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Antimicrobial and bioactive property monitorization of a polymer-based food contact material

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LIST OF ABBREVIATIONS

***A. parasiticus* :** *Aspergillus parasiticus*

ANOVA : Analysis of variance

***B. cereus* :** *Bacillus cereus*

BC: Before Christ

CFU: Colony-Forming Units

CIE: International Commission on Illumination

CMYK : Cyan, Magenta, Yellow, and Black

Cp: Specific heat Capacity

DNA: Deoxyribonucleic Acid

DP: degree of polymerization

DPPH: 2,2-Diphenyl 1-picrylhydrazyl

DRBC: Dichloran Rose Bengal Chloramphenicol

DSC: Differential scanning calorimetry

E. coli : *Escherichia coli*

EC_{50} : Half maximal effective concentration

Eq : Equation

EU: European Union

FCM: Food Contact Material

FDA: Food and Drug Administration

HAT: Hydrogen Atom Transfer

HEX : Hexadecimal

ISO : International Organisation for Standardisation

LOQ: Limit of Quantification

MDA: Malondialdehyde

MIC: Minimum Inhibitory Concentration

Mw: Molecular Weight

OCCs: Old Corrugated Containers

ONPs: Old Newspapers

P- value: Probability value

PCA: Plate Count Agar

PDA: Potato Dextrose Agar

pH: Hydrogen Potential

RAF: radical adduct formation

RGB : Red, Green, Blue

ROO : peroxide radicals

Rpm: Number of revolutions per minute

SD: standard deviation

SET: single electron transfer

SPLET: sequential proton loss electron transfer

TBA: Thiobarbituric Acid

TBARS: Thiobarbituric Acid Reactive Substances

TG : Glass transition temperature

TGA: Thermogravimetric Analysis

Tm : Melting temperature

TPA: Texture Profile Analysis

TSB: Tryptic Soy Broth

UAE: Ultrasound-Assisted Extraction

US: United States

USEPA: United States Environmental Protection Agency

UV : Ultra-Violet

VRBL : Violet Neutral Red Bile Lactose

***Z. rouxii* :** *Zygosaccharomyces rouxii*

ι-carrageenan : Iota-carrageenan

κ-carrageenan : Kappa- carrageenan

ABSTRACT

Plastic has become one of the key environmental issues of our time, over 8 million tons of plastic is cast into the ocean every year (UN Environment report ,2018). Majority of it being plastic packaging, from food packaging. Plastic waste is considered a global problem; therefore, solutions are in need to reduce the pollution resulting from the overuse of this material (Jung H., 2014).

In this context, this work explores the possibility of manufacturing a solution from a natural biopolymer to use as a spray to coat and protect food as an alternative barrier. The contact between the spray and food is harmless for human health. The Spray solution is composed of a primary solution of a natural biopolymer iota-carrageenan and a mixture of different natural antioxidants and extract from the plant of *Rosmarinus officinalis. L*, the extraction was made using two techniques, one with infusion extraction and the second is Ultrasound-Assisted Extraction and then mixed on an aseptic environment. The current work focuses on monitoring antioxidant activity using two methods (the DPPH and TBARS) and antimicrobial activity using a shelf-life span of 6 months, divided in 7 different times (0, 7, 15 days and 1, 2, 4, 6 month), in 4 different conditions 2 in refrigeration (± 3°C) and 2 at room temperature, each temperature with a pool of light protected and another one without light protection. And the study of Physico-chemical properties of the first formulation of Spray Safe food Solution and after the reformulation with the K-carraeenan polymer.

According to the obtained results, both extraction technique is excellent with advancing the infusion extraction because of the low coast. Also, the results showed that the spray safe solution have a good antioxidant and antimicrobial activity with some microorganisms.

In this study, reports within our research group have indicated that the solution could help protecting certain type of foods. And indicates that natural polymers combined with natural antimicrobials and antioxidants can be employed to reduce the dependence on synthetic polymers and offer feasible solutions to be applied at an industrial level.

Keywords: *Rosmarinus officinalis L,*, bioactive properties, Antimicrobial, antioxidant, Physico-chemical properties, polymeric complex, food contact material.

RESUMO

O plástico tem se tornado numa das principais preocupações ambientais do nosso tempo, com mais de 8 milhões de toneladas de plástico a serem são lançadas ao oceano todos os anos (UN Environment report ,2018). Os resíduos de plástico são considerados um problema globa, sendo portanto, necessárias soluções para reduzir a poluição resultante do uso excessivo desse material (Jung H., 2014).

Nesse contexto, este trabalho explora a possibilidade de produção de uma solução a partir de um biopolímero natural aplicado como "spray" para revestir e proteger alimentos, como uma barreira alternativa ao plástico. O contato do spray com os alimentos é inofensivo para a saúde humana, sendo A solução composta por uma solução primária de iota-carragenina, uma mistura de diferentes antioxidantes naturais e extrato de alecrim (*Rosmarinus officinalis. L*). A extração foi realizada usando duas técnicas, uma com extração por infusão e a segunda, extração assistida por ultrassons. O presente trabalho foca a monitorização da atividade antioxidante (DPPH e TBARS) e da atividade antimicrobiana utilizando um tempo de prateleira de 6 meses, dividido em 7 tempos diferentes (0, 7, 15 dias e 1, 2, 4, 6 mês), em 4 condições diferentes, 2 em refrigeração (± 3 ° C) e 2 em temperatura ambiente, um debaixo de luz, e outro protegido. Foram estudadas as propriedades físico-químicas da formulação de SpraySafe, sendo testadas posteriormente diferentes reformulações com K-carragenina.

Os resultados mostraram que a solução tem uma boa atividade antioxidante e antimicrobiana, e que a solução pode ajudar a proteger certos tipos de alimentos. Polímeros naturais combinados com antimicrobianos e antioxidantes naturais podem ser usados para reduzir a dependência de polímeros sintéticos e oferecer soluções viáveis para aplicação a nível industrial.

Palavras-chave: *Rosmarinus officinalis. L*, propriedades bioativas, propriedades físico-químicas, polímeros nature

1. INTRODUCTION

1.1 Packaging

The packaging industry is the third biggest industry in the world ($420 billion) after the aeronautics and clothing industries, being the food packaging sector one of the major commerce segments (Datamonitor, 2010). The food industry companies focus on two essential points for perfect packaging. The 1st point is the appearance of the packaging which provides the information about the food, and the 2nd point is the material and quality of the packaging: paper and cardboard, wood, glass, metals, plastics, among others. Packaging is the last step in the food production chain just before the products embark to the customers, and its main role is to preserve the food from microorganisms or other sources of contamination ensuring the hygiene and quality of the food till the consumer's tables. (Jeantet et al. 2016).

1.1.1 Definition and principles

The European directive 94/62/EC gives the official definition of packaging and its field of application and refers to packaging as meaning "All products made of any materials of any nature to be used for the containment, protection, handling, delivery, and presentation of goods, from raw materials to processed goods, from the producer to the user or the consumer". With the prior definition stated, packaging could understand further consisting of:

- sales packaging or primary packaging, that is packaging conceived to constitute a sales unit to the final user or consumer at the point of purchase.
- grouped packaging or secondary packaging consists of a certain number of sales units intended for the final user or consumer; it can be removed from the product without affecting its characteristics.
- transport packaging or tertiary packaging, that is packaging conceived to facilitate handling and transport of several sales units or grouped packaging to prevent physical handling and transport damage (Jeantet et al. 2016).

1.1.2 Relevance of food packaging

Before the development of technology to preserve the edibility of foods, as well as their taste and nutritional properties, food preservation was performed by several traditional techniques such as heat and salting, and other methods which could include diverse ways of employing natural ingredients such as animal or vegetable fats, alcohol, sugar, etc. With the development of different technologies all over the world, the methodologies of food preservation have also evolved, adding techniques such as aseptic processing, sterilization, and deep freezing, to avoid contamination and the growth of microorganisms. Following these developments, the food industries are constantly looking for innovative methods to preserve and transport processed food at lower costs keeping high-quality standards. Knowledge and technologies have allowed the transformation of products supply, for instance, technologies such as pasteurization offer a great improvement in the handling of products, allowing them to be transported, maintained, and sold without the need for extra conditions such as refrigeration. Improvements of this kind are beneficial and desirable for processors, retailers, and consumers, and would not be possible without proper packaging backing up the previous preservation processes. Additionally, the total energetic balance is also reduced by removing the need for refrigeration, which also allows maximizing the cost/benefit of the final products. (Bartkowiak et al. 2016)

"Contain, secure, impart, protect and transport ". These 5 words depict the essential components of a package. Its major article is to contain your food. "Contain" implies protecting it from outside squander: shocks, bugs, gases, etc. The correspondence space given by the bundle on the thing is used by creators and makers to demonstrate the supportive, major, and mandatory information that should appear on all food assortments, similar to the instruction of use, the utilization date, or the best before date (already the date of ideal use). The upkeep work is the most critical. The packaging extends the shelf-life of the contained item by its creation, due to the packaging advancements that make the packaging "vacuum" or " adjusted air ". It can in this manner accept a huge part in the fight against food waste.

1.1.3 Type of food packaging

The study of packaging is the study of materials. Therefore, the proprieties of the packaging materials used influences greatly the choice of the packaging, these proprieties are

expressed chemically and physically, different aspects are relied on when it comes to their usability. further details of the most conventionally employed materials in the food packaging industries will now be detailed.

1.1.3.1 Paper and paperboard

Food packaging uses a wide type of paper and paperboard. The most popular being the paperboard, used for food packaging for its rigidity. Produced mainly from primary fiber and secondary fiber derived from old, corrugated containers (OCCs), or old newspapers (ONPs); 100% virgin fiber can be used to produce kraft paperboard (Han, 2013).

For a long time, the entire packaging industry mainly consisting of paper and paperboard. Categorized by weight or thickness of the product, paper is used for general commodities such as writing and printing papers, tissues, and newsprint but also for packaging, such as wrapping paper, grocery bags, and shipping sacks, being lighter than paperboard which falls into two major subcategories, folding carton and corrugated packaging, and has been used for packaging since 1831 when George Shyrock installed the first cylinder type machine in a plant in Pennsylvania (Twede and Selke, 2005).

The use of paper and paper boards is flourishing, despite the advent of the digital era, and is playing a significant role in modern society. Currently, the largest share of global packaging materials is comprised of paper and paperboard with a value of $370 billion and a volume of 390 million metric tons in 2011, equal to around 40% of the market. Paper and paperboard are major packaging materials for food products around the world. (Han, 2013).

1.1.3.2 Glass

In approximately 3000 BC, the first use of glass as packaging material occurred (Robertson, 2006). The blowing technique was used to produce glassware, the use of the sand molding technique around 1000 BC, enabled the development of transparent glassware after the production of opaque glass bowls and cups. Due to the lack of mass-production techniques, glass was expensive until the early 20th century.

However, in 1904 Michael J. Owens was granted a patent for a fully automated glass-shaping machine for producing glass bottles. Used for food packaging, glass has displayed flaws, such as being prone to breakage upon physical impact and high pressure. On the other

hand, it presented many advantages like its durability and the fact that it has good barrier properties against gases and chemicals, providing insulation that keeps food fresh during storage, in addition to being suitable for heat processing at higher temperatures.

Most of the glass used for food and beverage containers can be easily reused and recycled. According to the USEPA (2012) (Han, 2013).

Glass packaging typically includes bottles, jars, flasks, glasses, and tumblers. It is used in many different sectors of the food industry (beverages, preserves, jams, condiments, baby food, dairy products, etc.). A distinction is made between glass varieties depending on their ability to absorb heat radiation and block ultraviolet light: clear glass for water, certain juices, jams, and yogurts; green/blue glass for beer, wine, and oil; brown/amber glass for beer and certain juices (Jeantet et al. 2016).

1.1.3.3 Metal's packaging

Metals are broadly utilized for food packaging given their reasonable properties: forming capacity, unbending nature, robustness, impermeability, darkness concerning light beams, and heat conduction, therefore, they are mostly utilized in the packaging of canned products since they are especially appropriate for long term storage (strength and impermeability). Besides, metal packaging is for the most part recyclable. Two metals are utilized in packaging: steel as tinplate (tin covered steel) or chromium covered steel and aluminum as composites (Jeantet et al. 2016).

The notable 'tin can' is the first and most unmistakable subtype of metal compartment for food applications. Fundamentally, steel materials can be found available on the market of metal containers in various forms, contingent upon the exceptional piece and insurance measures against the metallic corrosion (Brunazzi et al. 2014).

1.1.3.4 Plastics packaging

Plastic materials are employed for the assembling of various food and non-food packages. Concerning food and drink items, there are numerous potential arrangements including likewise "hybrid" packages: all things considered, the entire gathering of metal compartments can be characterized as the exemplary illustration of plastic/metallic holder in

light of the synergic concurrence of metal backings and plastic coatings, enamels, gaskets and other natural parts, including printing inks (Coles et al. 2003).

Plastics are manufactured materials made principally out of macromolecules and can be formed under warmth and pressure. Chemically, a plastic contains a macromolecular natural stage (polymer or resin), fillers or building up agents (glass, filaments, and so on), and added substances (plasticizers, heat stabilizers, against UV specialists, colorants, among others) All in all, there are two kinds of resin: thermoplastics, which mellow when warmed and solidify when cooled and can be continually remolded, and thermosets, which must be formed once. Plastics are fundamentally gotten from the petrochemical fabrication except for cellophane, which is acquired by the synthetic treatment of cellulose (Jeantet et al. 2016).

1.1.4 Drawbacks of plastics usage in the food industries

The properties of plastics are primarily influenced both by the substance creation of the crude materials and by their natural and actual states. Since plastics are made from monomers that contain different kinds of atoms, the arrangement, configuration, conformation, and several molecules and atoms are key components for the qualities of individual plastics. Plastic polymers are regularly characterized by their linearity: linear polymer, branched polymer, cross-linked polymer, or network polymer. Because of the various properties dependent on environmental and physical states, such as linearity, molecular weight, and its distribution, degree of density, crystallinity, humidity, and varying temperatures, plastics give truly usable and multi-adaptable usefulness for food packaging frameworks. For instance, as sub-atomic weight increments, different properties of polyethylene like elasticity, sway strength, clearness, and extreme lengthening additionally increment, and as thickness builds, these properties, aside from rigidity, decline. As the morphological properties of a plastic packaging polymer, for example, crystallinity change, different properties are generously influenced (Han, 2013).

Plastic packaging creates critical negative externalities, assessed by United Nations Environment Program at $40 billion yearly, a bigger whole than the benefits of the plastic packaging industry. Notwithstanding the monetary misfortunes, the subject of the effect of plastic on the environment and human wellbeing is a significant part of the present discussions about plastic. Researchers have shown that the harmfulness of contamination from plastics, including nano-sized plastics, affects marine life. The danger of getting a sickness increment from 4% to 80% for corals that have encountered plastic. Plastic waste is a vector for

microorganisms and miniature organic entities engaged with the spread of infections by obtrusive species and causing utilitarian issues in biological systems. (Jeantet et al. 2016).

1.1.5 Food contact material

Food packaging is rarely latent. The combination of new materials and new uses of packaging has led to previously unknown interactions between food and the packaging that contains it. These interactions can occur in different ways. By the known phenomenon of migration, the components of the packaging can move in the food. The packaging can also absorb food components, and when the aromatic compounds are absorbed through the packaging, the process is called scalping (Rish and Hotchkiss, 1990). Interactions can be grouped into three categories:

- Material transfers between the product, the material, and sometimes the environment, again called migration, can occur in the liquid, gas, or solid phase.
- Heat transfers such as conduction, convection, and radiation.
- Contamination due to the passage of microorganisms through the material, for plastic materials.

These interactions are probably going to affect the nature of the food. Since synthetic substances are probably going to relocate from these materials to food, the wellbeing of food contact materials ought to be evaluated. These materials should be made according to EU guidelines including great assembling practice with the goal that an expected exchange to food does not cause a health issue, does not change the quality of the materials or food in an unsuitable manner, or does not cause an antagonistic impact on the taste and/or the smell of the food (Barnes et al. 2007).

Migration and interaction phenomena are highly regulated and depend on the material used in contact with food. There is an important principle: the inertia of the material and the food, that the latter must not give up to the food compounds in quantities likely to present a danger to healthy humans, nor lead to an unacceptable modification of the composition of foodstuffs or an alteration of the organoleptic characteristics. However, with the development of technologies and materials that can come into contact with food, this principle can be called into question. If we talk about active packaging, for example, the latter may have the ability to release molecules that enhance the product, such as vitamins. European regulations are

changing through orders specific to certain materials, in particular plastics. However, there is nevertheless a need for inertia if the substances can harm the health of the consumer (Gaquerel and Costes, 2004).

1.1.6 Polymeric materials in the packaging industries

The creation and utilization of packaging made of petrochemical macromolecules in ceaselessly expanding amounts have brought about the accumulation of plastic in our environment, a wellspring of visual nuisance, blockage of landfills, and soil and sea contamination. This concentrated misuse during the last years presents huge issues for the environment.

The second half of the twentieth century saw a phenomenal mechanical blast in engineered polymers, to where they are omnipresent in our day-by-day lives today, they are found in the form of convenience polymers (packaging, bottles, toys, etc) (Cirillo et al. 2015).

1.1.6.1 Definition

A polymer is a grouping of basic units called monomers that are not necessarily identical. The quantity of monomer units establishing the macromolecule is known as the level of polymerization. (Cirillo et al.2015). The level or degree of polymerization (DP or Xn) is characterized as the number of monomer units in the polymer. It is determined as the ratio of molecular weight of a polymer and molecular weight of the repeat unit. Average number and weight are the two main types used for measuring the DP. Higher DP is desired for better mechanical properties. Other abbreviations usually employed are Mw as the average molecular weight of the polymer and Mo as the molecular weight of the repeating unit or monomer. (Johansson, 1993)

1.1.6.2 Natural polymers in the food packaging industries

Biopolymer or biodegradable plastics are polymeric materials in which at a certain stage in the degradation cycle is through the metabolism of natural organisms (Peelman et al. 2013). As indicated by the European Bioplastics Organization, bioplastics can be characterized as plastics depending on renewable resources or as plastics that are biodegradable and/or compostable. Under proper conditions of moisture, temperature, and oxygen, biodegradation

prompts discontinuity or deterioration of plastics with no poisonous or ecologically hurtful remnant (Chandra and Rustgi, 1998). Biopolymers can be divided into various classifications depending on the origin of the raw materials and their manufacturing processes They include.

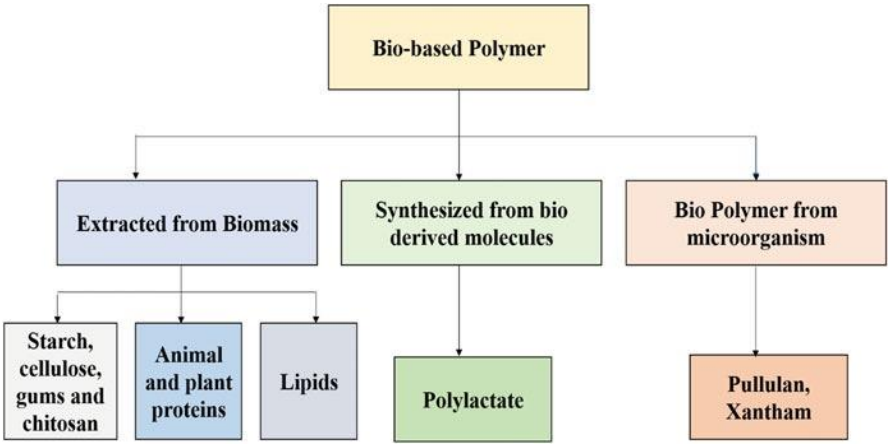

Figure 1-1 Biopolymers available from different sources (Shakeel, 2018)

1.1.6.3 Classification of bio-based materials

The utilization of biobased materials, filaments, paper, plastic coatings, and articles made of solidified sugar such as corn, starch, and other inexhaustible raw materials, has increased lately. Biopolymers have adaptable applications in drug conveyance systems, surgical implant devices, scaffolds for tissue engineering and packaging, food containers, agriculture film, waste bags, and packaging material.

Biopolymers often have a firm and definite internal structure, though this may not be the main feature (e.g. lignocellulose) (Shakeel, 2018).

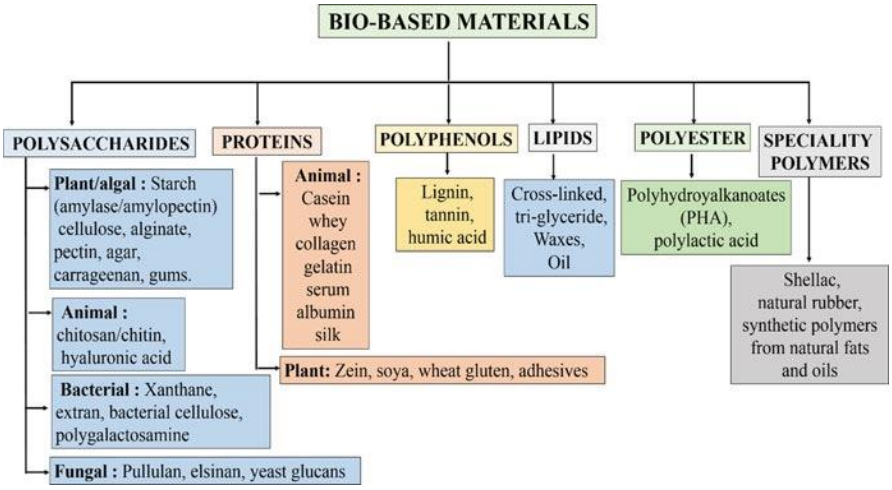

Figure 1-2 Classification of bio-based materials (Shakeel, 2018)

1.1.7 Polyphenolic compounds and their bioactive properties

Polyphenols, also called phenolic compounds, are part of a division of secondary metabolites with high antioxidant capacity, found naturally in most edible and inedible plants (Patricia, 2017). synthesized by shikimate pathway and known by the presence of an aromatic ring carrying free hydroxyl groups or engaged with a carbohydrate (Charpentier and Boizot, 2006). These phytonutrients are liable for pigmentation (leaf tone, fruit, and flower color) (Serrano et al. 2010) and assume a part in the development, reproduction, and protection of plants against pathogenic animosities (Nichols and Katiyar, 2010)

Polyphenols are naturally present in our food in various structures, for example, vitamins A, C, or E, carotenes, and certain minerals like selenium and (Farrukh and Santosh, 2012). They are found abundantly in fruits, vegetables, and grains. Until this point, researchers have identified more than 8000, going from basic molecules to exceptionally complex compounds. The essential underlying component that portrays them is the presence of one 6-carbon phenolic core to which is directly connected a minimum of one free hydroxyl (OH) group or engaged with another function: ether, ester, or heteroside (Perron and Brumaghim, 2009) Polyphenols display an extremely solid cancer prevention agent against oxidative stress due to excessive production of reactive kinds of oxygen (EROs), thus protecting the cells of our body against the damage brought about by maturing or prolonged exposure to elements such as infections, UV rays. from the sun, pollution, or tobacco smoke. As per the aftereffects of specific examinations lately, polyphenols might be associated with the avoidance of

cardiovascular illnesses and maybe likewise different pathologies, for example, neuro-infections. (Farrukh and Santosh, 2012).

1.1.7.1 Classification of polyphenols

The qualitative and quantitative studies of bioactive compounds from plant materials depend mostly on the selection of proper extraction methods. (Bravo, 1998)

- Hydrophilic or polar compounds (e.g., phenolic acids, flavonoids, organic acids, sugars); and
- Lipophilic or nonpolar compounds (e.g., carotenoids, alkaloids, terpenoids, fatty acids, tocopherols, steroids).
- They can also be classified according to their distribution in nature:
- Narrowly distributed (simple phenols, pyrocatechol, aldehydes);
- Widely distributed (flavonoids, phenolic acids); and
- The least abundant polymers (tannin and lignin). (Bravo, 1998)

1.1.7.2 Antioxidant power

The immediate addition of nutritional and antioxidant compounds into food systems may be disadvantageous because of the interaction with food molecules, so bioactive mixtures have been consolidated into consumable films to deliver antioxidant-active packaging (Ganiari et al. 2017).

An antioxidant compound is any substance that can delay, counteract, or eliminate oxidative damage to an objective molecule in any event when the antioxidant agent is available at a lower concentration than the oxidizable substrate (Halliwell, 2007). Antioxidants behave differently: they can (1) prevent free lipid radical formation and thus inhibit free radical oxidation reactions (preventive oxidants); (2) interrupt autoxidation chain reaction propagation (chain-breaking antioxidants); (3) quench singlet oxygen; (4) operate synergistically with other antioxidants; and (5) convert hydroperoxides into stable compounds (Carocho and Ferreira, 2013).

The food business has utilized antioxidants as additives substances to prevent lipid peroxidation. Although manufactured antioxidants have been widely applied in food

processing, scientists have reevaluated them to distinguish conceivable toxic and cancer-causing molecules that result in their degradation (Maeura et al. 1984; Ito et al. 1985).

1.1.7.3 Antimicrobial capacity

The utilization of antimicrobial mixtures including antimicrobial nanostructures into food packaging has been broadly examined and investigated (Azeredo, 2013; Sung et al. 2013).

Antimicrobial specialists can hinder or eliminate microorganisms (e.g., bacteria and fungi) by breaking their cell walls, intruding on their metabolism, or binding to their DNA and inhibiting their replication. Subsequently, these agents stop the propagation of microorganisms in the body. It is possible to classify antimicrobial molecules by their action against a specific microorganism; for instance, antibacterial (usually known as antibiotics) and antifungal agents act against bacteria and fungi, respectively. These molecules can likewise be divided by their capacity: antimicrobials that kill microbes are called microbicidal; those that just repress microorganism development are assigned microbiostatics (Bryskier, 2005).

Most microbiologists divide antimicrobial molecules into two groups: antimicrobial natural substances that specific groups of microorganisms produce and manufacture chemotherapeutic agents. A hybrid substance consists of a semisynthetic antibiotic molecule that is modified chemically to accomplish the ideal properties. Besides, a synthetic blend can yield some antimicrobial mixtures initially found as microorganism products (Bryskier, 2005)

1.1.7.4 Synergistic and antagonistic effects in packaging

Food packaging assumes an indispensable part in food processing and conservation. The developing concern of consumers in safety and quality has conferred a lot of consideration in understanding the interaction of food and packaging materials, especially, polymers interactions are a significant concern for the most part in polymer-based packaging materials. Polymers are favored for food packaging use because of their adaptability, strength, solidness, low density, simplicity of preparing, diminished expense, and controlled hydrophobicity. In any case, packing food items in polymers can induce inconvenient quality changes in packed food. For example, the level of browning and ascorbic acid degradation of orange and grapefruit juice was discovered to be higher when packed in polyethylene-covered containers than in glass (Mannheim et al. 1987). Interaction is the mass exchange event involving migration (from packing material to product), permeation (from the external environment to

food contact environment through packaging material), and sorption (adsorption/ingestion of food parts on the packaging material), either in mixture or separately interaction of packaging material with the food and the environment assumes a significant part on nature of the product just as the integrity of the package.

These impacts affect the market, sensory attributes of products, and health effects of consumers. In addition, severe enactments are set for 'zero tolerance' of carcinogenic migrants (Gilbert et al. 1980), and the compounds that relocate into food are considered as indirect food additives by FDA. Consequently, information on factors influencing interaction phenomena and their impact is of high significance.

1.1.8 Bioactive food packaging

The growing consciousness of the consumers and the increasing interest for quality food sources are developing advancement and new product improvement in the food business globally and are additionally liable for the extending overall interest in utilitarian food varieties. For the most part, a food item marketed as functional contains added ingredients, technologically created, that give a particular advantage to human health. (Alzamora et al. 2005)

Bioactive packaging is an even newer concept of packaging which, based on the same principles as active packaging, seeks to have an impact on consumer health through the controlled incorporation of bioactive or functional substances, initially contained within the package walls or biopolymeric structures, to the packaged food products (Rubio et al. 2006). The bioactive packaging concept embraces a series of technologies that can be used for the protection or stabilization of functional ingredients and can be grouped as follows:

1. Integration and controlled release of bioactive components or nanocomponents from biodegradable and/or sustainable packaging systems.
2. Micro and nanoencapsulation of these active substances either in the packaging and/or within foods.
3. Packaging provided with enzymatic activity exerting a health-promoting benefit through a transformation of specific food-borne components.

2 OBJECTIVES

In this work, a monitorization of a Food Contact Material (FCM) containing a biopolymeric structure incorporated with a mix of lipophilic and hydrophilic antioxidants conceived to be used as spray has been evaluated on their efficiency capacity against pathogenic microorganisms and oxidant factors. The development of the solution intends to fulfil two purposes, 1) reducing food waste and 2) combating plastic pollution.

Several components and parameters were evaluated, namely, polymeric complex and antioxidant compounds which possess several health attributes.

The tests carried out in the solution (Spray Safe) were designed for the adaptation to random food matrices as well as expanding the knowledge on their properties. Thus, the specific objectives of this work consisted of:

- Comparison of the leaf extraction from the *Rosmarinus officinalis L* by infusion and ultrasound-assisted extraction (UAE).
- Shelf-life screening of the Spray Safe solution.
- Determination of the antioxidant activity of the different compounds, synergistic and antagonistic effects.
- Antimicrobial performance of the Spray Safe solution.
- Characterization, and analysis of the main Spray Safe attributes and properties.

3 MATERIALS AND METHODS

3.1 Plant Material

Rosmarinus officinalis L or rosemary is a species of shrubs of the Lamiaceae (or Labiee) family, this plant has a strong antioxidant effect, mainly attributed to rosmarinic acid (the major compound found), carnosic acids, luteolin glycoside derivatives, and a caffeic acid derivatives, among others (Ribeiro et al. 2016). The rosemary used in this work was purchased from a Vila Nova de Gaia (Portugal) company "Cantinho das Aromáticas". The plant was collected, crushed, and sieved to obtain a fine and homogeneous powder and was stored in a sealed container in a cool and dry environment until use.

Figure 3-1 Rosemary leaves

3.2 Extraction process

In order to recover the phenolic compounds from *R. officinalis L.* powder for the spray solution, two extraction techniques were used: infusion and ultrasound-assisted extraction (UAE).

3.2.1 Infusion extraction

This type of process is used when the active ingredients of the plant are water-soluble and can easily be obtained from the tissue of the plant. Therefore, the infusion is ideal for leafy extractions. For this methodology, 25 g of rosemary powder were infused in 1000 mL of boiling water (100 °C), heated with a hot plate (for 5 min), then, the mixture was allowed to cool for another 5 min, and filtered with a filter paper (Whatman No. 4).

Figure 3-2 Infusion extraction steps. A. Rosemary powder, B. Boiled water, C. Rosemary powder in boiled water (infusion)

After freezing, the solutions were frozen and freeze-dried until the extract was completely dry.

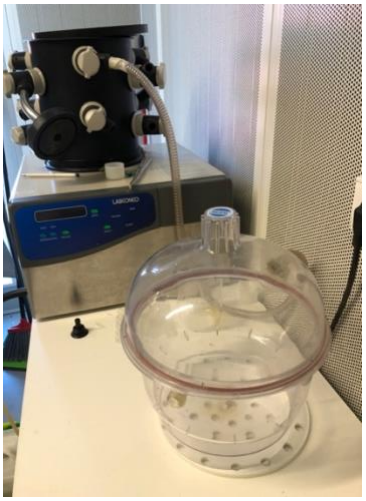

Figure 3-3 Lyophilizator (FreeZone 4.5, Labconco, Kansas, USA)

3.3 Ultrasound-assisted extraction (UAE)

Conventional methods of extracting secondary metabolites from plants often have drawbacks, namely low extraction yields, very long extraction times, large amounts of solvents and energy. Low frequency ultrasonic (20 kHz to 100 kHz) extractions are known to have significant effects on reaction kinetics, helping to reduce extraction time and increase yields. The cavitation induced by the ultrasonic probe is at the origin of these effects, creating a jet of

bubbles by acoustic depression which implodes on contact with the membrane surface releasing the bioactive molecules present in the plant cell (Veillet, 2010; Vilkhu et al. 2008).

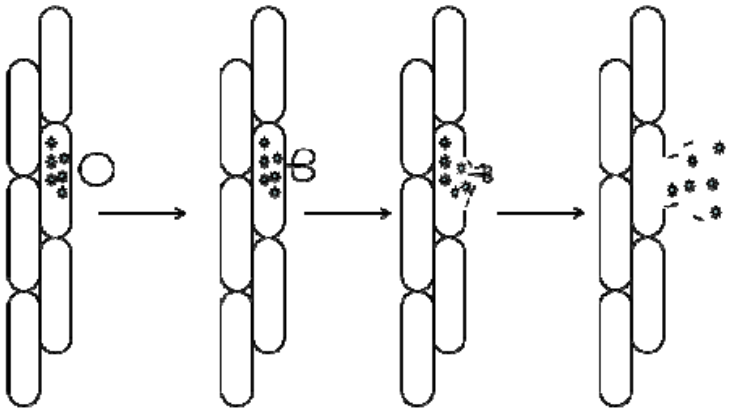

Figure 3-4 Evolution of a cavitation bubble near a plant cell (Veillet, 2010)

In addition, the series of compressions and rarefactions generated when the sound wave passes through the medium creates an acoustic pressure that generates stress on the surface of the plant membrane (Figure 3.4). The resulting ultrasonic energy, through this stress, makes it possible to improve the transfers of heat, matter or even to increase the quantity of movement, thus accelerating the kinetics and the quality of the reaction (Sališová et al. 1997, Cárcel et al. 2012; Chemat, 2011; Mason and al. 1996).

The used ultrasonic device (Qsonica sonicators, model CL-334, Newtown, CT, USA) was a low-frequency probe (20 kHz) at a power of 500 Watts to perform the extractions of the bioactive molecules of *R. officinalis L*.

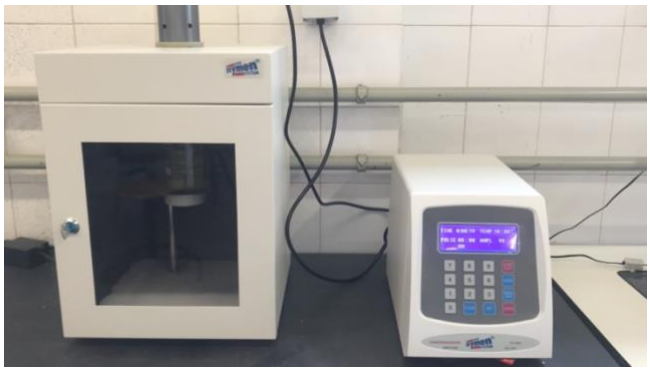

Figure 3-5 Qsonica sonicators, model CL-334, Newtown, CT, USA

In this extraction, the powdered *R. officinalis L* (7 g) were placed in a beaker with 200 mL of solvent (ethanol 80% in water) and processed by submerging the sonicating probe in the solution (Qsonica sonicators, model CL-334, Newtown, CT, USA) (figure 3.6) for 1 min with power of 99%.

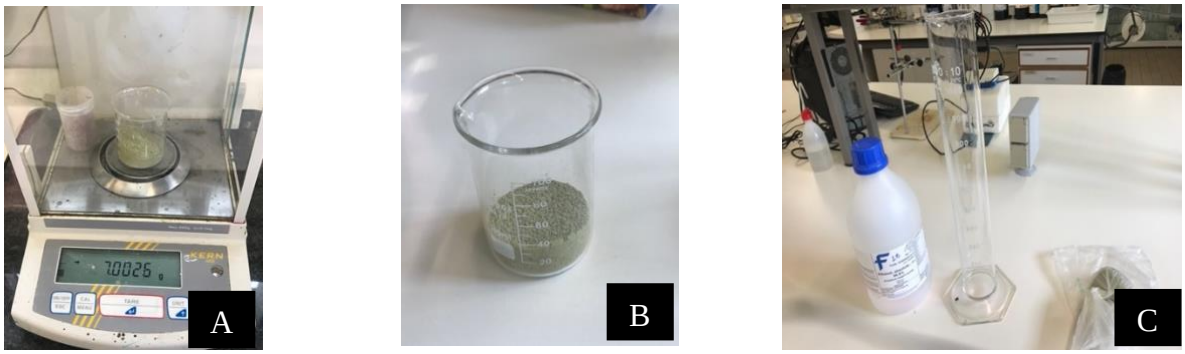

Figure 3-6 Steps of the UAE extraction: A. weight at electronic scale, B. Rosemary powder, C. Ethanol addition

The mix was filtered through paper filters (Whatman No. 4) into a flask then evaporated at 40-60 °C to remove the ethanol from the extract. After complete ethanol evaporation, the same procedure from infusion extraction of freeze-drying was applied.

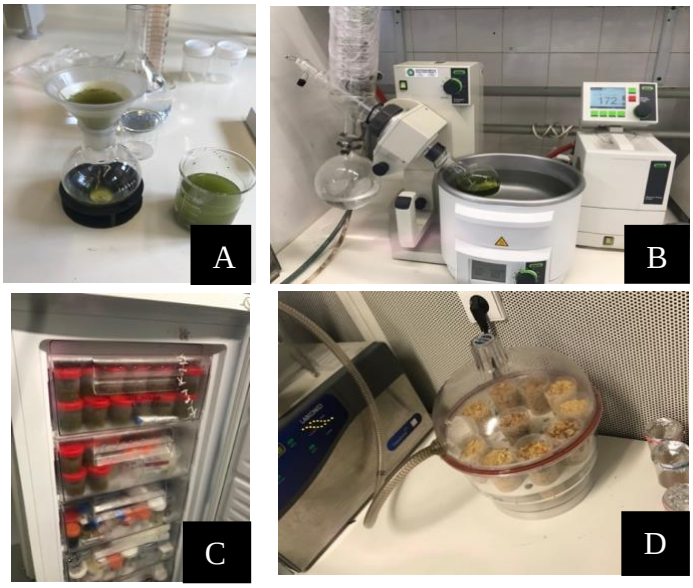

Figure 3-7 Sample preparation after the UAE: A. Filtration, B. Evaporation, C. Freezing, D. Lyophilization

3.4 Spray safe solution composition

The Spray Safe solution is composed of six 6 compounds that must be added in specific amounts and order. This solution is currently patented, and the compounds are added in the following order.

3.4.1 Carrageenan (polymer)

Is a polysaccharide (galactan) extracted from red algae used as a thickening and stabilizing agent in the food industry. It carries the E407 code of the classification of food additives. Carrageenan was first extracted in 1837 from seaweeds, and thereafter, the process of extraction continuously took place mainly from the Rhodophyceae family (Bemiller et al. 2012). This polymer makes it possible to form hot gels (up to 60 ° C.) and is therefore of interest compared to traditional animal gelatins. Iota-carrageenan was used in this study (Alfa Aesar, Haverhill MA, USA), and the last part of the experiment employed kappa- carrageenan.

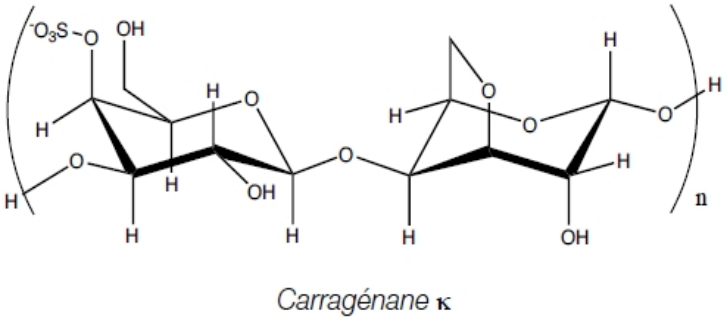

Figure 3-8 Chemical structure of carrageenan

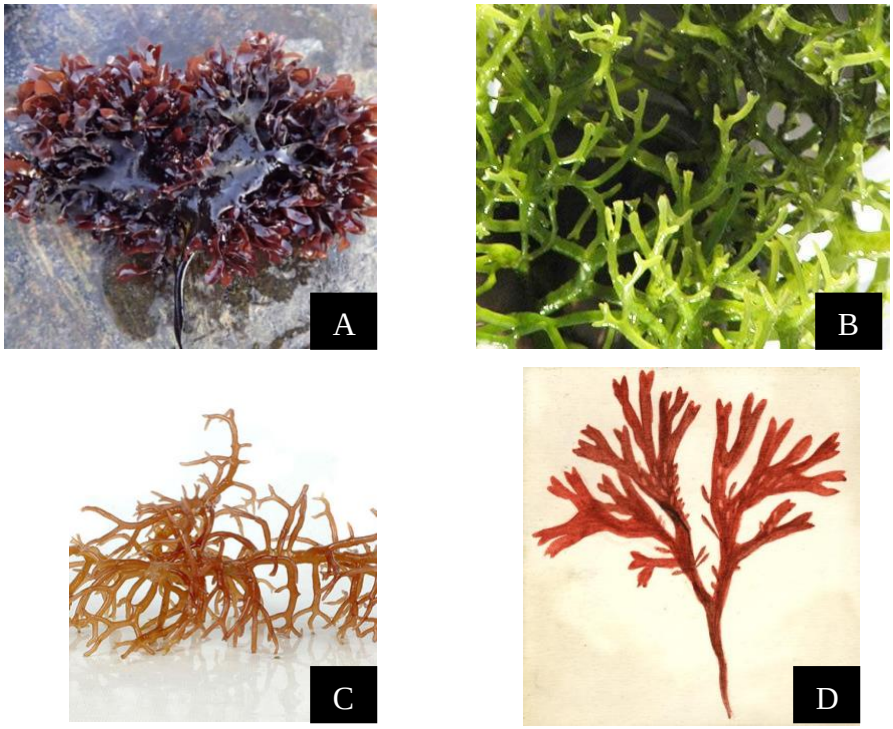

Figure 3-9 Different types of seaweeds used as a source of carrageenan's. (A) Chondrus crispus, (B) Euchema denticulatum, (C) Gigartina stellate, and (D) Kappaphycus sp.

3.4.2 Rosemary extract (*R. officinalis L*)

The extract of *R. officinalis L* was obtained with the two extraction techniques mention in section 3.2 and 3.3, infusion and UAE techniques.

3.4.3 Alpha-tocopherol (vitamin E)

Is a liposoluble vitamin covering a set of eight organic molecules, four tocopherols, and four tocotrienols. The biologically most active form is α-tocopherol, and the most abundant in the diet being γ-tocopherol. These molecules are present in large quantities in vegetable oils. They act, alongside vitamin C and glutathione, essentially as antioxidants against reactive oxygen derivatives produced by the oxidation of fatty acids. The tocopherol employed in the assays was the isomer α (Thermo Fisher Scientific, Hampton, NH, USA).

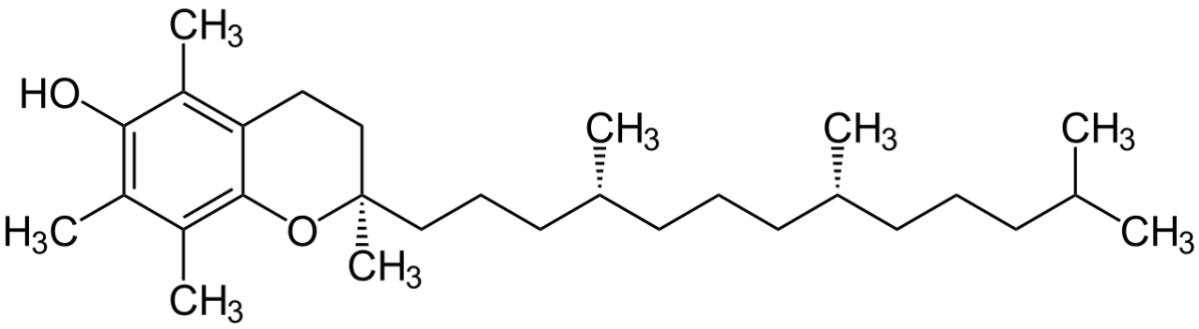

Figure 3-10 Chemical structure of α-tocopherol

3.4.4 Ascorbic acid (vitamin C)

Ascorbic acid or oxo-3-glucofuranolactone acid (enolic form), is an organic acid with antioxidant properties. A diacid (pKa of 4.1 and 11.8) and reducing agent with high antioxidant activity, working as a regenerator of tocopherols.

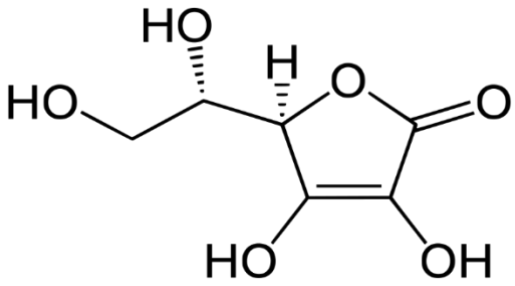

Figure 3-11 Chemical structure of Ascorbic Acid

3.4.5 Calcium chloride

Calcium chloride ($CaCl_2$) (molar mass M = 111 g/mol), occurs at standard thermodynamic conditions as a white solid (molten or anhydrous sodium chloride). It is easily

soluble in water and alcohol. It is a strongly hygroscopic compound, which means that in the presence of water it reacts to form a hydrate, with high heat release.

3.4.6 Glycerol (glycerine)

Glycerol is a chemical compound with the formula $HOH_2C–CHOH–CH2OH$. It is a colorless, viscous, and odorless liquid with a sweet taste. The glycerol employed was reagent grade (Thermo Fisher Scientific, Hampton, NH, USA).

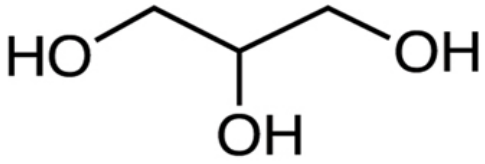

Figure 3-12 Chemical structure of Glycerol

3.5 Preparation of spray safe

The Spray Safe solution was prepared following a specific protocol (patented) in a sterile environment using autoclaved materials. In a laminar airflow chamber (Telstar BIO II A), the procedure was initiated by heating the polymer solution at 1% in water at c.a. 60 – 80 °C employing a digital magnetic stirrer with heating and ceramic coated plate (LBX H2OD series) under continuous electromagnetic stirring.

Once the polymer was completely dissolved, the temperature was dropped to incorporate the bioactive compounds, starting by the addition of rosemary extract (0.5%). At the same time, 1% of α-tocopherol was diluted in 10 mL of ethanol, protected from light, and then added to the main solution, followed by ascorbic acid (0.012%), and 0.1% of calcium chloride, finally, the solution was smoothed up with 30 mL of glycerol until it was clear and homogeneous.

Figure 3-13 Aseptic preparation in Laminar Air Flow chamber

Figure 3-14 Final Spray Safe solution

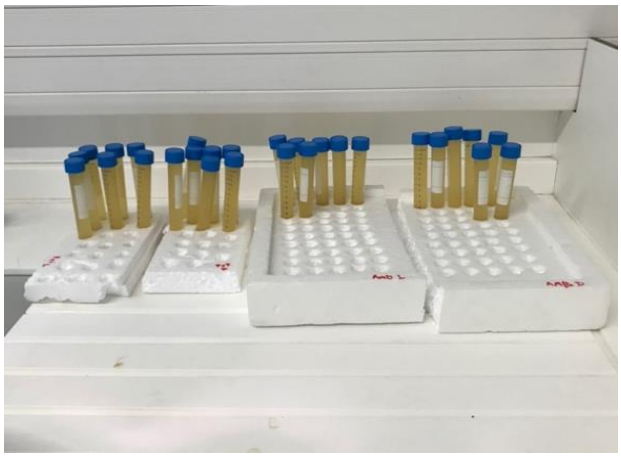

Figure 3-15 Spray Safe solution on test tubes for experimental procedures

3.6 Bioactive characterization of the spray safe solution

3.6.1 Antioxidant activity

The antioxidant power of the solution was measured during 7 different consecutive times, namely (T0-T6), 0 days, 7 days, 14 days, 1 month, 2 months, 4 months, and 6 months. Using different temperature conditions (refrigeration and room temperature) and light protection (protected and unprotected).

Two techniques were performed for the antioxidant activity: 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and thiobarbituric acid reactive substances (TBARS).

3.6.1.1 Radicals scavenging activity or 2,2-Diphenyl-1-picrylhydrazyl (DPPH)

DPPH was used to measure the antioxidant activity of a substance by its ability to scavenge free radicals from DPPH.

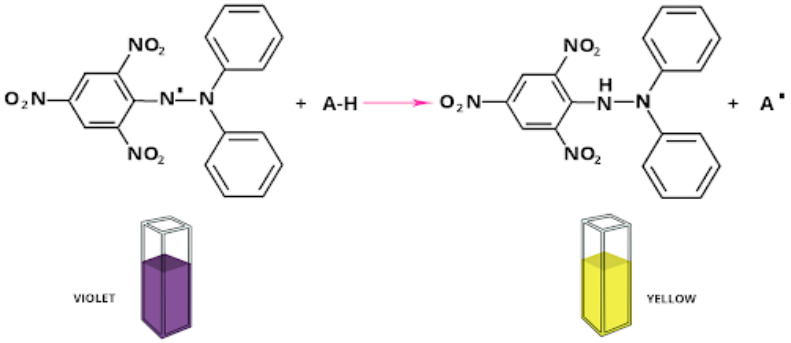

Figure 3-16 DPPH reaction representation

The mother solution of DPPH was prepared with methanol 99% at a concentration of $6x10^{-5}$ M. The reaction mixture consisted of one of the different concentrations (2,5µg/ml, 12,55µg/ml, 6,255µg/ml, 3,1255µg/ml, 1,565µg/ml, 0,785µg/ml) of the diluted solutions (33 µL) and the DPPH solution (260 µL). The reaction was made on a 96 well microplate which was then stored in the dark at ambient temperature for 30 minutes, and finally the absorbance was measured by spectrometertry (SPECTROstar Nano) at 450 nm.

The results were expressed in mg/mL as the extract concentration responsible for 50% of DPPH radical scavenging activity (EC_{50}).

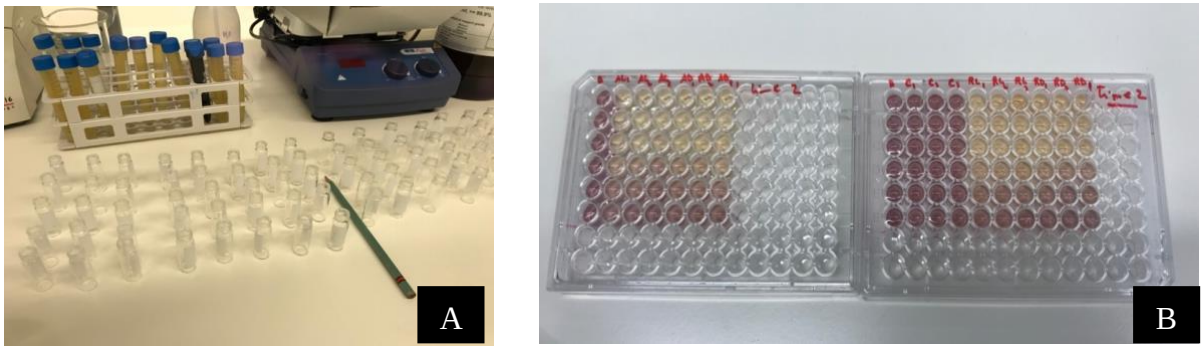

Figure 3-17 A: Serial dilutions representation. B: Reaction of samples against DPPH in microplate

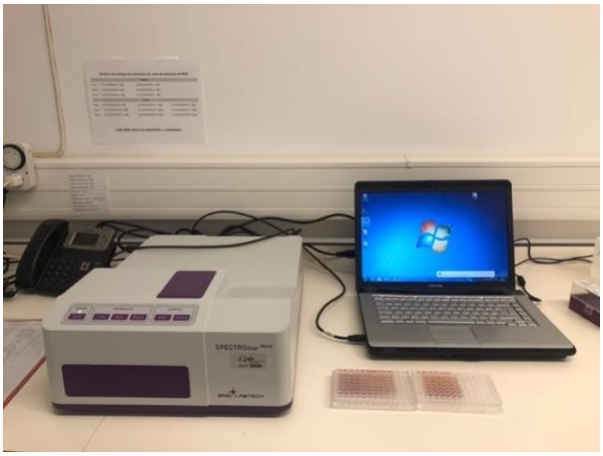

Figure 3-18 Spectrophotometer (SPECTROstar nano) used to read the absorbance

3.6.1.2 Thiobarbituric acid reactive substances (TBARS) assay

TBARS is a colorimetric assay that measures the malondialdehyde (MDA) production via lipid peroxidation, in which the thiobarbituric acid (TBA) reacts with MDA to produce oxidative MDA-TBA complex, responsible for the formation of a pink pigment which can be measured spectrophotometrically at 532 nm (Kaur and Geetha, 2006).

For the assay, porcine brains were obtained from a local slaughterhouse, weighted, and dissolved in Tris-HCl buffer (20 mM, pH = 7.4). The solution was then centrifuged at 3500 g for 10 minutes. Each dilution of the sample solutions (200 µL) was pipetted into Eppendorf tubes, adding to them 100 µL of FeSO4 (10 mmol/L), 100 µL of ascorbic acid (0.1 mmol/L), and 100 µL of the supernatant of brain tissue homogenate. Two blanks were prepared, one Tris-HCl buffer and the other with deionized water. The Eppendorfs were incubated at 37.5 °C for 1h. After the incubation, trichloroacetic acid (500 µL, 28% w/v) was added to stop the reaction, and at the same time 380 µL of thiobarbituric acid (TBA, 2% w/v) was added, followed by the

subsequent incubation of tubes at 80 °C for 20 min. Then, to eliminate the precipitated protein, the tubes were centrifuged at 3000 rpm for 5 min, and the supernatant samples were measured at 532 nm

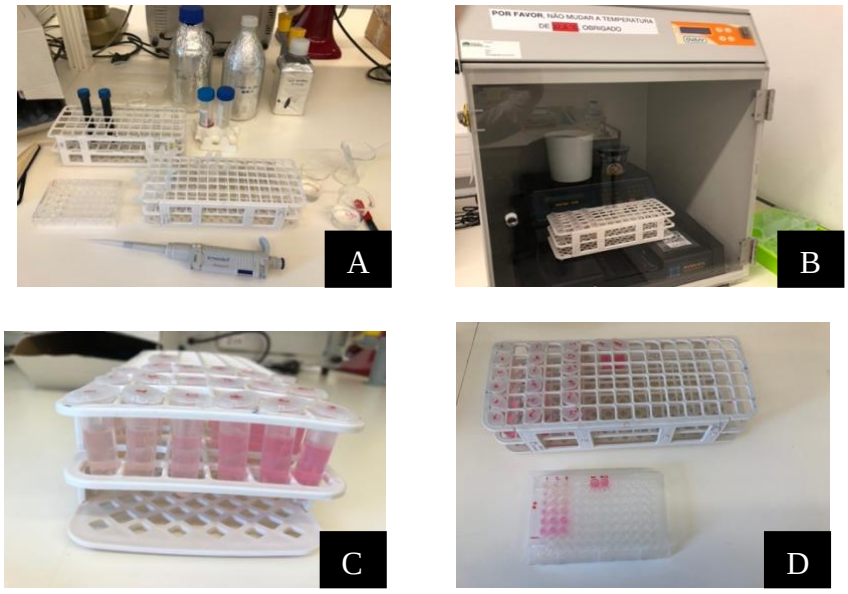

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Figure 3-19 TBARS test. A: Dilution. B: Incubation prior addition of TBA. C: complex color formation from MDA-TBA. D: Microplates for the absorbance reading

The equation, inhibition ratio (%) = [(A−B)/A] × 100; (A- absorbance of the control and B- absorbance of the sample solution) was used to calculate the percentage of inhibition, then transformed to EC_{50} values (concentration of extracts responsible for 50% of lipid peroxidation inhibition) in mg/mL of extract solution.

3.6.2 Spray safe Shelf-life screening

3.6.2.1 Spray safe microbial load

The microbiological analysis was assessed by mixing 1 g of the samples with 9 mL of peptone water. These solutions were diluted until achieving 10^{-12} and the following counts were performed: aerobic plate count (total viable count; ISO 4833-2:2013), coliforms (and *Escherichia coli*; ISO 4832:2006), yeasts and molds (ISO 21527-1/2:2008), and *Bacillus cereus* (ISO 7932:2004).

The test consists of using three concentrations of the solution. It was set up by introducing into a sterile hemolysis tube in 200 ml of the Spray Safe solution. The obtained suspensions were further diluted to obtain dilutions from 10^{-1} to 10^{-3}. Each solution was analyzed in duplicate.

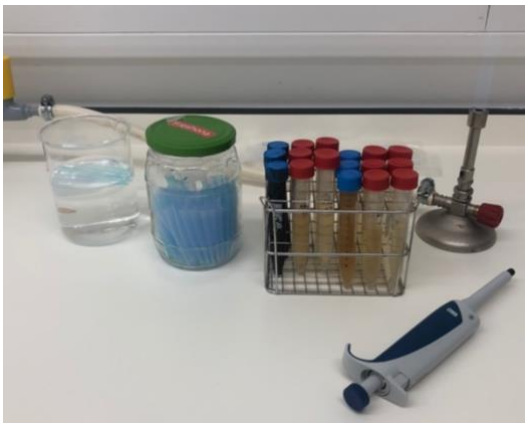

Figure 3-20 Sample preparation for the microbial activity assay

3.6.2.1.1 Determination of coliforms

The Crystal Violet Neutral Red Bile Lactose Agar (VRBL) (Liofilchem Co., Roseto Degli Abruzzi, Italy) was used. By the pour plate technique, in duplicate (Limit of Quantification (LOQ) = 1 log (Colony-Forming Units) CFU/g): 1 mL of solution coating food was pipetted into the petri dish and 15 mL of melted VRBLA (kept at 50 ◦C in a water bath or incubator) were poured prior to homogenization and left to solidify. The plates were incubated at 30 ◦C for 48 h, in reversed position. Counting was performed in plates that had between 10 and 150 colonies.

3.6.2.1.2 Determination of yeasts and molds

The Dichloran Rose Bengal Chloramphenicol Agar (DRBC Agar) is a media for the enumeration of yeasts and molds in food and animal products. By the spread plate technique, in duplicate (LOQ = 1.7 log CFU/g): 0.2 mL of the solution coating food were pipetted onto a Petri dish containing 15 mL of the solidified medium and were spread with a disposable spreader. Incubation was set at 25 ◦C for 5 days, in the upright position. In the plates having less than 150 colonies, the count of yeast and mold colonies were performed separately after 2 and 5 days of incubation.

3.6.2.1.3 Determination of aerobic mesophilic microorganisms

The Plate Count Agar (PCA, Liofilchem Co., Roseto Degli Abruzzi, Italy) is a media used for the enumeration of the revivable portion of the total aerobic mesophilic flora (AFNOR NF T 90-401 and 402 standards). By the pour plate method, 1 mL of each prepared solution coating food was mixed with 15 mL of PCA (in duplicate (LOQ = 1 log UFC/g). Incubation was performed at 30 ◦C for 72h and counted according to ISO 4833- 2:2013, in reversed position. All counts of the microbial load were performed at the same intervals as all other

assays of this work, namely T0 immediately after preparation, T2 after 30 days which was the shelf-life of the spray safe after opening, and T6, which was beyond the shelf-life.

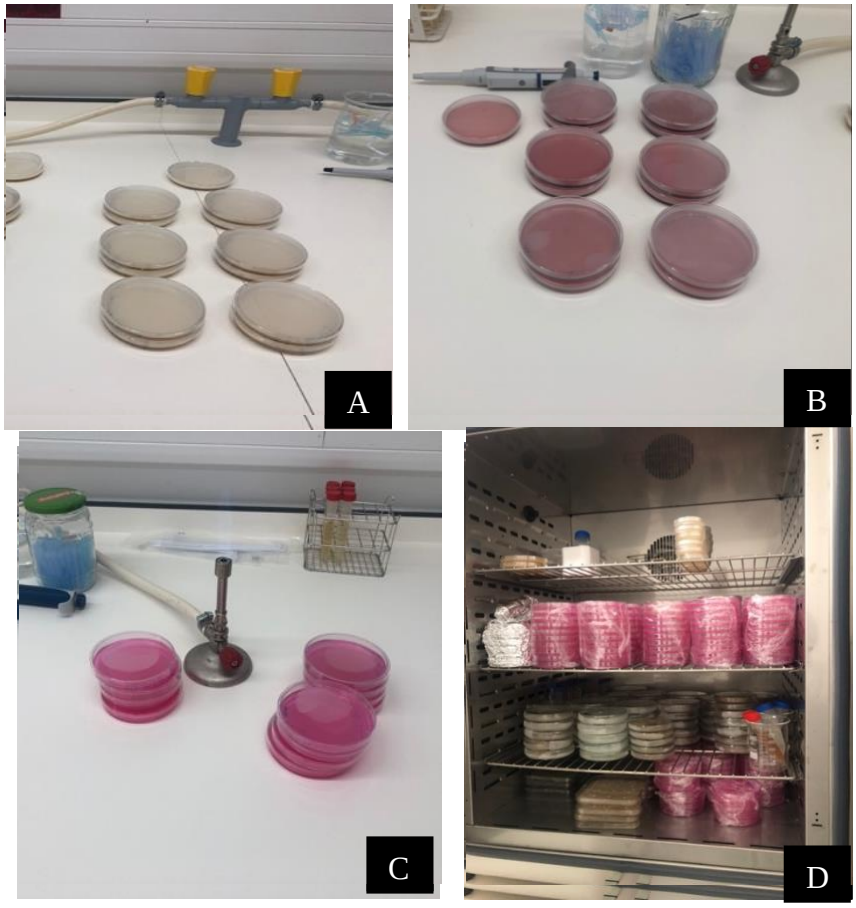

Figure 3-21 Different medias used in microbial activity screening. A: PCA, B: VRBLA. C: DRBC. D: Incubation.

3.6.2.2 Contamination procedure

In order to evaluate the spraysafe stability over the time, four different. microorganisms were selected to contaminate the coating solution (microorganisms that usually are food contaminants). Therefore, a fungus, *Aspergillus parasiticus*, and yeasts, *Zygosaccharomyces rouxii*, and 2 bacteria, *Eschericia coli* and *Bacillus cereus* were used. All of them are common foodborne pathogens in the food system. The experiment was applied in sterilized conditions to avoid contamination.

E. coli are Gram-negative microorganisms, is from the thermotolerant coliforms group a facultative inhabitant of the large intestine (Feng and Hartman, 1982). *Bacillus cereus* is a Gram-positive, spore-forming, facultative aero-anaerobic bacilli (Logan and De Vos, 2009).

For the preparation of the pre-inoculate, these two microorganisms were put in Tryptic Soy Broth (TSB) for 24 hours at 37°c. The yeast of *Zygosaccharomyces rouxii*, is among the most recognized species in nature, xerophilic, and very resistant to low water activities (Pitt and Hocking, 2009) and was placed in Tryptic Soy Broth (TSB) for 24 hours at 25°c and *Aspergillus parasiticus* is an infectious fungus was put in PDA Potato Dextrose Agar for 7 days at 25°c in the dark.

In order to do the antimicrobial susceptibility, a solution of microorganisms was prepared using these mediums with microorganisms and a McFarland densitometer BEN-1B (Biosan, Riga, Latvia) to measure solution turbidity in a wider range (up to 15.0 McFarland units), is used for measurement of cell concentration (bacterial, yeast cells), Cell concentration was estimated to be equal to 10^9 cells/ml. Isolated colonies from an overnight culture *Zygosaccharomyces rouxii*, *E. coli*, *Bacillus cereus* were dilute in broth to a turbidity comparable to that of a 0.5 McFarland turbidity standard (approximately 1.0×10^8 CFU/mL).

For the fungal spores, they were washed from the surface of agar plates with distilled water. The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^8 with a Neubauer chamber.

Figure 3-22 Material of the contamination experiment

The coating solution was prepared in sterilized conditions and put in 3 different bottles with a volume of 200ml in each one. And the microorganism's suspension was added to each bottle to contaminate the coating solution. All samples were contaminated with approximately 10^6 cell/mL of each microorganism and the number of viable cells (in CFU/mL) was confirmed inoculation of the contaminated samples in selective media.

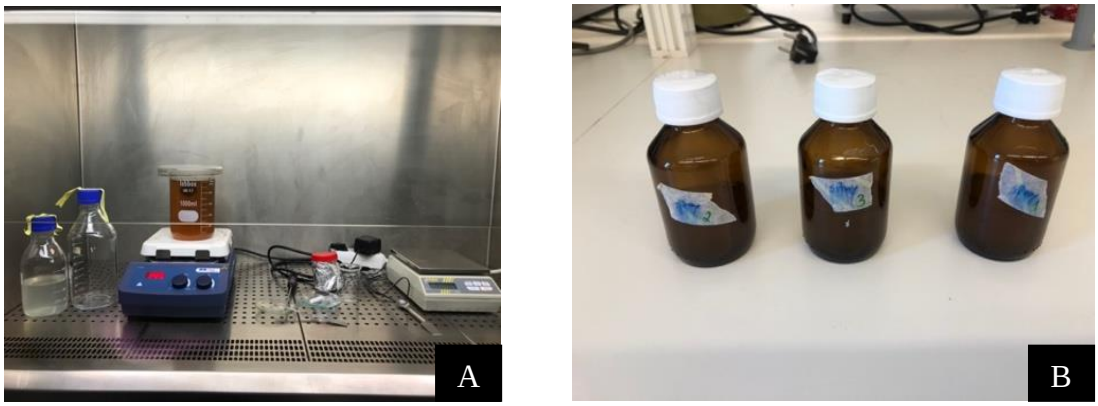

Figure 3-23 A. Solution prepared, B. Bottles of the solution for the contamination

The analytical unit must be diluted and homogenized with an adequate diluent, to allow inoculation into or onto culture media. Each solution was analyzed in duplicate.

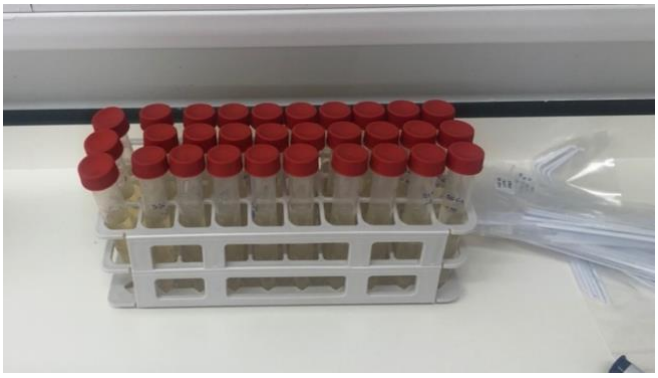

Figure 3-24 Different dilutions of contaminated spray safe solution

Figure 3-25 Petri plates with the medias used in antimicrobial power experiment.

The counting was performed following the following formula, corresponding to the subsequent formula, the colonies count was expressed in colony-forming units (CFU) per gram:

$$N = {}^{\Sigma c/}\,(v\,(n1 + 0.1n2)/d) \qquad \text{Eq. 3.1}$$

In which:

N: number of colonies per g or mL of sample;
Σc: sum of the colonies in the counted plates;
V: Volume of the suspension used;
n1: number of plates counted in the first countable dilution.
n2: number of plates counted in the second countable dilution; d: first countable dilution.

3.7 Physical properties of the spray safe solution

3.7.1 pH

The pH measurements were made on the inside of 3 different points of the solution, using a digital Hanna pH-meter, as shown in figure 3.26.

Figure 3-26 Digital pH-meter Hanna

3.7.2 Colorimetric analysis

The external color was measured on the surface of the coating solution, performed with a digital camera using a focal length of 4.71 mm, aperture f/1.7, ISO 100 and exposure time of 1/389 seconds. A blank background was used, as well as a distance of 10 cm from the target. Natural light was used, and the blank was subtracted from the samples. To obtain the CIE Lab L* a* b* color coordinates the processed pictures were analyzed on Photoshop 2021 (Adobe Systems Incorporated, California, USA) within a grid that considered the average of 20 random points. In the coordinate system, L* represents the lightness, a* represents the redness (red-green), and b* represents the yellowness (yellow-blue).

3.7.3 Texture analysis

Texture analysis was achieved with a Stable Micro Systems' (Vienna Court, Godalming UK) TA.XT Plus Texture Analyses with a 30 Kg load cell, using the P/45 45 mm aluminum cylinder probe. A Texture Profile Analysis (TPA) was performed with 5 mm/s as the pre-and post-test speed, and 3 mm/s as the test speed. The texture of the coating solution was measured with different dilutions; 4%, 2%, 1%, 0.5% and 0,25% of kappa carrageenan. The target mode

was set to “strain” and causing 25% strain to the samples for 5 consecutive seconds, while the trigger was set to “force” and starting the measurement at 50 g of force. After the analysis, a macro was performed to measure various dimensions of texture, namely hardness, adhesiveness, springiness, cohesiveness, chewiness, and resilience. The texture results were achieved through the Exponent program, proprietary of Stable Micro Systems. The moisture variation was calculated based on water loss during the storage time by weighing the samples at the different storage times.

Figure 3-27 Stable Micro Systems’ (Vienna Court, Godalming UK) TA.XT Plus

3.7.4 Viscosity

Viscosity is defined as the resistance to flow. In a flowing fluid, shearing stress arises from friction between adjacent layers of fluid, which are sliding past each other. Viscosity was measured on a rotational viscosimeter B-ONE Plus (Lamy Rheology Instruments, Champ. au Mont d’Or, France) at room temperature and sheer rate of c.a. 90%.

3.7.5 Thermogravimetric analysis

The thermogravimetric analysis traces the evolution of the mass of the sample during a temperature rise. This measurement provides information about physical phenomena, such as phase transitions, absorption, adsorption, and desorption; as well as chemical phenomena including chemisorption, thermal decomposition, and solid-gas reactions (Carsalade, 2009).

3.7.5.1 Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) is used to study the structure and behavior of materials when they are subjected to a thermal cycle. It allows understanding the temperatures of transition, fusion, and crystallization, specific heat, rate of crystallinity of polymers, and their thermal conductivity (Osa, 2005).

In order to make the DSC test, the DSC 204 F! Phoenix equipment of Netzsc (Selb, Germany) was employs, 5-10 mg of spray safe solution were sealed in aluminum pans and subjected to a heating scan from - 75 to 200 °C at a heating rate of 10 °C min^{-1}. Glass transition temperature (Tg) was referred to the inflection point of the heat capacity change whereas the maximum of endothermic peak was settled as the melting temperature (Tm) being the area under the peak the melting enthalpy (ΔHm).

3.7.5.2 Thermogravimetric analysis (TGA)

The thermogravimetric analysis leads to the quantitative analysis of the variation in mass as a function of time and temperature. The principle is to continuously weigh a sample subjected to a temperature ramp. This characterization provides information on the kinetic aspects of chemical reactions, the mechanisms of degradation chemical compositions of a compound. The percentage of mass, as well as the derivative of the mass as a function of the temperature, are represented as a function of this temperature. The thermogravimetric analysis (TGA) was performed using a NETZSCH model TG209F3 Tarsus (Selb, Germany) equipment.

3.8 Texture reformulation

To improve the texture of the coating solution, another polymer was used, namely “kappa” carrageenan, which has different textural properties to iota carrageenan, the original polymer used for the Spray Safe solution

Code	Iota (mg/100mL)	Run	Kapa (mg/100mL)	$CaCl_2$ (mg/100 mL)	Glycerol (mL)
STD-1	1000	1	0	0	7,5
STD-2	1000	2	125	50	3,5
STD-3	1000	3	375	0	1,5
STD-4	1000	4	63	25	5,5
STD-5	1000	5	0	150	1,5
STD-6	1000	6	250	25	2,5
STD-7	1000	7	188	75	1,5
STD-8	1000	8	0	75	4,5
STD-9	1000	9	63	100	2,5
STD-10	1000	10	188	0	4,5
STD-11	1000	11	0	0	7,5
STD-12	1000	12	375	0	1,5
STD-13	1000	13	0	150	1,5
STD-14	1000	14	188	75	1,5

Table 3-1 The different preparations to improve the texture of the coating solution

For the reformulation assay, a simplex centroid mixture designed was performed in a random order. The design was created on Design Expert software 11.1.2 (Stat-Ease Minneapolis, USA) employing 3 factors X_1: κ-carrageenan, X_2: Calcium chloride and X_3: Glycerol, and up to 5 levels depending on the analysis (Table 3.1)

3.9 Statistical analysis

Throughout the document, all values are represented as mean ± standard deviation (SD), and all analyses are carried out using a significance level of 0.05. To aid the interpretation of the results, the data were transformed when needed and treated through different statistical analysis such as 2-way analysis of variance (ANOVA) for multiple comparison or T-test for comparison of two samples using Prism 9 (GraphPad Software, San Diego CA, USA), while the correlation analysis and its graphical representation was performed on R studio 1.4.1106 (R Studio, Boston MA, USA) using the libraries corrplot and RColorBrewer.

For the mixture design, a comparison was made between the different samples through simple analysis of variance (ANOVA) using F-test, together with the Fisher's Least Significant Difference test, using the Design expert and Statgraphics Centurion XVI software (StatPoint Technologies, Inc. Warrenton, VA, USA) software.

4 Results

4.1 Solvent screening of the *R. officinalis L* extract

The solvents employed for the different extractions of bioactive compounds will yield diverse profiling of the same bioactive compounds embedded within the matrix analyzed, this divergence relies on the chemical properties of the solvents, specifically on the polarity of them, therefore, it is important to make various considerations for selecting the proper solvent of extraction. Selection dilemmas will always be present, and the researcher must consider and test as many as possible to understand the final outputs and select not only the best yielding solvent but also examine the cost/benefit, toxicity, availability, etc.

This thesis focuses on the monitorization of properties and optimization of a new product conceived as a Food Contact Material (FCM) which intends to partially replace the plastic film in the food packaging industries. Briefly, the composition of this new product named "Spray Safe" is constituted by GRAS labeled 100 % natural ingredients, and the composition incorporates a polymeric base, bioactive molecules, and texturizing ingredients.

Within the bioactive molecules, the extraction of *R. officinalis L* was selected due to the performance of the rosmarinic acid as an antioxidant compound, although, according to the previous analysis performed by Carocho et al. (2019), maceration on the water was arbitrarily chosen, therefore, an ethanolic extraction seemed necessary to discard better bioactive performance from another solvent that it is employed usually.

In figure 4-1A a representation of the scavenging activity of DPPH is displayed, from both EC_{50} values of the rosemary with aqueous and ethanolic solvents. The aqueous extraction showed an EC_{50} mean value of 4.849 mg/mL, exceeding the performance of the ethanolic extraction (7.787) by almost 0.5 fold lower and exposing a significant P value of 0.0181 of the total 18 values analyzed. It is worth noting at this point those lower values of EC_{50} correspond to a better performance, in this case of the extract antioxidant activity.

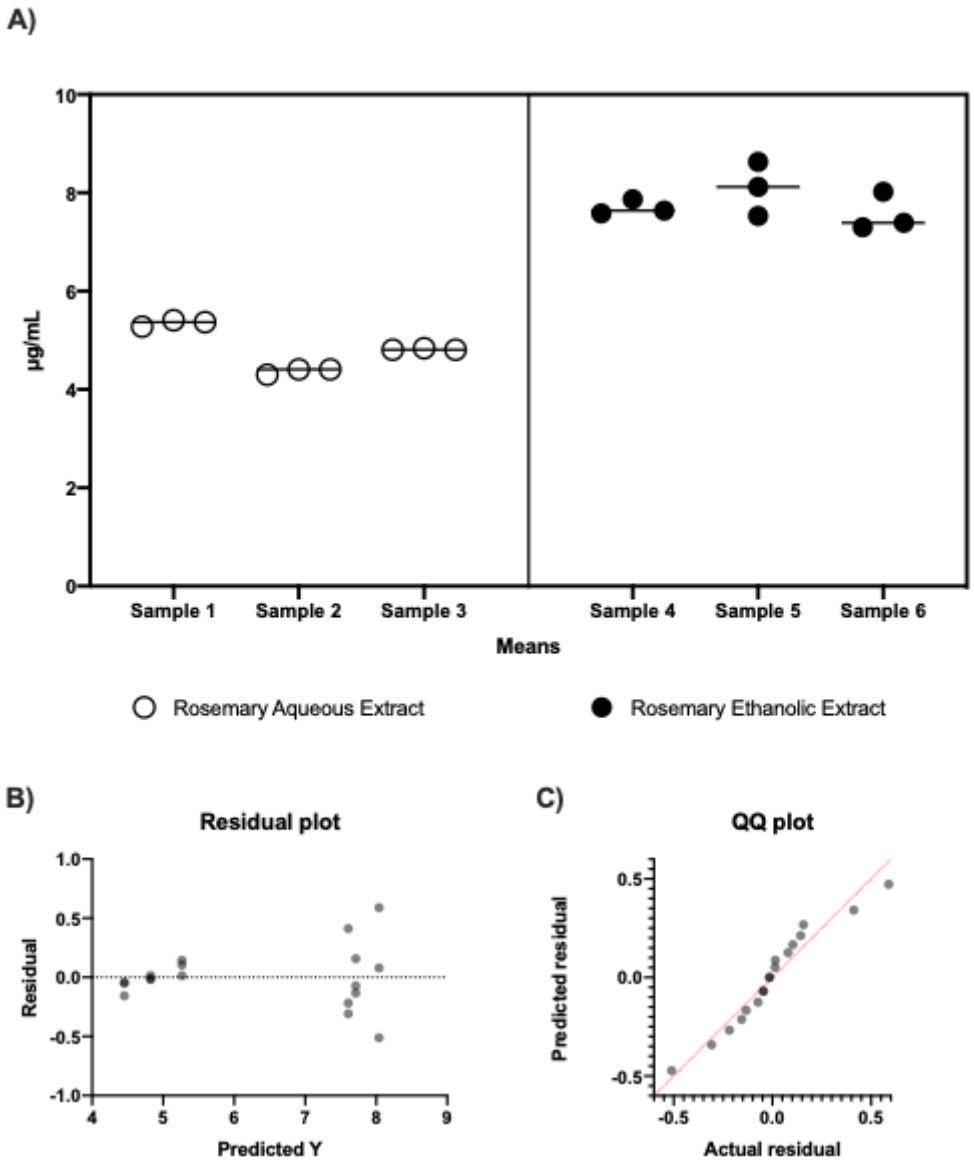

Figure 4-1 DPPH EC_{50} Aqueous vs Ethanolic Rosemary Extraction

The rest of the graphical representations on figures 4-1B and 4-1C, illustrate first the residuals of the data, where there is no abnormal variability and it is approximately the same across the chart, showing no systematic curvature or nothing indicating non-normality, and a fairly random scattering of points where variability is lower within the first 3 samples on the mean range of 4.8. Finally, the last graphical representation of the quantile-quantile plot of residuals, confirmed the normal distribution of points that results in an approximately straight-line, therefore the residual plots do not indicate any problems with our assumed model. Perhaps the little variability on the last 3 samples with a mean of 7.7 could be thought of as a heteroscedasticity trend, although analyzing all the data points we decided to ignore it due to the low number of points and due to the spread created mainly on sample 5.

Therefore, after comparing the DPPH EC_{50} values of both extracts, the suggestion of continuing with aqueous infusion was still recommended since positive significant differences were encountered, specifically for this antioxidant bioactivity, nevertheless, the decision is not considering energetic or equipment tolls, which could switch the final decision based on time or economic issues.

4.2 Antioxidant effect of the Spray Safe ingredients

To understand the composition of the newly developed product, assessment on their antioxidant activity was determined as described in the methodology section, testing all the individual components and their interaction, and as shown in figure 4-2, comparing their DPPH EC_{50} values throughout graphical representation backed up by the one-way ANOVA with multiple comparisons statistical test.

First, it has to be stated that antioxidant activity of ι-carrageenan, calcium chloride, and glycerol was also measured, but these compounds did not show any antioxidant activity, therefore, the active compounds are the only ones presented with their respective combination, and also, although "a+b+c" is bioactive, it has to disclosed that in the latter, all the ingredients of the solution are present, while in the other, just the combination of the 3 antioxidants is present. For every ingredient or combination, equal ratios of preparation according to the original formulation of Spray Safe were followed.

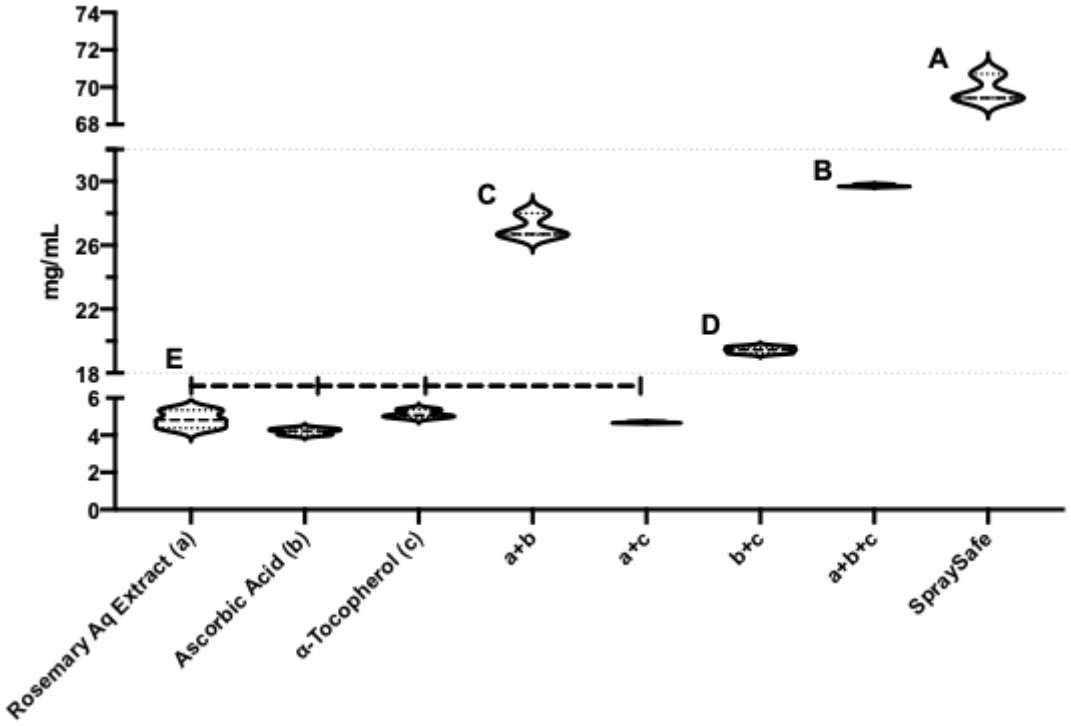

Figure 4-2 EC_{50} Spray Safe Ingredients

Surprisingly the isolated ingredients performed better as antioxidant compounds and the results on the mixtures showed antagonistic behavior, except for the rosemary extract and α-tocopherol combination. It is well known that not only plants are major contributors of exogenous antioxidants, that act as a defense system against reactive species and oxidative challenges, but also that combination of compounds such as ascorbic acid and α-tocopherol work synergically. First, both vitamins possess different solubility and while vitamin C is highly soluble in water, vitamin E is liposoluble, thus their pattern of transport, excretion, action, and storage, behave differently. Second, while tocopherol acts as an antioxidant by donating a hydrogen atom to ROO•, ascorbic acid reacts as an optimal polyunsaturated fatty acid protector. Consequently, a synergistic effect was expected on the graph combination "b+c", but this effect did not occur.

Nonetheless, antioxidants are considered "double-edge swords", their type, dosage, and matrices are determining factors impacting the balance between the beneficial (antioxidant) or deleterious (pro-oxidant) effects (Paramasivam et al., 2012) therefore, a pro-oxidant effect could be attributed to the combinations "a+b" and "b+c", confirmed with the greater EC_{50} of "a+b+c". Although to provide a statement of this magnitude, diverse antioxidant properties evaluation must be performed, considering both, single (Hydrogen Atom Transfer (HAT),

single electron transfer (SET), radical adduct formation (RAF), etc) and multiple-step mechanisms (sequential proton loss electron transfer (SPLET), single electron transfer (SEPT), SPLHAT) analytic procedures.

The other interesting results are shown in graph 4-2, in which the EC_{50} value of Spray Safe is higher than the combination "a+b+c", it is interesting to observe this effect that confirmed that antioxidants get protected within the polymeric-salt complex formed, which could benefit the product protecting and extending the antioxidant effect once applied. If the antioxidants get attached within the polymeric complex, then it will be also interesting to further understand this phenomenon and be directed towards our defined purposes.

Finally, it is also interesting to acknowledge that every single bioactive compound employed in the formula, possess similar scavenging performance and that for future directions, it will be important to test diverse ratios of every single one to optimize the effect of them finding the best synergistic points to be considered for the reformulation of the Spray Safe coating.

4.3 Antioxidant chemical mixed-mode (ET/HAT) vs cell-based assays

Due to the unexpected results obtained in the previous section, quick verification of antioxidant activity was tested on a cell-based methodology (TBARS), in figure 4-3 a comparison of the antioxidant activity expressed in EC_{50} value is shown, on the left, sample 1-3 belongs to DPPH, and on the right, sample 4-6 to TBARS methodologies. DPPH measurements accounted for a mean value of 71.84, a third fold higher than TBARS with a mean value of 54.62 μg/mL. This difference of 17.22 ± 11.07 is not large enough to show significant differences among both assays with a *p*-value of 0.1948 on the two-tailed t-test, although it is planned to verify the behavior of the individual compounds and their mixtures in at least 2 more analytical procedures for measuring their antioxidant capacity differences in future tests.

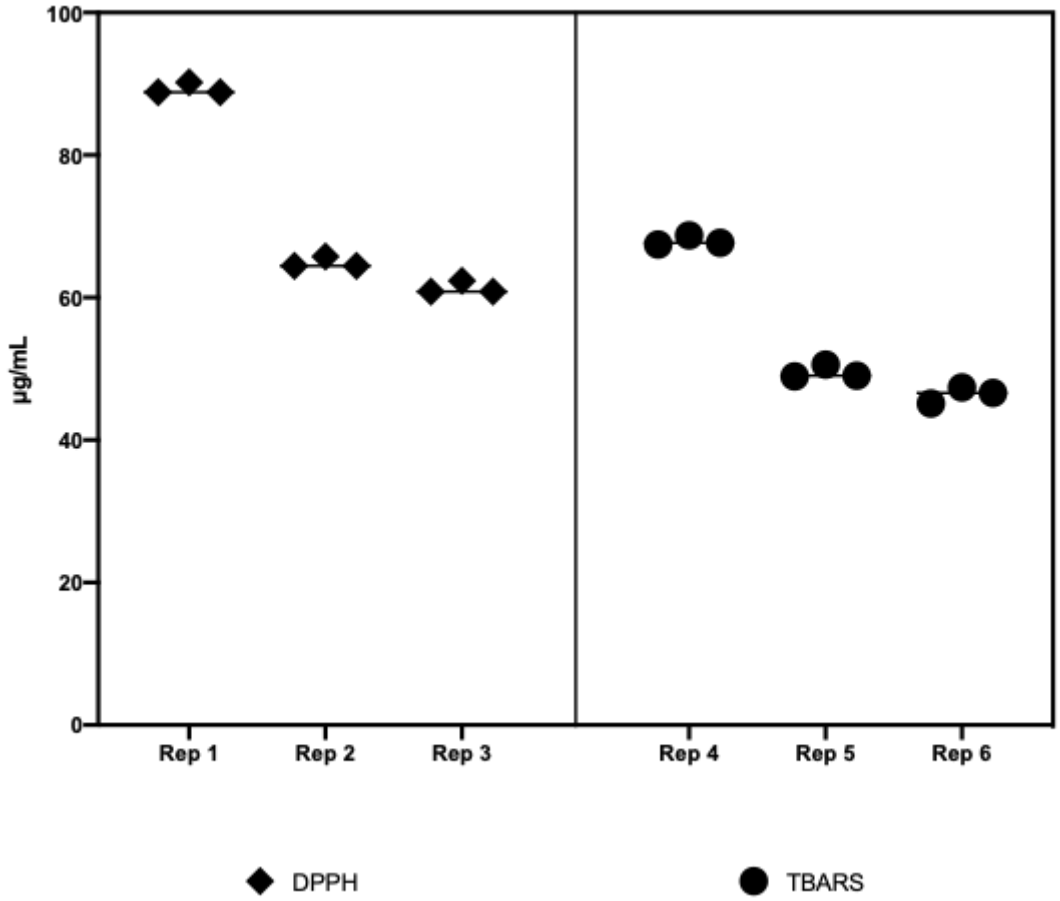

Figure 4-3 Spray Safe EC_{50} values on chemical vs cellular assays

4.4 Spray Safe antimicrobial activity against specific pathogenic strains

To understand the antioxidant properties of the Spray Safe ingredients, an intentional contamination assay was performed with 4 common pathogenic microorganisms: *Bacillus cereus* (bacteria Gram +), *Escherichia coli* (bacteria Gram -), *Zygosaccharomyces rouxxi* (yeast), and *Aspergillus parasiticus* (mold).

In figure 4-4, a global comparison of the original sample without contamination (outer left row) vs contaminated samples (rows 2,3,4,5) is presented. Axis "Y" belongs to the elapsed days of the test while axis "X" displays the different treatments and the second "Y" axis displays the logarithmic growth(+)/inhibition (-). Data shown followed 2 transformations, first, the number of colony-forming units (CFU/g) was converted to logarithmic scale for numerical reduction and representation according to Eq 4.1, then, starting from the initial inoculum the percentage of reduction was calculated according to Eq 4.2.

$$\text{Log Reduction} = \log10\ (A/B) \qquad \text{Eq 4.1}$$

$$\text{Percent Reduction} = (A-B) * 100 / A \qquad \text{Eq 4.2}$$

Column “0 days elapsed” has been normalized to 0 to quantify growth (red color) or inhibition (green color), where color intensity represents magnitude. Thus, it is worth noting that Spray Safe did not show efficiency against yeast but was effective against bacteria and mold. Ordered from the highest to the lowest inhibition effect, these were respectively *E. coli* (3.15 log reduction or 99.93%), *A. parasiticus* (2.4 log or 99.6%), and *B. cereus* (0.84 log or 85.64%) at the end of 16 days trial period. The growth in the first two days of *E. coli* is interesting because if the Spray Safe product was applied on a highly contaminated sample within the span of a couple of days, Spray Safe will not show any improvement unless the contact with the contaminated product would extend the contact period over 5 days. Similarly, *B. cereus* starts acting as microbiocidal on the 5th day while in the days before it behaves as a microbiostatic, therefore, the Spray Safe solution would be suitable for any product with a low concentration of this microorganism since the moment of application. Finally, for *A. parasiticus* the Spray Safe solution behaves as a great fungicidal and could be reliable from the moment of application.

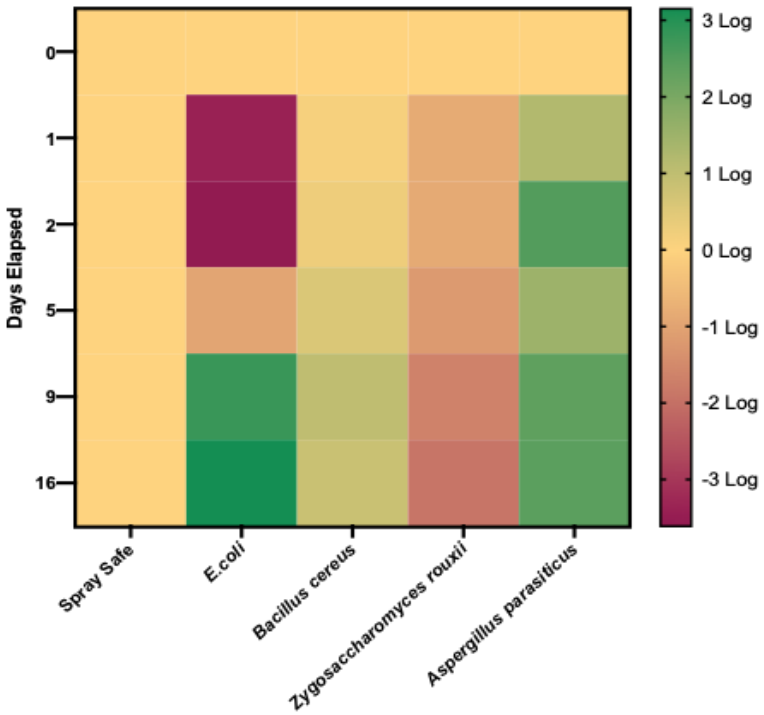

Figure 4-4 Applied antimicrobial effect of Spray Safe against certain pathogenic strains

Future changes on the antimicrobial compounds should verify the efficiency against yeast such as *Z. rouxii* and should extend the number of pathogenic strains to be tested. Meanwhile, the current formulation has proved to display good antimicrobial attributes on 3 out of 4 tested strains.

4.5 Spray Safe shelf-life

After testing both antioxidant and antimicrobial bioactivities on the Spray Safe solution, a screening assay with the duration of 6 months was carried out. This technology, still in the validation stage before starting the market tasting phase, requires shelf-life tests to understand the bioactive molecule degradation hypothesis. Thus, these tests focused on temperature (refrigeration and room temperature) and light exposition (protected and unprotected).

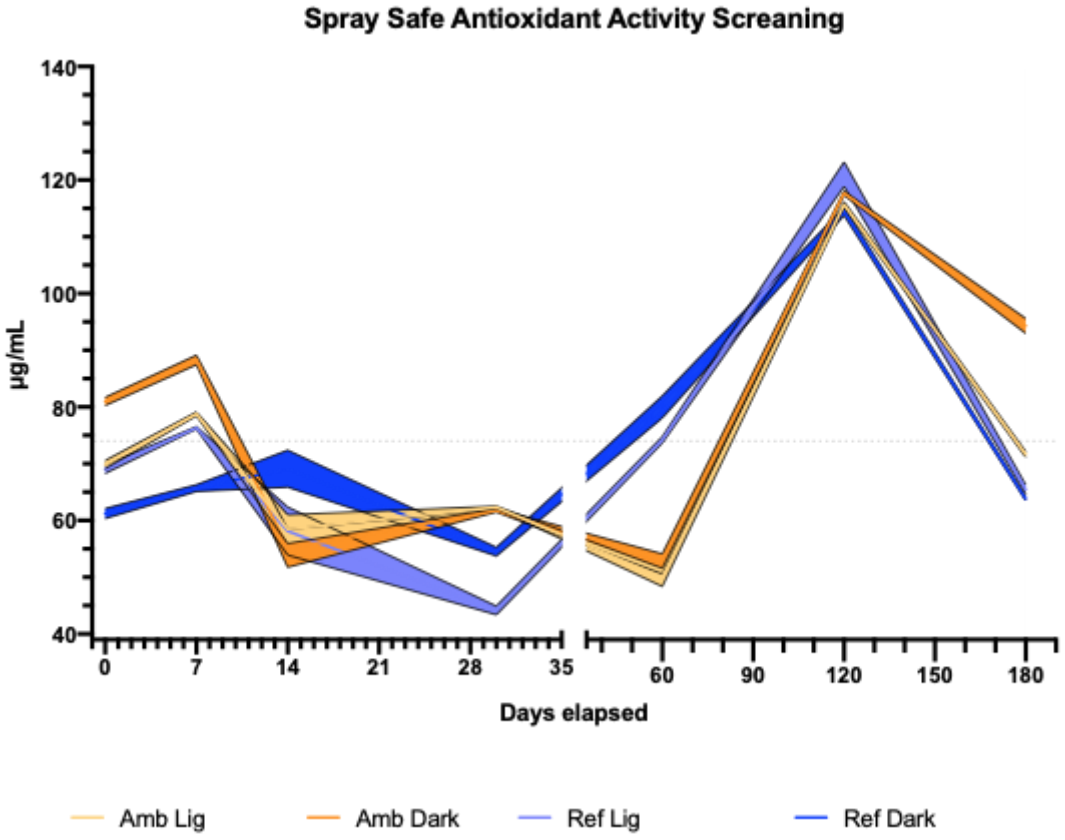

Figure 4-5 Shelf-life graphical representation at the 4 different conditions

In figure 4-5 a representation of the values monitored at different times for antioxidant activity is displayed, although, instead of graphing the averages on their own, the positive and

negative standard deviation shown in the same line, therefore, width varies through their ranges. The highest deviation was observed in the third time (14 days) and the rest of the times behaved similarly. There is no specific pattern along the experiment in any condition tested, with values going up and down randomly. Further analysis to find the cause of this are expected to start shortly, as well as testing other conditions for estimation of a shelf-life.

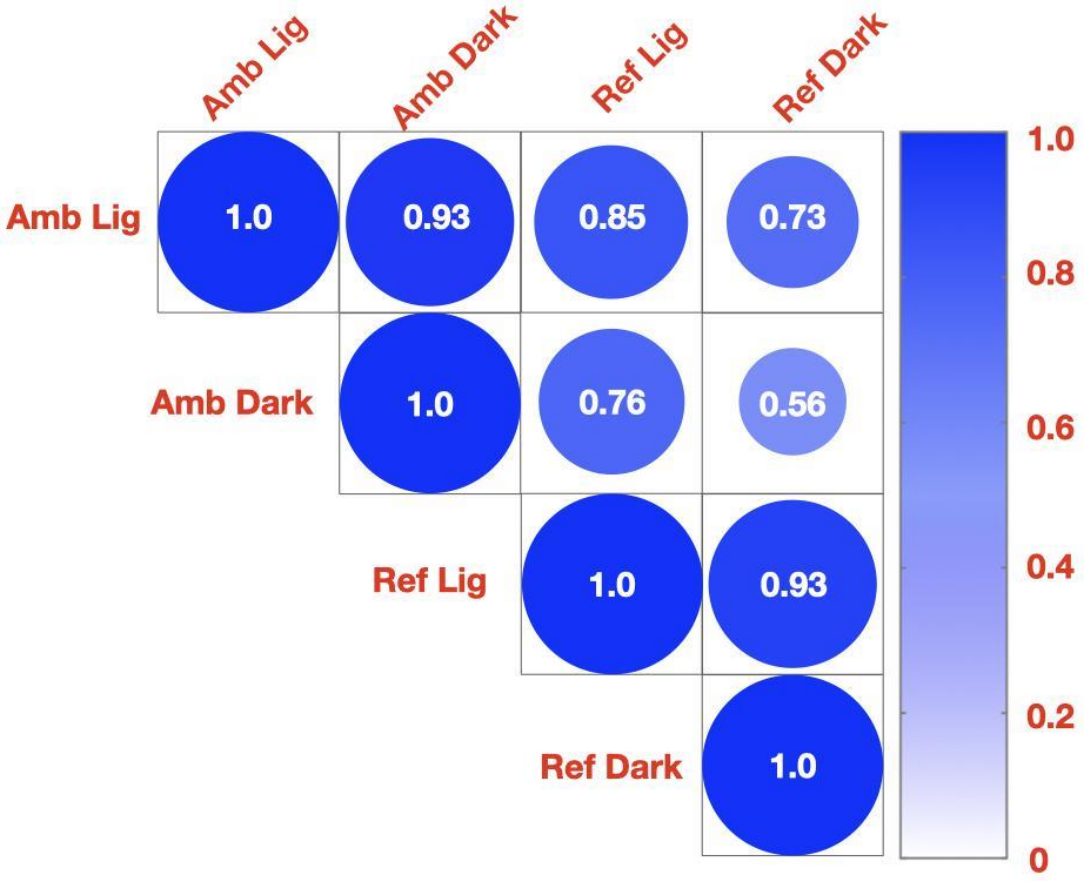

Figure 4-6 Correlation analysis of the conditions tested along the different times tested

Therefore, to extract as much information as possible, a correlation analysis was done between the different treatments, presented in figure 4-6. It paired treatments higher according to temperature rather than light protection. When comparing ambient dark vs refrigeration dark, the correlation coefficient was 0.56, the lowest value in the whole correlation, and in the other end of the analysis, when comparing the sample temperature, the correlation value raised to 0.93 in both cases, therefore, it is important to focus more on temperature rather than light protection.

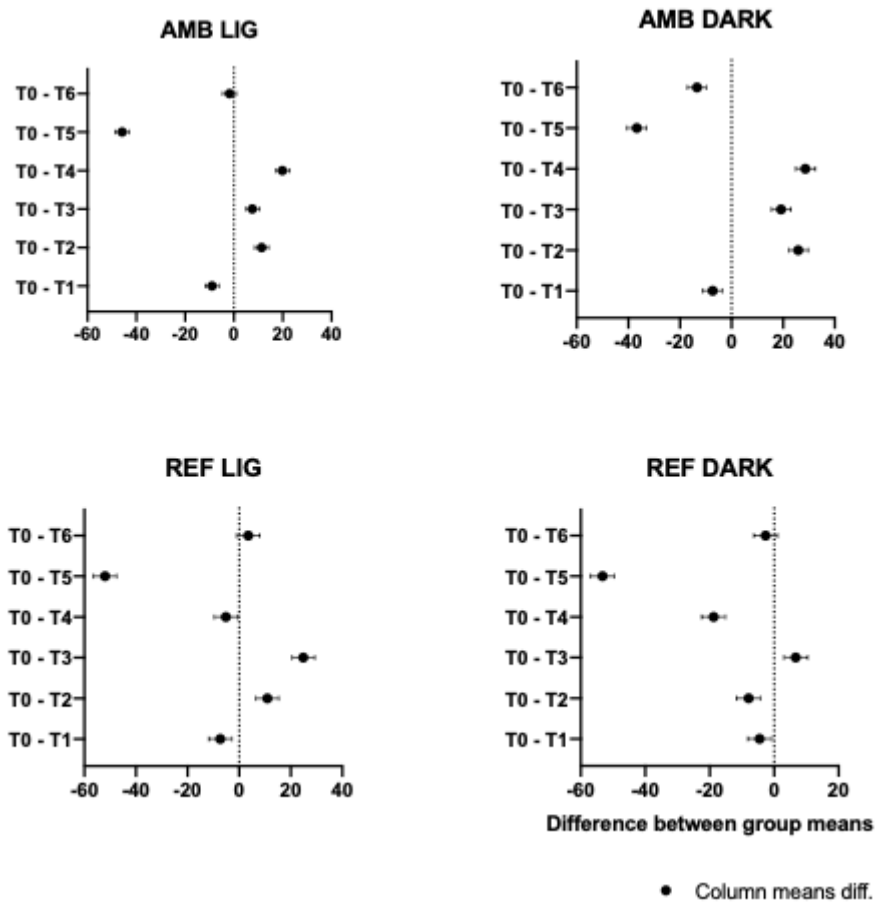

Figure 4-7 Comparison of group means at the different times tested

Finally, on the antioxidant activity shelf-life analysis, an ordinary one-way ANOVA was performed and analyzed on multiple comparisons, displayed in figure 4-7, although, due to the intra-variability a decision to focus on the comparison of T0 and the final time T6 was made. No significant differences were sought in any treatment, except for ambient dark, which increased around 14 μg/mL at the end of the 6 months.

Following the same time pattern, microbiologic tests were performed as described in the methodology to understand possible growth of microorganisms, nevertheless, until the 6th month, no growth was spotted in any treatment or sample. Thus, the preparation of the Spray Safe can be considered innocuous and the timespan of six months tested could guarantee the integrity and efficiency of the bioactivities of the coating product, and from the economical point of view the packaging and conservation methodology of Spray Safe, the recommendation for this formulation would be the storage of the product at room temperature without the need of a special type of protection against light, however, since bioactive molecules could react on light stress, it would be advisable to employ at least amber packaging, since the condition tested

on the laboratory where for a small volume of sample and with and unique source of artificial light.

4.6 Spray Safe thermogravimetric and texture properties

As a new product, Spray Safe was lacking depth description of its inner properties other than bioactive activity, thus, to explore further characteristics of the new product, thermogravimetric such as Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA) were performed, complemented with rheological properties such as viscosity and texture properties such as firmness, consistency, and cohesiveness, plus pH and colorimetric determinations (Table 4-1).

Properties	Values	Units	Analytic methods
Dynamic viscosity	**201**	**mPa.s (25°C)**	**Rotational viscosity**
pH	**9.18**	**22.75 °C**	**AOAC 981.12**
Glass Transition	**N/A**	**°C**	**DSC**
Cold crystallization	**100.9**	**°C**	**DSC**
Polymer decomposition	**83.9**	**°C**	**TGA**
T max	**236**	**°C**	**TGA**
Shelf-life	**6**	**Months**	**Antioxidant and antimicrobial assays**
Color L* parameter	**70**	**-**	**CIE Lab colorimetry**
Color a* parameter	**-7**	**-**	
Color b* parameter	**79**	**-**	
Firmness	**34.16**	**g**	**Back extrusion**
Consistency	**234.4**	**g.s**	
Cohesiveness	**-19.38**	**g**	
Index of viscosity	**61.6**	**g.s**	

Table 4-1 Physico-chemical properties of Spray Safe Solution

The final and homogeneous dissolution of Spray Safe presented an average pH of 9.18 at 22.75 °C ± 0.49, which fits within the basic pH ranges, just 2 units above neutrality. While colorimetric parameters registered were reported on CIE L*a*b* coordinates with an average luminosity of 70 and values of -7 and 79 for coordinates a* and b* respectively. If this coloration wanted to be replicated, it would be necessary to mix the component red on the

intensity of 185, green on 173 and absence of the blue component as shown in figure 4-8, the result of this coloration came mainly from the extract and α-tocopherol. The dynamic viscosity of the Spray Solution was 201 mPa.s (25 °C, 50 rpm, and 88.9% torque gage), and through the solution properties, Spray Safe can be defined as a pseudoplastic non-Newtonian fluid at room temperature.

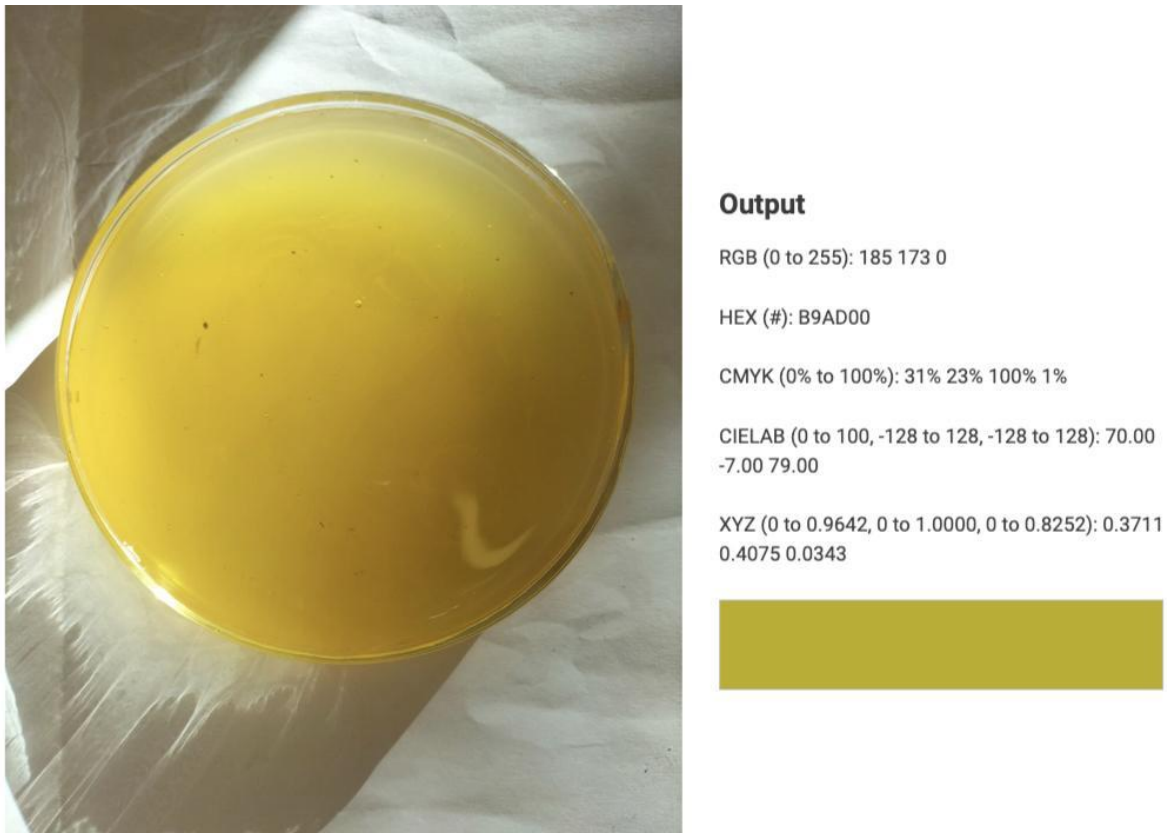

Figure 4-8 Visual appearance of Spray Safe solution and colorimetric parameters

In table 4-1 and figures 4-9 and 4-10, the main thermic attributes are summarized from both DSC and TGA analysis. From the differential scanning calorimetry, only cold crystallization was spotted in figure 9, displaying a temperature of 100.9 °C, normally the glass transition is spotted just before the crystallization peak, although, the crystallization area is too broad and might overlap this parameter.

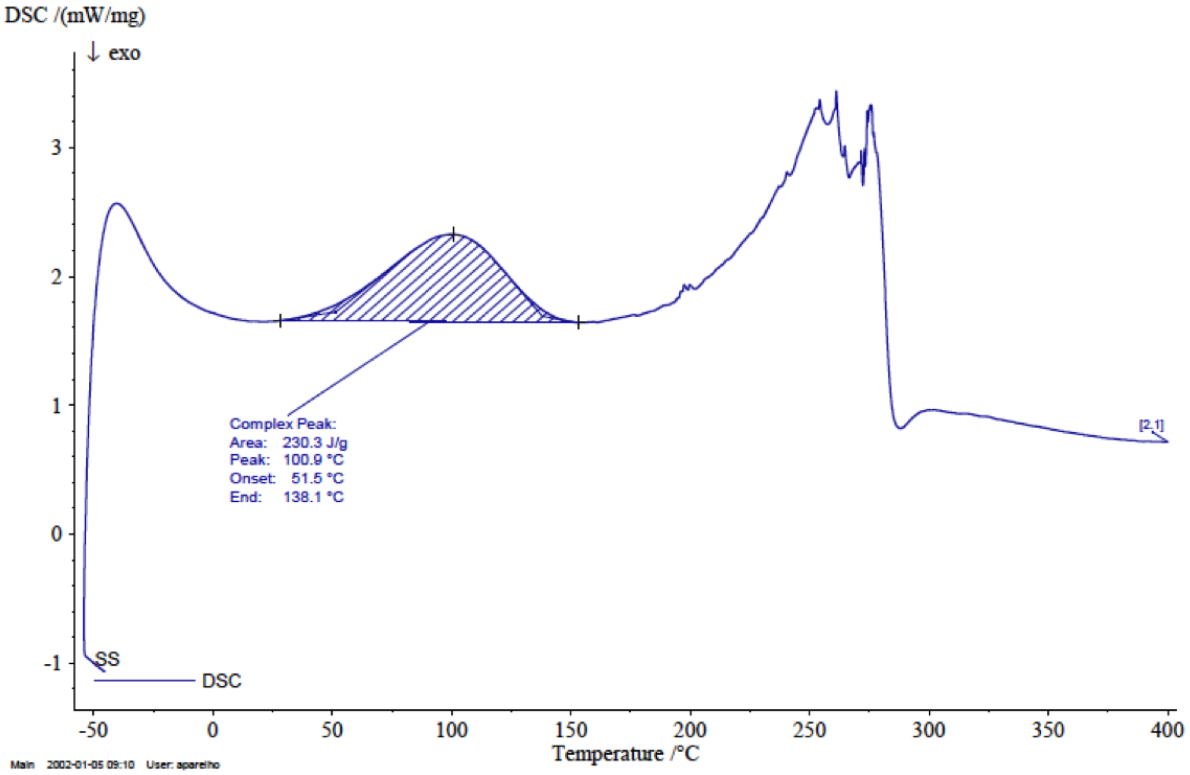

Figure 4-9 Differential Scanning calorimetry graph of Spray Safe

Then in figure 4-9 in the red line 2 important parameters can be observed, the starting point of polymer decomposition at 83.9 °C, just before the crystallization point, and then 236.6 °C the lowest inverse peak is shown, which by the percentage of mass change reflected on the green line, we observed a decrease of 55.03% of mass change, this point provide to us the information that the polymer has completely modify its properties and has to reach its total decomposition. Therefore, to avoid damage to the polymeric mixture, the temperatures employed do not have to exceed 83.9 °C.

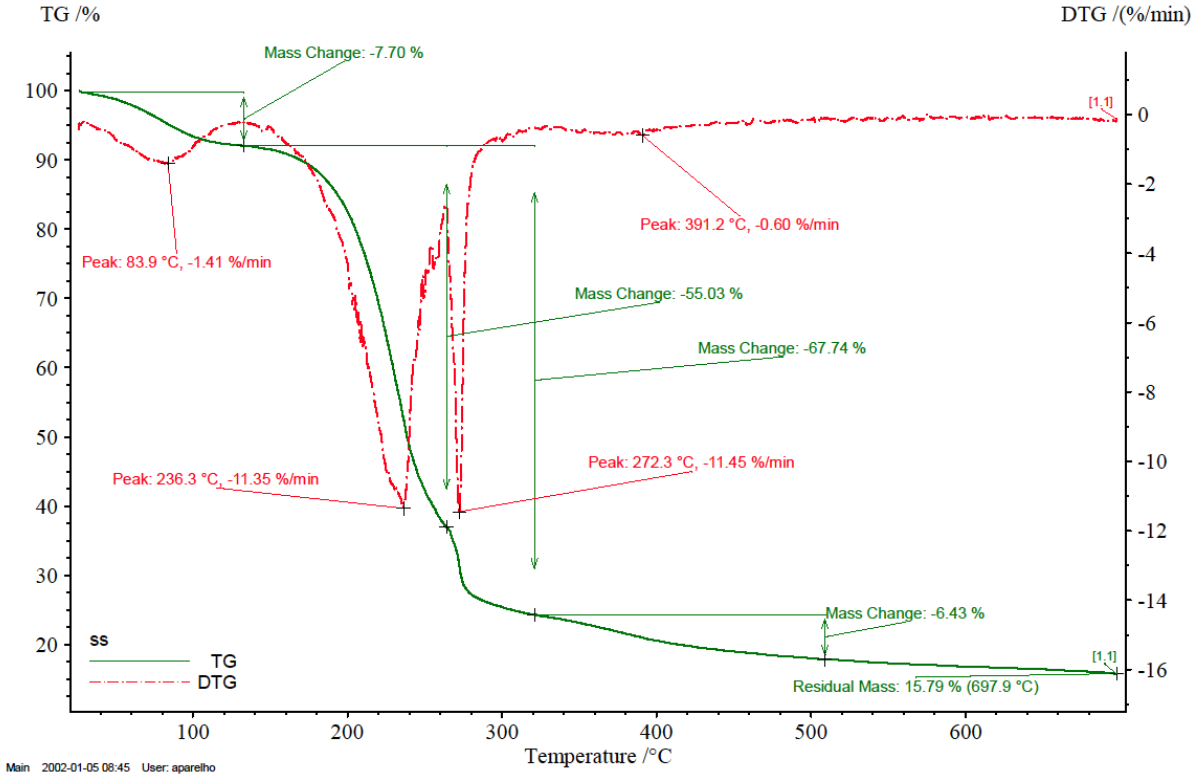

Figure 4-10 Thermogravimetric analysis graph of Spray Safe

Finally, texture properties were also analyzed at 4 °C to understand the flux behavior of the solution and as reported in table 4.1 and shown in figure 4-10, the solution reported a firmness of 34.16 g that is the highest force applied to the sample, hence, the highest point of the positive area on the graph, then consistency was reported as the mean of the pressure applied during the whole positive area, displaying a result of 234.4 g/s. This texture analysis consists of two parts, penetration, which measured the positive areas of the force applied, and removal, which measured the negative areas of the force necessary to release the probe from the sample and measures 2 parameters, cohesiveness, the opposite value reference of firmness with a value of -19.38 g and index of viscosity with a value of -61.6 g/s.

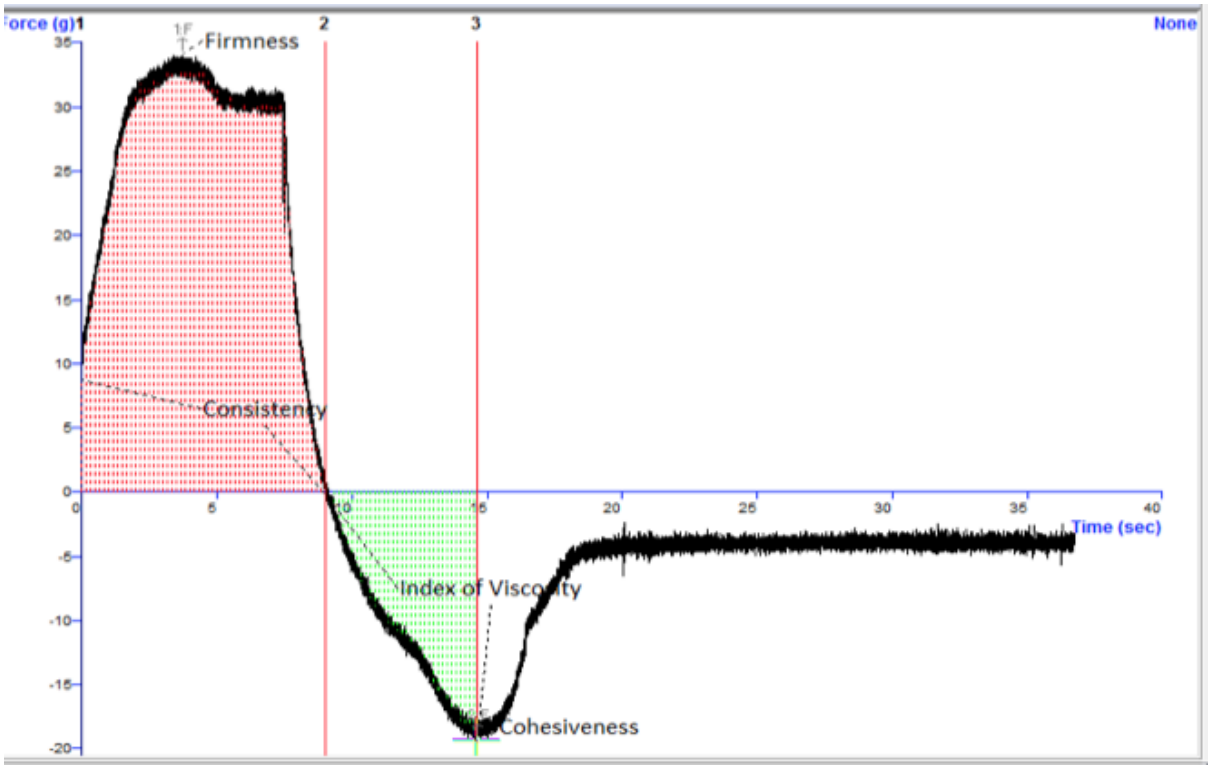

Figure 4-11 Texture properties of Spray Safe

The main reason for measuring texture at refrigeration temperature to the physicochemical properties of Spray Safe changing to a smooth gel-like solution with higher consistency and because the main application of the product is for use at refrigeration temperatures. Although the properties of the product were given at room temperature due to being the temperature of application, handling and storage (of the solution), texture was given at refrigeration temperature which will be the temperature of its use when applied.

4.7 Texture reformulation of the original Spray Safe formulation

One of the most important parameters of the production, conservation, and applications is the texture of the Spray Safe solution, therefore, a first broad exploration was performed on the solution at room temperature to identify the firmness properties (Y1) of every compound and at the same time, explore the addition of a carrageenan isomer (kappa). The analysis design consisted of a simplex centroid mixture design shown in Table 4-2. According to previous experiments, the selection of levels was made in the following way, levels 0 and 1 were for κ-carrageenan from 0 to 3.75 g/L, calcium chloride 0 to 1.50 g/L, and glycerol 15 to 75 mL/L, maintaining ι-carrageenan always constant at 10 g/L in every run.

Run	Space type	κ-carrageenan (mg/100mL) X1	Calcium Chloride (mg/100mL) X2	Glycerol (mL/100mL) X3	Firmness (g) Y1
12	Vertex	0.0	0.0	7.5	0.58
1	Vertex	375.0	0.0	1.5	1.26
10	Vertex	0.0	150.0	1.5	209.62
3	Vertex	0.0	0.0	7.5	0.67
13	Vertex	375.0	0.0	1.5	1.42
11	Vertex	0.0	150.0	1.5	266.14
2	CentEdge	187.5	75.0	1.5	9.12
6	CentEdge	0.0	75.0	4.5	6.8
4	CentEdge	187.5	0.0	4.5	0.73
7	CentEdge	187.5	75.0	1.5	10.41
14	AxialCB	62.5	25.0	5.5	1.85
8	AxialCB	250.0	25.0	2.5	1.78
9	AxialCB	62.5	100.0	2.5	8.91
5	Center	125.0	50.0	3.5	2.87

Table 4-2 Chronological order of runs of the mixture design

Graphical representation of the firmness result can be observed in figure 4-11, although table 4-2 already shown in the vertex runs 10 and 11 the higher results for firmness, proving that this response is almost 100 percent dependent on the factor X2 (calcium chloride), as the X2 increased its level of magnitude, the growth is almost exponential, displaying a 200-fold increase (c.a. 23,000%) from the lowest to the highest level. The original content of X2 in the Spray Safe solution was established as 1 g/L and in this experiment, as far as 1.5 g/L was tested. Surprisingly, κ-carrageenan did not show an important change on the Y1 attribute but its properties at different temperatures should not be overlooked, and thus being analyzed in subsequent assays. Perhaps the huge increase of X2 also shadows the firmness effect of X1, thus being important to test different ranges and conditions.

Model	90258.36	4	22564.59	37.79	< 0.0001	significant
[(1)]Linear Mixture	59182.05	2	29591.02	49.55	< 0.0001	
AB	17774.69	1	17774.69	29.76	0.0004	
BC	13236.01	1	13236.01	22.16	0.0011	
Residual	5374.53	9	597.17			
Lack of Fit	3776.42	5	755.28	1.89	0.2785	not significant
Pure Error	1598.1	4	399.53			
Cor Total	95632.89	13				
Std. Dev.	24.44		**R^2**	0.9438		
Mean	37.3		**Adjusted R^2**	0.9188		
C.V. %	65.52		**Predicted R^2**	0.8726		
			Adeq Precision	16.9417		

Table 4-3 Statistical terms of the mixture analysis

In table 4-3 the statistical approach of the experiment is shown after excluding irrelevant terms (AC). It is clear that the obtained model fit is reliable, since the term and the model are significant and the lack of fit was higher than 0.05, also showing a high (0.9438) coefficient of determination. Therefore, from the built model, the coefficient terms were estimated and the final equation for firmness is displayed in Eq 4.3.

Firmness = 7.42*κ-carrageenan + 229.31*Calcium Chloride + 6.93*Glicerol - 437.77*κ-carrageenan*Calcium Chloride - 450*Calcium Chloride*Glicerol Eq 4.3

Once the statistics are correct and the equation was built, it becomes handy to make a 3D graphical representation as shown in figure 4-12, employing three X-axis, (a representation of an acute triangle (equilateral) is drawn, where every axis starts at the middle of the base (level 0) and with termination at the vertex angle (level 1) confronting with the response obtained in the Y1 axis. Besides, attributing red color to lower and green to higher values, the final visual representation is easier to understand.

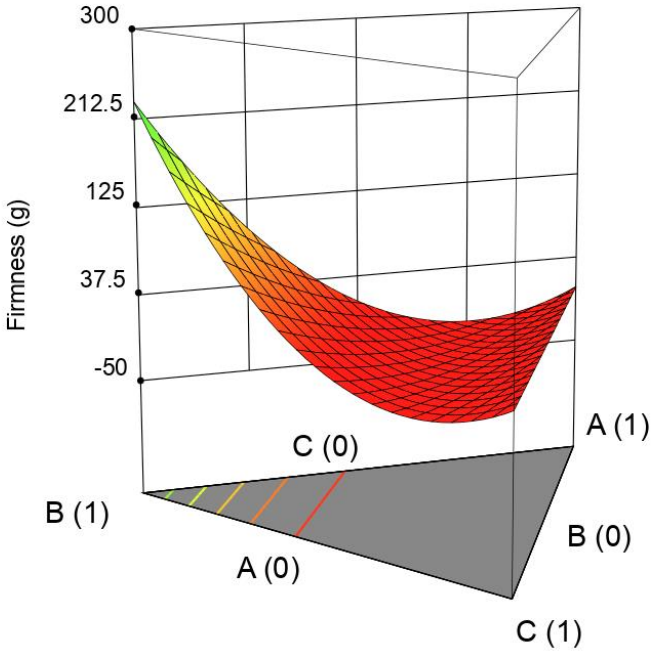

Figure 4-12 Graphical representation of the data from the simplex centroid design on Firmness (factors A, B, and C, belong to κ-carrageenan, calcium chloride, and glycerol respectively)

Finally, with the data and Eq 3 obtained, the optimization of the response could be done and tested on the wanted values (minimum, maximum, or target), although this first approach did not intend to optimize, but to understand the behavior of the formulation components. Future work will be directed towards specific values and parameters to properly optimize the Spray Safe formulation according to the temperature required.

5 Conclusions and future perspectives

The Spray Safe solution is an innovative formulation that intends to aid the food coating sector, helping to replace the use of high amounts of plastic and in this way minimize the effects of pollutants produced by retailers, by means of a biodegradable film which also possesses bioactive attributes.

The solution displays overall antioxidant and antimicrobial properties which will help in the storage of products protected with the product, although, the antioxidant results suggest that improvement of the formulation against yeast and optimization of bioactive ingredients must be performed to maximize the effect while reducing costs.

Bioactive stability was identified on the shelf-life analysis, although further physicochemical properties should also be analyzed in the future, to ensure all the expected properties of the product, namely texture on the different ranges of temperature, possible aromatic interferences due to the extract, among others.

The aqueous extraction is proven to be more effective than ethanolic extraction which at the same time helps reduce extraction solvents costs.

Both antioxidant assays tested proved to display no significant differences among them, therefore, these could be employed arbitrarily while analyzing Spray Safe dissolution, although, to warrant the effect, different methodologies must be tested.

Finally, the information compiled on the polymeric composition of the product has just scratched the surface of a deep knowledge that has to be generated to optimize its usability. Therefore, with the data acquired and the techniques learned, further exploration of synergistic effects and polymer interaction must be performed.

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