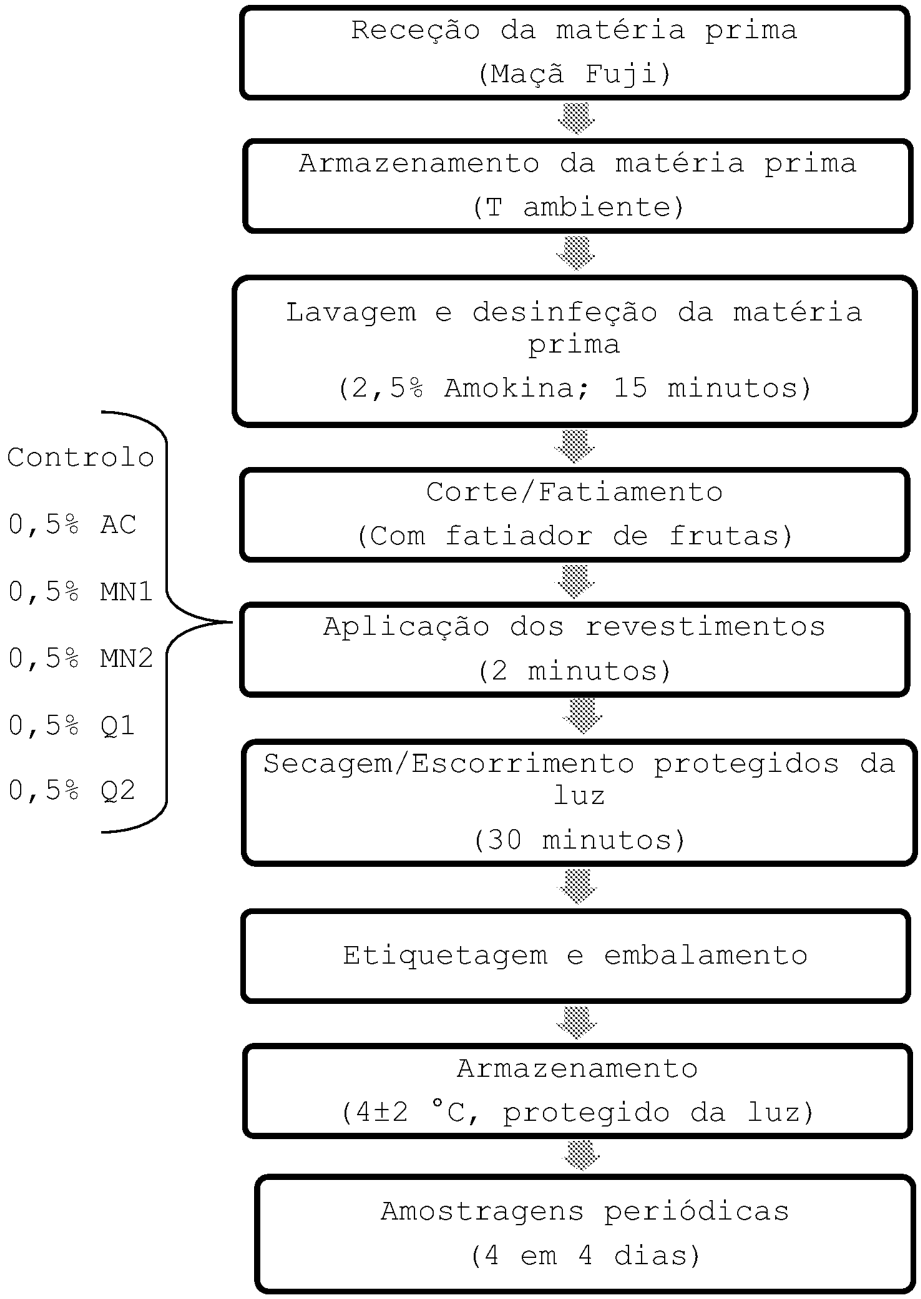
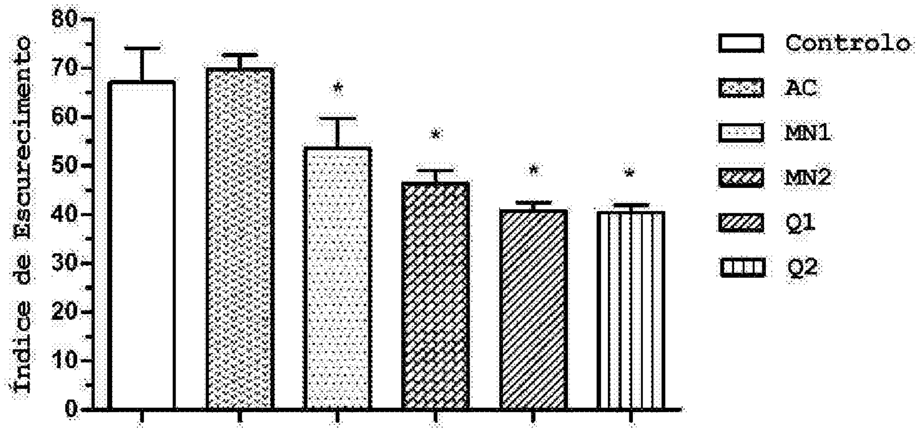

Receção da matéria prima
(Maçã Fuji)
Armazenamento da matéria prima
(T ambiente)
Lavagem e desinfeção da matéria prima
(2,5% Amokina; 15 minutos)
Corte/Fatiamento
(Com fatiador de frutas)
Aplicação dos revestimentos
(2 minutos)
Secagem/Escorrimento protegidos da luz
(30 minutos)
Etiquetagem e embalamento
Armazenamento
(4±2 °C, protegido da luz)
Amostragens periódicas
(4 em 4 dias)
Controlo
0,5% AC
0,5% MN1
0,5% MN2
0,5% Q1
0,5% Q2

Figura 1



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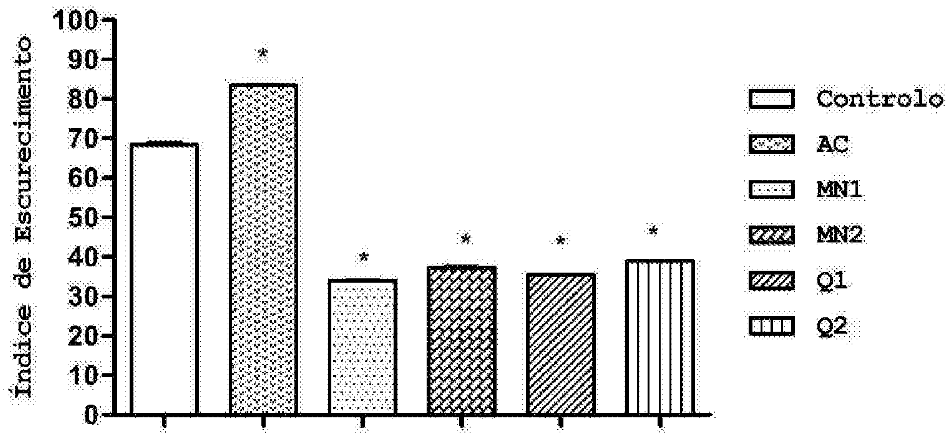
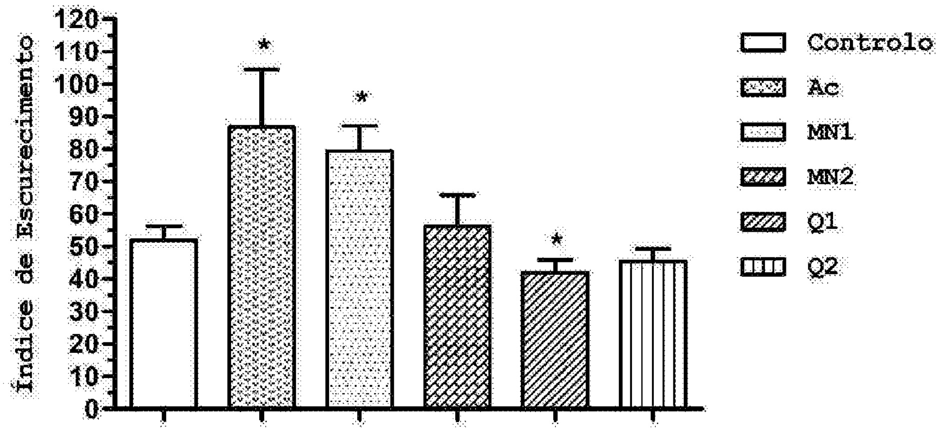


Figura 3



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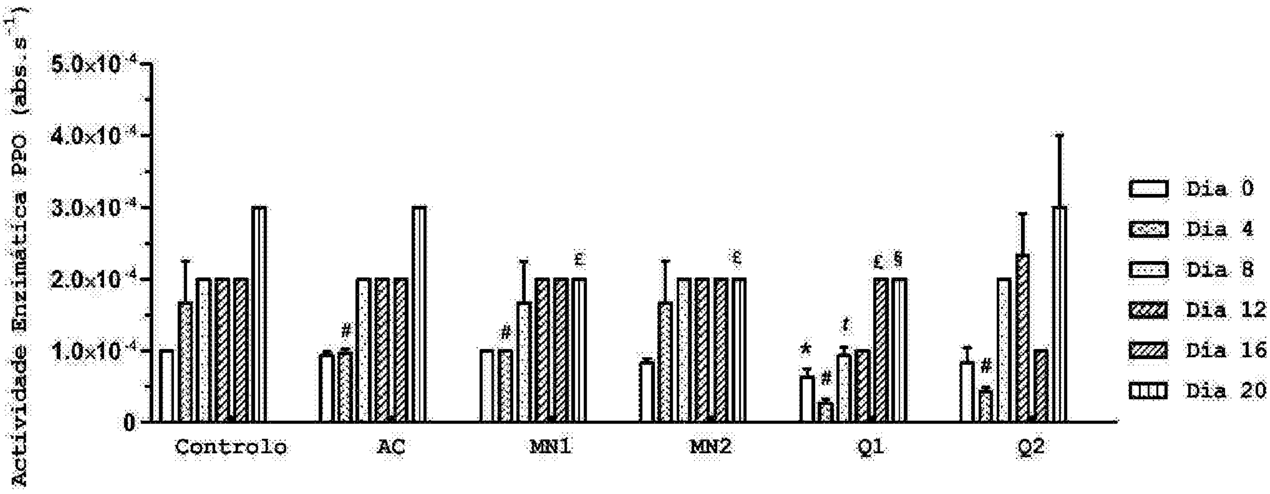

Figura 5

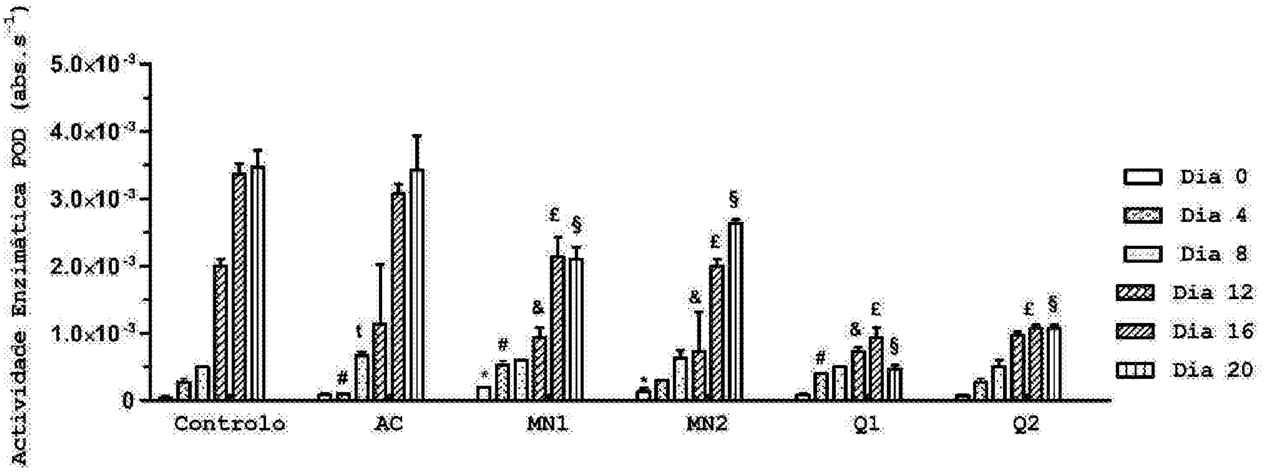

Figura 6

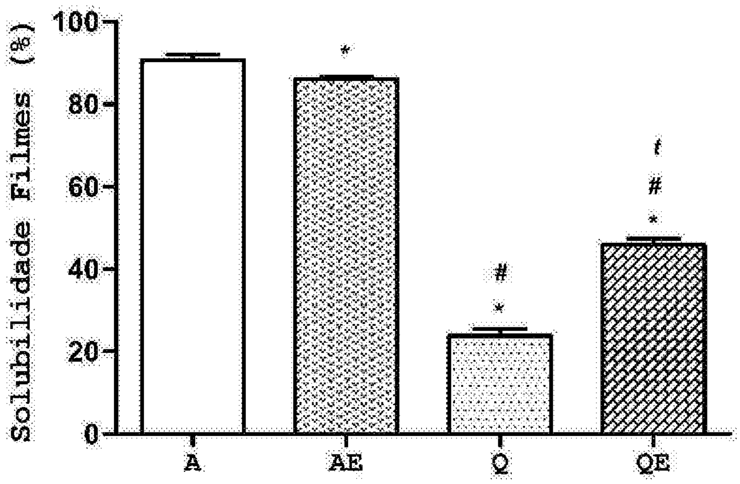

Figura 7

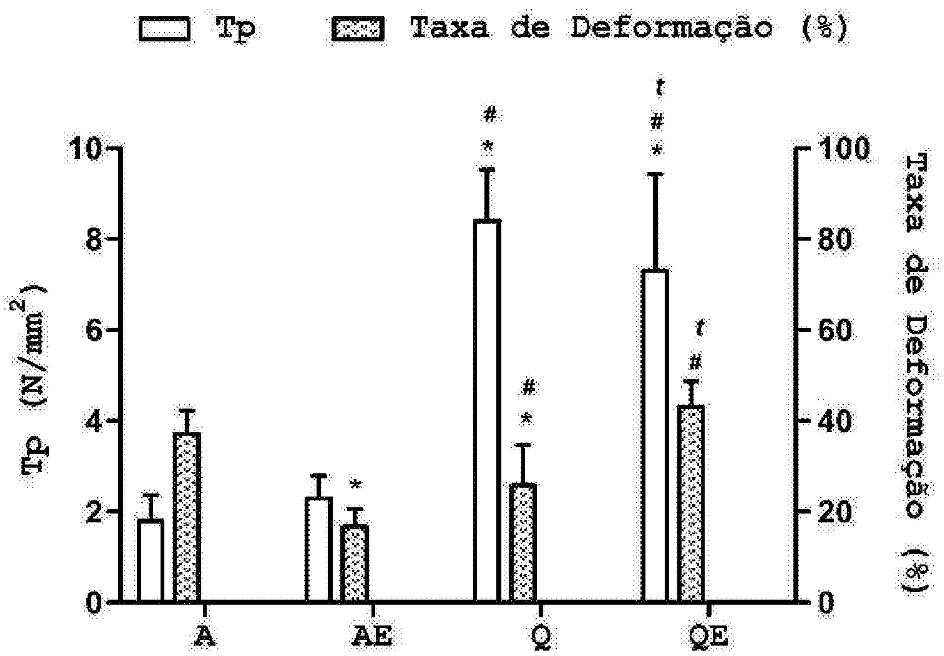

Figura 8

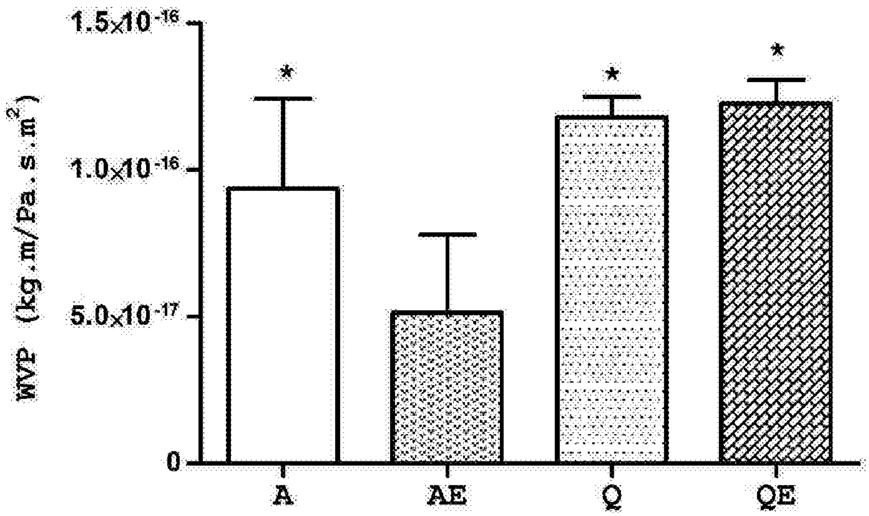

Figura 9

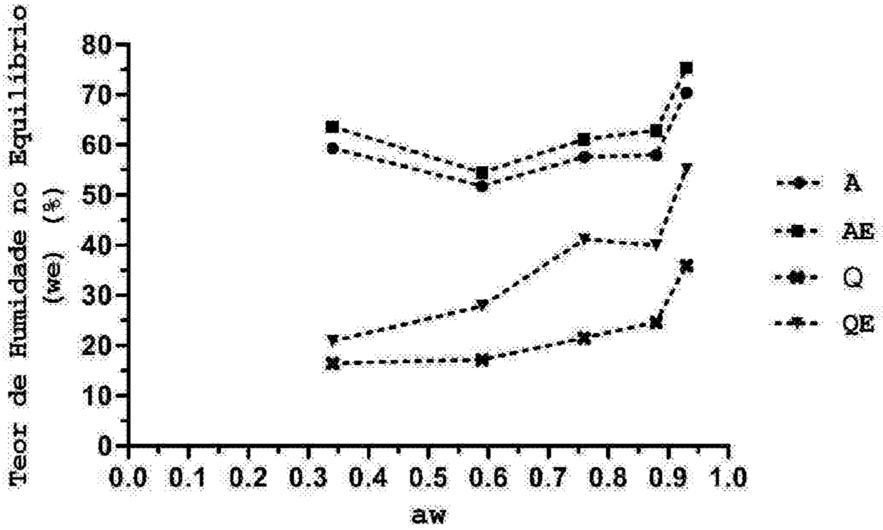
Teor de Humidade no Equilíbrio (we) (%)
80
70
60
50
40
30
20
10
0
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
aw
A
AE
Q
QE

Figura 10