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Nanocellulose films activated with essential oils for active packaging applications

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Abstract

Due to the extremely high consumption of packed foods and the strong environmental impact of the traditional plastic materials used, in the last years there is a strong interest in the research on biodegradable and natural systems to be used in the active packaging field. For this reason, this study was focused on the analysis of the state of the art regarding the active packaging and on the development of an innovative active packaging system based on the use of nanocellulose matrix embedded with essential oils.

After detailed research aimed to deeply know the topic of interest and select the best biopolymer and active agents, the solubility and diffusivity of thyme, cinnamon and oregano essential oils in three nanocellulose (NFC) films, endowed with different carboxymethylation degree, were analysed. Solubility and diffusion of liquid essential oils resulted to increase with the carboxymethylation degree for both thyme and oregano. Cinnamon, on the other hand, partially dissolved the nanocellulose matrix, making it impossible to obtain clear data about its uptake in the different materials. Diffusivity was studied both in liquid sorption and vapor desorption experiments. The release of the oils in vapor phase was characterized by two different kinetics: a faster one dominating at short times and a slower one visible at long times. Fickian diffusion was able to describe the behaviour of most of the samples even if in some case (such as for Oregano), short time data were better fitted by using an exponential process. Also in desorption, the carboxymethylation degree results to affect the obtained results, suggesting that the release of antimicrobial essential oils from nanocellulose can be tuned by appropriate choice of the carboxymethylation degree of the material.

Then, the antimicrobial and antioxidant activity of those films was also analyzed. These experiments have been performed in two sequential experimental phases. Firstly, the activity against model pathogenic bacteria was tested and the minimum inhibitory concentration (MIC) of each oil was determined (0.37 – 0.68 mg/mg of matrix). This initial validation was then followed by experimental settings aimed at testing the system directly on clamshell type packed raspberries. The tests on the bacterial strains were based on the use of microbiological agar plates and showed that the dimensions of the nanocellulose films and the quantity of essential oil have a strong influence on the antimicrobial effect. For all the oils, the major effect was demonstrated against the *Staphylococcus aureus* strain rather than *Escherichia coli* strain. From the tests on the fruit it was observed that thyme and oregano essential oils were more effective in maintaining firmness and reduce weight loss than cinnamon essential oil or controls, through 12 days storage at 1ºC. There was no significant effect of the essential oils on the other quality parameters measured. The effectiveness of essential oils was more evident after 12 days storage, especially for fungi, with oregano showing the best results, followed by thyme or their combination. From the results obtained, it is possible to conclude that the dispersion of thyme and oregano essential oils in nanocellulose matrix is a promising technology to improve shelf-life of raspberries or other fresh fruits.

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Introduction

In the last decades, the fast development of society has led to a substantial increase in the consumption of all types of goods (1) and vast amounts of products are shipped everyday all around the world to meet the consumer's demand (2). Among the others, the food market is one of the most important and globalized, as it is now possible to find in every city food coming from any part of the globe (3). The increase of the food market brought to a parallel increase of the importance of food packaging, both in terms of quantity and efficiency (4). The global food packaging market (growing from 293 to 423 billion dollars in the period 2018 - 2020, according to the Zion Market Research (5)) covers, indeed, 42% of the polymer global market. It reached production rates of up to 100 million tonnes/year (6) and caused many well-known environmental problems. Thus, the increasing international consciousness about the environmental crisis we are facing, led the interest to sustainable food packaging systems with additional functionality, able to increase the shelf-life of the products. All over the globe, we have been exploiting in different ways the Earth's resources for commercial and economical purposes and for decades this has been done without considering the consequence of these activities. Fortunately, in the last years, the governments and the population started to be more aware of the impact that human activities have on the planet ecosystem. Thus, a lot of research activity is addressed to develop new sustainable systems and technologies to minimize human impact on the Nature.

For all these reasons, in the recent years, the research on more effective packaging systems has gained much more importance in the attempt to obtain better performance utilizing biodegradable and bio-based materials to replace oil-based polymers (7–9). New types of packaging have been developed following the need to increase the shelf-life of the products, to ensure no damage during transport and storage (10) and to decrease environmental impact (11). Among the different approaches, then, particular interest has been devoted to the development of smart and active systems (12,13). These innovative packaging systems extend the classical concept of sustainable packaging by coupling the use of renewable materials with the shelf-life increase (14–16), thus reducing at the same time environmental impact and the food waste (17). Therefore, several new composite materials produced from biobased and/or biodegradable polymers have been investigated, and coupled with different active agents, strengthening the idea of circular economy (6,18).

Among the different possibilities, cellulose is one of the most studied biopolymers for active packaging applications (19–22). In fact, cellulose is the most abundant biopolymer on Earth, and its biodegradability and the possibility to combine its sustainability with the properties of the nanoscale made it a very fascinating biopolymer (23). Nanocellulose is then obtained directly from cellulose as nanofibrils (also called micro/nanofibrillated cellulose) or nanocrystals, or it can be directly produced

by bacteria through a biotechnological process. The raw material from which nanocellulose is obtained, the pre-treatment applied and the process itself can variate, conferring different properties to the final material (24–27). Moreover, the possibility of many surface modifications makes it very versatile and able to adapt its properties in view of its final application (28–30). Several studies take into consideration these variations and how they affect the structure and the possible application of the final material, both as nanofiller, to improve the mechanical and barrier properties of other biopolymers and as matrix itself, alone or with the use of other fillers or additives, to adapt its features to particular applications (31–34).

The use of nanocellulose resulted very promising in the field of active packaging, where the strengthening effect of the fibres can be coupled with the extension of the shelf-life due to the antimicrobial properties of the active agents (35). Regarding this specific application, natural antimicrobial agents are more and more favoured with respect to inorganic ones, such as metals or metal oxides, due to the intrinsic sustainability of the solution (36,37). Among these, the essential oils aroused certain attention, for the enhancement of the quality and safety of food products, thanks to their well-known antimicrobial activity against a broad spectrum of food pathogens, their natural origin and the fact that they are generally approved for food contact applications (38). Also, their addition to packaging materials helps to reduce their adverse flavour while prolonging the action time, which will greatly improve their effective utilization rate in food preservation. (39,40).

Essential oils are volatile and aromatic oily liquids obtained from flower, leaves, or other parts of the plants, where they have the role of secondary metabolites (40,41). They exhibit antimicrobial and antioxidant properties against the most diffused gram-positive and gram-negative bacteria making them interesting compounds to be included into food packaging formulations (42–44). The most common plants from which they are derived are Rosemary, Oregano, Lemongrass, Thyme and others (45). These oils contain different components, which depend not only on the plant type, but also on the harvesting and growing conditions and the geographical area. In general, the main components typical of a certain essential oil are always present, but their quantity variates with the parameters cited, affecting the antimicrobial activity (46). Each essential oil has particular characteristics, also depending on the plant from which it was extracted and the method of extraction itself. Thyme, cinnamon, and oregano essential oils are the ones that present the highest antimicrobial and antioxidant activity and are amongst the most studied essential oils in the active packaging field. For example, cinnamon essential oil effect has been studied in various bio-matrices, such as nanocellulose (47), chitosan (48) and others (49,50). Also, thyme and oregano are very common active agents for active packaging applications which have been studied in combination with different materials, such as soy-protein edible coatings to be used with fresh beef and cellulose nanocrystals reinforced chitosan (51,52). For these reasons, various methods to incorporate

essential oils into active packaging had been studied: from the utilization of edible coatings containing essential oils to the application of nanoemulsions to the food (53,54); from the use of nano-encapsulation to obtain antimicrobial surfaces (55) to the incorporation into films for successive release in the package headspace (56,57).

In addition to the antimicrobial activity, essential oils may also significantly affect other material properties such as mechanical (tensile strength), barrier (reducing water vapor permeability) and optical properties (58). Many studies are available on the essential oils' mechanical and chemical effects on biobased polymers, such as nanocellulose alone or coupled with other biopolymers (21,44). The main aim is to give to the composite material antimicrobial and antioxidant effects thanks to essential oils properties, while not endangering the mechanical or barrier properties of the base materials (34,59,60).

Currently, most of these studies utilize a very pragmatical approach, based on the production and testing of new composites materials in terms of mechanical, barrier and antimicrobial properties, by considering the effect on specific bacteria or on the shelf life of well-defined food. There is, instead, a limited amount of works focusing on fundamental aspects, such as the study of the diffusion properties and the release rate, which, however, have a great importance on the final efficiency of the active packaging solution. For example, K. Kuorwel et al. considered the migration of carvacrol, thymol and linalool from starch-based films into a food simulant (61), while L. Sánchez-González et al. studied the release of limonene present in chitosan films enriched with bergamot oil in food simulants (62). In another study, Tunc and Duman tested the release of carvacrol from methyl cellulose/montmorillonite nanocomposite (63), whereas Nostro et al. investigated the effect of carvacrol and cinnamaldehydes from EVA copolymers (64). Finally, some modelling work has also been considered to describe the release kinetics of these substances (65,66). However, no values for diffusion coefficient of essential oils have been obtained considering a pure nanocellulose matrix. For this reason, the development of the present research work started from this kind of analysis, which represents a fundamental starting point to understand the antimicrobial effect of essential oils, as a function of its concentration, and to proceed to an informed optimization of the systems on the base of the desired application.

So, trying to give a contribution to this matter, the focus of this PhD project was the development of biodegradable packaging systems based on nanocellulose support activated with essential oils for the increase of food shelf-life. As said above, the analysis of mass transport properties of thyme, cinnamon and oregano essential oils kept in direct contact with different types of nanocellulose was the first step to characterize the system. The untreated whole oils have been used to mimic the most common applicative condition and tests were made to evaluate both the sorption and the desorption kinetics, which are fundamentals to obtain information on how to control and optimize the

antimicrobial effect of the final active packaging solutions. After that, an analysis on the antioxidant and the antimicrobial activity of nanocellulose films activated with thyme, cinnamon and oregano essential oils had been performed, to have a complete evaluation of the effectiveness of the system. The experiments were performed both on gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*) bacterial strains, and directly on raspberries. In the first case the goal was to obtain information on the minimum inhibitory concentration for each oil, which was then considered as a reference to carry on the tests on the fruit. The latter were carried out monitoring all the product parameters of interest to define the state of conservation over time (firmness, colour weight loss etc.). A sensory evaluation was also performed, to understand the acceptability of this technique by the consumers.

Following the different activities, the present thesis is divided into four main chapters. The first one, contains a complete overview of the different active packaging technologies, where the main features of nanocellulose and other biopolymers and of the different essential oils used in the field of active packaging are introduced and described. The latest findings about the nanocellulose based active packaging will be presented and the antimicrobial effectiveness of the different solutions proposed in the open literature is discussed, focusing also on their effect on other material properties. The effect of the different inclusion strategies is also reviewed, considering both *in-vivo* and *in-vitro* studies, trying to understand more promising solutions and pathways for further developments. The focus, in line with the thesis topic, is on antimicrobial systems containing nanocellulose as support for the incorporation of essential oils, but also other renewable solutions are considered, to give a complete overview of the current research on the topic.

After the state-of-the-art analysis, the second chapter will be devoted to the presentation of the different materials and experimental methods considered in the present thesis. Solvent casting technique was adopted to create nanocellulose and PLA membranes which were then functionalized applying essential oils. Mechanisms of NFC-PLA pairing were also investigated, such as casting together the materials or hydraulic press post-fabrication pairing. The transport properties of the oils in such matrices were investigated through absorption in liquid phase and desorption in gas phase, by measuring the mass variations over time and utilizing a quartz spring microbalance. Then, the analysis of the antimicrobial activity against most common pathogenic bacteria was investigated with Petri dish colonies counting. Finally, the antimicrobial activity and the effect on the quality of the fruit of the active films was studied by direct application on packed raspberries, monitoring the fruit parameters and the colony growth over time.

In the last two chapters, the different experimental results will be presented and discussed starting from those about the characterization of the membranes and the oils transport properties and continuing with those related to the antimicrobial activity and the effect on the fruit. In particular, this

latter section is divided into tests on bacterial strains and tests in real-case conditions, since they were performed in different laboratories and also regard different types of experiments and outputs.

1. Active packaging systems: theoretical background and state of the art

Active packaging systems belong to the more general category named Smart Packaging, which refers to technologies aimed at prolonging the shelf-life, monitoring freshness, displaying information on quality of the packaged product and improving the product and consumer safety (67). Smart Packaging could identify both Intelligent Packaging, mainly used to monitor the condition of the packed food through the use of indicators and sensors, or Active Packaging, which interacts with the internal environment of the package to prolong the shelf-life of the final product (68). For the aim of this study, major attention is devoted to Active Packaging technologies.

Figure 1.1 shows a possible classification of the different types of Active Packaging Technologies (14,69). They can be divided into active scavenging systems, active release systems and non-releasing systems. The first group includes Modified Atmosphere Packaging (MAP) as well as other packaging involving the use of absorbents. For example, oxygen or moisture scavengers (70,71) aim at reducing bacterial growth by maintaining an adverse environment for their development within the package. The second type acts, instead, through the release of molecules (72) which prevent food spoilage (12). Finally, many of the existing antimicrobial packaging belong to the third type and are based on metal nanoparticles (silver, gold, and zinc), metal oxide nanomaterials (silicon and magnesium, titanium, zinc etc.) (73) and carbon nanotubes, which result highly effective in developing the antimicrobial effects once immobilized on the package surface (6,69,74).

Figure 1.1. Active packaging. These systems can be applied through different techniques such as controlled release packaging, antimicrobial packaging and antioxidant packaging.

In every case, the food product will be in contact with packaging materials. Thus, it is very important to have a detailed safety analysis regarding the effect of this interaction. Particular attention is required since the use of nanomaterials is involved, and they could easily migrate into the food. The impacts of using nanoparticles on human body and on the environment should be evaluated (18). The consumer's perception is another important factor to take into consideration, since their attitude could influence the spread use of nanomaterials (75).

The legislation regarding the use of nanotechnologies for food application has been updated in line with the new discoveries. The main EU regulatory framework regarding materials used for food application is the Regulation (EC) 1935/2004, which ensures "the effective functioning … of materials and articles intended to come into contact directly or indirectly with food, whilst providing the basis for securing a high level of protection of human health and the interests of consumers". Special requirements are specified for active and intelligent materials. For example, they shall not lead to changes in the composition or organoleptic characteristics of food, and they shall be adequately labelled to allow identification by the consumer of non-edible parts. Moreover, the Commission Regulation (EC) 2023/2006 compliments the previous one by indicating the good manufacturing practice for materials and articles intended to come into contact with food. While the Commission Regulation (EC) 450/2009 on "Active and Intelligent materials and articles" provides a list of authorized components that can be present in the food environment and may interact with it. There is a derogation to this regulation which refers to intelligent packaging materials, that are usually not used in direct contact with the food from which they are separated by the primary packaging. Anyway, all the substances which have nano-dimensions should always be reported in the product description, since the risk assessment of a certain substance does not cover its application in the nano-dimension scale. This is because the nanoparticles characteristics, their mechanisms of mass transfer in the final matrix and their behavior in contact with the food environment could variate with respect the conventional particle size scale case (76).

The regulation (EU) 10/2011 regulates the plastic materials which are in contact with food products. It imposes a limit of 10 mg of constituent per dm^2 of surface area to all substances that can migrate from the packaging to the food (77). Moreover, it lists all the monomers, macromolecules, additives and auxiliary substances which can be used for polymerization, together with the migration limit and, in some cases, other specific restrictions. This regulation is also a reference for the substances that

can be used in antimicrobial packaging. Each substance with potential antimicrobial effect to be used inside an active packaging needs to be approved as food additive by the EU commission (78).

Indeed, it is important to recall that, with the increase in the food packaging market, not only the food quality issues were addressed, but also the environmental concerns regarding the materials used. The use of sustainable solutions has been a priority, both in terms of active substances, such as EOs, and of packaging matrix, using renewable and biodegradable materials such as, for example, biopolymers (79,80). The wide range of these promising bio-derived materials can be categorized by their method of production, as shown in figure 1.2 (81). If they are directly extracted from biomass, they can be divided into polysaccharide films (such as starch, cellulose (82–84) and derivatives, chitosan (85,86) and alginate), protein films (soy protein and wheat gluten), lipid films (fatty acids and resins) and composite films (9). Otherwise, they can be synthesized from bio-derived monomers (such as PLA) or produced directly from microorganisms (like polyhydroxyalkanoates) (79).

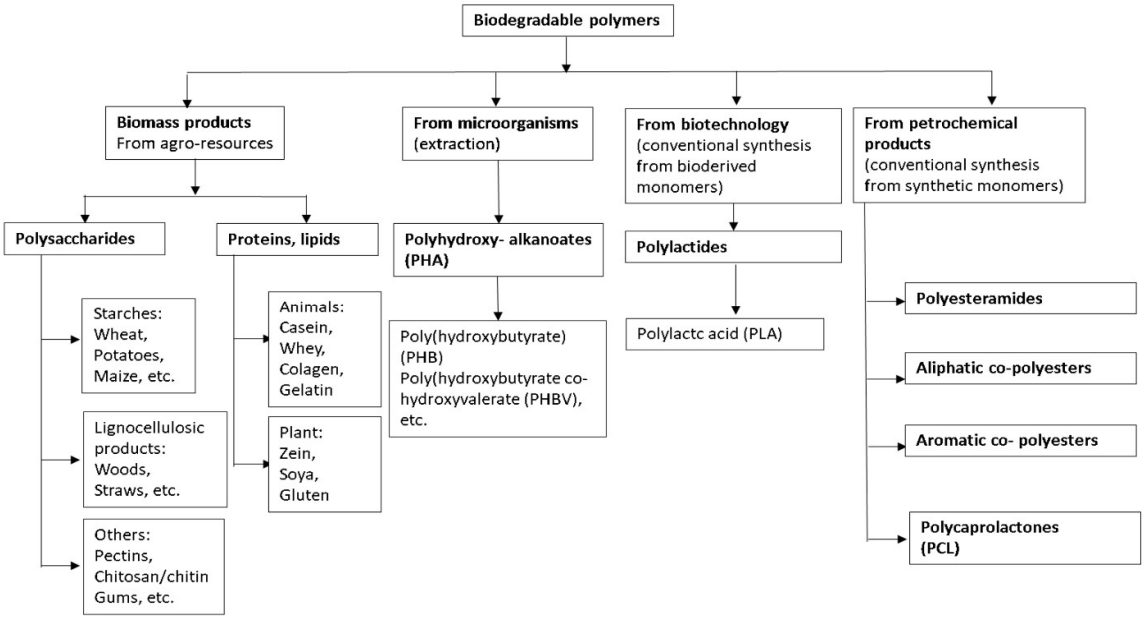


Figure 1.2. Classification of biodegradable polymers (81). *Biodegradable polymers can be obtained from different sources such as biomass, microorganisms and petrochemical products.*

In the following paragraphs, the most interesting current trends in active packaging research are reported. The focus is on the latest outcomes concerning nanocellulose based active packaging and on the use of different essential oils as active agents and their effects on the mechanical and barrier properties of the final material. Other biopolymers such as PLA, chitosan and alginate will also be considered, as they are often composite together and are used as packaging materials. Also, the antimicrobial effects against the most common food spoilage bacteria and the direct application to some food products will be presented.

1.1 Active packaging biopolymers

In the recent years a wide range of biomaterials have been considered for packaging applications, to reduce the use of oil-based plastics and to increase the sustainability of the packages (87). In the following paragraphs a brief description of some of them, especially considering those more commonly used for active packaging, will be given.

Cellulose

Cellulose is the most abundant and renewable biopolymer in the biosphere. Its global market size was USD 346 million in 2021 and it is expected to reach 963 million by 2026, according to Markets and Markets report (88). It is widely distributed in vegetable organisms such as vascular plants, where it has a structural role for the cell walls. Cellulose is a linear homopolysaccharide composed by repeating glucose units (see Figure. 1.3a) and it has been obtained for many years mainly from plant materials. The structure and properties of native cellulose are determined by the isolation process used, which affects the number of inter- and intra-molecular hydrogen bonds, the chain length, the chain length distribution, the crystallinity and the distribution of functional groups within the repeating units and along the polymer chains (89). The hydrogen bonding patterns inside and among cellulose fibers, in particular, are the main factor which determines its physical and chemical properties (31).

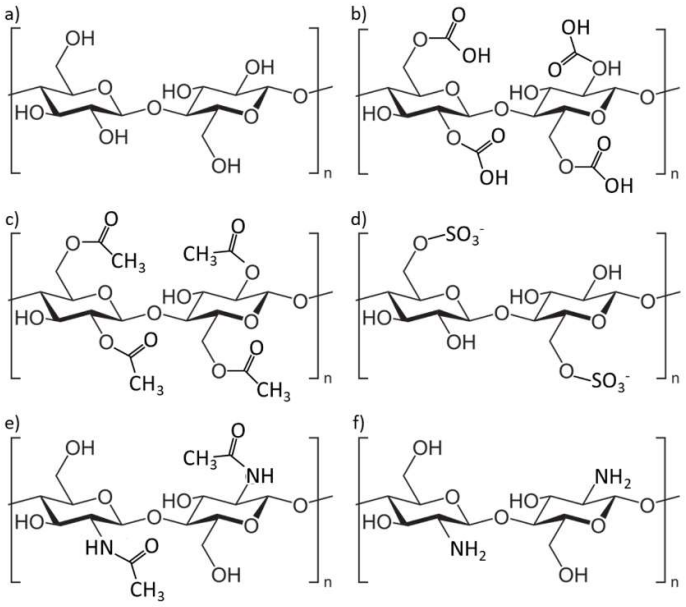


Figure 1.3. Chemical structure of cellulose materials and chitosan a) Cellulose, b) Carboxymethyl Cellulose (CMC) c) Cellulose Acetate (CA) d) Cellulose sulphate (CS) e) Chitin f) Chitosan.

Traditionally, cellulose had been extracted from plants and used to obtain veterinary foods, wood and paper, fibers and clothes, cosmetic and pharmaceutical products (90). In the recent years, in agreement with its green potential, cellulose has been obtained also from wastes, through different pretreatments of the raw material. For that reason, its composition and physical properties could vary, influencing the further steps of the process (20).

Accordingly with the increasing interest in nanomaterials, cellulose has also been produced in nanoscale dimension (19). Several types of nanocellulose that have raised great interest in a wide range of applications are: Cellulose Nano Crystals (CNC), Cellulose Nano Fibrils (CNF) and Bacterial Nanocellulose (BNC) (25,91). Briefly, CNF are usually obtained through mechanical homogenization of pristine cellulose fibers, which were previously treated chemically or enzymatically (26). CNC, on the other hand, can be obtained from cellulose by strong acidic treatment, able to hydrolyze the amorphous part of the fibers, thus leaving only the crystalline part of the original fibers (92). Finally, BNC comes directly from bacterial metabolism, so it is free from hemicellulose and lignin. This reduces the purifying costs and the environmental damage derived from the use of chemicals (36,93,94). Obviously, the different processes can be optimized depending on the characteristics of the source material and on the final chemical structure needed. Figure 1.4 shows a comparison of the different nanocellulose types described above. Passing from cellulose nanocrystals, to nanofibrils and consequently to bacterial nanocellulose a stretching of the filaments can be noticed.

In fact, the singular nanocrystals, short and very rigid, are then connected among them by sections of amorphous cellulose, creating a more complex net of interconnected fibers (figure 1.4c).

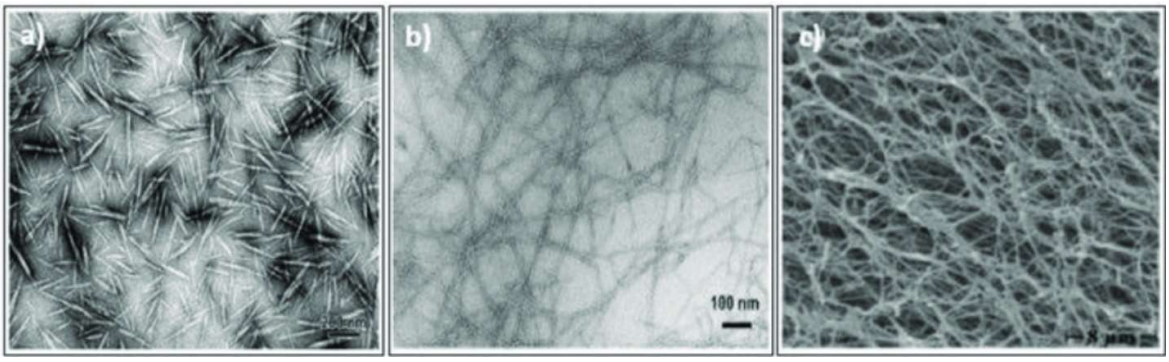


Figure 1.4. Microscopic image of (a) cellulose nanocrystals, (b) cellulose nanofibrils and (c) bacterial nanocellulose (95).

In fact, nanocellulose is a very versatile material which exhibits good mechanical strength, high barrier properties when in dry conditions and good biodegradability. It is therefore a strong candidate in food packaging to replace the petroleum-based product in favor of renewable and biodegradable materials (96). A vast literature on the use of nanocellulose in packaging is present, both in pure form as film or coating (97) or as reinforcing filler in bio-composite materials (36,98). In the latter case, it is used to increase the strength and the modulus of the matrix and to reduce the water vapor permeability and the oxygen permeability (99) or as stabilizing agent for emulsions (100). Moreover, it was also shown to increase the thermal stability and the water resistance of some biopolymers, such as chitosan (101), PLA and thermoplastic starch (95,102).

Apart from their direct use, cellulose and nanocellulose, thanks to the high number of hydroxyl group present of the fiber surface, can be easily modified (103). So it is possible to obtain a wide variety of materials with modified surface properties that are suitable for many different specific functions (104), such as esters or ethers. They found wide use in different sectors (90,105,106), including active packaging (107). Some of the more common cellulose derivatives are shown in Figure 1.3b,c,d. For example, cellulose acetate (figure 1.3c) is produced replacing the hydroxyl groups from cellulose backbone chain with acetate groups by means of a reaction of native cellulose with acetic anhydride (108). While cellulose sulphate is obtained by substituting, partially or completely, the hydroxyl groups of the nanocellulose by sulfate groups (SO_3^-; Figure 1.3d) (109). Also, etherification is a widely used chemical pretreatment method that facilitates cellulose defibrillation to prepare CNF. Cellulose ethers can be obtained by a first activation of the fibers with an aqueous alkali hydroxide, such as NaOH, and then converting the hydroxyl groups to carboxymethyl moieties (83). All these various possibilities of surface modification widen the horizon towards more applications of NFC in advanced

and new functional materials. In this way it will be possible to use natural and renewable resources for the development of sustainable and environmental-friendly products (110).

Regarding the environmental impact, the use of nanocellulose can be evaluated through Life Cycle Assessments with a focus on its production. Hervy et al. compared the environmental impact of bacterial cellulose and NFC reinforced epoxy composites with neat PLA and glass fibre-reinforced polypropylene composites. It resulted that, considering as use phase an automotive application and as end-of-life landfill or incinerator, the nanocellulose composites were comparable to that of neat PLA. While when the composite contains more than 60% vol. of nanocellulose the global warming potential and the abiotic depletion potential of fossil fuels are even lower than neat PLA (111). Focusing on the production method itself, among the different process studied, the TEMPO-oxidation followed by homogenization had the lowest environmental impact regarding the global warming potential, the energy consumption and the overall impact (112). While Arvidsson et al. compared different pretreatments to produce NFC and found that the enzymatic and no pretreatment routes had lower environmental impacts than the carboxymethylation one and the other carbon nanomaterials (113).

Other biopolymers

Besides cellulose, many other biopolymers arouse the interest of the researchers and the companies, to obtain biocomposites with enhanced properties through synergetic effects for active packaging and other several applications. In this section, the ones mostly used in combination with nanocellulose will be briefly introduced to make the reader understand their importance and their main characteristics. Moreover, other biopolymers not analysed in this work are for example starch-based materials and polyhydroxyalkanoates. For more detailed information about them and their applications, the reader is addressed to the reviews in the literature (114–117).

The second most abundant biological material after cellulose is chitin, the precursor of chitosan. This is a linear highly acetylated polymer (Figure 1.3e), which is referred to as chitosan (Figure 1.3f) when the degree of N-acetylation is lower than 50% (118,119). Chitin is obtained from crustacean wastes, through acid and alkaline treatments (101). Distinct factors, such as alkali concentration, incubation time, chitin to alkali ratio, temperature and atmosphere play a role in the alkaline N-deacetylation of chitosan, thus affecting the final properties of the obtained polymer.

Chitosan and its derivatives became extremely useful in many fields like cosmetics, pharmaceuticals, food, agriculture, biomedical and material science due to its biological activity. In fact, those materials are biocompatible, non-antigenic, non-toxic, intrinsically antimicrobial and have a good film-forming ability (120,121). Chitosan-based active films have been widely studied in recent years since they

can be used as antimicrobial agents (122–124) and polymer substrates at the same time (125,126). The intrinsic antimicrobial activity of chitosan seems to be addressed to three different mechanisms, which comprehend the ionic surface interaction, the penetration of the chitosan in the nuclei of the microorganisms and the creation of an external barrier inhibiting the nutrients contribution (127). Moreover, they resulted to depend on the polymer molecular weight and on the degree of acetylation (128,129).

Regarding the food packaging applications, chitosan has been classified as "Generally Recognized as Safe" by the US Food and Drug Administration (FDA) in 2001 (130) and several studies analyze different methods for chitosan film production, in relation with the specific food packaging systems (85,131,132). As far as active packaging is concerned, the effects of chitosan as an antimicrobial preservative turned out to be limited to food products with low protein and NaCl content. So, the incorporation of antimicrobial agents needs to be considered to extend the protection to all kinds of food products (133,134).

Continuing the list, polylactic acid (PLA, Figure 1.5) is surely one of the most studied biopolymers for food packaging applications. It is biobased and biodegradable, as the lactic acid monomer can be produced from completely renewable resources, and it also results biocompatible (135). In fact, it received approval by the FDA for applications with food contact.

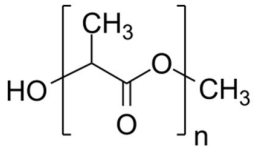


Figure 1.5. Chemical structure of PLA.

Considering its high transparency and the optimal organoleptic characteristics, PLA has an enormous potential for food packaging application. In general, also the mechanical and barrier properties of PLA are remarkable (136), but still insufficient to meet the performance of many oil-based polymers. For this reason, several studies have been focused on incorporation of fillers, such as nanocellulose, and active agents to impart additional functionalities (137). For example, PLA films with 550 g/kg of NFC showed an increase of the tensile strength and tensile modulus of 59% and 47%, respectively, compared to pure PLA films (138). While applying a cellulose coating on a PLA substrate allowed to decrease the oxygen transfer rate of the film of about one order of magnitude even in humid environments (up to 60% RH) (97).

Besides PLA, alginate (Figure 1.6) is another interesting biopolymer due to its low toxicity and biocompatibility (139). It is an example of polysaccharide commonly obtained from the cell wall of brown algae and extracted from seaweed for commercial purposes (140). In fact, alginate has already been used to create antibacterial (141) and antifungal films (142) and stimulus response drug releasing materials, but their application for food packaging is limited for the lack of mechanical strength as for many other biopolymers (143). Several reinforcements were studied in order to improve alginate's mechanical properties, such as cellulose nanofibrils (144) or inorganic fillers (145). The use of nanocellulose resulted effective not only for the tensile strength (TS) but also for the water vapor barrier properties (143,146,147).

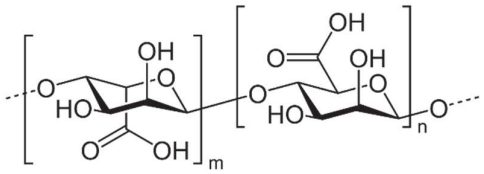


Figure 1.6. Chemical structure of alginate.

Furthermore, agar (Figure 1.7) is a linear polysaccharide extracted from red algae of the class *Rhodophyceae*. It has been used to produce biodegradable films due to its good film-forming ability, high biocompatibility and moderate water-resistant properties (148–151). Besides that, agar-based films still have some limitations due to the low thermal stability and poor mechanical properties. In order to improve its properties, it has been studied in combination with fillers, such as metallic nanoparticles and nanoclays (152), and in presence of gelatin (153) or plant extracts (154). Also, BNC was used to reinforce agar-based edible films (148).

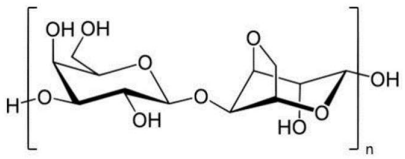


Figure 1.7. Chemical structure of agar.

1.2 Antimicrobial agents - essential oils

Antimicrobial agents are compounds used to provide safety assurance, shelf-life extension, and quality maintenance on food. In fact, when incorporated into the packaging, they are able to inhibit spoilage and suppress pathogens, responsible for food-borne diseases, that can potentially contaminate the food product (155). Antimicrobial agents can be inorganic compounds such as metals (156) or metal oxides nanoparticles (157), which release antimicrobial ions while directly interacting with microorganisms. However, the most commercialized products available in the market contain antimicrobial agents such as chlorine dioxide, ethanol and sulfur dioxide, that act in the gas phase of the package (155).

In the recent years there has also been a growing interest in natural antimicrobial agents, also due to the lower risk perceived by consumers in their use. The natural compounds used in antimicrobial packaging are biologically-derived components, like bacteriocins, enzymes, and plant extracts (158,159).

In particular, essential oils (EOs) are lipidic extracts from plants which have been studied for many years as additives in films and coatings, to replace synthetic preservatives. In fact, they naturally possess antioxidant and antimicrobial properties, due to the presence of bioactive compounds, such as phenols and terpenoids (160). The antimicrobial activity of the essential oils is related to the presence of hydroxyl groups that can damage the cell membrane of the pathogens, which results in the release of the cell constituents and in the death of the microorganisms (161). For this reason, EOs show a broad antimicrobial spectrum against different pathogenic and spoilage microorganisms, including gram-negative species such as *Escherichia coli* (162).

As previously explained, EOs widely differ in chemical composition, depending not only on the characteristics of the plant of origin, but also on the part of the plant from which they are extracted and from the extraction process itself (163). The qualitative and quantitative differences that could be present may further influence and increase biological effectiveness. In this regard, Table 1.1 offers an overview of different essential oils used as antimicrobial agents incorporated in filler/matrix systems to create active biomaterials. These works will be further explained in the next paragraphs considering the antimicrobial effects as well as the other possible influences on the biocomposite mechanical and barrier properties.

Table 1.1. Different uses of antimicrobial agents incorporated in filler/matrix systems. The first column indicates the antimicrobial agent responsible for the activity of the film, while the second one reports the filler and the matrix where the agent is incorporated. The third column refers to the effects of this integration on the mechanical and chemical properties of the composite, while the fourth one shows the antimicrobial properties and the possible use for active packaging systems. Last column is for references.

Antimicrobial agent – Essential oil	**Filler/matrix**	**Effect on mechanical and chemical properties**	**Antimicrobial activity and use**	**Ref.**
Oregano EO	CNC (Pickering emulsion)	Good chemical and thermal stability	*S. aureus*, *S. cerevisiae*, *E. coli* and *B. subtilis*	(164)
Cinnamon EO	CNC/CNF (Pickering emulsion)	Long term emulsion stability	*B. subtilis*	(165)
Ginger EO, Citric acid	CNF (edible coating)	Improved taste, odor, texture, and overall acceptability of the samples	Increase of meat shelf-life	(34)
Oregano, Thyme, Cinnamon EOs	Cellulosic pads	Acceptable taste and odor (sensory evaluation)	Meat bacterial species, like *S. aureus*	(166)
Oregano, Thyme, Cinnamon, Sweet fennel EOs	Cellulose Acetate	Increased flexibility, Reduction in water vapour transmission rate	*Penicillum spp, E. coli, S. aureus*	(167)
Pink pepper EO	Cellulose Acetate		*S. aureus,* *L. monocytogenes* microbial growth decreased in sliced cheese	(60)
Rosemary EO	Cellulose Acetate		Pathogenic microorganisms on chicken breast cuts	(168)
Rosemary EO, Aloe Vera	Cellulose Acetate	Decreased tensile strength, water uptake and contact angle; increased	*E. coli, B. subtilis*	(169)

Antimicrobial agent – Essential oil	**Filler/matrix**	**Effect on mechanical and chemical properties**	**Antimicrobial activity and use**	**Ref.**
		hydrophobicity and free radical scavenger activity		
Rosemary EO, Oregano EO	Cellulose Acetate (electrospinning)		*E. coli, C. albicans* and *S. aureus* and anti-biofilm effects	(170)
Thymol	Cellulose Acetate	Reduced transparency, mechanical, OTR, and WVTR properties	*L. monocytogenes, S. aureus, E. coli, P. aeruginosa, Klebsiella pneumoniae, and Salmonella enteritidis*	(171)
Thymol + organoclay	Cellulose Acetate	Enhanced optical and mechanical properties	*Listeria innocua*	(172)
Thymol	Cellulose Acetate (supercritical CO_2 impregnation)	Decreased glass transition temperature; disappearing of crystalline structure	23 tested strains, in particular *S. aureus*	(66)
Thymol	Cellulose Acetate	Anti-adhesion surface properties	*P. aeruginosa, S. aureus*	(173)
Oregano EO + Montmorillonite clay	Cellulose Acetate	increased oxygen and water vapour barrier properties, rigidity, thermal stability, and elongation	Phytopathogenic fungi: *Alternaria alternata, Geotrichum candidum, and Rhizopus stolonifer* – Postharvest conservation	(108)
Mustard EO	Cellulose Sulphate (edible film)	Reduced TS, Water sorption; increased elongation	*L. monocytogenes, E. coli, S. aureus, B. subtilis, A. niger*	(174)
Murta fruit extract	Methyl Cellulose	Increased mechanical strength; decreased swelling index; affected thermal properties	*L. innocua*	(175)

Antimicrobial agent – Essential oil	**Filler/matrix**	**Effect on mechanical and chemical properties**	**Antimicrobial activity and use**	**Ref.**
Mexican Oregano EO	Carboxymethylated Cellulose (CMC) - (edible film)		*L. monocytogenes, S. aureus*	(176)
Mentha spicata EO	CMC, Chitosan (edible film)		Increased strawberries shelf-life; *L. monocytogenes*	(177)
Mentha spicata EO, *Ziziphora clinopodioides* EO	CMC (edible film)		Increased fresh and sauced chicken breast fillets shelf-life; *C. jeuni*, *L. monocytogenes, S. aureus, E. coli, S. typhimurium*	(178)
Bay EO	CMC (edible film)	Increased antioxidant activity, WVP, UV-light barrier effect;	*Escherichia coli, Candida glabrata*	(72)
Zataria multiflora Boiss EO	CMC (edible film)	Increased total phenol content, antioxidant activity; reduced transparency and solubility in water	*S. aureus, B. cereus*, *E. coli*	(179)
Thymus daenensis EO	Hydroxyl-propyl-methyl cellulose (edible film)	Reduced tensile strength and Young's modulus	Gram positive and Gram negative bacteria	(180)
Mentha spicata EO	Chitosan (edible coating)	Good appearance at sensory analysis	Increased fresh strawberries shelf-life; *L. monocytogenes*	(177)
Red thyme and Oregano extracts	Chitosan (edible coating)		Increased fresh strawberries shelf-life; antifungal activity	(181)
Rosemary essential oil	Chitosan	Decreased solubility, WVP and UV-light transmission	*L. monocytogenes, Streptococcus agalactiae, E. coli*	(182)
Ziziphora clinopodioides EO	Chitosan (edible film)	Decreased solubility, WVP, UV-light transmission, swelling index and TS	*L. monocytogenes, S. aureus, B. cereus*	(183)

Antimicrobial agent – Essential oil	**Filler/matrix**	**Effect on mechanical and chemical properties**	**Antimicrobial activity and use**	**Ref.**
Apricot (*Prunus armeniaca*) kernel EO	Chitosan	Decreased solubility, WVP and UV-light transmission and transparency	*E. coli, B. subtilis,* inhibition f fungal growth on bread	(184)
Lemongrass EO	Chitosan	Increased elongation; decreased moisture content, WVP, solubility and TS	*B. cereus, E. coli, L. monocytogenes, Salmonella typhi*	(185)
Thyme EO	Chitosan (edible film)	Decreased water condensation in the head space of the packaging; good odor	Reduced yeast population – cooked ham	(186)
Thyme EO, Cinnamon EO, clove	Chitosan (edible film)	Increased moisture content, solubility in water, WVP, elongation at break. Opposite behaviour for Cinnamon EO	*L. monocytogenes, S aureus, Salmonella enteritidis, Ps. aeroginosa*	(187)
Cinnamon EO	Chitosan (edible film)	Decreased moisture content, solubility in water, WVP and elongation at break; Increased TS	*L. monocytogenes, Lactobacillus plantarum, Lactobacillus sakei, Pseudomonas fluorescens, E. coli*	(86)
EOs, gelatin	Chitosan	Increased UV-light barrier properties, moisture absorption and WVP; Decreased transparency	*C. jejuni, E. coli, L. monocytogenes, Salmonella typhimurium*	(188)
Cinnamomum zeylanicum EO	Chitosan nanoparticles		Increased shelf-life and physicochemical quality of cucumbers; *Phytophthora drechsleri*	(189)

Antimicrobial agent – Essential oil	**Filler/matrix**	**Effect on mechanical and chemical properties**	**Antimicrobial activity and use**	**Ref.**
Zataria multiflora EO	Chitosan nanoparticles		Prevention of pre or postharvest fruit from decay – strawberries treatment; *Botrytis cinerea*	(190)
Carvacrol	Chitosan nanoparticles		Increased shelf-life of fresh-cut carrots	(191)
Cinnamon EO	Chitosan nanoparticles	Physicochemical quality maintained; sensory analysis	Increased shelf-life of chilled pork; Psychrotrophic aerobic bacteria, Lactic acid bacteria, Enterobacteriaceae	(48)
Frankincense EO	CMC/Chitosan biguanidine hydrochloride (edible film)	Decreased WVP, increased TS, EB;	*S. pneumonia, B. subtilis, E. coli*	(192)
Cinnamon EO	CMC/Chitosan	Increased TS, WVP, EB and antioxidant properties; Decreased water solubility	*L. monocytogenes, P. aeruginosa*	(193)
Cinnamon and ginger EOs and oleic acid	CMC/Chitosan	Increased EB; Decreased WVP	*A. niger*	(194)
Thyme, Oregano, Tea tree and Peppermint EOs	CNC/Chitosan	Sensorial evaluation; Increased TS, EB, WVP	Increased shelf-life of rice; *A. niger, Aspergillus flavus, Aspergillus parasiticus, Penicillum chrysogenum*	(52)
Thyme, Oregano EOs	CNC/Methyl cellulose	Increased TS, EB, WVP	Increased shelf-life of rice; *A. niger, A. flavus, A. parasiticus, P. chrysogenum*	(195)
Thyme EO	CNF/Chitosan (edible coating)	Decreased weight loss, preserved anthocyanin content, better appearance	Sweet cherry storage	(196)

Antimicrobial agent – Essential oil	**Filler/matrix**	**Effect on mechanical and chemical properties**	**Antimicrobial activity and use**	**Ref.**
Oregano EO - *Carum copticum* EO	CNF-LCNF/ Chitosan	Increased water vapour barrier, water solubility and opacity; release controlling effect of CNF – LCNF	*E. coli, B. cereus*	(197), (198)
Oregano EO	CNC/PLA	Increased EB; Decreased TS, TM;	*L. monocytogenes;* mixed vegetables	(59)
Tanacetum balsamita EO, propolis ethanolic extract	CNC/PLA	Increased TS, elastic modulus; Decreased elongation	E. coli, B. cereus, S. aureus, S. Typhimurium; vacuum-packed cooked sausages	(199)
Ziziphora clinopodioides EO	CNC/PLA	No alteration of organoleptic properties	*Pseudomonas spp.*; Increased shelf-life of minced beef	(200)
Zataria multiflora EO, propolis ethanolic extract	CNF gel/PLA	Increased WVP, TS, EM; Decreased transparency	S. aureus, E. coli, Vibrio parahaemolyticus, L. monocytogenes; Vacuum-packed cooked sausages	(201)
Rosemary EO	Chitosan/PLA	Increased EM, TS; Decreased elongation; antioxidant activity; colour change	Fresh minced chicken breast	(202)
Oregano, Cinnamon, Winter savory EOs	Alginate (edible film)		*Salmonella Typhimurium*, *L. monocytogenes;* ham slices	(203)
Cinnamon EO	CMC/Sodium alginate	Increased WVP, oxygen permeability and elongation; Reduced moisture content and TS	*S. aureus; E. coli;* Bananas	(49)
Savory EO	Cellulose nanoparticles/ Agar	Decreased TS, water solubility; increased EB, WVP, opacity;	*L. monocytogenes, B. cereus; S. aureus*	(149)

Antimicrobial agent – Essential oil	**Filler/matrix**	**Effect on mechanical and chemical properties**	**Antimicrobial activity and use**	**Ref.**
Summer savory EO	CMC/Agar	Increased mechanical flexibility, hydrophobicity; Reduced transparency	S. aureus, B. cereus, L. monocytogenes, E. coli	(204)

Essential oils incorporation in the matrix

In active packaging applications, EOs are particularly interesting since they can be released as vapors from the film, sterilizing both the headspace and the food surface. Moreover, they are approved by the FDA for food applications and are recognized as safe. So, they are an attractive alternative to conventional antimicrobials, which have experienced a continuous increase of resistance from microorganisms (205). Furthermore, while introducing a new agent inside the packaging material, not only the antimicrobial properties, but also the mechanical, barrier and thermal properties of the final composite will be influenced and possibly improved (9,206). As an example, EOs incorporation can reduce the water vapor permeability (WVP) of hydrophilic materials and can also decrease the tensile strength (TS), while increasing the elongation at break. This is possible thanks to the partial replacement of stronger polymer–polymer interactions by weaker polymer-oil interactions in the film network (44). Comparable results were obtained when different cellulose esters were tested as matrix for the incorporation of EOs. In fact, lemongrass, basil and rosemary pepper EOs behaved in the matrix like plasticizers, affecting the Young's modulus, the tensile strength and the elongation at break of the films (207).

The incorporation of the EOs in a polymer matrix such as nanocellulose, PLA or chitosan, could be done with different methods, which are chosen based on the materials characteristics and on the release kinetics requested by the final product. As an example, the active molecules or scavengers could be directly dispersed inside the matrix (166) or encapsulated (48) inside a carrier prior to the addition in the packaging material. The latter procedure, indeed, allows more flexibility in terms of active substance dispersion in not compatible polymers and in terms of release rate control. In fact, microencapsulation increases the stability of these compounds and allows to obtain a controlled and continuous release (208), which leads to antimicrobial activity without altering the organoleptic properties of the food product (209). Essential oils can be microencapsulated into the matrix following several different technologies, such as spray-drying, simple or complex coacervation and extrusion (208). The mentioned dispersion methods, indeed, result among the most effective techniques for protecting these compounds against volatilization, oxidation, and thermal degradation (210,211). In addition, encapsulation not only protects the antimicrobial compounds from the effects of the outside environment (212), but also allows to control the influence of the mechanical and transport properties of the film (213,214). Active ingredients could also be incorporated through a Pickering emulsion (215,216), which stabilizes the oil-in-water solution interface by using solid particles (50) or through nano-liposomal systems (217). On the other hand, direct application of EOs on the film could lead to inactivation of the

antimicrobial compounds due to the interaction with the matrix and makes control of its release difficult. For that reason, it is mainly considered for edible polymer films (44,218,219).

While incorporating these active agents inside the matrix, it is fundamental to take into consideration that the concentration of the EOs active compounds depends on their origin. Since different compounds could act against different microorganisms, some EOs can be more active in certain types of food products. For this reason, mixing different EOs can be a strategy to widen their active spectrum and increase their effectiveness. EOs, indeed, often show synergistic interactions and, when used in combination, they show increased antimicrobial properties with respect to the single components (220). However, since the antimicrobial activity of the EOs could decrease after the dispersion in the polymer and the microbial population could vary depending on the food product, *in vitro* and *in situ* analysis are usually required to define the real efficiency of the chosen solution (161,221).

A vast variety of essential oils and other plants extracts has been considered for food packaging applications (222,223). However, oregano (224–227) and thyme (228,229) demonstrated the strongest antimicrobial effect due to the presence of the phenolic compounds thymol and carvacrol (230). These compounds have received substantial attention as useful natural antimicrobial agents. They exhibit a broad antimicrobial spectrum against different microorganisms (231) and possess sufficient heat stability to withstand the incorporation into packaging materials (42,232).

Due to these multiple possibilities, each active packaging system needs to be adapted based on the food product, not only in terms of materials used but also in terms of mechanism of release and action. In fact, the antimicrobials present in the film could have a diffusive release in the headspace, with a decreasing effect over time, or act when in direct contact with the food, in case they are immobilized on the packaging surface or used as edible films (12,233).

Lastly but not least, it is important to consider the sensorial impact that the essential oils have on the food, since they contain terpenes. It is well known that these molecules convey characteristic smell and taste, which is also one of the main reasons why they are used since many years in different applications. Regarding the food packaging, although the use of natural agents such as essential oils is a valid option to preserve the food in a safe way, the main constraint remain the sensory impact and perception owed to their strong flavor (234). In fact, the intense aroma could interact with the complex system of the food-packaging and alter the natural taste of the food. Active packaging systems are already a solution to this, because the incorporation of the essential

oils into the matrix or their encapsulation prevent the direct contact with the food and the immediate dispersion of the volatile molecules in the head space of the package. Anyway, since the essential oil at some point will diffuse and be present in the head space, it is fundamental to perform a sensorial evaluation of the system and to be aware of their potential toxicity (235). This evaluation must be case specific because the EOs' minimum inhibition concentration, mode of action and sensory effects variates with their source, method of extraction and regulatory requirements. Several studies have been performed to understand the EOs effects on the sensory attributes of different types of food, but larger scale tests are still necessary to propose this technology to the consumers (166,236). Moreover, the legal framework concerning the use of EOs in food, refers to them either as additives and flavors or as part of the packaging material. In the first case, they are categorized following the Commission Regulation (EU) 1130/2011 on food additives (237) and the list of flavoring substances provided for by Regulation (EC) 2232/96 of the European Parliament and of the Council (238). While the second case is included in the legislation framework previously explained regarding the food packaging materials.

1.3 Nanocellulose based active packaging

As already discussed, cellulose is one of the most used polymers for active and sustainable packaging production (239). Many different natural antimicrobial additives have been studied in combination with cellulose (240–243). In the present section an overview of the latest applications in the field of active packaging based on essential oils (EOs) will be given. In particular, the focus will be on cellulose nanocrystals (CNC) and nanofibrils (CNF) and on nanocellulose derivatives such as cellulose esters (like cellulose acetate and sulphate) or cellulose ethers (such as methylcellulose and carboxymethylcellulose).

Cellulose nanocrystals (CNC) and nanofibrils (CNF)

As said above, nanocellulose crystals or fibrils, obtained from pristine cellulose fiber (19), have found several applications in the field of food packaging, thanks to their unique properties. They are often used as fillers to improve the tensile strength of the composite material (96) and many applications are also found where they helped the dispersion of the essential oils into the matrix (174). This type of solution will be discussed further in one of the following paragraphs, while this section is more focused on the direct application of CNC and CNF as matrices to produce antimicrobial films.

In some case nanocellulose was used to stabilize and protect the essential oil. Souza et al. (2021), for example, obtained films with high activity against *B. subtilis* by preparing nanocellulose-based Pickering emulsions with cinnamon essential oil (165). Zhou and coworkers (2018), instead, analyzed the antimicrobial activity of oregano EO Pickering emulsion stabilized by CNC (164). Good stability at higher CNCs concentration and pH values, or at lower oil/water ratio and salt concentration was demonstrated, together with a slightly higher antimicrobial effect. In fact, the OEO Pickering emulsion exhibited an inhibitory effect against *Staphylococcus aureus*, *Saccharomyces cerevisiae*, and *E. coli,* with a minimum inhibition concentration (MIC) of 12.5 µL/mL. The highest antimicrobial effect was obtained against *Bacillus subtilis*, with a MIC of 6.25 µL/mL.

The direct incorporation of ginger EO and citric acid in CNF edible coatings (20 g/kg and 10 g/kg, respectively) was instead studied by Khaledian et al. and increased the meat shelf life up to 6 days (34). The combined effect of the EO and citric acid resulted then in an increase of the antimicrobial properties and the overall acceptability of the food samples. Also, the antimicrobial activity of cellulosic pads amended with oregano (OEO), thyme (TEO) and cinnamon (CEO) EOs were demonstrated (166). They were effective against meat bacterial species and other common foodborne pathogens like *S. aureus.*

Direct incorporation of the essential oils' active compounds was also considered for the addition of polyphenols in a cellulose dispersion obtained through mechanical fibrillation. The resulting films had low porosity and high compactness, thus good barrier properties and improved hydrophobicity (244). The addition of tannin to CNF reduced the air permeability more than 6 times with respect the pure film, reaching a value of 3.1 mL/min, which is comparable to the polypropylene one. Also, the release of tannins ensured antioxidant activity for 48 hours. These films were thermally stable until *ca*. 230°C and chemically resistant against common organic solvents. A possible application could be dried food packaging (like rice and pasta), as well as for preserved fruits, vegetables and meat.

Direct incorporation therefore results in a very viable and simple method to produce nanocellulose based films. Indeed, the antimicrobial activity does not seem to be reduced by the interaction between the fibers and the EOs. This is, however, not the only option available and other methods were used with satisfactory results. For example, microencapsulation is a common technique used to protect the sensitive compounds inside a carrier. Saini et al. (2017) studied the microencapsulation of carvacrol in beta-cyclodextrin (β-CD), directly grafted on the carboxyl

groups of TEMPO-oxidized CNF (245). These films showed a sustained release of the active molecule over 150 hours and then reached the equilibrium in water. Moreover, the carvacrol antimicrobial activity against *B. subtilis* was increased by the presence of β-CD from 3 to 50 hours. This is due to the three-dimensional shape of the β-CD, which forms an inner hydrophobic cavity with an outer hydrophilic wall (246). In this way it can entrap molecules and create complexes by either hydrogen bonds, hydrophobic or Van der Waals interactions. Thus, the release rate of the included molecules is strongly influenced by those interactions (247).

Cellulose esters

In the field of active packaging, the most studied cellulose esters resulted to be cellulose acetate (CA) (248) and cellulose sulfate (CS) (174). Several EOs (Oregano, OEO, Cinnamon, CEO, and Sweet Fennel) were incorporated in CA films in different combinations in order to test the antimicrobial effectiveness of the biodegradable composite (167). OEO combined with CEO showed good results in terms of reduction of water vapor transmission rate and antimicrobial activity against *Penicillum spp.* and *E. coli*, with diameters of inhibition zones of 2.74 and 1.14 centimeters, respectively. Films incorporated with pure OEO were more effective against *S. aureus*, with 3.75 centimeters of inhibition zone. All the other combinations of EOs were effective against these microorganisms, but with a much lower inhibition zone.

Also, the effect of pink pepper EO (PPEO) in CA was studied (60), with films active against *S. aureus* and *Listeria monocytogenes*. The antimicrobial effect started from a concentration of 20 g/kg of the PPEO, that demonstrated the capacity to diffuse in solid, liquid and gas phase until reaching the contaminated cheese used for the tests. Cellulose acetate active films incorporated with Rosemary EO were produced to control the pathogenic microorganisms on chicken breast cuts (168). The films showed increasing antimicrobial activity at increasing EO concentration. However, it was necessary to reach a concentration of 500 g/kg to obtain an effective result. Also, the effect of Rosemary and Aloe Vera EOs on CA films was studied (169). The presence of these essential oils decreased the tensile strength, the water uptake and the contact angle, but increased the free radical scavenging activity. The antimicrobial activity against *E. coli* and *B. subtilis* increased as Rosemary and Aloe Vera oil percentage increased in CA membranes. In particular, the films containing 80% (v/w) of EOs (based on CA weight) showed no bacterial growth over 7 days of storage. Moreover, the electrospinning technique has been used to create CA nanofibers with 1 and 5 mL/L of Rosemary and Oregano EOs (170). The fibers with 5 mL/L of

oregano EO showed the best antimicrobial and anti-biofilm effects, especially for *E. coli* and *Candida albicans*.

Harini and Sukumar (2019) studied the direct incorporation of thymol, the major active compound present in the polar fraction of oregano EO, inside CA (171). The films were produced through vacuum drying and the difference between bulk dispersion and surface immobilization of the active compound was studied. The obtained films were transparent and showed, respectively, > 90% and *ca*. 65% of thymol retention. The UV-assisted surface immobilization decreased the mechanical and barrier properties of the CA films. Good antioxidant and antimicrobial properties were obtained in general, even if films with the thymol dispersed in the bulk showed higher activity. These films were active against *L. monocytogenes, S. aureus, E. coli, P. aeruginosa, Klebsiella pneumoniae,* and *Salmonella enteritidis* with a minimum inhibition concentration of 20 mg/L. The effect of thymol was also studied when used together with organoclay, which resulted to increase the antimicrobial effect against *Listeria innocua* (172).

In terms of addition method, among other techniques, it is interesting to mention the study concerning the release of thymol from a cellulose acetate film impregnated using supercritical carbon dioxide ($scCO_2$) (66). This technique was already studied on CA (249) and other polymers (250) and it is already used at industrial scale for the extraction of low volatility and/or thermal sensitive compounds. Due to its low viscosity and surface tension, indeed, $scCO_2$ can easily penetrate into a solid matrix, facilitating the impregnation processes and ensuring a good distribution of the active molecules (173). The CA structure and morphology of the obtained films resulted dependent on the thymol content. Increasing the thymol content above 137 g/kg, indeed, led to a decrease of the glass transition temperature up to 29 °C, while the crystalline arrangement of the CA disappeared. In general, the thymol release, which depends on the concentration and on the release medium, was up to 3 days. The thymol was detected on the CA surface as well, thus allowing antimicrobial activity through direct contact. The impregnated CA showed antibacterial activity against 23 tested strains, in particular against methicillin-resistant *S. aureus*, cause of fatal infections in animals and humans (66). The optimal thymol loading for an efficient reduction of biofilm formation resulted to be in the range from 26 to 30% wt. (with respect to polymer mass) (173). In particular, the film containing 30% wt. of thymol exhibited antiadhesion properties on its surface. They resulted active against all tested strains, including antibiotic resistant *Pseudomonas aeruginosa* DM50 and methicillin-resistant *S. aureus.* Furthermore, CA incorporated with OEO and montmorillonite clay were used to control the growth of phytopathogenic fungi (108). It was demonstrated that contemporary addition of active oils and

nanoclays allowed to obtain active films with a decreased water vapor transmission rate and an improved thermal stability.

CS was also investigated as a potential matrix for the development of food packaging films, although to a lower extent with respect to CA (251). Cellulose sulfate-based films with slow release of mustard EO due to the presence of β-CD were tested (174). The mustard essential oil (MEO) had already been studied as active agent in edible films against *L. monocytogenes* (252). The addition of MEO to CS reduced the TS and the water sorption without affecting the WVP and increased the elongation at break (EB). Also, the films showed great antimicrobial activity against *E. coli, S. aureus* and modest activity against *B. subtilis* and *Aspergillus niger*, which was attributed to a lower antimicrobial effect of MEO for these bacterial strains.

Cellulose ethers

In the field of active packaging, the functionality of biocomposite based on methyl cellulose and carboxymethyl cellulose in combination with plant extracts, such as murta fruit (*Ugni molinae*) (175) or curcumin (253) was investigated. The mechanical strength and water vapor barrier properties of the films improved, as well as the antioxidant and antimicrobial activity.

Studies on carboxymethyl cellulose (CMC) were done by incorporating Mexican OEO at different values of pH (176). It was found that antimicrobial treatment against *L. monocytogenes* and *S. aureus* were more effective at lower pH values (pH = 5 and 2.5 g/kg of Mexican OEO). Also, the *Mentha spicata* EO (MSEO) was investigated (177). The treatment of fresh strawberries with CMC with 2 g/kg MSEO resulted in a decrease of *L. monocytogenes* population, while physicochemical and organoleptic properties were maintained. Moreover, MSEO was used together with *Ziziphora clinopodioides* EO (ZiEO) to create CMC active coatings for the extension of fresh and sauced chicken breast fillets shelf-life (178). In fact, the application of CMC with ZEO (2.5-5 g/kg) and MSEO 5 g/kg increased the shelf-life up to 14 days and completely inhibited the growth of *Campylobacter jejuni*, while the growth of *L. monocytogenes, S. aureus, E. coli* and *Salmonella typhimurium* was retarded.

The effect of bay EO on CMC was also considered (72) reporting high antioxidant activity (up to 99%) and inhibition of microorganisms' growth (*E. coli* and *Candida glabrata*). Good barrier properties against water vapor (50% improvement with respect to CMC in films containing 150 g/kg of EO) were observed and UV-light barrier effect was increased (almost 100% of protection). Higher water solubility (93%) was finally found, which ensured material biodegradability. CMC was also incorporated with *Zataria multiflora Boiss* EO (ZaEO) (179). The increase of ZaEO

content led to a decrease in transparency and an increase in total phenol content and antioxidant activity. Figure 1.8 shows the SEM images of the surface (left) and cross-sections (right) of CMC films containing different concentrations of ZaEO (1,2, and 3% v/v based on the CMC solution). Pure CMC film appeared homogeneous and smooth, while the presence of ZaEO led to a more heterogeneous structure. The porous structure could be due to the evaporation of the EO during drying or to the entrapped air bubbles during membranes fabrication. The films with the highest ZaEO content (3% v/v) had the best essential oil dispersion in the matrix. They also showed the highest microbial inhibition, in particular against *S. aureus, B. cereus* and *E. coli*. Raeisi and coworkers added to the same composite the grape seed extract (GSE) and observed the effect on the shelf life of rainbow trout fillets (254). The minimum number of total viable bacteria (lactic acid bacteria and *Pseudomonas spp*.) were determined in the fillets coated with CMC plus 20 mL/L ZaEO and 10 mL/L GSE. While the fillets containing 10 mL/L of both compounds resulted to have the best organoleptic properties.

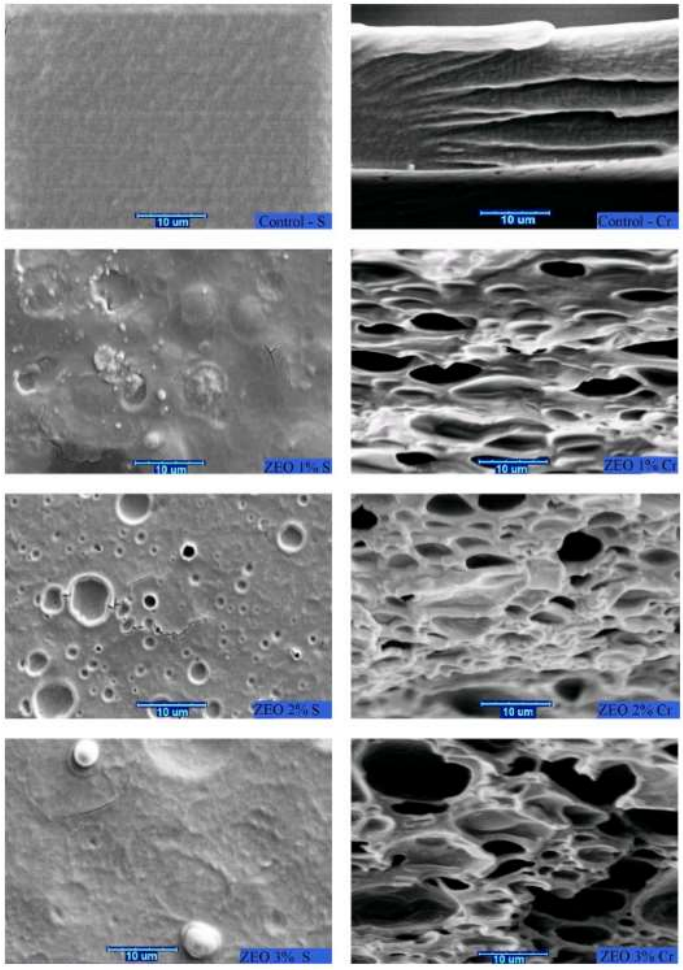


Figure 1.8. SEM images of the surfaces (left) and cross-sections (right) of CMC films containing different ZaEO concentrations (from the top: control, 10, 20 and 30 g/kg of content) (179).

Edible films containing *Thymus daenensis* EO from wild and cultivated plants loaded in hydroxyl propyl methyl cellulose (HPMC) were also produced (180). The uniform incorporation of the nanoemulsions into the matrix led to a plasticizing effect and in a relevant antimicrobial effect against several microorganisms. In particular, the EO from the wild plant showed better antimicrobial activity against gram-positive bacteria. While the EO from the cultivated plant was more effective against the gram-negative ones. This difference is due to the various quantities of components in the EOs: the wild EO was indeed poorer in thymol and carvacrol, but richer in ρ-cymene. The latter is a precursor of carvacrol, and it was found to have a synergistic effect with it. In fact, when combined, these two substances can cause the swelling of the cytoplasmic membrane (42).

1.4 Chitosan based active packaging

Chitosan, as previously explained, demonstrated to be suitable and convenient to develop novel food packaging systems due to its intrinsic antimicrobial activity and its film forming ability (120,121). In addition, the mechanical and barrier properties of chitosan have been largely reviewed in comparison with synthetic plastics (255).

The most important studies regarding chitosan in packaging application are related to antibacterial packaging based on the material intrinsic properties (158) or on their synergetic effect with other active substances (256–258). In the literature it is possible to find many studies on the incorporation of natural antimicrobials such as nisin (257,259), polyphenols (260–262) and various plants extracts (263–270). These advances are related to the use of chitosan-based materials in various fields such as wound healing, food packaging, textile and biomedical sectors (271).

Active Chitosan films

As in the case of nanocellulose, most of the studies involving chitosan/EOs active packaging were focused on the analysis of antimicrobial activity on different gram-positive and gram-negative pathogens. The influence on the physical and mechanical properties of the films was also studied. As an example, the incorporation of rosemary EO up to 15 mL/L was able to produce chitosan films with antimicrobial activity against *L. monocytogenes*, *Streptococcus agalactiae* and *E. coli*. The treatment also decreased their light transmission in UV light and the solubility and the water

uptake by about 25% and 85%, respectively (182). In other works, fennel, peppermint (272) and *Citrus limonia* (273) essential oils were tested showing that they also help in decreasing the moisture content and protect from UV light. The same trends were observed with the use of *Ziziphora clinopodioides* EO, red grape seed extract (183), and with the use of Apricot (*Prunus armeniaca*) kernel EO (184). Also, the incorporation of lemongrass essential oil had similar results, with a 101% improvement in the elongation at break and 15% reduction in water vapor permeability (185). Other authors tested thyme EO in chitosan and found it more effective as antimicrobial than clove and cinnamon EOs (187). Moreover, the presence of thyme and clove EOs in chitosan films led to an increase of the moisture content (+23%), the solubility in water (+28%), the water vapor transmission rate (0.004 g/s/m^2), and finally on the elongation at break (+34%). Interestingly, cinnamon-enriched films showed an opposite behavior, with a decrease of EB and an increased tensile strength. The same trend was observed also by other authors, which suggested that these results are due to the cross-linking effect of CEO components within the chitosan matrix (86). Although, the antimicrobial effect against several gram-positive (*L. monocytogenes, Lactobacillus plantarum, Lactobacillus sakei*) and gram-negative (*Pseudomonas fluorescens, E. coli*) bacteria was satisfactory for a concentration of 20 mL/L of EO.

Chitosan films enriched with essential oils have been recently amended with gelatin and characterized (188). They were active against *C. jejuni, E. coli, L. monocytogenes* and *S. typhimurium*. In addition, they showed good barrier properties against UV light and an increase in moisture absorption and water vapor permeability. Chitosan films were also used in combination with propolis extract instead of essential oils. Since the extract has a high polyphenols content, the antimicrobial and mechanical improvements observed were similar to those obtained with essential oils (274).

Considering direct application on food products, fresh strawberries are among the most common foods used to test antimicrobial properties in active packaging. Their treatment with chitosan and *Mentha spicata* EO 2 g/kg resulted in a decrease of *L. monocytogenes* population while physicochemical and organoleptic properties were maintained (177). Fresh strawberries were also treated with edible bioactive chitosan films containing red thyme and oregano extracts substantially increasing the shelf-life, as visible in figure 1.9 (181). Thyme EO was also tested as antimicrobial agent inside a chitosan matrix used for cooked ham packaging. Its presence reduced the water condensation inside the package and the odor was perceived as desirable in the food product. Moreover, the yeast population was reduced by the antimicrobial agent, while

the aerobic mesophilic bacteria, the lactic acid bacteria and the enterobacteria were not affected (186).

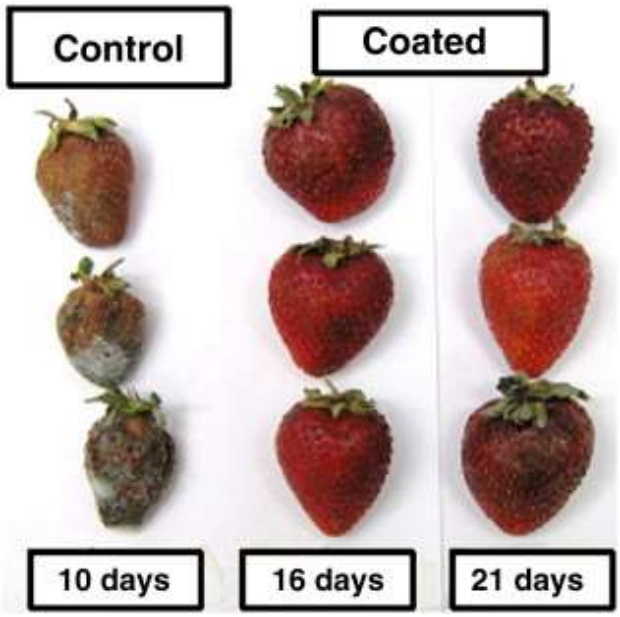


Figure 1.9. Appearance of strawberries coated with modified chitosan-based formulation during shelf-life test. The control (on the left) after 10 days shows worst conditions compared with coated strawberries after 21 days (on the right) (181).

Chitosan nanoparticles

In addition to its function as matrix, chitosan has also been considered to produce nanoparticles (CSNs) (275), alone or in combination with nanoclays (276) and nanofibers (277). They can be homogenously mixed with different essential oils to enhance the shelf life of various food products. This procedure is usually applied to fruit or vegetables, which are dipped in the coating solutions containing the EOs entrapped into the chitosan nanoparticles. The effect of chitosan nanoparticles incorporated with *Cinnamomum zeylanicum* EO on the cold storage of cucumber was observed by Mohammadi et al. (2015) (189). The CEO encapsulated by CSNs provided a better antimicrobial activity against *Phytophthora drechsleri*, improving the shelf-life and the physicochemical quality of cucumbers. The same research group also encapsulated *Zataria multiflora* EO in chitosan nanoparticles through ionic gelation. They studied the release rate and the performance *in vitro* and *in vivo* against *Botrytis cinerea,* the major cause of gray mould disease (190). The findings revealed a promising technique to prevent postharvest fruit from decay and extend the storage life, without the use of synthetic fungicides. Carvacrol-loaded chitosan

nanoparticles were also tested to maintain quality of fresh-cut carrots (191). The vegetables dipped in the washing treatments presented a 2-6 lower log CFU/g units compared to control samples and better sensory and physicochemical quality after 13 days at 5°C. Similarly, the effect of cinnamon EO in chitosan nanoparticles on the conservation of chilled pork was object of study (48). In this case, the food product was wrapped with low density polyethylene films, whose inner surface was coated with layers of chitosan nanoparticles of distinct size, loaded with the EO. After 15 days of storage at 4°C, a significant decrease of microbial growth, pH and peroxide concentration was observed for films containing microparticles (527 nm).

1.5 Biocomposite films

Bio-nanocomposite materials consist of a matrix composed of a bio-based polymer and a dispersed nanometric phase which is meant to improve the properties of the base material. Most nanocomposites are focused on the improvement of the mechanical and structural properties of the matrix. It is quite common, for example, to use cellulose nanowhiskers or cellulose nanocrystals as reinforcements, due to their high tensile strength and modulus (84). They can be easily dispersed in hydrophilic polymers (101) and can be modified to increase the compatibility with non-polar matrixes, improving their mechanical, physical, thermal and optical properties. At the same time, they help the homogeneous homogenous dispersion of the active molecule previously integrated (36). The contemporary incorporation of nanoparticles and essential oils, therefore, has become a common technique to endow the final biocomposite materials with additional antimicrobial properties while maintaining sufficient mechanical strength (18). The incorporation of essential oils in nanocomposites, in addition, allows to modify properties not related to the antioxidant and antimicrobial activity, such as film transparency or hydrophobicity.

Nanoparticles can be treated or coupled with the EOs before or after the addition to the matrix. Several techniques have been used to create biocomposite films, depending on the type of materials involved and the final application of the composite. Some of the most used are, for example, extrusion, solvent casting, impregnation, layer-by-layer deposition and spin-coating (96). Among the several existing techniques, then, the creation of nanoemulsions of the EOs and nanoparticles prior to dispersion in the active matrix represents a step forward for the food packaging applications, in particular for the incorporation of active compounds in films and coatings (278).

As in the previous cases trying to give an organic overview of the many studies available the section has been divided following the main materials considered as a matrix for the composite, starting from chitosan-cellulose composite to then consider other biopolymers added with EOs, chitosan and/or nanocellulose.

Chitosan – cellulose composites

Biocomposite films where chitosan and cellulose in different forms are used together as matrix and/or reinforcement for active food packaging applications are rather common in scientific literature (279–281). The focus, in this concern, is not only on antimicrobial activity, but also on the influence that the different components have on the mechanical properties of the film. In this case, most often *in vitro* studies were conducted, without direct testing on food products.

For example, CNC incorporation in chitosan matrix allowed to obtain films with antifungal properties, active for chicken meat shelf-life increasing (281). Also, many works on their combination with other polymers and with agricultural or food process wastes are reported. For example, the production of biodegradable nanocomposites from carrot minimal processing waste (CMPW) was optimized in combination with HPMC as ligand and high-pressure microfluidized cellulose fibers for the mechanical reinforcement (282). While a composite film based on bacterial cellulose and chitin has been recently developed (283), also considering the case of multi-nanofiber system (284). The nanofibers allowed a good dispersion of curcumin nanoparticles in the films, which reduced the TS and increased the WVP. However, these modifications were counterbalanced by the presence of chitin nanofibers, which positively affected both mechanical and barrier properties of the material. The produced films also showed antioxidant and antibacterial activity against *E. coli* and *S. aureus* with an inhibition ratio of 65% and 75%, respectively. Conversely, the incorporation of curcumin extract in pure chitosan resulted in opposite effect on mechanical properties (285). This indicates that even if these materials have certain properties and known effects on the matrix, their combination strongly influences the final properties of the composite.

Moreover, binary edible films made from CMC and chitosan biguanidine hydrochloride (CGg) activated with frankincense oil (FO) or titanium oxide nanoparticles (286) were prepared and analyzed by Salama et al. (2019) (192). The presence of FO resulted in a lower WVP, higher TS and EB, without any change in transparency. These films exhibited antibacterial activity against *S. pneumonia, B. subtilis* and *E. coli*. The same biopolymers were used to produce chitosan/CMC

films incorporated with glutaraldehyde, cinnamon EO and oleic acid (OA), in order to study their simultaneous effect (193). In fact, the cross-linkage by glutaraldehyde improved the mechanical properties, and its use together with the CEO increased the film bioactivity. Also, the presence of OA increased the antimicrobial and antioxidant activity. While the inclusion of both CEO and OA significantly increased the WVP, due to significant changes in the microstructure of the biocomposite. This could be due to the covalent interactions between the essential oil constituents and/or OA with the biopolymer chains.

Equivalent results were found investigating the effect of cinnamon and ginger EOs on chitosan/CMC films emulsified with oleic acid (194). Clear differences appeared between cinnamon EO and ginger EO incorporated films. As the amount of each essential oil increases, the crystallinity decreases with the former essential oil, while it increases with the latter one. In fact, the cinnamaldehyde present in cinnamon could interact with the network created by CMC, chitosan and oleic acid, acting as plasticizer and inhibiting close packing in the polymer chains. Moreover, the cinnamon-incorporated films showed higher antifungal activity *in vitr*o against *A. niger* and higher increase in elongation at break percentage: +328% compared to +111% of the ginger films.

Chitosan based films reinforced with CNCs and encapsulated with thyme, oregano, tea tree and peppermint EOs nanoemulsions showed improved mechanical properties and better release of the active compound (52). They were tested *in vitro* and *in situ* against *Asperigillus niger, Aspergillus flavus, Aspergillus parasiticus* and *Penicillum chrysogenum*, reducing their growth by 51-77%. *In situ* experiment on inoculated rice during 8 weeks of storage resulted in a 2 log reduction of the fungal growth. The irradiation of the materials with a dose of 750 Gy of ionizing radiation further increased the antifungal and mechanical properties. The same research group also considered Methyl cellulose (MC) reinforced with CNC and amended with a blend of oregano and thyme EOs (195). The optimal conditions were found to be 75 g/kg CNC into MC containing 5-7.5 g/kg EO. The films exhibited the same antifungal activity as the previous study (52) and the irradiation treatment resulted again in improved antifungal and mechanical properties. The presence of different matrices and EOs concentration in the two cited works led to different characteristics in the final films. Respectively, the chitosan-based and the CM-based films treated with irradiation had a tensile strength of 57 and 64 MPa, an increase of the elongation at break of 36% and 26% and an increase in water vapor permeability of 24% and 5%. Chitosan films enriched with cellulose nanoparticles were also studied in combination with ethanolic propolis extract (287).

Chitosan matrix incorporated with 10 g/kg nanocellulose fiber and 10 g/kg thyme EO was tested on sweet cherry quality during storage (196). After 5 weeks of fruit storage within the edible coating, the nanocomposite affected the fruit's water retention, decreasing the weight loss and preserving the anthocyanin content. Moreover, the total sugar content increase indicates dehydration and decomposition of organic acids in the fruit during the storage time.

The effect of cellulose and lignocellulose nanofibers (LCNF) (40 g/kg) as nanoreinforcement on *Origanum vulgare* EO (50 g/kg) – loaded chitosan films was investigated (197). The films without nanoreinforcement, containing only the EO, showed higher antioxidant and antimicrobial activity against *E. coli* and *B. cereus* than bionanocomposite films, where the release controlling effect of CNF and LCNF is present. The incorporation of EO and CNF/LCNF, on the other hand, improved the solubility and the water vapor barrier but affected the color properties. This is due to the new hydrogen bonds created between the chitosan chains, the EO and the nanofibers. LCNF resulted to better disperse the EO into the chitosan matrix, which led to better properties of the composite. The same investigation was done also with the use of *Carum copticum* EO which gave similar results with respect to the previous study (198).

Combination of PLA and nanocellulose or chitosan

PLA-CNC nanocomposite films containing OEO were tested against *Listeria monocytogenes* in mixed vegetables (59). It was observed that the presence of the OEO did not affect the WVP, but increased the EB while reducing the TS and the tensile modulus. These films demonstrated a strong antimicrobial activity through the continuous release of phenolic compounds over the tested period.

The fabrication of a PLA composite with the addition of CNC is expected to significantly increase the mechanical properties of the material. Interestingly, however, Khodayari et al. showed that such improvement was more pronounced when it was coupled with different concentrations of *Tanacetum balsamita* EO (TBE) and propolis ethanolic extract (PEE) (199). While PLA films containing PEE, alone or coupled with CNC, could not inhibit the growth of bacteria, the presence of TBE allowed to affect gram-positive and gram-negative bacteria, especially *B. cereus*. All films containing TBE showed significant antibacterial effects against aerobic mesophilic bacteria, lactic acid bacteria and psychrotroph. The same nanocomposite was created using *Ziziphora clinopodioides* EO to improve beef meat shelf life (200). The microbial population after 11 days of storage of minced beef decreased 1 to 3 log CFU/g and films containing 20 g/kg EO extended the shelf-life without any alteration of the organoleptic properties.

PLA was also used in combination with CNF gel, through the incorporation of *Zataria multiflora* essential oil (ZaEO) and propolis ethanolic extract (PEE), by solvent casting method (201). The gel was obtained directly from wood particles by a mechanical method. The addition of ZaEO and PEE made the films more flexible. The presence of CNF improved the WVP, the TS (+32%) and the elastic modulus (+19%). The maximum antibacterial effect was recorded in the film containing both ZaEO (5 mL/L) and PEE. In particular, the PLA/10 g/kg of ZaEO/PEE composite was able to increase the shelf life of sausages up to 40 days, addressing the antimicrobial activity against *S. aureus, E. coli, Vibrio parahaemolyticus* and *L. monocytogenes.*

PLA has also been combined with chitosan-based materials. For example, Fiore et al. (2021) tried to coat PLA film with chitosan enriched with rosemary essential oil for the development of fresh minced chicken breast application (202). With a concentration of 20 g/kg of essential oil in the coating it was possible to reduce by 25% the water vapor permeability and increase the antioxidant activity. In general, those films demonstrate the ability to improve the shelf-life of fresh meat products.

Alginate antimicrobial biocomposites

Alginate edible films were incorporated with oregano, cinnamon or winter savory EOs and their antimicrobial activity was tested against *Salmonella Typhimurium* or *Listeria monocytogenes* in ham slices (288). The films were pretreated with different concentrations of calcium chloride. In fact, the formation of ionic bonds produced an insoluble gel, which affected the release rate of the active compounds. Cinnamon-based film pretreated by immersion in a 20% $CaCl_2$ solution was the most effective against both pathogens.

The application of sodium alginate (SA) films activated with CMC, CEO as antimicrobial agent, glycerol as plasticizer and Tween® 80 as surfactant were studied by Hal and al. (2018) (49). At the highest CEO concentration of 15 g/L, the inhibitory effect against *S. aureus* increased with the increase of the Tween® 80 concentration. The incorporation of Tween® 80 in the SA/CMC matrix may facilitate the release of CEO from the film matrix and promote its diffusion into the surroundings, thus increasing the antimicrobial activity of the film. These film forming solutions were coated on banana fruits to test possible shelf-life extension. The bananas coated with SA/CMC containing 15 g/L CEO deteriorated more rapidly than those with control coating. This was probably caused by the increase in oxygen permeability of the films that, associated with the higher oxygen solubility in CEO, favored the oxidation of phenolic compounds. Water content, on

the other hand, was decreased in presence of CEO and, due to the increased hydrophobicity of the films, also WVP was reduced for SA/CMC films incorporated with 15 g/L CEO. Concerning the mechanical properties, finally, it is interesting to notice that the effects of Cinnamon EO are different to the ones observed in other matrices such as chitosan (187). Due to the specific interactions between the fillers and the matrix itself, the presence of CEO led in this case to an increase of EB and the decrease of TS of the films.

Agar antimicrobial biocomposites

Another example of biocomposite used in active packaging applications is the agar film reinforced with cellulose nanoparticles in presence of *Savory* EO (SEO) studied by Atef et al. (2015) (149). The addition of SEO decreased tensile strength, young's modulus, and water solubility, while increased the elongation, WVP and opacity of the nanocomposite film. In addition to these changes, the agar/cellulose-based nanocomposite showed a noticeable antimicrobial activity. In particular, the film containing 15 g/kg SEO demonstrated the highest inhibition zone especially against *L. monocytogenes* and *B. cereus*, while *E. coli* was more resistant. To improve the mechanical flexibility and the thermal stability of agar, summer savory EO was incorporated into a CMC-agar film (204). The biocomposite showed good inhibition against gram-positive bacteria and an improvement in mechanical flexibility and hydrophobicity, at the price of a reduced transparency. The same biocomposite was created with grapefruit seed extract. An increase in UV barrier properties, moisture content, water solubility and water vapor permeability was observed, with a decrease in tensile strength, elastic modulus and surface hydrophobicity (154,289) .

General considerations about the state of the art for active packaging

The literature research that has been conducted at the beginning of this work allowed to have a wide and clear map of the various studies conducted on this topic in the last years. Anyway, after this evaluation it is necessary to make some general considerations about the types of tests made for the different materials and systems analyzed.

For example, EOS added cellulose-based matrixes are effectively a promising green alternative for food active packaging applications. Almost all the works cited demonstrated the effectiveness against gram-positive and gram-negative bacteria independently from the method used for coupling. Only a fraction (about 30%) of them, however, tried to implement the developed solution on real packaging systems, mostly on cheese and meat products, and an even lower part (only 10%) focused also on a complete sensory evaluation. Also, the incorporation in chitosan of

various essential oils at different concentrations can be remarkably effective for the shelf-life elongation in fresh food products like vegetables and meat. In fact, these active components not only allowed the reduction of microbial growth, but also showed a decrease in water vapor permeability of the films, together with an increase in thermal stability and elongation at break. Interestingly, compared to what is seen for nanocellulose, in the case of chitosan-based active packaging, there was a higher number of works (about 60% of the articles analyzed) dealing with direct application on food products. They were mostly vegetable products or fruit, but a certain attention was given also to meat, cheese and bakery products.

In all these works the deterioration of the product was monitored through laboratory analyses, while only a small percentage of them also presented a comprehensive sensory evaluation of the packaged food. This kind of assessment, however, has significant importance for the development of the final product and should be treated with more attention.

In the current analysis, the most used essential oils resulted to be, in the order, oregano, cinnamon, thyme and rosemary. However, many other plant-derived compounds, also different from essential oils, were considered and resulted effective against a wide range of microorganisms. In fact, these natural compounds showed antimicrobial activity against the most known gram-positive bacteria (*S. aureus, B. subtilis, L, monocytogenes*), gram-negative bacteria (*E. coli*), and fungi (*S. cerevisiae, A. niger*).

In general, however, despite the extremely high number of works and the potential of most of the materials considered, it is difficult to draw general guidelines in the production of active packaging based on the combination of renewable materials and essential oils. Many of the available studies are indeed focused on a very specific application, to test the performance of certain essential oils, incorporated in each material, on the preservation of the quality of specific food. The use of a combination of essential oils in the same packaging, for example, is seldom considered, leading to ample space for further analysis and optimization of the currently tested solutions.

It can be noticed that, while most of the materials considered base their activity on the release of the active compounds, very few information is given about the release rate and kinetics, which are essential in order to design and adapt an active packaging solution for the desired shelf-life. For this reason, the first experiments of this research project were focused on the transport properties of essential oils into nanocellulose matrix as a basis for further development of these types of systems.

2. Materials and Methods

The present study focused on the analysis of the absorption and desorption kinetics of cinnamon, thyme and oregano essential oils into different nanocellulose matrices. Also, the development of an active system to be tested both directly on bacterial strains and on food product was targeted. In this section, the materials and the characterization techniques adopted for the kinetics and antimicrobial analyses will be presented.

2.1 Materials for film preparation

The Nanofibrillated Cellulose (NFC) utilized in this work was kindly provided by INOFIB in water suspension and was obtained from eucalyptus pulp. The NFC was purchased both in water and in acetone suspensions. Pure NFC and surface modified carboxymethylated NFC (CMC-NFC) were used. Three different types of NFC were selected: pure NFC (indicated in the following as NFC1), CMC-NFC-780 (NFC2) and CMC-NFC-1600 (NFC3), which had, respectively, a final superficial charge density of *ca.* 30, 780 and 1600 μequiv/g. The CMC-NFC was synthetized by alkali-catalysed reaction of cellulose from eucalyptus. The fibers were impregnated with a solution of monochloroacetic acid in isopropanol and then heated for 6 hours under the boiling temperature in a reactor fitted with a condenser. Then they were filtered and washed. After the mechanical treatment at 1500 rpm, a gel with a concentration of 1.7 wt.% was obtained. The fibers had final diameter of 80-150 nm (290).

Pellets of PLA4060 and PLA3100 from Natureworks were used for the fabrication of PLA membranes through solvent casting technique. Ethanol, chloroform, acetone and DMSO were purchased from Sigma Aldrich and used as solvents.

Cinnamon, Thyme, and Oregano essential oils were kindly provided by Destilerías Muñoz Gálvez, S.A. (Murcia, Spain). They were 100% pure oils, with respectively 74.7 % v/v of *Eugenol,* 55.5 % v/v of *Thymol* and 71.5 % v/v of *Carvacrol* as major active compounds*,* as indicated in table 2.1, which also reports the main properties of the different compounds. Figure 2.1 shows the chemical structure of the major components of these essential oils. Both the nanocellulose and the essential oil have been used without any further purification, as this is the way they are often used in active packaging application. Deionized water available in the laboratory was used to dilute the solutions during the film preparation procedures.

Table 2.1. Major compounds present in Cinnamon, Thyme and Oregano essential oils. The molecular weight, the boiling point and the vapor pressure are taken from https://pubchem.ncbi.nlm.nih.gov/ (291)

Cinnamon essential oil				
Compound name	**Composition (% v/v)**	**Mol. weight (g/mol)**	**Boiling Point (°C; @P atm)**	**Vapor pressure (mmHg @25°C)**
Eugenol	74.70	164.20	252	0.02
β-caryophyllene	3.97	204.36	245	0.30
Linalool	3.11	154.25	198	0.16
α-Pinene	1.45	136.23	155	4.36
Benzyl benzoate	1.32	212.24	324	$2.24x10^{-4}$
Safrole	1.24	162.18	232	$8.70x10^{-2}$
Eugenyl	1.13	206.24	281	~0
Thyme essential oil				
Compound name	**Composition (% v/v)**	**Mol. weight (g/mol)**	**Boiling Point (°C; @P atm)**	**Vapor pressure (mmHg @25°C)**
Thymol	55.50	150.22	232	0.02
p-cymene	16.80	134.22	177	1.50
γ-terpinene	5.77	136.23	183	1.09
Carvacrol	4.46	150.22	237	$2.96\ x10^{-2}$
Linalool	3.36	154.25	198	0.16
Thujene	1.21	136.23	151	4.77
Oregano essential oil				
Compound name	**Composition (% v/v)**	**Mo. weight (g/mol)**	**Boiling Point (°C; @P atm)**	**Vapor pressure (mmHg; @25°C)**
Carvacrol	71.50	150.22	237	$2.96x10^{-2}$
p-cymene	4.71	134.22	177	1.50
Linalool	4.32	154.25	198	0.16
γ-terpinene	3.98	136.23	183	1.09
β-caryophyllene	3.11	204.35	245	0.30
Thymol	2.19	150.22	232	0.02
Borneol	1.32	154.25	213	0.05

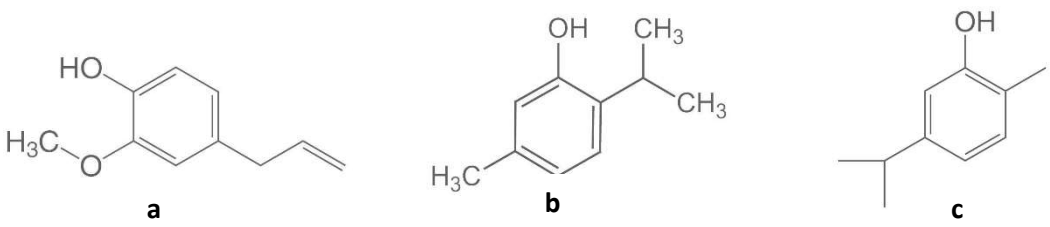


Figure 2.1. Chemical structure of the major compounds present in the Cinnamon, Thyme and Oregano essential oils: (a) Eugenol, (b) Thymol, (c) Carvacrol

2.2 Nanocellulose films

Solvent casting technique was adopted to prepare a series of thin films for the different tests performed during this work, both related to transport phenomena and antimicrobial essays. The NFC or CMC-NFC suspension was weighted with a standard laboratory balance. Then distilled water was added until reaching the desired weight concentration (*ca.* 0.5-1.7 wt.%), which was chosen based on preliminary experiments to obtain the correct viscosity for each type of nanocellulose used. After that, the solution was homogenized with a high-speed homogenizer (IKA - T 18 digital ULTRA-TURRAX®) and sonicated in order to eliminate the embedded air. In the case of pure NFC, the solution was also put in vacuum oven at ambient temperature for 10 minutes, since some air bubbles were still present after sonication. Finally, the solutions were casted in a PTFE Petri dish of 9 cm of diameter and dried at 35-40 °C for about 72 h. The final films were then removed from the Petri dish and stored in a plastic bag until they were used for tests. The weight (Mettler Toledo AE 240 Analytical Balance) and the thickness were measured as well. The thickness of the final samples was in the order of 30-50 μm, measured with a flat plate micrometer (Mitutoyo Absolute Series 227-221). In table 2.2 it is possible to find the specific parameters used to create each sample.

Table 2.2. Nanocellulose casting parameters

Name	Material	Conc. (wt.%)	Homogenization (Time and speed)	Drying Temperature (°C)
NFC1	NFC	0.50	10’ x 10k rpm	35
NFC2	CMC-NFC-780	1.24	15’ x 13k rpm	40
NFC3	CMC-NFC-1600	1.70	15’ x 13k rpm	35

2.3 PLA based films

PLA films were considered in the experimental analysis in the attempt of obtaining a completely biodegradable for the controlled release of the oils in active packaging applications. PLA was used as it resulted to be the most interesting biopolymer for this purpose, since it is already industrially used, easy to handle and process and completely safe.

In particular PLA films were casted alone as well as in mixture with nanocellulose. In the first case the aim was to build a multilayer system with nanocellulose films, with the polymer simulating the lid of a box on which the nanocellulose pad amended with the essential oils could be attached. In addition to that mixture PLA-Nanocellulose were also considered in the view of future application of this active packaging system, to understand if the oil saturated NFC fibres could be integrated in the biopolymer to enhance at the same time mechanical, barrier and antimicrobial properties.

2.3.1 Pure PLA films

For the fabrication of PLA membranes, different concentrations of PLA material were used in different solvents, in order to optimize the procedure and obtain homogeneous films. Also, two different grades of PLA were tested: PLA4060 and PLA3100 with the latter having a higher crystallinity. During preparation, the selected amount of PLA was dissolved in the selected solvent using a magnetic stirring and increasing the temperature. Since the solution had a very low viscosity, any further homogenization process was not needed, and the solution was directly poured into glass petri dish of 9 cm of diameter and let dry for 24-48 hours.

The table 2.3 resumes the crucial casting parameters used for these tests. In particular, for the two types of PLA tested, the concentration and the casting parameters (stirring velocity, temperature and time) are reported. Different parameters were tested in order to optimize the process and obtain homogeneous membranes.

Table 2.3. Parameters for the casting optimization of pure PLA membranes.

Sample name	Material	Solvent	Concentraion (wt.%)	Stirring (rpm)	Temperature (°C)	Time (min)
PLA1	PLA4060	Chloroform	2.99	300	60	60
PLA2	PLA 3100	Chloroform	3.24	300	60	60
PLA25	PLA4060	Acetone	3.02	500	60	40
PLA28	PLA3100	Chloroform	3.03	500	60	40
PLA41	PLA4060	Chloroform	2.94	500	60	25

PLA42	PLA4060	Acetone	2.99	500	60	30
PLA43	PLA3100	Chloroform	2.99	500	60	30

2.3.2 PLA-NFC pairing

In the view of future application of this active packaging system, it is important to understand how the NFC active pads could be integrated in a packaging system made from a biopolymer. After the literature research. PLA resulted to be the most interesting biopolymer for this purpose, since it is already industrially used, easy to handle and process and completely safe.

PLA-NFC pairing through casting

After obtaining pure PLA membranes, NFC was also included in the casting in different concentration, in the view of pairing these two materials for the active packaging membrane development. NFC was used both in aqueous solution and in acetone solution and carboxymethylated NFC was tested too (292). First, the PLA was dissolved in the selected solvent as explained in the previous paragraph. Then, the NFC solution was added to the PLA one and they were homogenized (homogenizer) for 10 minutes at 12000 rpm. Once the final solution appeared homogeneous with no solid suspension, it was poured into PTFE petri dishes and let dry at ambient temperature for 24-48 hours. Table 2.4 reports the parameters used for the casting optimization of the composite membranes. The concentrations of PLA and of NFC to PLA wt% are reported. Also, the stirring velocity, the temperature and the time are shown in the last columns of the table.

Table 2.4. Parameters for the casting optimization of NFC-PLA membranes.

Sample name	Material	Solvent	Conc. (wt.%)	NFC	NFC conc. (wt.%)	Stirring (rpm)	Temp (°C)	Time (min)
PLA3	PLA3100	Chloroform	3.80	NFC in acetone	20.17	500	60	60
PLA5	PLA4060	Acetone	2.39	NFC in acetone	10.56	600	50	60
PLA6	PLA3100	Chloroform	3.81	NFC in acetone	4.75	600	50	60
PLA10	PLA4060	Acetone	2.45	NFC in acetone	6.07	700	50	30
PLA12	PLA4060	Acetone	0.58	NFC in water	10.24	700	50	30
PLA13	PLA4060	Acetone	1.94	NFC in water	1.00	700	50	30
PLA14	PLA4060	Acetone	1.96	CMC-NFC 1600 in water	1.02	700	50	30
PLA15	PLA4060	DMSO	1.00	NFC in water	16.85	700	50	120
PLA16	PLA4060	Chloroform	3.83	NFC in acetone	20.41	600	50	60
PLA17	PLA3100	Chloroform	3.86	NFC in acetone	40.28	500	60	35

PLA18	PLA3100	Chloroform	3.78	NFC in acetone	40.63	500	60	35
PLA19	PLA3100	Chloroform	4.03	NFC in acetone	9.90	600	50	45
PLA20	PLA3100	Chloroform	3.94	NFC in acetone	19.58	600	50	40
PLA31	PLA4060	Acetone	2.48	NFC in Acetone	1.19	500	60	40
PLA32	PLA4060	Acetone	2.57	NFC in Acetone	0.54	500	60	40
PLA33	PLA4060	Acetone	2.45	NFC in Acetone	0.50	500	60	40
PLA34	PLA4060	Acetone	2.38	NFC in Acetone	0.11	500	60	40
PLA35	PLA4060	Acetone	3.57	NFC in Acetone	0.14	500	60	40
PLA36	PLA4060	Acetone	3.74	NFC in Acetone	0.28	500	60	35
PLA37	PLA4060	Acetone	3.56	NFC in Acetone	0.12	500	60	35

PLA-NFC Pairing through hydraulic press

PLA films casted as explained before were used for the tests of NFC-PLA pairing through the use of hydraulic press. Moreover, the commercially available extruded film of PLA Polybio 212 was used as well for the same purpose in order to detect any possible difference with the casted PLA films.

The hydraulic press allowed to tune the pressure, the temperature and the time in which the materials were processed at those certain conditions. Various conditions of the system were tested, as shown in table 2.5. Also, the essential oils were inserted in the membranes before the hydraulic press stage, to investigate the possibility to scale up their integration inside the packaging.

Table 2.5. Hydraulic press processing parameters for NFC-PLA pairing

Sample PLA	Sample NFC	Temperature (°C)	Time (min)	Pressure (bar)
PLA4060	NFC	140	15	126
PLA3100	NFC	140	5	126
PLA3100	NFC	130	4	168
Polybio	NFC	140	15	209
Polybio	NFC	100	15	209
Polybio	CMC-NFC	100	10	209
Polybio	CMC-NFC	140	15	209

2.4 Sorption and desorption measurements

Several small pieces of about 10 mg in weight were obtained from both PLA and NFC casted films and put in vacuum conditions at 35°C for 2 hours, in order to remove all the water still present inside the matrix. After the weight and the thickness of each piece were measured, they were put inside 20 ml glass vials, each one filled separately with 2 ml of a different essential oil, namely, thyme, oregano or cinnamon. As a reference, one of the films was kept in the same conditions but without any essential oil. The samples were stored at ambient conditions and the weight was measured at fixed time intervals to monitor the absorption of the essential oil inside the polymer. Before each measurement, the sample was taken out from the vial and quickly dried with absorbing paper until no trace of oil was detected on the paper. In this way, the excess of oil was removed from the surface. The balance used for experiments was an analytical balance Mettler Toledo with a precision of 1 $x10^{-4}$ g. Tests were always performed in duplicate.

Desorption tests were made using a Quartz Spring Microbalance (QSM) schematized in Figure 2.2 and already described elsewhere (293). The apparatus is formed by a quartz spring of known elastic constant, placed inside a thermostatic glass column. The column temperature is controlled by a water jacket, while the tubes connecting the different parts of the systems are insulated and heated by a heating tape. The temperature was set at 35°C. The spring had a sensitivity of 2 mm/mg and a maximum load of 100 mg. The sample was attached to the bottom of the spring and its weight variations were measured by monitoring the shortening of the spring, related to the essential oils diffusing outside the membrane, through a CCD camera. The system was connected to a PC where a dedicated software allowed to register the data and tune the different experimental parameters.

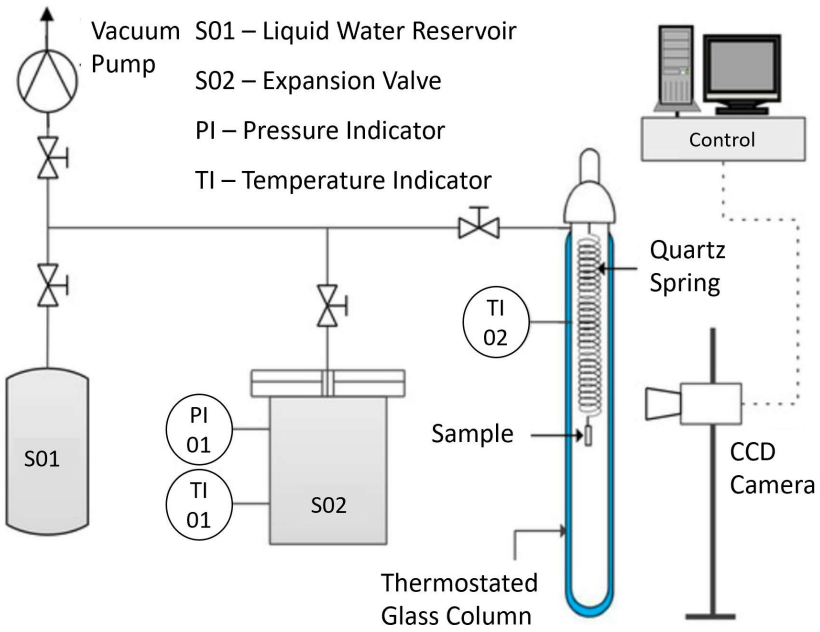


Figure 2.2. Quartz spring apparatus set up (290).

The experiment was started by hanging a sample, previously equilibrated with the liquid essential oils, to the spring and keeping it at atmospheric pressure and controlled temperature until the equilibrium conditions were reached, i.e. the column was saturated with the volatile compounds diffusing out from the sample. At that point, vacuum conditions were set inside the column, through the vacuum pump connected to the system and the data were again collected until a new equilibrium was reached. At each step the final mass of the sample can be calculated following Eq. (1):

$$m_f = m_0 - \left(h_o - h_f\right) \cdot k/g \qquad (1)$$

Where k represents the spring elastic constant, h_o and h_f the initial and final spring length and g is the gravity acceleration. Since the experiment was carried out at ambient to vacuum pressure, the buoyancy force had been neglected. The resulting error was not affecting the results more than temperature oscillations and column leaks; the overall precision of the system, considering the noise of the measurements and the other uncertainties can be considered in the order of ± 5 μg.

The mass variations of the samples, due to the interactions with the essential oils, in the adsorption and desorption tests were calculated as follows:

$$\frac{m_{ads}}{m_0} = \frac{(m_Q - m_0)}{m_0} \times 100 \quad (2)$$

$$\frac{m_{des}}{m_f} = \frac{(m_Q - m_f)}{m_f} \times 100 \quad (3)$$

Where m_0 is the initial mass of the sample, after being vacuumed; m_Q is the mass of the sample after being immersed in the essential oil, prior the QSM measurement and m_f is the final mass, after the QSM experiment and the final vacuum treatment. Theoretically, if no interactions and/or solubilization occur between the nanocellulose and the essential oils, m_f should equal m_0 within the experimental uncertainties. The latter are mainly related to the uncontrolled desorption of the essential oils during sample handling, which cause weight losses in the order of 5 %, as estimated by diffusivity data.

2.5 Diffusivity analysis

The information about weight change during time, as obtained from the sorption and desorption experiments, was used to investigate the essential oils diffusion through the membrane. Different approaches can be used to fit the experimental data, by considering the different transport mechanisms of the penetrating molecules within the nanocellulose matrix.

If a purely diffusive mechanism is considered, the Fick's law needs to be solved with the appropriate boundary conditions in order to obtain the weight change during time. If the surfaces of the planar sample are kept at constant concentration during sorption, as in the case of liquid sorption experiments, eq. 4 can be used to estimate the diffusion coefficient, D (294,295).

$$\frac{m_t - m_0}{m_{inf} - m_0} = 1 - \sum_n \frac{8}{(2n+1)^2\pi^2} \exp\left[\frac{-D(2n+1)^2\pi^2 t}{L^2}\right] \quad (4)$$

Where m_t is the mass of diffusing substance which has entered the sheet at time t, m_{inf} is the same mass at the equilibrium condition and L is the sample thickness. In the case of desorption in the gas phase, m_t will be the mass of essential oil which left the membrane at time t. Eq. (4) can also be written in a different approximated form, valid for short time, where linear relationship can be obtained, simplifying the fitting procedures. Eq. (5) relates the mass uptake for short times (linear when reported against the square root of time) (294)

$$\frac{m_t - m_0}{m_{inf} - m_0} = \frac{2}{L}\left(\frac{D}{\pi}\right)^{1/2} t^{1/2} \quad (5)$$

In the case of desorption experiments, instead, when the sample is suspended in a compartment of limited volume where the concentration of penetrant changes with time due to diffusion process, a different equation has to be used. If the external volume is well stirred, in particular, eq. (6) can be used to estimate the diffusion coefficient, D (294,295):

$$\frac{m_t - m_0}{m_{inf} - m_0} = 1 - \sum_{n=1}^{\infty} \frac{2\alpha(1+\alpha)}{1+\alpha+\alpha^2 q_n^2} \, exp\left(-\frac{D q_n^2 t}{L^2}\right) \quad (6)$$

Where m_t represents the mass of essential oil exiting the membrane at time t and m_{inf} corresponds to the mass of the sample at the end of the tests, prior to the vacuum step needed to recover the initial weight. Also, q_n are the non-zero positive roots of the equation $\tan q_n = -\alpha q_n$, with α representing the ratio of the volumes of solution and of the sheet rescaled with the partition coefficient, which was needed to account for the differences in the equilibrium concentration of solute within the surface of the sheet with respect to the solution.

The previous relationship holds when, as said above, the diffusion is Fickian. However, this is not always the case and more complex diffusion behaviours can be encountered. When a single diffusion coefficient is not sufficient to describe the process, a different approach may be used to describe the experimental data. As an example, a second diffusion coefficient can be taken into consideration, by assuming that different species are diffusing contemporarily and independently in the sample, thus changing the eq. (6) into eq. (7) describing a Dual Fickian Sorption (DFS) process:

$$\frac{m_t - m_0}{m_{inf} - m_0} = 1 - (1-\beta) \sum_{n=1}^{\infty} \frac{2\alpha(1+\alpha)}{1+\alpha+\alpha^2 q_n^2} \, exp\left(-\frac{D_1 q_n^2 t}{l^2}\right) - \beta \sum_{n=1}^{\infty} \frac{2\alpha(1+\alpha)}{1+\alpha+\alpha^2 q_n^2} \, exp\left(-\frac{D_2 q_n^2 t}{l^2}\right) \quad (7)$$

Where the factor β indicates the relative influence of the two diffusion coefficients on the process. In particular, the first diffusion coefficient (D_1) usually represents the diffusion at short times, while the latter (D_2) represents the diffusion at long times. β, therefore, represents the weight of the slow kinetics in the overall desorption process.

Another possibility is, instead, related to considering a different modelling approach such as the one based on the “Parallel exponential kinetics” (PEK), which has been already used to describe transport of water in nanocellulose (296) as well as in natural fibres such as lyocell, cotton (297), flax, hemp and others (298).

The model assumes two parallel independent first order processes that refer to different sorption/desorption sites. In the case of nanocellulose, it has been suggested to target the fast sorption sites in the amorphous region of the films. While the slow sorption sites were considered

to be embedded in the crystalline parts of the sample (296). According to the PEK model, the mass sorption at time t over the total mass absorbed at $t = inf$ is related to time as the equation (8) shows:

$$\frac{m_t - m_0}{m_{inf} - m_0} = \varphi_{PEK}\left(1 - e^{-t/\tau_1^{PEK}}\right) + (1 - \varphi_{PEK})\left(1 - e^{-t/\tau_2^{PEK}}\right) \quad (8)$$

Where φ_{PEK} is a parameter which indicates the relative weight of each process in the overall sorption, τ is a characteristic time of the sorption process, and the subscripts 2 and 1 refer to the fast (small τ) and the slow (high τ) kinetic processes, respectively.

2.6 FTIR-ATR spectroscopy

IR analysis was carried out on the different films before and after the absorption of the essential oils, to ensure the complete removal of the essential oil from the nanocellulose after the desorption and to check for possible interaction among the fibres and the oil which could lead to matrix modification.

An AVATAR 380 Infrared spectrometer (Nicolet, Thermo Fisher Scientific Inc.) provided with an Attenuated Total Reflection tool (MIRacle™ Single Reflection, Pike Technologies) was used for the tests. The spectra were acquired by pressing the film at room conditions directly on the ATR ZnSe crystal, with a calibrated pressure in order to ensure repeatability of the results. Each acquisition employed 32 scans per spectrum with a resolution of 4 cm^{-1} and was elaborated via Omnic™ software package. As said above, the samples were analysed before and after the sorption/desorption experiments, in order to see how the contact with the essential oil could impact the structure of the film. All analyses were performed at least in duplicate.

2.7 Preparation of the active nanocellulose films and anti-bacterial activity assays in the vapor phase

The nanocellulose films were prepared from the water dispersion of pure nanocellulose (NFC1) through a solvent casting technique, as explained in section 2.2. Once the films were dried, a controlled amount of the essential oil of interest was added on the surface of the films with a micropipette to reach the desired concentration. The reference concentration firstly used was related to the maximum oil absorption rate of the nanocellulose, that is when the nanocellulose is saturated with the oil. This value was determined through experiments done in the previous work

and depends on the specific oil. Then, lower and higher concentrations (from 20% up to 85% wt.) were also tested in order to correlate the concentration effect on the antimicrobial activity. Also, nanocellulose films of 9 cm and 4 cm of diameter were used, to test the size effect on the antibacterial activity.

A summary of the various systems tested can be found in table 2.6. There, both the quantity of oil used (µL) for each membrane and the ratio between the volume of the oil used and the mass of the film are reported. By maintaining this ratio constant for the membranes of 4 cm and 9 cm, it was possible to analyse the impact of the dimension of the films, regardless the quantity of oil present. In the last column of the table the ratio between the quantity of oil used and the volume of the headspace of the packaging (volume of air) is reported. For the sake of clarity in the text, the samples in the various experiments will be reported with the dimension of the film (either 9 or 4 centimetres) followed by name of the oil present in the membrane, and the ratio between the oil quantity and the mass of the film. For example, NAP9_T-0.19 refers to the nanocellulose membranes of 9 cm of diameter, embedded with thyme essential oil in a ratio of 0.19 (mg)/ per mass of the film (mg).

After preparation, the active film was attached to an aluminium foil paying attention to not having a leakage of the oil from the borders. In particular, the active surface was directly in contact with the aluminium foil, while the borders of the film were sealed with hot glue. In this way, it was certain that the oil could only diffuse through the nanocellulose film before to be dispersed in the headspace of the testing system (Petri dish). The aluminium foil was needed only to prevent the oil to evaporate before the nanocellulose film was correctly placed inside the system for the test.

The bacterial strains used for the tests were gram-positive *Staphylococcus aureus* and gram-negative *Escherichia coli*. A volume of each bacterial suspension was taken from 5 mL cultures grown overnight up to a concentration of 10^{-6} – 10^{-8} CFU/mL and spread onto LB agar plates. Immediately after the spreading, the active film (either of 4 or 9 cm diameter) was attached over the plate lid to test antimicrobial activity associated with the oil volatilization. The agar plates containing films activated with the same oil were incubated at 37°C in static conditions after their insertion into a humid box. Aluminium foils with only nanocellulose without the oils were used as a control of the experiment. The bacterial growth was detected and enumerated as CFU/mL counting after an incubation of both 24 hours and 168 hours (i.e., one week). These numbers were compared with those counted on agar plates that did not have any films added but that were spread with the same inoculum and incubated in the same box at the same conditions. The active films of 4 cm activated with thyme oil were also tested in petri dishes that were sealed with parafilm

to limit the external dispersion of the essential oil and to evaluate its effect on the antibacterial activity observed. This particular case will be indicated with an asterisk (NAP4*_T-0.38) in the results and discussion part. All the tests were performed in duplicate.

Table 2.6. Nanocellulose-oil systems used for in vitro tests. The dimension of the films, the type of oils and their quantity and concentration are reported in the table. The experiment indicated as "Thyme" represents the sample sealed with Parafilm.*

Name	Diameter (cm)	Oil	Quantity (µL)	m,oil(mg)/m,film(mg)	V,oil (µL)/liter of air
NAP	9	No oil	0	0	0
NAP9_T-0.19	9	Thyme	25	0.19	393
NAP9_T-0.37	9	Thyme	50	0.37	786
NAP9_T-0.60	9	Thyme	80	0.60	1258
NAP9_T-0.75	9	Thyme	100	0.75	1572
NAP9_C-0.68	9	Cinnamon	80	0.68	1258
NAP9_C-0.85	9	Cinnamon	100	0.85	1572
NAP9_O-0.62	9	Oregano	80	0.62	1258
NAP4_T-0.19	4	Thyme	5	0.19	79
NAP4_T-0.38	4	Thyme	10	0.38	157
NAP4*_T-0.38	4	Thyme*	10	0.38	157
NAP4_T-0.57	4	Thyme	15	0.57	236
NAP4_C-0.65	4	Cinnamon	15	0.65	236

2.8 Real case application: raspberry case-study system

Raspberry fruit (*Rubus idaeus L.*) used for the real condition tests were purchased from a local producer (cultivar Eros) and immediately transported to the postharvest laboratory at the University of Algarve, where they were selected for the experiments. The media used for antimicrobial analyses on fruit were Plate Count Agar (PCA), Buffered Pepton Water (Oxoid) and Dichloran Rose Bengale purchased from Biokar diagnostics.

The set-up was prepared as for the tests directly on bacterial strains. In this case the active nanocellulose films (NFC1 of 9 cm of diameter) were attached directly on the inner upper surface of the raspberries packages (as shown in figure 4.4), that was commercial clamshell type PET boxes (125g) with 13.5x11x3 cm dimensions. So, the main difference from the previous tests was that, instead of having the films on an inoculated Petri, they were placed directly on the fruit package, containing the fruit from the cultivation.

Four replications were done for each oil used, plus a control with the pure nanocellulose membrane without the oil and another control without the nanocellulose film, for a total of 4

treatments plus the control. The oil was added on the inner surface of the lid of the packaging box (i.e., between the nanocellulose film and the package itself) using a micropipette. Then, the nanocellulose film was sealed with the use of hot glue, to avoid the oil to be dispersed from the sides (292)

The fruits were conserved in a separate box for each treatment, at 1°C. The measurements were performed every 6 days, until reaching 12 days of total storage. Two sets of experiments were performed to optimize the quantity of the oils and select the most effective treatment. In the first test the quantity of the oils was chosen based on the MIC resulting from the tests performed directly on bacterial strains. It was 0.68 mg/mg, 0.37 mg/mg and 0.62 mg/mg for cinnamon, thyme and oregano essential oils (mg of oil per mg of nanocellulose film), respectively (table 2.6). Also, a treatment with nanocellulose without any oil was tested to measure the effect of the biopolymer alone. In the second experiment, only thyme and oregano essential oils were used, and the mixture of them (38% and 62%, respectively). In the second experiment, only thyme and oregano essential oils were used, alone and in combination, with a higher concentration (5 times higher than MIC) in order to further test their potentiality and possible synergetic effects.

For both experiments, the control was considered as the fruit box without any film nor essential oil. Table 2.7 shows the quantity of oils used in the experiments (µL) and the ratio between the mass of the oils used and the mass of the nanocellulose films. The ratio between the volume of the oil used and the headspace of the packaging is also reported in the table, as a reference for future application that may be applied to different fruit/packaging conditions.

Table 2.7. Essential oils and their quantity used for the two experiments.

Experiment	**Essential oil**	**Treatment name**	**Quantity (µL)**	**m,oil (mg)/m,film(mg)**	**V,oil (µL)/liter of air**
Test 1	No oil, nanocellulose film	NAP	0	0	0
	Cinnamon	NAP_C	80	0.68	359
	Thyme	NAP_T	50	0.37	224
	Oregano	NAP_O	80	0.62	359
Test 2	Thyme	NAP_T	250	1.87	1122
	Oregano	NAP_O	400	3.09	1796
	Thyme + Oregano	NAP_TO	325	2.48	1459

2.8.1 General quality parameters

The general quality parameters were measured on 10 fruits per each box/replication. The colour of the raspberries was measured using a Minolta Chroma meter CR-300 (EC Minolta, Japan) using the CIELab scale (L*, a* and b*). The L* represents colour lightness (0 = black and 100 = white). Hue (h°) was calculated as h° = arctan (b*/a*), while Chroma (C*) was calculated as C* = $\sqrt{a^{*2} + b^{*2}}$ and indicated the degree of departure from gray toward pure chromatic color34.

The firmness is another very important parameter to define the quality attributes of the fruits. The tissue softening affects not only the visual aspect, but also it reduces the shelf-life of the fruits. The firmness of the fruit was determined by compression with a Chatillon TCD200 and a Digital Force Gauge DFIS 50 (Jonh Chatillon & Sons, Inc., USA) fitted with a cylindrical plate pressing on the fruit at a maximum depth of 10 mm. For the determination of the total soluble solids content (SSC) the °Brix was measured by a digital refractometer HI 96801 (Hanna instruments), in the fruit juice. Weight loss was expressed as percentage of the initial weight (299).

2.8.2 Evaluation of the antimicrobial activity of the nanocellulose films applied to a real setting

Microbial counts were determined for each treatment starting from the fruit obtained from the cultivar (no inoculum had been applied in this phase). The microbiological parameters that were considered, included counts of aerobic mesophilic and psychrophilic bacteria and molds and yeasts and were determined according to Guerriro et al. (300), using the Plate Count Agar medium for bacteria and Dicloran Rose-Bengal Cloranfenicol Agar for the others. Ten gram of each sample was transferred to 90 mL of peptone water and homogenized. Decimal dilutions were prepared using the same diluent. The incubation temperature for yeasts and molds was 25±1 °C during 48-72 h, for aerobic mesophilic bacteria was 37 ± 1 °C during 24–72 h and was 6.5 ± 1 °C during 5 to 10 days for psychrophilic bacteria. Experiments were done in duplicate. Results were expressed as Log_{10} CFU (Colony Forming Unit) per gram of fresh weight.

2.8.3 Total phenolic content

Phenolic compounds are phytochemicals present in fruits and vegetables where they have nutritional and sensory role (301). Total phenolic content was determined according to the Folin–Ciocalteu colorimetric modified for microplates (302). Raspberry juice was obtained after squeezing raspberries with an UltraTurrax T 18 (IKA, Germany) for 2 min, then centrifuge for 5

min at 5000 rpm. The sample (20 µL raspberry juice) and 80 µL of sodium carbonate (75 g L^{-1}) were added to 100 µL of 10% (w/v) Folin–Ciocalteu reagent. After 30 min of reaction at room temperature, the absorbance was measured at 765 nm (Tecan Infinite M200, Swiss). Gallic acid was used as standard for calibration curve (303).

2.8.4 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

Capacity for scavenging DPPH free radicals was evaluated by mixing 1850 µL of ethanolic solution of DPPH 25 µM to 150 µL of raspberry juice at different concentrations. After 30 minutes of incubation at room temperature, the absorbance of samples was read at 515 nm. Analyses were run in triplicate. The DPPH radical scavenging activity was expressed as: [(A0 - A1 /A0) ×100], in which A0 is the absorbance of the control reaction (without sample), and A1 is the absorbance of the sample. The values were compared with the curve for several Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) concentrations and expressed as µM Trolox equivalent antioxidant capacity.

2.8.5 Anthocyanins

The total anthocyanins content was measured using a modified pH differential method (304). Absorbance of anthocyanins at 520 nm and 700 nm in different pH buffers (pH 1.0 and 4.5) was measured, respectively. Absorbance readings were converted to total mg of cyanidin 3-glucoside per 100 g fresh weight of fruit sample (299) using the molar extinction coefficient of 26,900 and the absorbance read at different wavelengths, defined by A (305).

Anthocyanin pigment concentration was, therefore, expressed as Cyanidin-3-glucoside equivalents, as follows:

$$\frac{A \times MW \times DF \times 10^3}{\varepsilon \times l}$$

In which:

$$A = (A_{520nm} - A_{700nm})_{pH1.0} - (A_{520nm} - A_{700nm})_{pH\ .5}$$

MW = 449.2 g/mol = value of the molecular weight of Cyanidin-3-glucoside

DF = dilution factor

ε = 26,900 molar extinction coefficient for Cyanidin-3-glucoside, in $L \times mol^{-1} \times cm^{-1}$

10^3 = factor for conversion from g to mg

l = path length in cm.

2.8.6 Sensory evaluation

A taste panel was performed with 15 trained panelists, after 6 days storage for test 1. The same test was performed by 21 panelist after 12 days of storage for test 2. Panel members were asked to evaluate the appearance, aroma, texture, sweetness, acidity and flavour on the base of a 7-point hedonic scale: 1 – dislike very much; 2 – dislike; 3 – dislike slightly; 4 – either like nor dislike; 5 – like slightly; 6 – like; 7 – like very much. Overall liking was calculated as a mean of the sensory parameters evaluated.

2.8.7 Statistical analysis

The experimental design was a complete randomized block design. Statistical analysis was performed using the SPSS 24.0 software (IBM, Inc., Armonk, N.Y., USA). Two-way analysis of variance (ANOVA) was done using treatments and storage time as factors. ANOVA is one of the most widely used statistical method for hypothesis testing currently in use in which data sets are compared and measured to determine their significance (306) Duncan's multiple-range tests ($p < 0.05$) for means comparison were done. This procedure is based on the comparison of the range of a subset of the sample means with a calculated least significant range. This least significant range increases with the number of sample means in the subset. If the range of the subset exceeds the least significant range, then the population means can be considered significantly different (307). In our case, the significant range was set at a value of $p=0.05$.

3. Results and Discussion of Transport properties

3.1 Film characterization

Pure NFC films

As can be observed in figure 3.1 (NFC1), the films obtained by casting are uniform and opaque, with a whiteish colour. They have a thickness in the range of 30 - 50 µm and are all very similar, making it impossible to distinguish the different type of nanocellulose by simple observation. They all appear brittle during the handling, especially after the vacuum step which substantially reduced the amount of water within the samples.

Figure 3.2 shows the IR analysis comparison of the different nanocellulose types. NFC1 refers to pure nanocellulose, while NFC2 and NFC3 refer to different carboxymethylation degrees, 780 µeq/mol and 1600 µeq/mol, respectively. The spectra are rather similar but with clear variations in the peaks, which can be related to the variation of the chemical structure going from the pure nanocellulose to the carboxymethylated one. For example, the broad absorption band at 3333 cm^{-1}, attributed to the stretching frequency of the –OH group, is more evident in the NFC1 and tends to decrease increasing the carboxymethylation degree, though it is not completely disappearing because it is overlapping with the broad absorption band at 3307 cm^{-1}, due to the stretching frequency of the –COO– group (308). The small peak detected around 1726 cm^{-1}, on the other hand, corresponds to the C=O stretching frequency of carboxylic acid groups and results to increase going from NFC1 to NFC3 (33). Same behaviour was found for the bands around 1425 cm^{-1}, assigned to the presence of O-H bending related to a carboxylic acid. While the band at 1028 cm^{-1}, due to C–O asymmetric bridge stretching, seems shifted to lower wavenumber with the increase of the surface charge in the sample. Also, the band at 862 cm^{-1}, appearing for the carboxymethylated nanocellulose, is related to C-O-C stretching (309).

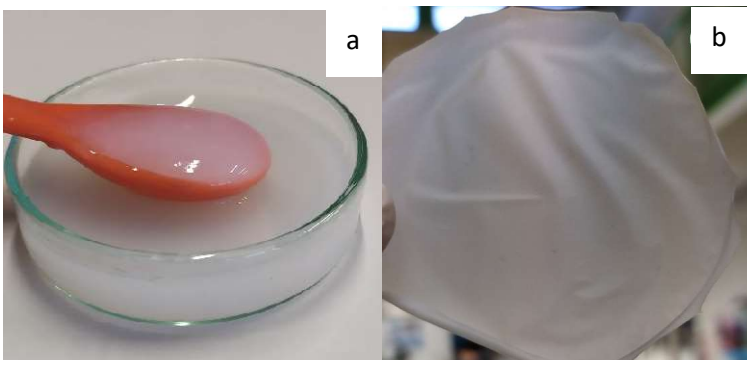


Figure 3.1. Nanocellulose solution (NFC1) in water (a) and nanocellulose casted film (b)

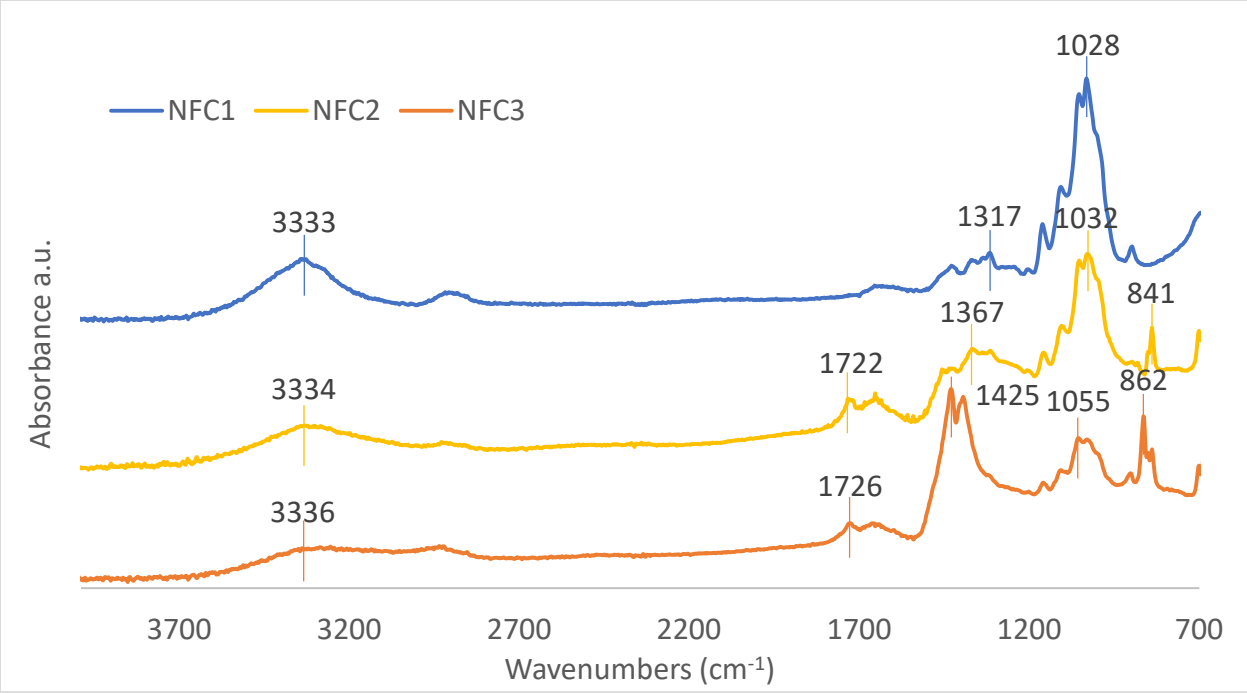


Figure 3.2. FT-IR analysis of the three different types of nanocellulose. NFC1 refers to pure nanocellulose, while NFC 2 and NFC3 refer to different carboxymethylation degrees, 780 µeq/mol and 1600 µeq/mol, respectively.

Pure PLA films

The figure 3.3 shows the results from the casting of pure PLA membranes. The films appeared as homogeneous and completely transparent. As it is possible to observe, it was not possible to distinguish the two types of PLA only from direct observation of the samples.

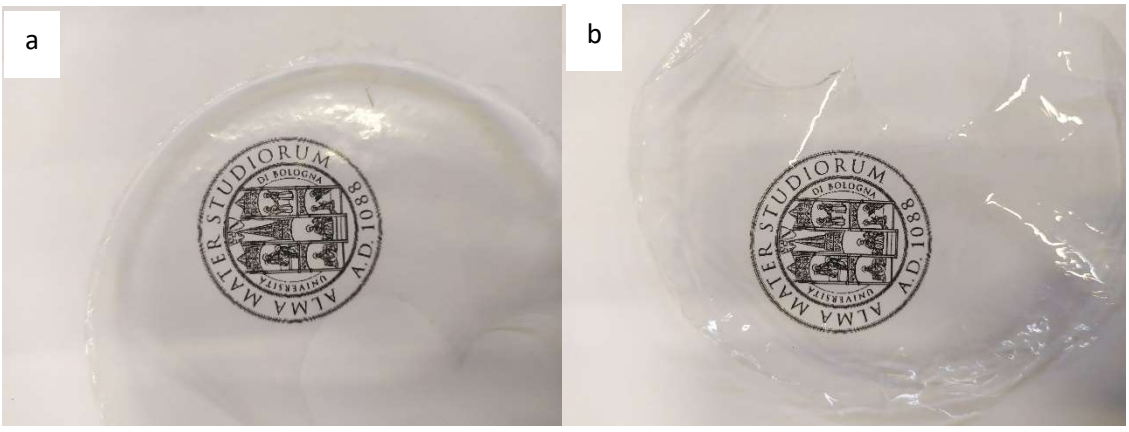


Figure 3.3. PLA films obtained from solvent casting of (a) PLA1 and (b) PLA2.

NFC-PLA films

From the casting of NFC-PLA membranes it resulted that the NFC in water solution was impossible to use to create homogeneous membranes, due to PLA insolubility in water. In fact, even if the PLA was firstly dissolved separately and the final solution with NFC was homogenized, then during the casting the two phases separated and the membrane resulted having holes, as shown in figure 3.4a, which shows PLA4060 dissolved in acetone + NFC in water suspension. Instead, figure 3.4b and c show PLA incorporated with NFC in acetone solution. In particular, figure 3.4b reports PLA3100 incorporated with 20% wt. of NFC, while figure 3.4c is PLA4060 + 0.12% 2T of NFC. They both appear with a withish colour and more brittle than pure PLA by handling.

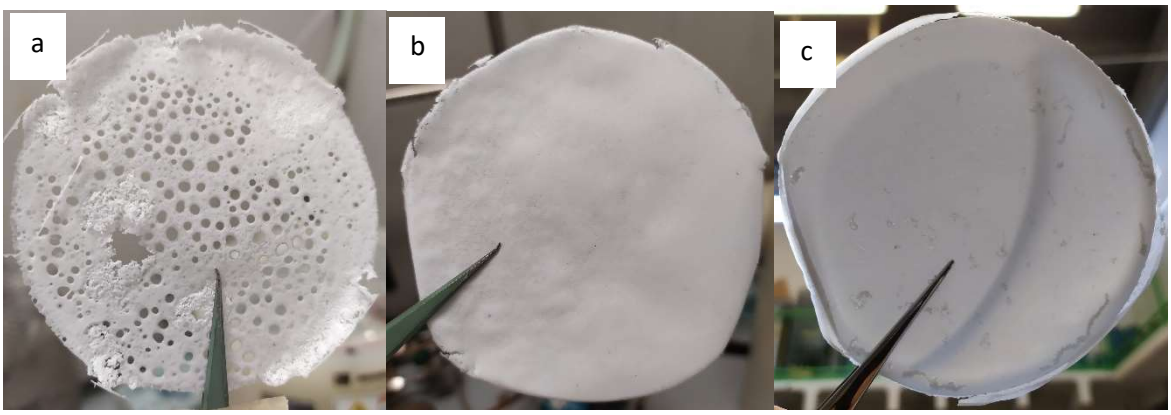


Figure 3.4. Different NFC-PLA films from solvent casting. (a) PLA4060 + 1% wt. NFC in water; (b) PLA3100 + 20% wt. NFC in acetone; (c) PLA4060 + 0.12% wt. NFC in acetone.

The figure 3.5 shows some of the results from the NFC – PLA membranes pairing by using the hydraulic press. In particular, figure 3.5a shows the appearance of the best solution, which was the pairing between Polybio and NFC, pressed for 15 minutes at 100°C and 209 bar. Instead, figure 3.5b shows a case in which the NFC resulted to be partially degraded by the high temperature and appeared to be with a yellowish colour. The last photo represents a different configuration: the NFC membrane was pressed inside two PLA membranes. This case was studied since the PLA, being a thermoplastic polymer, was more easily melting and attaching to itself rather than NFC films. The idea was to have pocket with high mechanical resistance and formed at lower temperature with respect to NFC-PLA films. Indeed PLA, being a thermoplastic polymer, can be easily melted and readily attached to itself rather than NFC films.

In general, the NFC membranes demonstrated to effectively attach to the PLA ones, by mean of the high temperature and pressure. They also remained attached after a long period of time (more than 6 months) in which the films were stored in a plastic bag in the laboratory. However, this procedure was not further investigated during the present study, since the temperature needed for the operation was that high to degrade the essential oils components, thus inactivating their antimicrobial and antioxidant properties. In addition to that, the essential oils showed to dissolve the PLA, making it impossible to include them between the NFC and the PLA films even working at low temperature as in the case of the PLA/NFC/PLA films.

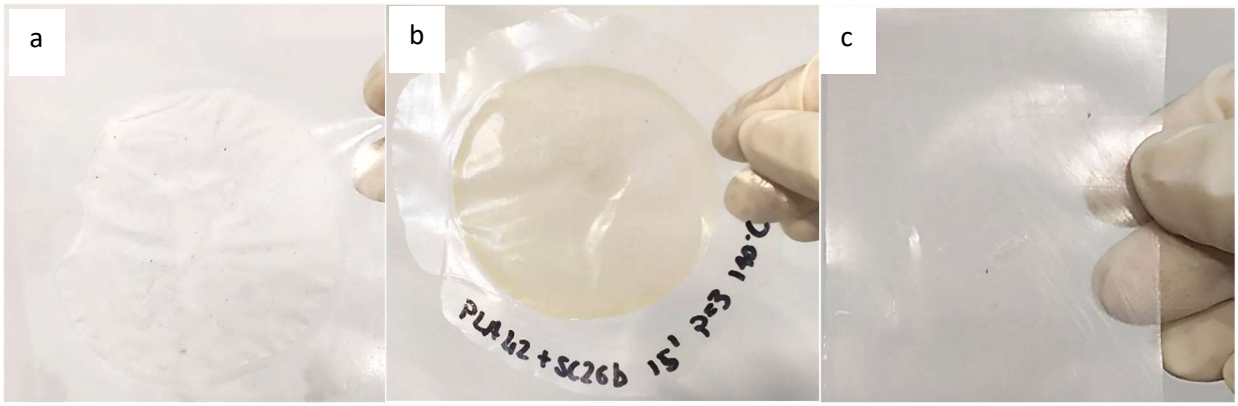


Figure 3.5. (a)Polybio+NFC, 100°C,15',209 bar; (b) PLA3100+NFC, 140°C,15',209 bar; (c) Polybio+NFC+Polybio, 140°C, 15', 209 bar.

The coupling of NFC, PLA and essential oils, in view of the application in active packaging solutions was therefore more complex than expected and could not be completed it the present study. Some solutions to the encountered problems could be found: for example by testing a specific hydraulic press system which only operates in a specific area. In that case, it would be possible to apply the pressure and the high temperature only in the circular area needed in order to pair the NFC with the PLA, but not in the middle, where the essential oil eventually would be. Furthermore, it would be possible to test solutions in which the NFC and PLA films are paired before the essential oils is applied, like in the case of the figure 3.5. Then it could be sprayed onto the NFC surface, avoiding its contact with the PLA film. Moreover, the solution illustrated in figure 3.5c could be further developed by variating the thickness of the PLA film only from the side in which the oil permeation has to be avoided. Unfortunately, as said above, due to time constraint, this kind of analysis could not be completed as only pure NFC films could be tested also for antimicrobial activity. Therefore, the optimization of this systems is left to future investigation.

3.2 Absorption and desorption measurements

The tests on essential oils absorption into pure PLA membranes showed that the oils could readily solubilize the biopolymer. After the addition of the oils, the PLA films lost their dimensional stability and could not be extracted in order to complete the weight procedure. Then, few hours from the beginning of the experiments, they completely dissolved into the oils. For this reason, PLA was excluded for applications in direct contact with the oils and no analysis of sorption and diffusion were completed in this material nor on the nanocellulose/PLA composites.

Considering therefore cellulose films, the comparison between the absorbed and the desorbed mass by different NFC types when immersed in the liquid essential oils is shown in figure 3.3, as calculated from equations (2) and (3). In particular, equilibrium data at the end of the sorption are compared with data obtained by weighting each sample after the desorption of the essential oil in the QSM and after an additional vacuum treatment was applied in the attempt to remove all the remaining oils. All the tests were repeated at least two times and the values reported are obtained by averaging the different results.

It is possible to observe very different behaviours for the various oils and nanocellulose types, in view of the amount of oil absorbed and of the difference between data obtained from sorption and desorption. Indeed, thyme and oregano results are substantially stable, and the mass values obtained in the two tests are rather similar, within the uncertainty of the measurements. For cinnamon, on the other hand, a rather different behaviour is observed, with the desorbed mass substantially lower than the absorbed one in the case of NFC1, but definitely higher in NFC2 and NFC3, which actually showed final value of mass uptake very close to 0. The observed behaviour is likely due to a difficulty in completely remove the cinnamon from the pure nanocellulose at the end of the first experiment, and to a non-negligible solubilization of the carboxymethylated NFC during the sorption tests in the second and third, as it will be better discussed in the following section. For this reason, in the current analysis, only the desorption values observed for cinnamon will be considered for NFC2 and NFC3 as a measure of the total mass uptake in the sample. While for NFC1 the final value at the end of the sorption will be considered as the more consistent result.

In view of the previous consideration, by analysing the solubility of the oils in the different nanocellulose matrixes, it can be seen from figure 3.6 that solubility of thyme and oregano in nanocellulose has a clear dependence on the carboxymethylation degree. Indeed, the absorbed mass monotonously increases for both oils going from NFC1 to NFC3. In particular, then, thyme shows a higher solubility, ranging from 5 to 20%. While oregano, that has solubility similar to

thyme in NFC1, results less affected by surface charge, reaching a value of only 10% for NFC3. Cinnamon behaviour is instead substantially different, with values of solubility in the three samples in the range of 15-20%, therefore rather similar to each other considering the uncertainty of the measurements.

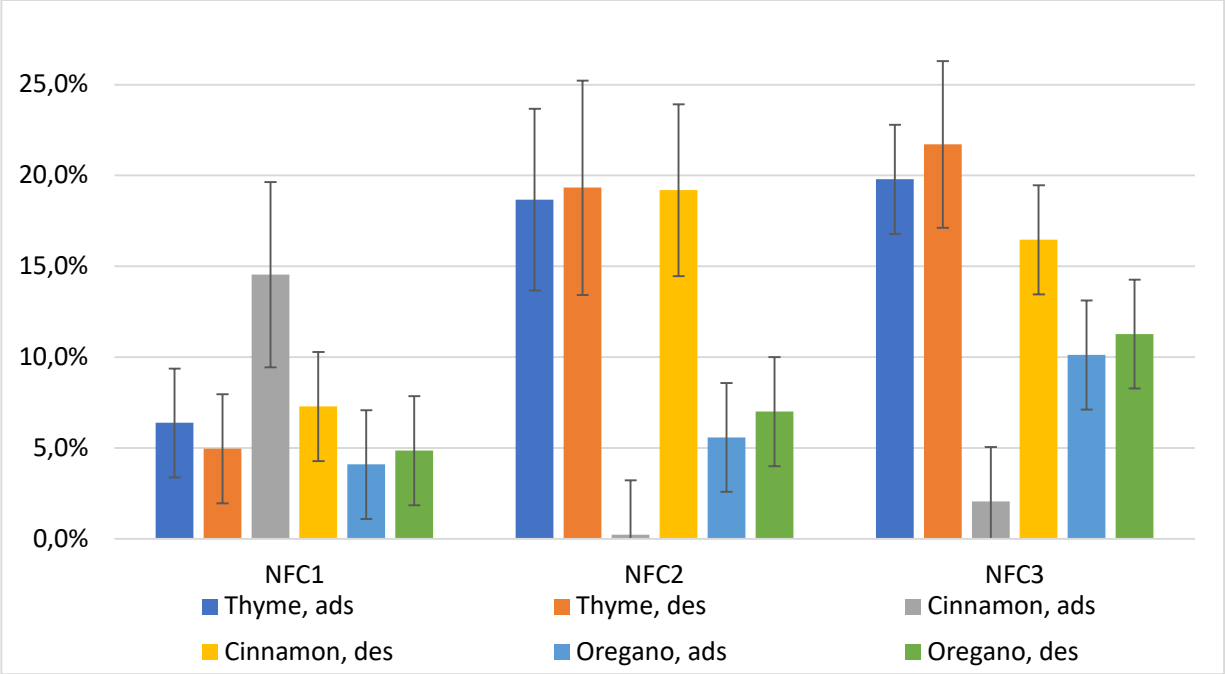
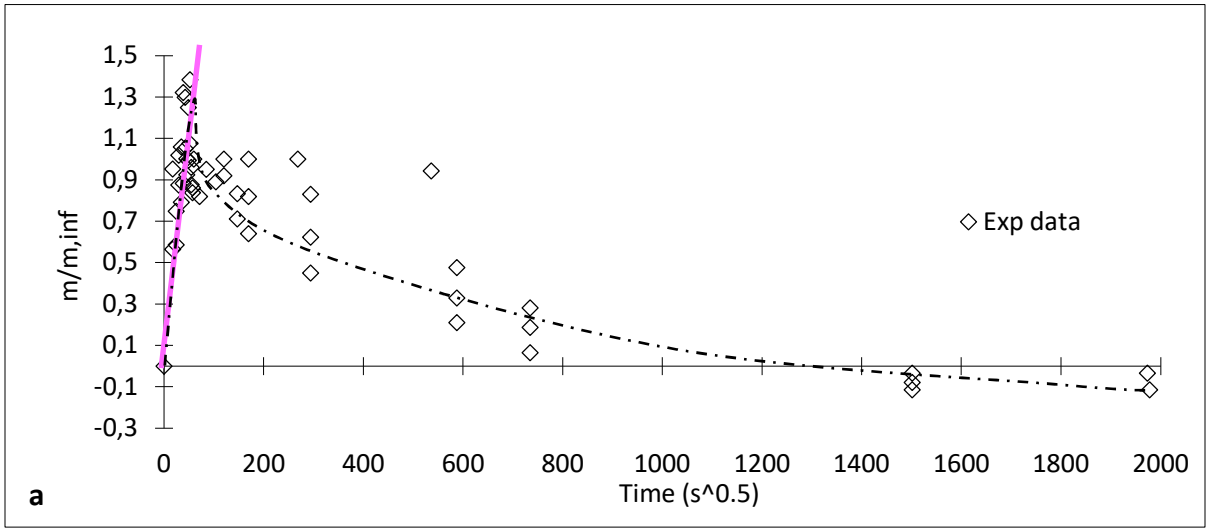
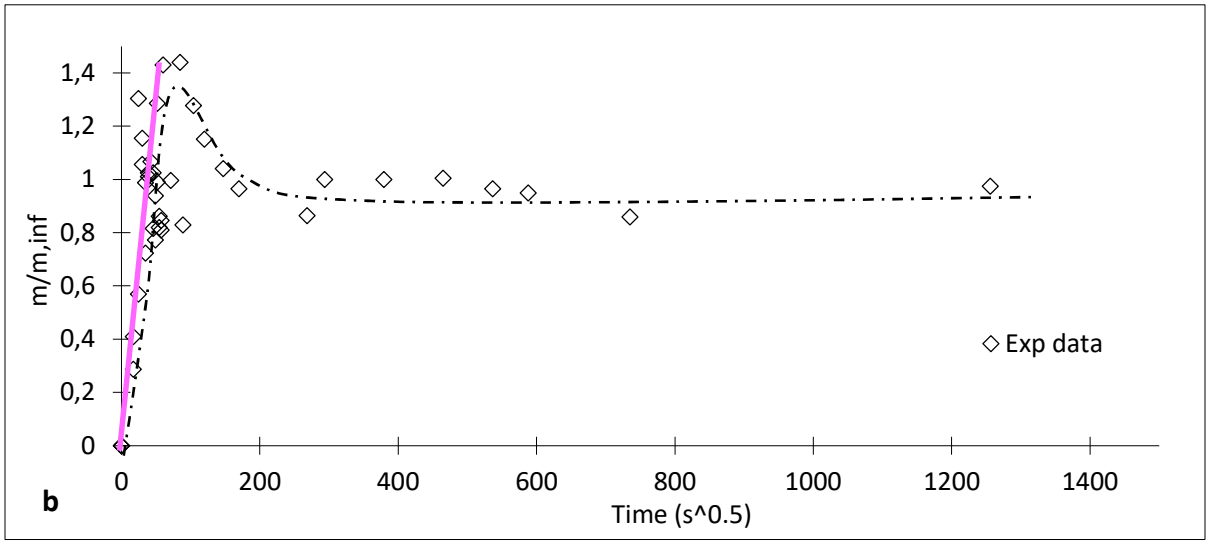


Figure 3.6. Comparison between the absorbed and desorbed mass, considering the mass lost during the experiment set up. The data are presented for the three different oils and the three different nanocellulose types, as indicated in the legend.

In order to better explain the behaviour of the different samples and, more in particular, the cinnamon results, the mass sorption as a function of the time for, respectively, cinnamon, thyme and oregano essential oils in NFC2 (endowed with a surface charge of 780 µequiv/g) is reported in figure 3.7. The scattering of the data is rather high, in line with the difficulties of the measuring technique. However, the behaviour of the three oils in the NFC matrix is rather similar for short time, with a fast sorption up to a peak which, in case of thyme and cinnamon, is then followed by a slight decrease of the sorbed mass with time. While for thyme this desorption is rather limited, in time and mass loss, for cinnamon it continues also for long times, substantially reducing the sorbed mass at the end of experiments to values often very close to 0. This effect is likely due to a partial dissolution of the matrix, as also confirmed by the final mass of the sample measured after completing the desorption tests. Indeed, while for oregano and thyme differences in the order

of 2-3% were measured, well inside the experimental uncertainty, for cinnamon in NFC2 and in NFC3 reductions in the order of 13-16% were obtained, for all samples inspected.





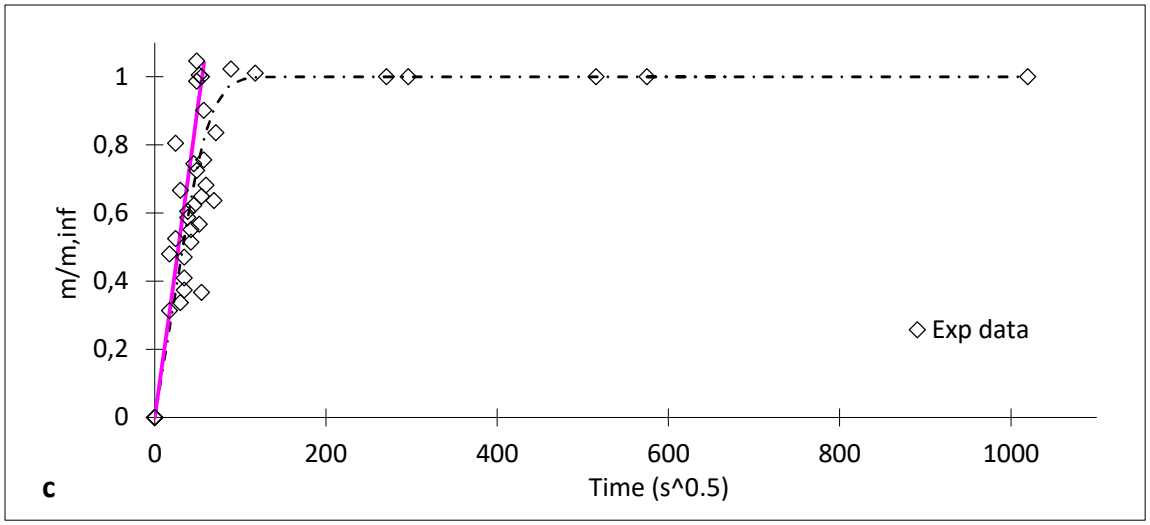
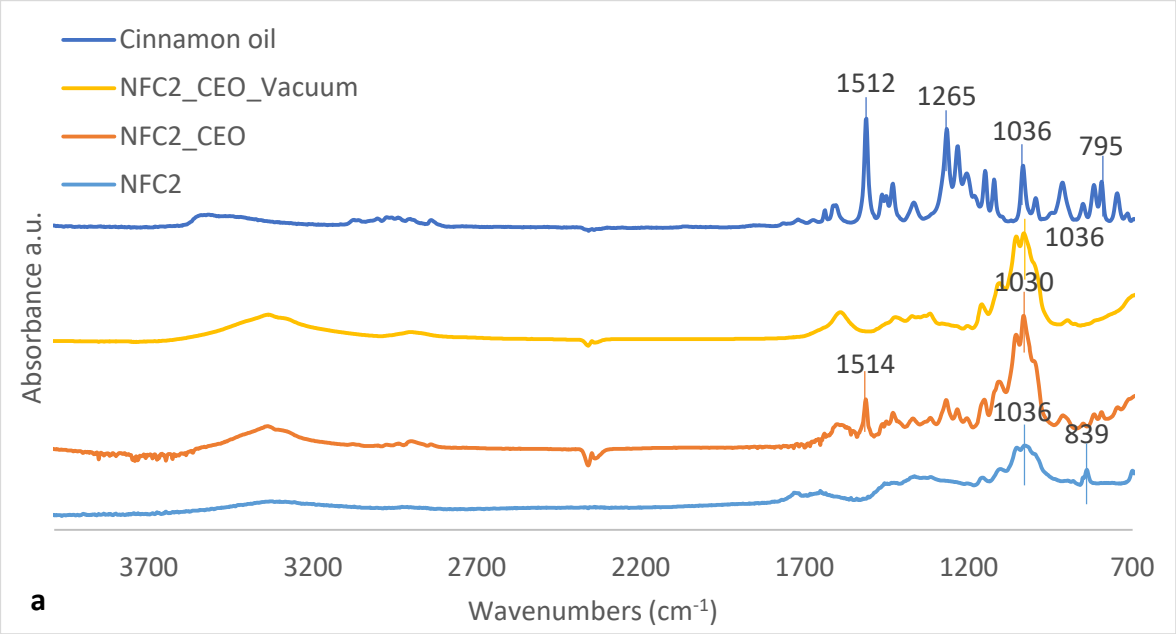


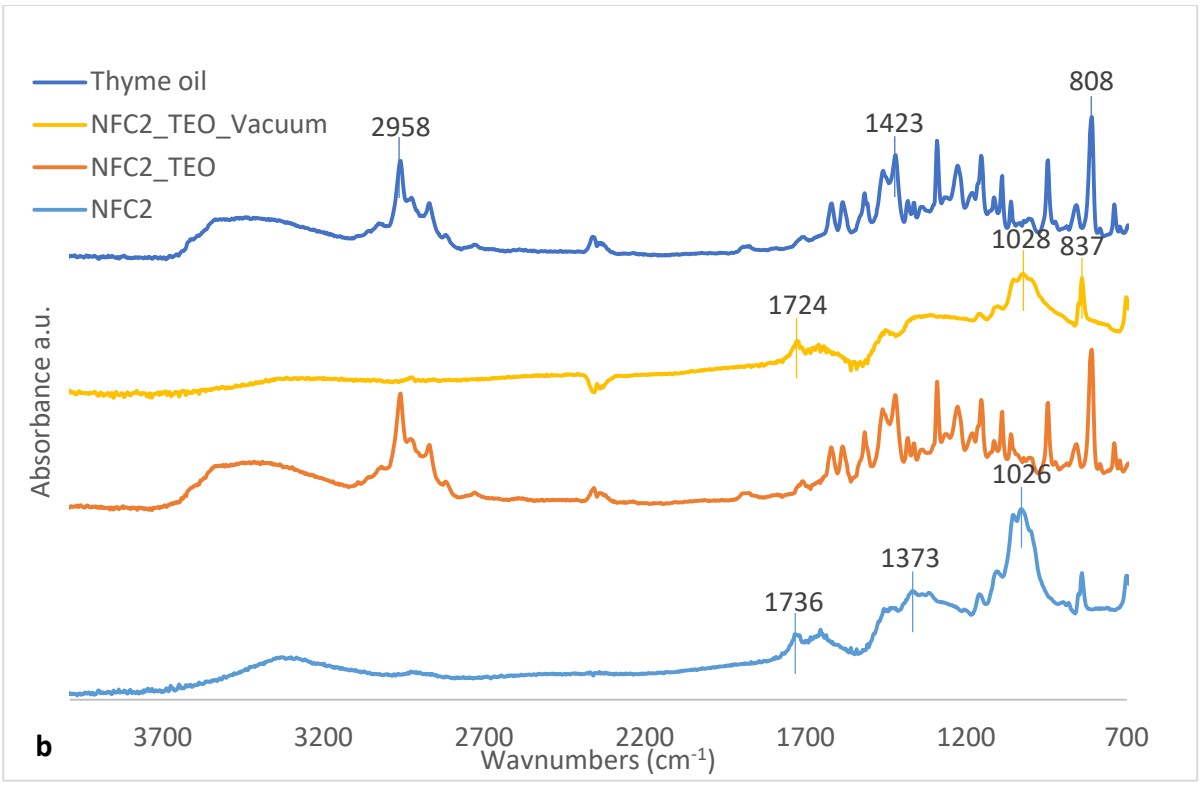
Figure 3.7. Cinnamon (a), Thyme (b) and Oregano (c) essential oil absorption in NFC2.

To better understand the results, the FTIR spectra of the different samples was repeated at the end of the sorption tests and after the final vacuum treatment following the desorption tests to enlighten possible changes in the film chemical structure. Figure 3.8a compares the NFC2 prior and after being in contact with Cinnamon essential oil. Compared to the pure NFC spectrum (light blue line), the NFC immersed in the oil (orange line) also contains the small but clear characteristic peaks of the cinnamon essential oil (blue line). Absorption peaks are indeed observed around 1512 cm^{-1} and 1265 cm^{-1}, which can be related, respectively, to the nitrogen compounds and the aromatic amine present in the oil (310,311)(305). Interestingly, however, other modifications are visible, which remain also after the vacuum is applied (yellow line in figure 3.8a). The peak near 839 cm^{-1} present in pure nanocellulose is indeed missing in the other spectra, as well as the ones around 1700 cm^{-1}, which seem to disappear upon cinnamon addition or somewhat shift at 1514 cm^{-1}, where a new peak is formed. All these modifications confirm the existence of strong and non-reversible oil-matrix interactions. As an example, the peak at 900 cm^{-1} is often associated with the β-glycosidic linkages between glucose units in cellulose, which stands for cellulose II crystals. Its disappearance after the contact with the oil strengthens the hypothesis of strong interaction between nanocellulose and cinnamon essential oil (312,313).

As a further confirmation, it is possible to observe figures 3.8b and c, which represent the IR spectra of NFC2 prior and after being in contact with thyme and oregano essential oils, respectively. In these cases, the NFC2 peaks remain substantially the same after the contact with the oil, even if with some differences, especially in the case of thyme, suggesting indeed the presence of some residue after the vacuum treatment. The spectra of the other types of

nanocellulose with the cinnamon, thyme and oregano essential oils can be observed in the Appendix, and show a very similar behaviour with respect to cinnamon, also for NFC3. Minor differences, instead, are observed for untreated nanocellulose NFC1 which, indeed, is the one that seems to better resist to solubilization. Being FTIR ATR a surface technique, sampling only few microns of the specimen thickness, it is not possible to use current data to estimate the possible loss of cellulosic materials during sorption. They, however, clearly indicate the strong interaction among cinnamon and carboxymethylated nanocellulose, which seems to cause more changes in the film chemical structure with respect to other oils.





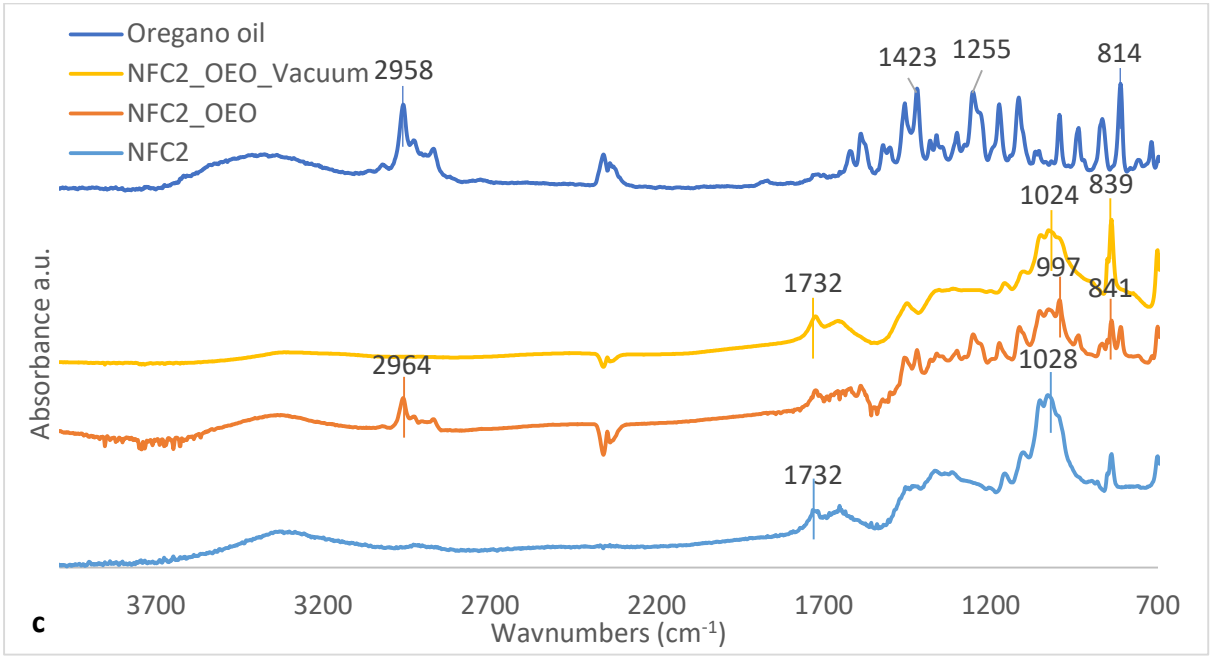


Figure 3.8. FT-IR analysis of NFC2 prior and after being in contact with the cinnamon (a) thyme (b) and oregano (c) essential oils. The spectra of pure oils have also been reported for comparison.

3.3 Diffusivity analysis

The absorption and desorption data are also used to perform the diffusivity analysis. The kinetics of the different processes, indeed, allowed to calculate the values of the different diffusion coefficients related to the three types of nanocellulose films, impregnated in the three essential oils.

The diffusivity values were obtained from sorption curves by hypothesizing a Fickian diffusion in line with the experimental evidence. From figure 3.7, indeed, despite the data scattering and the complex behaviour related to sample solubilization, it is quite evident that initial mass uptake is substantially linear with square root of time as obtained from Eq. 5, which holds when Fick's law is obeyed. Therefore, by considering the initial data and the mentioned equation, the diffusivity was calculated, and the results are shown in Figure 3.9. The chart clearly shows that for cinnamon and oregano the diffusion coefficient increases with the carboxymethylation degree of the film, while it is somewhat non monotonous in the case of thyme. In fact, for cinnamon the value of diffusivity for the pure nanocellulose films is in the order of $3.7 \times 10^{-9}\ cm^2/s$, increasing to $1.1 \times 10^{-8}\ cm^2/s$ for the carboxymethylated nanocellulose at 780 μeq/mol, and up to $2.0 \times 10^{-8}\ cm^2/s$ in the carboxymethylated nanocellulose at 1600 μeq/mol. Similar behaviour is observed for oregano that, however, has generally lower diffusivity, ranging from 1.0×10^{-9} to $4.4 \times 10^{-9}\ cm^2/s$ from NFC1 to NFC3. Thyme, on the other hand, does not show a clear trend as NFC2 and NFC3 have the same diffusion coefficient, in the order of $7.6 \times 10^{-9}\ cm^2/s$ which remains in any case more than three times higher than the one of untreated nanocellulose.

The surface modification, therefore, seems to speed up the diffusion process as if the higher surface charge is able to increase the spacing between fibres, thus facilitating the transport of the different oils across the films. This hypothesis was also considered to explain the behaviour of water diffusion into pure and surface modified nanocellulose films (32).

In general, then, cinnamon essential oil has the higher diffusion coefficient, followed by thyme and oregano. These effects can be associated with the interactions occurring between the essential oil and the nanocellulose and the ability of the former to relax the bonding within the cellulose chains. From this point of view, the high diffusivity of cinnamon can be easily related to its ability to cause major changes in the nanocellulose matrix up to a point where part of the chains results solubilized in the oil. Thyme, on the other hand, showed a solubility which is clearly higher

than the oregano one in all the samples inspected. So, the higher diffusivity can be directly related to the higher swelling of the nanocellulose matrix. It is well known, indeed, that in dry conditions nanocellulose is a quite barrier material and relaxation of the interfibrillar bonding is needed to allow different vapor and gases to penetrate its structure (314).

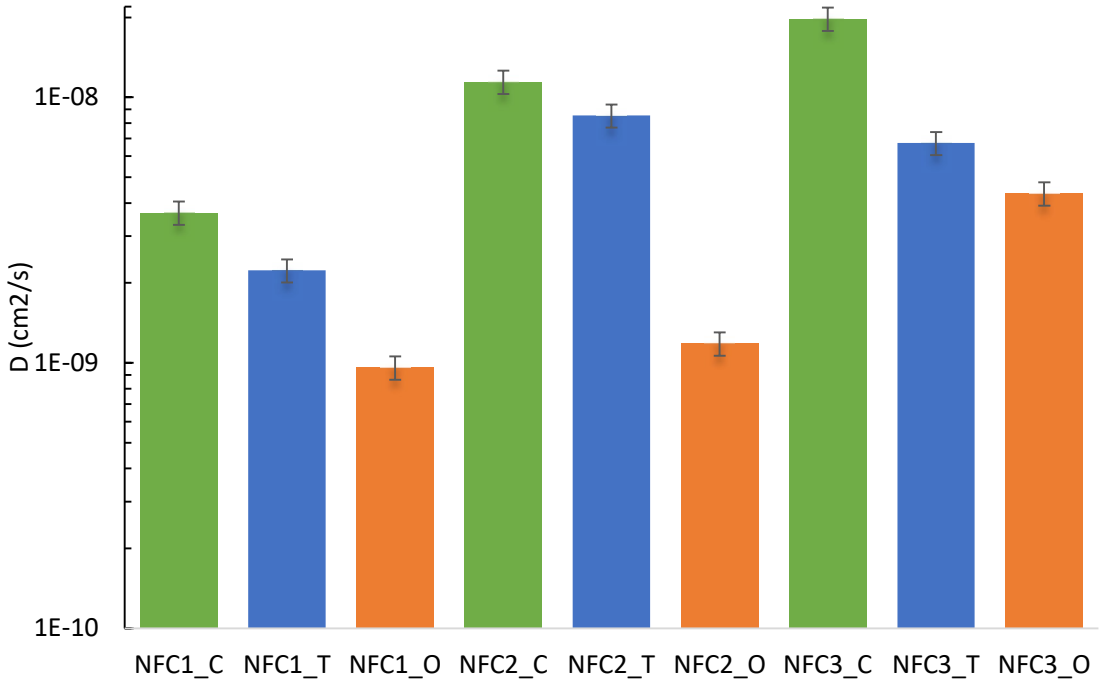


Figure 3.9. Diffusion coefficient estimated from absorption in liquid phase for Cinnamon (green), Thyme (blue) and Oregano (orange) essential oils in different nanocellulose matrices.

As made for the absorption, the kinetics of the desorption process was studied by considering the behaviour of the mass change as a function of time.

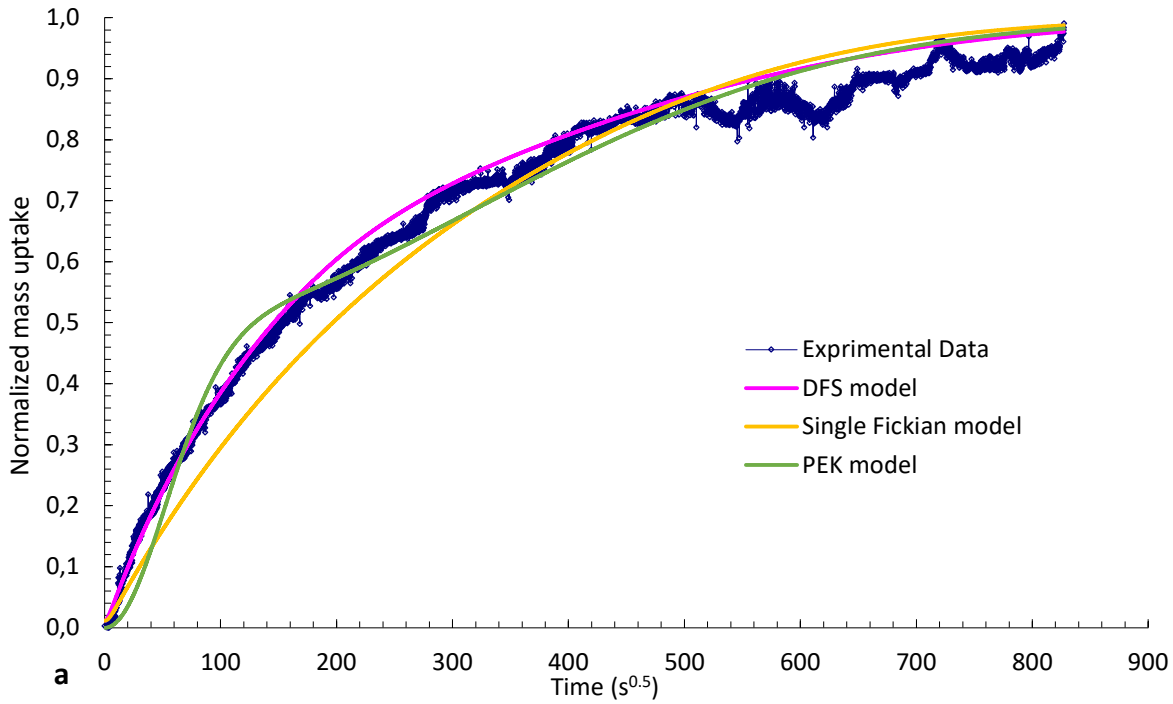
An example of the data obtained in this experiment is reported in figures 3.10a and b which show, respectively, the thyme desorption from NFC1 and oregano desorption from NFC2. In the plots, the vertical axis which reports the normalized mass uptake, refers to equation 9:

$$normalized\ mass\ uptake = \frac{m_t - m_0}{m_{inf} - m_0} \quad (9)$$

Data fitting was carried on with the different kinetic models previously described and it results that while DFS more suited to describe thyme desorption, PEK results more adequate for catching the oregano behaviour.

In both cases, the data suggest that two different stages exist: a first one where the oils desorb at a high rate from the swollen matrix, followed by a second stage where, likely due to the lower amount of oil, the packing density of the fibres increases, and the desorption become more difficult.

The type of kinetics that better describes the data is not common to all the systems, making it difficult to draw general trends. An overview of the fitting results is given by table 3.1, while charts of the data fitting for all the systems inspected are reported in the Appendix.



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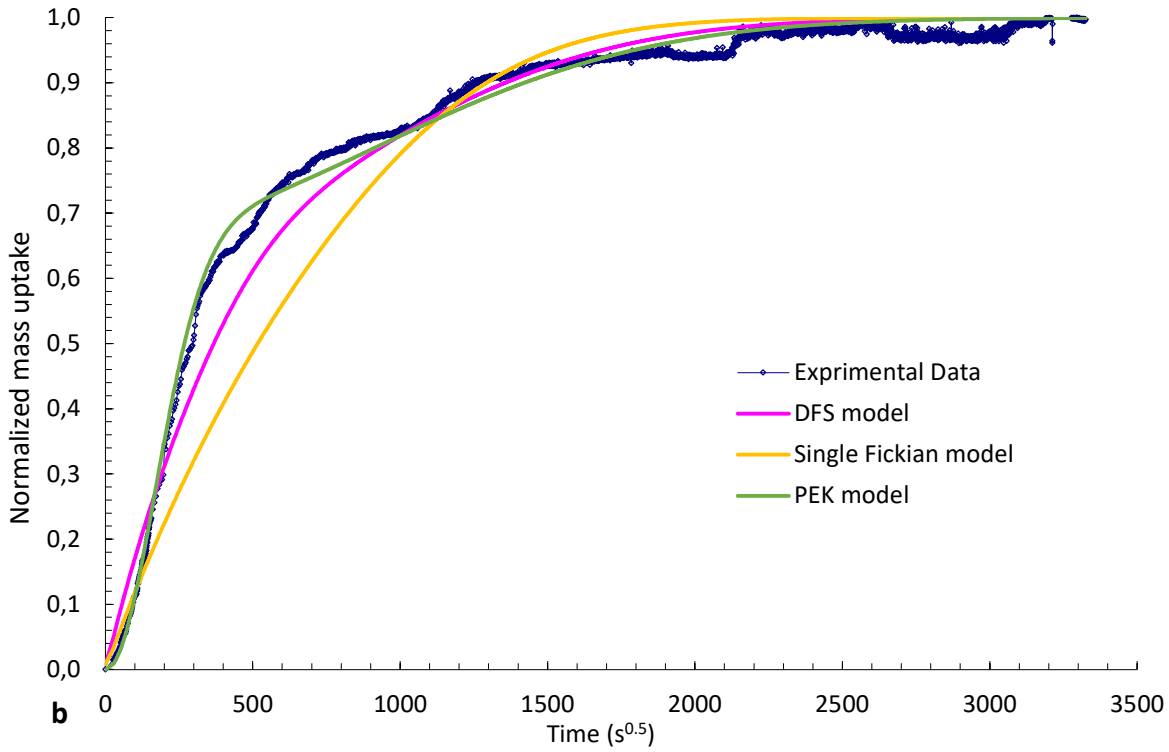

Figure 3.10. Desorption kinetics of Essential oils from Nanocellulose films: (a) thyme desorption from NFC1 (b) oregano desorption from NFC2.

By analysing the data from tables, it is interesting to notice that both φ and β parameters are generally spanning in the range of 0.3-0.7, confirming that two process are generally needed for the correct interpretation of the desorption behaviour. The only exception in this concern is represented by in thyme-NFC3 desorption data, that can be described very well using a single diffusion coefficient. In fact, D_1 and D_2 obtained from fitting of DSF model are substantially equivalent so that this curve tends to collapse to the results of the single Fickian processes. For all the other systems, instead, φ and β values are well correlated as systems of high φ show high β and vice versa. In particular, among the different oils, oregano is the one with lower φ (and β), while cinnamon and thyme show very similar values. Oregano, therefore, is the oil for which the fast desorption has a higher importance with respect to the slow one. Interestingly it is also the oil for which PEK usually shows a better fit of the experimental data, as it can be seen from the last two columns of table 3.1, which reported the global and short time average relative error defined by Eq. 10:

$$err\%_{average} = \sum_{n=1..N}\left[\left(\frac{|m_{model} - m_{\exp data}|}{m_{exp\ data}}\right)_n \frac{1}{n}\right] \quad (10)$$

Where N represents, respectively, the total number of data points for the total error, and the short time data points for short time errors. Short time indicates the time interval needed for the desorbed mass to reach the amount assigned by the given model (PEK or DSF) to the fast process (proportional to 1-φ and 1-β, respectively).

This latter error was introduced as the total errors are usually dominated by long time data and could not properly describe the initial stage of the desorption, where the main differences between the two models are encountered.

From the tables it can be seen, indeed, that the obtained total errors are generally low and comparable for the two models, since long time data are generally described fairly well by both PEK and DFS approach. Short time errors, on the other hand, show well defined differences reflecting the Fickian (as in Fig. 3.10a) rather than the exponential kinetics of the data (clearly visible from Fig. 3.10b).

Therefore, oregano short time data, as said above, are in general well described by an exponential behaviour, since the short time errors for PEK never exceed 30% against those observed for DFS which are spanning between 38 and 55%. For the other oils, however, the situation is less clear; both cinnamon and thyme data, indeed, are mainly Fickian, even if short time exponential behaviour was dominant in some of the films considered, namely NFC2 for thyme and NFC1 for cinnamon.

Table 3.1. PEK and DFS parameters for all the samples. The column MSE indicates the mean square error of the model compared to the experimental data.

PEK model data					
Sample	**τ1**	**τ2**	**ϕPEK**	**% Relative error (total)**	**% Relative error (short time)**
NFC1_C	5.0×10^5	3.6×10^3	0.49	5%	11%
NFC1_T	2.0×10^5	5.3×10^3	0.52	10%	22%
NFC1_O	4.0×10^4	1.2×10^3	0.22	2%	16%
NFC2_C	6.0×10^4	1.7×10^3	0.60	3%	28%
NFC2_T	3.8×10^4	1.4×10^3	0.55	3%	8%
NFC2_O	1.7×10^6	5.6×10^4	0.33	3%	29%
NFC3_C	2.6×10^5	1.6×10^4	0.50	4%	32%

NFC3_T	6.4×10^4	2.0×10^3	0.70	9%	18%
NFC3_O	5.2×10^4	1.7×10^3	0.25	1%	7%
DFS model data					
Sample	**D1**	**D2**	**β**	**% Relative error (total)**	**% Relative error (short time)**
NFC1_C	3.85×10^{-11}	3.80×10^{-13}	0.68	5%	40%
NFC1_T	2.50×10^{-11}	2.20×10^{-12}	0.67	6%	13%
NFC1_O	1.10×10^{-9}	4.00×10^{-11}	0.30	2%	38%
NFC2_C	1.00×10^{-9}	2.90×10^{-11}	0.73	2%	20%
NFC2_T	6.60×10^{-11}	1.10×10^{-11}	0.67	3%	39%
NFC2_O	1.17×10^{-11}	1.20×10^{-12}	0.57	4%	48%
NFC3_C	2.50×10^{-10}	1.50×10^{-11}	0.70	3%	9%
NFC3_T	9.94×10^{-12}	9.97×10^{-12}	0.23	7%	12%
NFC3_O	4.22×10^{-10}	3.50×10^{-11}	0.33	4%	55%

Despite the lack of general trends, the present data are well in line with other literature results as the complex transport behaviour in essential oils release is in general confirmed by several studies involving both polymers and fibres. Mishra et al. (2018), for example, studied the retention of lemongrass essential oil loaded on cellulose nanofibre-polyethylene glycol composite. The results suggested that Fickian diffusion makes the predominant contribution to release of major aroma compounds (315). Also, release studies conducted on ethylcellulose-encapsulated thyme essential oil showed a time-dependent Fickian diffusion (316). Instead, J. Ke et al. (2019) studied the diffusion kinetics of cinnamaldehyde from corn starch-based film into food simulant. The compound followed a Fickian behaviour with relevant differences between short and long times (317). Another interesting work was done by Montero et al. (2021) on polybutylene adipate-*co*-terephthalate films added with nanocellulose and functionalized with cinnamon essential oil (311). Here, as well, a pseudo Fickian diffusion was observed, with a faster release at short times, maybe due to the non-adsorbed oil on the nanocellulose surface, which quickly migrates on the polymer surface thus causing a burst effect at the beginning of the experiment. In addition to such results, the diffusion kinetics in microfibrillated cellulose is known to strongly depend on film swelling. As an example, non Fickian sorption behaviour was observed in many different types of nanocellulose, when equilibrated with water vapor at high relative humidity (296,314,318). In fact, the water causes the swelling of the matrix, which leads to a relaxation of the chains of the polymer and a consequent change in the diffusion mechanism (319).

In this concern the strong interaction of the essential oils with the nanocellulosic matrix is also confirmed by desorption data analysis which also suggests, following Montero et al. (311), that the higher fraction of mass desorbed in short time in case of oregano can be related a lower ability of this oil to bind with (adsorb on) the cellulose matrix.

Another consideration could be made by comparing absorption and desorption kinetics. Even with the limitation of the fitting approach, it clearly results that the diffusion coefficients calculated during sorption from liquid phase are definitely higher (usually 1 order of magnitude) with respect to the D_1 values obtained for short time desorption rates. Therefore, the transport of the different oils in the nanocellulose seems to be dominated by the relaxation and swelling of the fibrous matrix, higher in the liquid rather than in the gas phase, while other sources of complexity play a secondary role, such as, for example, of the effects related to essential oils compositions. As already discussed, indeed, essential oils are composed by a wide variety of compounds, which strongly differ in both molecular weight and volatility (boiling point or vapor pressure) and that are expected to have different diffusion behaviour.

In this concern, a detailed study of the gas leaving the film would be very useful, in order to confirm the present considerations and, more in general, to have a better description of the overall system behaviour. Unfortunately, this analysis was not possible in the present experimental set up. The kinetics analysis, however, suggests that, in view of the foreseen application related to active packaging, the quantity of oils present in the matrix can guarantee an antimicrobial activity even at long times. Indeed, based on φ and β values and on the concentration, molecular weight and the vapor pressure of the essential oils' components, the most active compounds should be released in both desorption stages.

4. Results and Discussion of antimicrobial and shelf-life properties

4.1 Anti-bacterial properties of the activated nanocellulose films

Figure 4.1 displays representative agar plates that show the antibacterial activity results obtained after exposing certain numbers of *E. coli* and *S. aureus* cells (spread on solid agar cultures) to the nanocellulose films embedded with cinnamon essential oil. It is possible to observe the different colonies growth among the control (inoculated plate with only aluminum foil), the plate with pure nanocellulose (NAP) and the one with the cinnamon essential oil (NAP9_C-0.68). The antibacterial effect can be noticed by the reduction or absence of microbial growth on plates exposed to activated films, still visible in all samples but the control, where it was removed to better visualize the colonies in the picture. Also, it is possible to observe that pure nanocellulose did not have any antimicrobial activity itself, since the colonies were present all over the plate. Instead, the film containing the cinnamon essential oils clearly had an antimicrobial effect on *S. aureus*, since there were no visible colonies. Moreover, it was noticed that, even in the cases of insufficient antimicrobial activity, the films still had bacteriostatic activity on the strains tested. This means that the colonies appeared in the Petri dishes, but they did not grow during the week of the test.

Furthermore, when the microbial colonies (indicative of microbial growth) were present, they were mostly concentrated in the external area of the Petri dish, near the borders. This could be because, in this region, the vapor part of the essential oil came out from the Petri dish and had less effect on the system itself.

To confirm this hypothesis, a specific test was performed in which the inoculated agar plates were sealed with Parafilm to limit the essential oil diffusion towards external environment (indicated with an asterisk: NAP4*_T-0.38). As a result, when the Parafilm was used, the microbial growth was absent also on the plate border areas. Although the use of parafilm was useful to confirm that there is a diffusion effect of the essential oil at the level of the plates border which is relevant in terms of antimicrobial activity, this experimental setting could not be used further application because it does not represent real packaging conditions. Indeed, the real fruit packaging conditions are characterized by boxes which have parts open to the external environment to prevent problems related with fermentation processes.

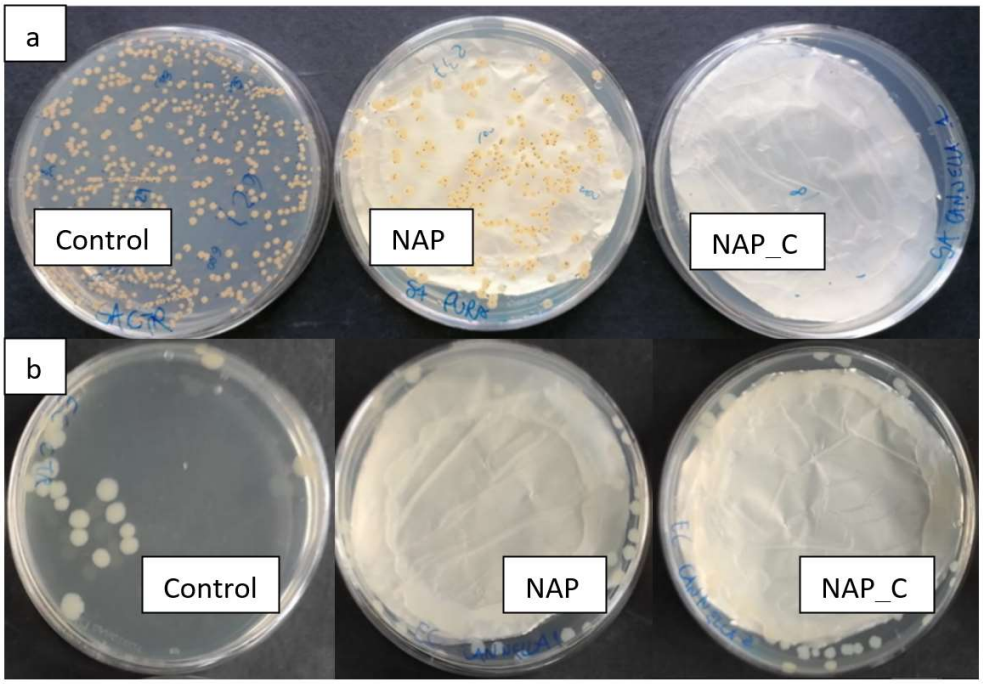

Figure 4.1. Appearance of the Petri dishes after the test with (a) S. aureus and (b) E. coli. The figure reports the control, the pure nanocellulose without any oil (NAP) and the nanocellulose with cinnamon essential oil (NAP9_C-0.68).

The overall results of the experimental campaign are reported in figures 4.2 and 4.3, which show the antimicrobial activity of the different samples tested as variation of colonies count with respect to the control. In particular, figure 4.2 refers to nanocellulose films with 9 cm of diameter, while figure 4.3 refers to the samples of 4 cm of diameter. In both cases, in the control experiments, pure nanocellulose had no effect on the microbial growth.

Concerning the films with diameter of 9 cm, comparing the results obtained with films containing the same amount of essential oil, the system was always more effective against *S. aureus* rather than *E. coli;* even if all the oils showed very high effectiveness for both microbial strains making difficult to observe major differences in the colonies count. This difference indeed never exceeded 10% and was therefore not statistically relevant. Only in the case of NAP9_T-0.19 the system had a higher antimicrobial effect against *E. coli*, but still very similar to the case of *S. aureus*, with values of 98% and 97%, respectively.

Oregano essential oil seemed to have the better antimicrobial activity, since it was the only one that had 100% of effect against both pathogens with a quantity of 0.62 mg/mg. It was followed by thyme and, lastly, cinnamon, which had the lowest effect at this concentration. This could also be

because cinnamon showed to strongly interact with the nanocellulose matrix (as proved from FTIR and sorption analysis), which somewhat reduced the tendency of the film to release the antimicrobial components. Cinnamon, indeed, showed the lower diffusivity (D) and higher characteristic times (τ) at long times with respect to both thyme and oregano.

Regarding the membranes of 4 cm of diameter, it was possible to notice an increasing trend of the antimicrobial efficacy with the increase of the essential oil quantity. Although, in this case the difference in the antibacterial effect was more evident, showing for all the oils at all the concentrations stronger activity against *S. aureus*, and confirming what observed with the membranes of 9 cm. In the case of the Parafilm sealing of the Petri (NAP4*_T-0.38) a higher antimicrobial effect against both pathogens compared to the normal set up (NAP4_T-0.38) was observed, with a difference of 98% for *E. coli* and of 30% for *S. aureus*. Also, in this case the microbial growth was absent on the borders, differently from all the other Petri dishes which were not sealed (figure 4.1). Moreover, for the same quantity of 0.57 mg/mg, thyme essential oil was more effective than oregano against gram-negative bacteria. While the opposite was true in the case of the gram-positive one, in which the difference was even more evident.

As can be seen in table 2.3, the volume of oil (µL) per liter of air in the packaging headspace is higher for the bigger samples than for the small one (to maintain the same ratio between the mass of the oil and the mass of the nanocellulose films). This resulted in the bigger films showing higher antimicrobial activity, in the range between 75% and 100% considering all the oils. While the smaller ones never showed an antimicrobial activity higher than 80% (the highest value of 76% was recorded for NAP4_T-0.57 against *S. aureus*). This behavior suggests once more that the volatilization of the oil and its dispersion to the external atmosphere due to the border effects is an important phenomenon to take into consideration while tuning the oils' concentration.

The lowest antibacterial effect (corresponding to 17% of bacterial growth inhibition) was observed with the small films containing thyme essential oil 0.19 mg/mg. This confirms the hypothesis that, due to the border effect, the dimension of the film itself is an important parameter to take into consideration when incorporating this kind of active system inside a real package. Bigger films seem to better support and enhance the antimicrobial properties of the essential oils. This is maybe since, even if the percentual load of oil in the film is the same, the absolute quantity of oil present in the system is higher in the case of bigger samples. And being bigger, the samples could probably release the oil for a longer period, resulting in higher antimicrobial efficacy. Also, regarding these results, it is possible to assume that the oil quantity per volume of headspace is more determinant for the antimicrobial effect than the oil quantity referred to the nanocellulose

film itself. In fact, since this system works thanks to the volatile part of the oils and border effects were observed, it is important to tune the quantity of oil in the film based on the headspace of the specific packaging used. For this reason, the membranes of 9 cm of diameter were used as reference for the MIC calculation and oregano essential oil was not tested in the small membranes. Also, since each type of packaging is different depending on the type of the fruit or, more in general, the food to preserve, in any case a specific evaluation of the system oil-film-packaging should be considered and tested to calculate the most efficient quantity of oil. In fact, tests directly on packed fruit were performed as well. The MIC concentration used in these tests was the minimum concentration of each essential oils which gave the higher antibacterial effect: 0.68 mg/mg for cinnamon, 0.37 mg/mg for thyme and 0.62 mg/mg for oregano (mg of oil/mg of nanocellulose membrane), respectively. Table 4.1 shows the antibacterial activity of all the membranes tested, which is a summary of the data that can also be visualized in figures 4.2 and 4.3.

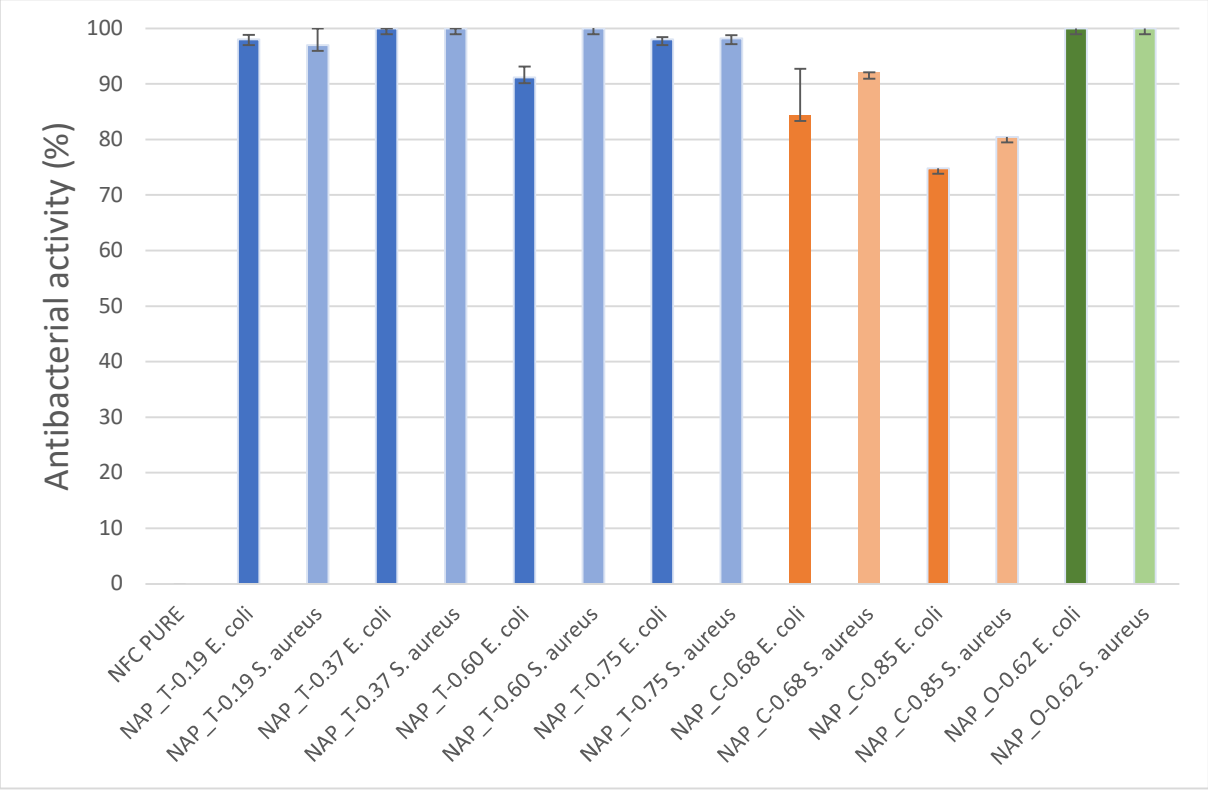

Figure 4.2. Antimicrobial activity against E. coli (darkest color) and S. aureus (lighter color) expressed through CFU counting from the nanocellulose samples of 9 cm of diameter embedded with thyme (blue), cinnamon (orange), and oregano (green) essential oils.

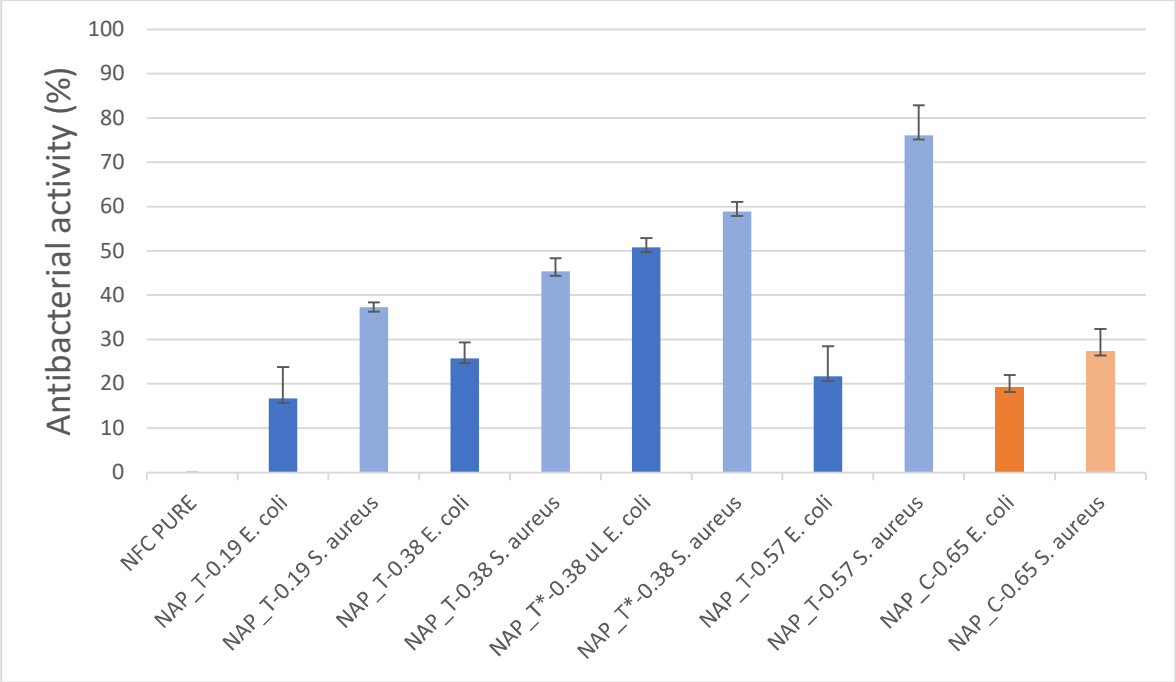

Figure 4.3. Antimicrobial activity against E. coli (darkest color) and S. aureus (lighter color) expressed through CFU counting from the nanocellulose samples of 4 cm of diameter embedded with thyme (blue) and cinnamon (orange) essential oils.

Table 4.1. Antibacterial activity of the membranes tested with the various essential oil at different concentrations.

Membrane name	**Antibacterial activity (%)**	
	E. coli	***S. aureus***
9 cm		
NAP (NFC PURE)	0	0
NAP_T-0.19	98	97
NAP_T-0.37	100	100
NAP_T-0.60	91	100
NAP_T-0.75	98	98
NAP_C-0.68	84	92
NAP_C-0.85	75	81
NAP_O-0.62	100	100
4 cm		
NAP (NFC PURE)	0	0
NAP_T-0.19	17	37
NAP_T-0.38	26	45
NAP_T*-0.38	51	59
NAP_T-0.57	22	76
NAP_C-0.65	19	27

4.2 Real condition application: raspberry case-study system

Figure 4.4 represents the active packaging systems during the test on packed raspberries. The nanocellulose films were attached to the inner side of the lid of the packaging, after being embedded with the selected quantity of essential oil. The image shows the appearance of the fruit after 12 days of storage with the treatment applied. It can be noticed that for all tests, the qualitative fruit appearance seemed comparable to normal fruit without treatment, while major differences were observed for others quality parameters as better detailed below.

An important factor to be considered was also the diffusion of the essential oils into the plastic constituting the packaging box used for the case-study system on raspberries (r-PET). Licciardello et al. (2013) studied the diffusion behaviour of some essential oils in plastic films obtaining a diffusion coefficient of the various oils' components in the range of $0.03\text{-}6.40\times10^{-11}$ cm^2/s. Also, the diffusion resulted to depend on the initial concentration of the EO's components, their molecular weight and polarity (320). These values can be compared with the diffusion of the oil in the nanocellulose matrices studied (table 3.1). In the initial phases of the desorption, the diffusion of the oils resulted to be higher in the nanocellulose than in the plastic film. Also, considering the plastic film had a thickness of 0.5 mm, versus the approx. 50 µm of the nanocellulose film, it can be stated that there was no problem of oil leaking through the packaging. So, the quantity of oil lost because of the permeation in the plastic film was considered negligible compared to the one diffusing through the nanocellulose film and having the antimicrobial activity.

Figure 4.4. Raspberries active packaging systems with pure nanocellulose after 12 days of storage at 1°C.

4.2.1 General and nutritional quality parameters

General and nutritional quality parameters of the fruit in the packaging with films embedded with the different essential oils were analyzed. Both sets of experiments are presented and compared. In particular, as it will be explained more in detail in the results discussion, in the test 2 cinnamon essential oil was discarded due to the less effectiveness compared to thyme and oregano essential oil. Instead, thyme and oregano were used again but with a higher quantity than test 1. Also, their combination was tested to study their synergetic effect.

An important indicator of fruit freshness is weight loss. A weight loss up to 4-5% is considered to not significantly affect the fruit aspect and the acceptance by the consumers (321). Figure 4.5 shows the weight loss (%) of the fruit during storage at 1°C. After 12 days all the treatments presented a weight loss within 5%, with cinnamon treatment at the limit of acceptance. In particular, regarding the first test, from figure 6a it is possible to see that NAP and NAP_C had higher weight loss compared to the control. While NAP_T and NAP_O had the lowest weight loss, with 3.50% and 3.40%, respectively. Instead, in test 2 (figure 4.5b) NAP_O had higher weight loss compared to the control, while NAP_TO resulted the best treatment overall with weight loss below 3% (2.5%).

Furthermore, the color is the first indicator of the freshness of the fruit perceived by the consumer. For this reason, it is very important to make conclusions about the storage conditions of the fruit. The color parameters lightness (L*), Hue and Chroma for tests 1 and 2 are reported in figure 4.6. For test 1, the lightness had a significant decrease in the first 6 days, which was smaller for NAP_O and higher for NAP_C than control and NAP. Then it was maintained along the 12 days of treatment without significant difference among the treatments. The same trend was observed in test 1 for Hue and Chroma (figure 4.6b and 4.6c). Though after 12 days of storage for test 1, the NAP and the NAP_O treatments were significantly different from the NAP_C one. This could be explained by the fact that in the initial phase the quantity of oil released was higher because its concentration in the film was still high, while along the days its quantity decreased.

In test 2 the results were more scattered with respect to test 1 and the data for different oils followed different trends. Apart from NAP_TO, indeed, no clear sign of parameter stabilization after day 6 was observed. In general, lightness was better maintained by the control in the first 6 days, with a significant difference from the NAP_O and NAP_TO. After 12 days of storage, the control resulted significantly different from both the treatments NAP_O and NAP_TO. The same trend was observed for Hue angle (h°), with a statistical difference only between the control and the NAP_TO treatment, indicating an increase in redness, and ripening of the fruits. While for Chroma (C*) the values of all the treatments increased in the first 6 days and then decreased over the 12 days, without statistically significant differences.

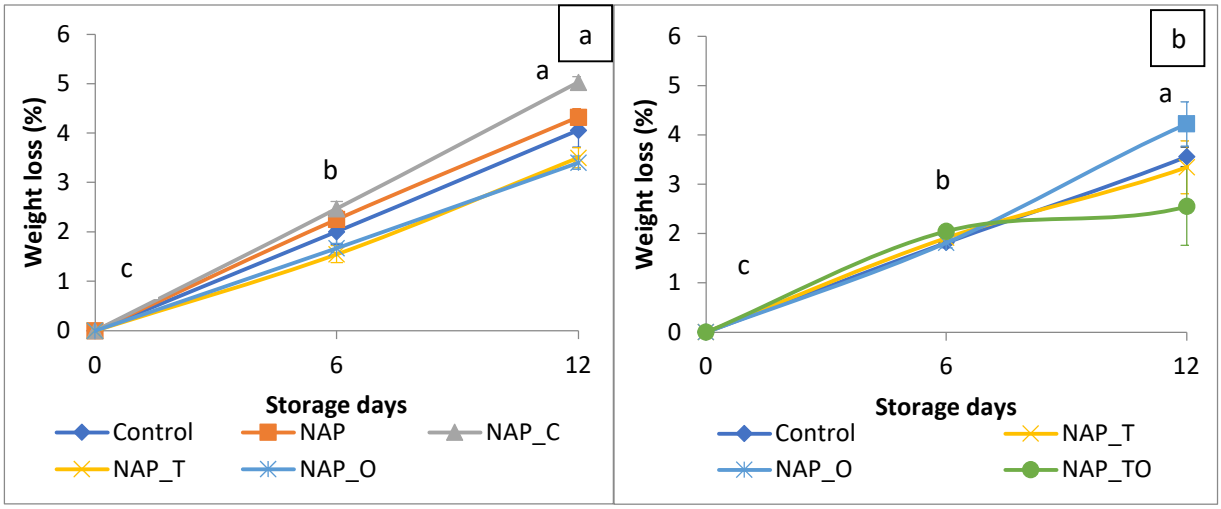

Figure 4.5. Weight loss (%) of the fruit during storage for (a) test 1 and (b) test 2. Values with different lower case letter are significantly different over time by Duncan's multiple range test ($P < 0.05$). No statistical differences were present for the different treatments for each time (0, 6th and 12th days).

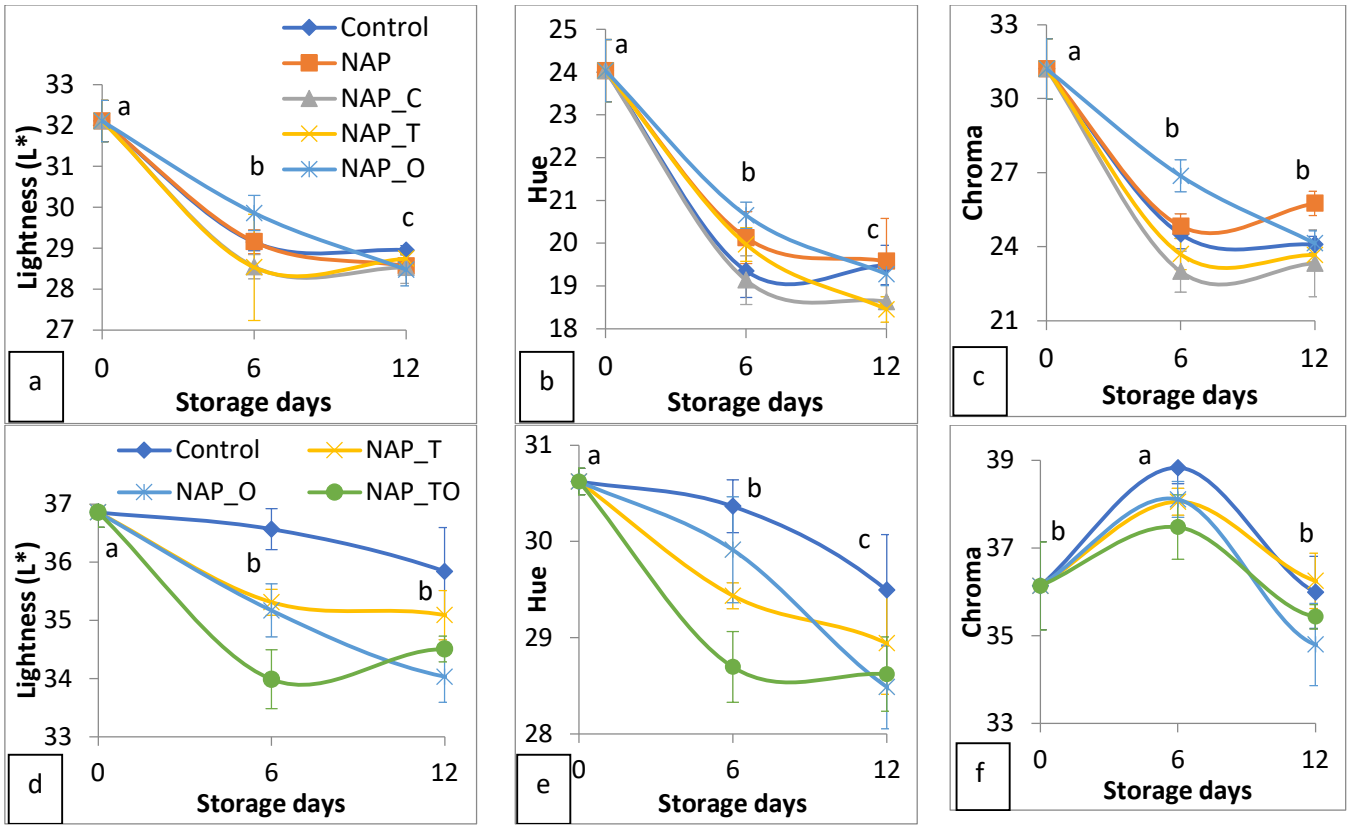

Figure 4.6. Color parameters of the fruit during the storage: lightness (a,d), Hue (b,e) and Chroma (c,f) for test 1 and 2, respectively. Values with different lower case letter are significantly different over time by Duncan's multiple range test (P < 0.05).

The table 4.2 reports other quality parameters analyzed during the experiments, such as the firmness and the soluble solids concentration (SSC). Also, it reports the total phenols content, the antioxidant activity calculated through the DPPH method and the anthocyanins concentration.

By a general statistical analysis for all the treatments, the soluble solid concentration (SSC) firstly increased (after 6 days) to then slightly decrease along the 12 days of the experiment 1. For experiment 2, on the other hand, it showed no significant differences. The results are in line with expectations, as raspberries are non climacteric fruit, which usually show only slight changes of SSC (54). More in detail, for test 1 the variations with respect to the initial time values are always lower than 4.8%, except for NAP and NAP_O, which, after 6 days, registered a statistically significant increase (+6.21% and +9.60%, respectively). After 12 days, the control had the highest SSC, while there was no difference between the other treatments. Regarding the second test, none of the treatments had significant variations along the time of the experiment. While comparing the treatments, after 12 days NAP_T had the highest value of 7.70 °Brix (7.69%

increase from the beginning), which means this treatment was the one related with the faster maturation of the fruit.

The firmness of the packed fruit considerably decreased after 12 days of storage for all the treatments, until reaching the limit of sensibility of the measurement instrument. In this case, NAP_C was the one with the highest decrease, passing from 4.75 N to 0.95 N after 12 days, followed by NAP_O and NAP_T, respectively. The latter films, however, showed firmness values similar to the ones of the control and NAP while cinnamon resulted below. In the second experiment, the initial values of firmness were almost the double of the ones of the test 1, 8.77 N and 4.75 N, respectively. After 6 days, NAP_TO had the highest decrease passing to 4.64 N (-47%), while NAP_T and NAP_O decreased significantly with respect to the control, with a difference of 37% and 34%, respectively. At the end of the experiments all the treatments were significantly lower than the control, with NAP_O having the lowest value.

The fruit softening during the post-harvest period is related to the hydrolysis of starch to sugar and to the degradation of pectin contained in the fruit cell wall associated with the fruit ripening (322). The present results are similar to the ones obtained by Cefola et al. (2022) studying the raspberries' application of an antifungal active package based on green tea and rosemary ethanolic extracts, suggesting that essential oils tend to negatively affect this parameter (323).

The phenolic content was also monitored since it is an indicator of the degradation of the fruit in the post-harvest period (295)The total phenolic content in raspberries never had significant difference along the time of test 1 for all the treatments. At the end of the experiment, the control and NAP_T had the highest values, with 114.67 and 107.72 mg of gallic acid equivalent/100g of fresh weight, respectively. While NAP_O continued decreasing up to 79.09 mg of gallic acid equivalent/100g of fresh weight (table 4.2). Instead, for test 2 (table 4.3) the trend was different. The control increased the total phenolic content from 60.25 up to the maximum value of 160.50 72 mg of gallic acid equivalent/100g of fresh weight after 12 days. All the other treatments had an increase at 6 days, followed by a decrease at the end of the experiment, without statistically difference among them. The fact that the concentration of phenols did not change significantly in the experiments could be associated to the absence of reactive oxygen species which would lead to oxidative reactions (324). The same trend was observed by Rahmanzadeh et al. (2019) which applied lemon verbena essential oil on raspberries (325). It could be explained by the high essential oil concentration, which lead to the breakdown of cellular structure more easily. In fact,

in the test 2, with higher essential oils concentration, this decrease was more evident than in the test 1, where the essential oil quantities were only equal to the MIC.

The measurement of the antioxidant activity by DPPH method showed a decrease after 6 days for NAP_T and NAP_O in test 1, with 2020 μM Trolox/g and 2088 μM Trolox/g, respectively. At the end of the experiment the values of the various treatments did not have significant differences, with the lowest value of 2397 μM Trolox/g from NAP_O. Also, for the test 2 no significant differences among the treatments were found during the storage of the fruits. In general, the values were higher than in the first experiments and after 12 days the lowest value was from NAP_TO with 4205 μM Trolox/g. In both the experiments, the highest value of antioxidant activity after 12 days was registered in the control. The application of the essential oil helped in maintain the levels of antioxidants but did not improve the effectiveness over time. This could mean that the antioxidant capacity assayed may be due to the presence of high amounts of antioxidants already in the fruits. The findings of Jin et al. (2012) confirmed the higher amount of antioxidants, such as anthocyanins, flavonoids and phenolic acids, in raspberries (326).

The anthocyanins quantity measured in the test 1 started to be significantly different only after 12 days, with NAP having the highest value of 86 mg/ml, compared to the control (70 mg/ml). For all treatments, the anthocyanins content was increasing over time, maybe due to the biosynthesis of phenolic compounds after harvest related to the ripening of the fruits (324). Instead, in the second experiment, their values increased significantly in the first 6 days and then decreased again after 12 days (table 4.3). Also, in this case the highest value was observed in the control (44 mg/ml) and the lowest from NAP_O (34 mg/ml), but without statistical difference. These results are in line with those by Guerreiro et al. (2015) which analyzed raspberries coated with pectin and alginate based edible coating enriched with essential oils components citral and eugenol (54).

Table 4.2. Soluble solid content (SSC), firmness, total phenolic content, DPPH, and anthocyanins of raspberries treated with cinnamon (C), thyme (T) and oregano (O) essential oils during storage at 1°C. NAP indicates the presence of the nanocellulose film, without any oil.

	Days	Control				NAP				NAP_C				NAP_T				NAP_O			
		Mean		SD		Mean		SD		Mean		SD		Mean		SD		Mean		SD	
SSC (°Brix)	0	8.85	±	0.13	aA	8.85	±	0.13	bA	8.85	±	0.13	abA	8.85	±	0.13	abA	8.85	±	0.13	bA
	6	9.18	±	0.17	aB	9.40	±	0.16	aAB	9.28	±	0.42	aB	9.10	±	0.28	aB	9.70	±	0.18	aA
	12	9.10	±	0.29	aA	8.70	±	0.14	bAB	8.98	±	0.39	abAB	8.48	±	0.55	bB	8.75	±	0.31	bAB
Firmness (N)	0	4.75	±	0.92	aA	4.75	±	0.92	aA	4.75	±	0.92	aA	4.75	±	0.92	aA	4.75	±	0.92	aA
	6	4.18	±	0.93	aA	3.22	±	1.20	bB	3.24	±	0.56	bB	3.76	±	0.79	bAB	3.02	±	0.20	bB
	12	1.61	±	0.25	bAB	1.88	±	0.33	cA	0.95	±	0.81	cB	1.60	±	0.71	cAB	1.51	±	0.23	cAB
Total phenolic content (mg GAE/100g)	0	92.42	±	18.68	abA	92.42	±	18.68	aA	92.42	±	18.68	aA	92.42	±	18.68	aA	92.42	±	18.68	aA
	6	97.73	±	15.32	abA	95.71	±	5.96	aA	81.59	±	8.89	aA	98.87	±	28.09	aA	87.26	±	6.53	aA
	12	114.67	±	28.61	aA	100.00	±	9.24	aAB	96.54	±	12.23	aAB	107.72	±	9.87	aA	79.09	±	8.01	aB
DPPH (µM Trolox/g)	0	3074.72	±	122.91	aA	3074.72	±	122.91	aA	3074.72	±	122.91	aA	3074.72	±	122.91	aA	3074.72	±	122.91	aA
	6	3255.65	±	212.99	aA	2884.69	±	280.36	abAB	2949.24	±	743.31	aABC	2020.00	±	148.68	bC	2088.20	±	440.28	bBC
	12	3024.71	±	1231.90	aA	2658.29	±	229.24	bA	2684.65	±	389.02	aA	2830.13	±	519.68	aA	2397.34	±	399.61	abA
Anthocyanins (mg/ml)	0	47.12	±	9.04	bA	47.12	±	9.04	bA	47.12	±	9.04	cA	47.12	±	9.04	bA	47.12	±	9.04	bA
	6	63.82	±	8.95	aA	79.10	±	13.45	aA	62.23	±	6.50	bA	81.16	±	17.07	aA	77.15	±	11.98	aA
	12	70.05	±	9.88	aB	85.89	±	1.49	aA	76.54	±	5.58	aAB	60.95	±	13.23	abB	61.90	±	5.05	abB

Values in the same column followed by different lower case letter and in the same row followed by different upper case letter, for each parameter, are significantly different by Duncan's multiple range test ($P < 0.05$).

Table 4.3. Soluble solid content (SSC), firmness, total phenolic content, DPPH, and anthocyanins of raspberries treated with thyme (T) and oregano (O) essential oils and their combination (TO) during storage at 1°C.

	Days	Control				NAP_T				NAP_O				NAP_TO			
		Mean		SD		Mean		SD		Mean		SD		Mean		SD	
SSC (°Brix)	0	7.15	±	0.59	aA	7.15	±	0.59	aA	7.15	±	0.59	aA	7.15	±	0.59	aA
	6	6.95	±	0.37	aAB	7.68	±	0.29	aA	7.48	±	0.60	aAB	6.78	±	0.56	aB
	12	7.58	±	0.13	aA	7.70	±	0.62	aA	6.98	±	0.33	aA	7.55	±	0.72	aA
Firmness (N)	0	8.77	±	0.33	aA	8.77	±	0.33	aA	8.77	±	0.33	aA	8.77	±	0.33	aA
	6	7.07	±	0.38	bA	5.49	±	1.00	bB	5.80	±	0.43	bB	4.64	±	0.71	bC
	12	2.67	±	1.05	cA	2.20	±	0.49	cAB	2.06	±	1.33	cAB	1.32	±	0.38	cB
Total phenolic content (mg GAE/100g)	0	60.25	±	7.41	bA	60.25	±	7.41	bA	60.25	±	7.41	cA	60.25	±	7.41	cA
	6	59.32	±	19.95	bC	99.88	±	26.92	aB	107.77	±	15.27	aB	130.76	±	7.76	aA
	12	160.50	±	69.97	aA	63.63	±	10.43	bB	84.02	±	2.64	bB	89.54	±	9.36	bB
DPPH (µM Trolox/g)	0	4743.87	±	36.02	abA	4743.87	±	36.02	aA	4743.87	±	36.02	aA	4743.87	±	36.02	aA
	6	4347.05	±	858.77	bA	5038.67	±	647.91	aA	5065.38	±	390.26	aA	4978.86	±	954.87	aA
	12	4708.62	±	207.73	abA	4331.56	±	44.44	bA	4558.01	±	254.87	aA	4205.52	±	728.82	aA
Anthocyanins (mg/ml)	0	29.08	±	2.22	bA	29.08	±	2.22	bA	29.08	±	2.22	cA	29.08	±	2.22	cA
	6	26.25	±	6.20	bC	37.80	±	10.13	aB	51.27	±	2.52	aA	49.62	±	1.80	aA
	12	44.03	±	17.86	aA	36.82	±	3.03	abA	34.48	±	6.60	bA	35.90	±	2.73	bA

Values in the same column followed by different lower case letter and in the same row followed by different upper case letter, for each parameter, are significantly different by Duncan's multiple range test ($P < 0.05$).

4.2.2 Microbial evaluation

The microbial evaluation was fundamental to establish the antimicrobial activity of the activated films. In fact, they not only have to preserve the physical and chemical quality of the fruits, but also must ensure a good protection against food pathogens. Here, the prevention from post-harvest decay was carried out by the vapors from essential oils which were released by the nanocellulose films. Other studies confirmed the effectiveness of this application against Fungi growth (327).

Figure 4.7 shows the microbial analysis results for Fungi (a,c) and mesophilic bacteria (b,d) for test 1 and 2, respectively. The results from the psychrophilic bacteria are not represented since they always showed no colonies for all the experiments and all the treatments.

In the first experiment, the control treatment showed a Fungi's growth of about 2 log (CFU)/g of fresh weight already in the first 6 days, ending in the highest value of 5.8. Instead, other treatments had an increase in the first 6 days, and then decreased. NAP_O showed the lowest value, followed by NAP_T and NAP_C, respectively. Also, in the second experiment all the treatments showed better results than the control over the 12 days of storage, even if without statistical differences. They always decreased over time, with NAP_T and NAP_TO reaching a log (CFU)/g of 0 at the end of the experiment.

Regarding the mesophilic bacteria, instead, the log (CFU)/g was maintained like the one at time 0 by all the treatments in test 1. After 12 days, NAP_C had the highest growth, while NAP_O and NAP_T showed the best results. In the second experiment there was a slight decrease in the first 6 days, followed by an increase in the whole time of the test. Nevertheless, this increase was reaching a value of the log (CFU)/g which was still lower than the one reached at the end of the first test. The best results were showed by NAP_O and NAP_TO.

This different trend in the two experiments was probably due to the different quantity of oils present in the matrix. Being present more oils in the test 2, it was possible to preserve the antimicrobial activity longer in time, and with a stronger effect on the reduction of the colonies since the beginning of the application. In any case, the microbial evaluation never exceeded the accepted limit of 6 log (CFU)/g according to Bierhals et al. (2011) (328).

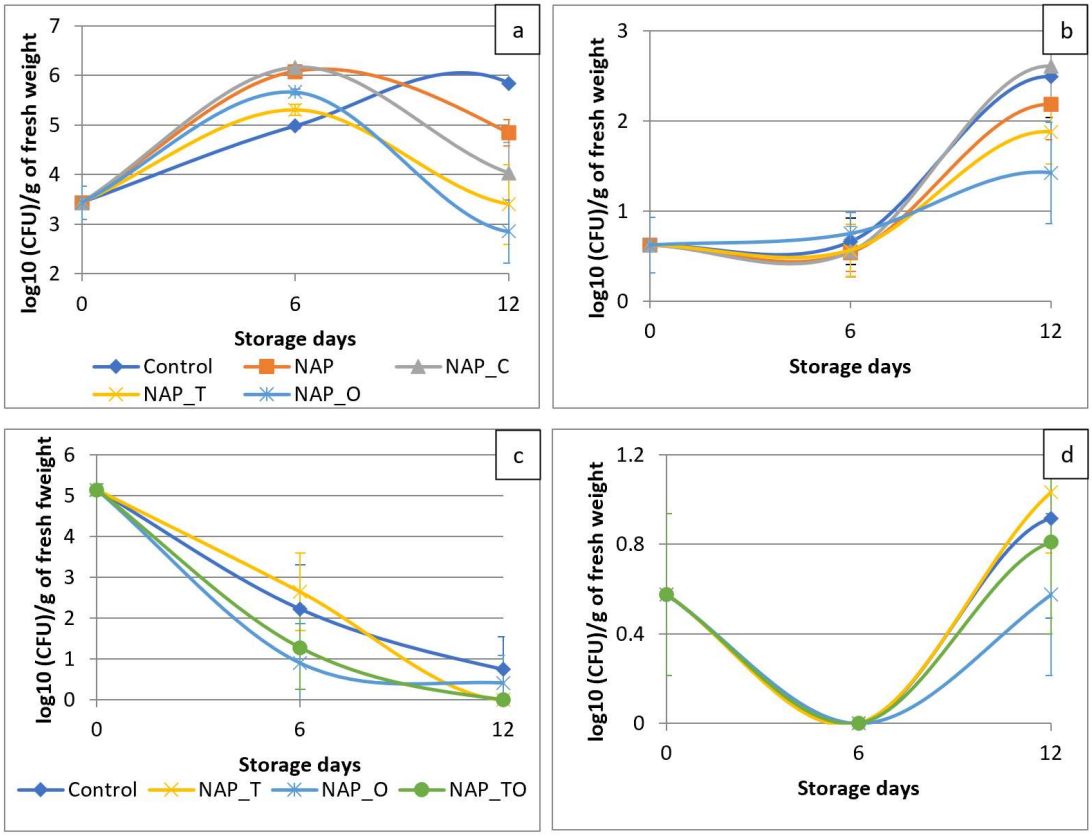

Figure 4.7. Microbial analysis of (a,c) fungi and (b,d) mesophilic bacteria on raspberries after 12 days of storage at 0.5°C, for test 1 and 2, respectively. Values with different lower case letter are significantly different over time by Duncan's multiple range test ($P < 0.05$).

4.2.3 Sensory evaluation

Antimicrobial properties of essential oils help in preserving the quality parameters and extend the shelf-life of packed food, but they could alter its sensory characteristics. For this reason, a sensory evaluation was also performed to test the consumer perception and acceptance of the proposed active packaging technology.

Figure 4.8 shows the results of the sensory evaluation performed with the panelist. The test was performed after 6 days of storage in the experiment 1 (figure 4.8a) and after 12 days of storage for experiment 2 (figure 4.8b), to see the differences of the consumer perception over various storage times.

In the first case, there were only slight differences between the treatments. In general, no significant alteration of the fruits was detected from the panel test: the general flavor and the fruit

appearance were similar for the different treatments. Most of the panelist slightly liked the texture of NAP_T and NAP_O. Nonetheless, NAP_T had the lowest sweetness, and this could be the reason it was perceived as less pleasant in the general taste.

Also, in the second test very limited differences were observed for the different oils. As it could be expected from the higher quantity of essential oils present, the general flavor was considered worse than in the first test, with the control being slightly more pleasant than other oils. The treatments with both thyme and oregano essential oils showed the best results in terms of fruit appearance, texture and acidity compared to those oils used alone.

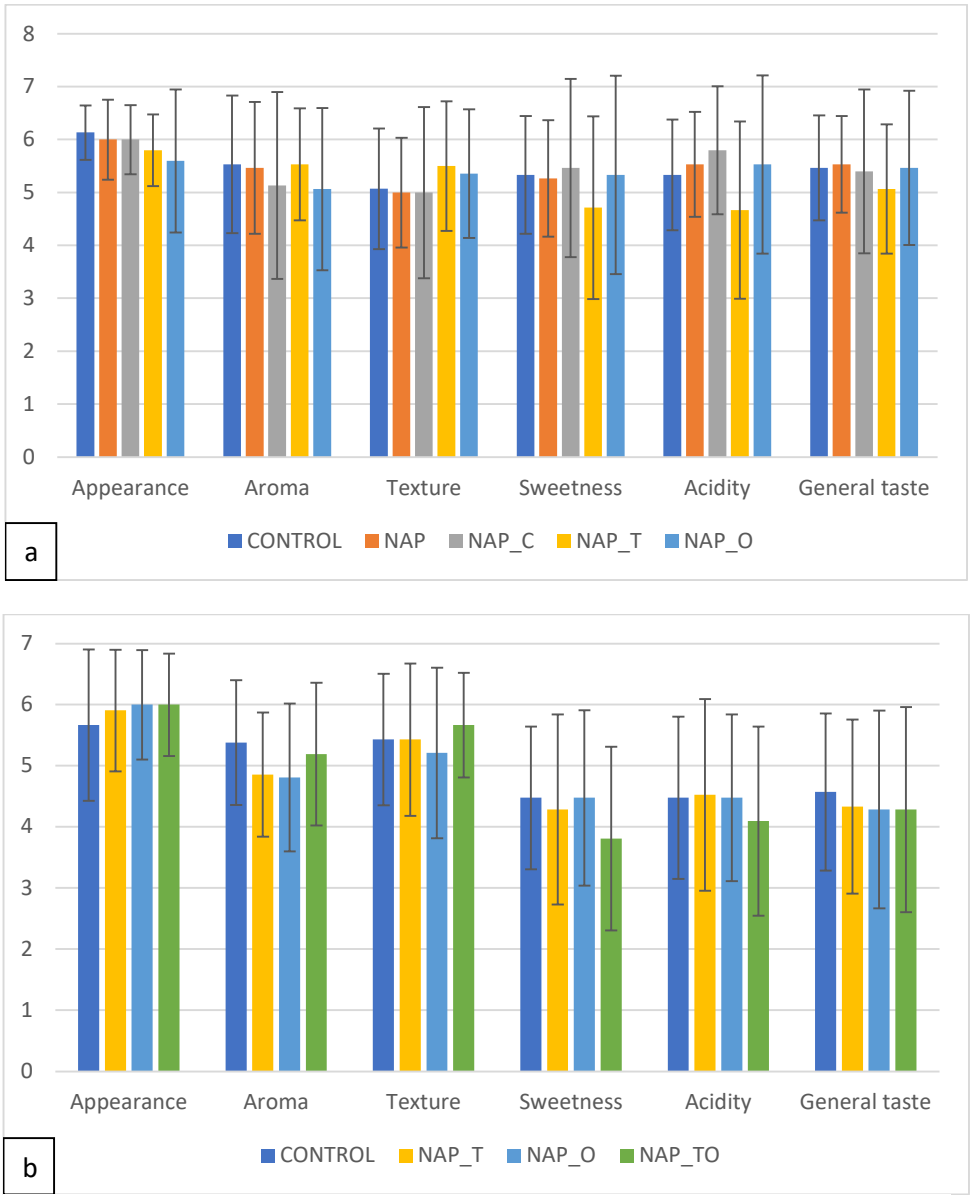

Figure 4.8. Sensory evaluation for (a) test 1 and (b) test 2.

5. Conclusions

In the recent years a strong boost in the research on packaging materials has been given by the need to increase the sustainability of packaging while further reducing the food spoilage. This has brought to an exceedingly high attention on renewables materials, such as chitosan and cellulose, and the use of natural compounds, such as essential oils, to impart antimicrobial features to the package, thus increasing the fresh product shelf-life.

The literature review present in this work has focused on this application, trying to report the use of the materials mentioned, alone or in combination with other biopolymers (such as PLA, agar and alginate) to obtain a completely renewable active packaging solution. Solutions involving the use of essential oils were highlighted due to the high interest in such natural antimicrobial substances in food applications. In such analysis, it clearly results that there is a strong interest in the use of cellulose and chitosan based active packaging.

In general, the analysis of the activity of the packaging solution is made on specific pathogens through tests on bacterial strains and is often coupled with considerations about mechanical strength of the final composite. This kind of considerations are common to about 80% of the reviewed studies, while tests on transport properties, such as water vapor and oxygen permeability of the active materials, are slightly less common. Interestingly, about half of the studies also consider direct application to specific food for in situ testing of the shelf-life increase. The interest is directed to meat products and fruit/vegetable products, which are the type of food that can benefit the most from this active approach due to their high perishability. Few examples of other foods such as cheese and bakery products were found. In all these works the deterioration of the product is monitored through laboratory analyses, while only a small percentage of them also present a sensory evaluation of the packaged food. This kind of assessment, however, has significant importance for the development of the final product and should be treated with more attention.

In the current analysis, the most used essential oils resulted to be, in the order, Oregano, Cinnamon, Thyme and Rosemary. However, many other plant-derived compounds, also different from essential oils, were considered and resulted effective against a wide range of microorganisms. In fact, these natural compounds showed antimicrobial activity against the most known gram-positive bacteria (*S. aureus, B. subtilis, L, monocytogenes*), gram-negative bacteria (*E. coli*), and fungi (*S. cerevisiae, A. niger*).

In general, however, despite the extremely high number of works and the potential of most of the materials considered, it is difficult to draw general guidelines in the production of active packaging based on the combination of renewable materials and essential oils. Many of the available studies are, indeed, focused on a very specific application, to test the performance of certain essential oils, incorporated in each material, on the preservation of the quality of specific food. The use of a combination of essential oils in the same packaging, for example, is seldom considered, leading to ample space for further analysis and optimization of the currently tested solutions.

It can be noticed that, while most of the materials considered base their activity on the release of the active compounds, very few information is given about the release rate and kinetics, which are essential in order to design and adapt an active packaging solution for the desired shelf-life. These kinds of studies, together with a more structured analysis of the interaction between the essential oils and the different matrices, would be of great interest in the selection of the best approach and components to be used for a given application.

Furthermore, even if some applications already exist in the market, the field of active packaging has a lot of space for further research and optimization. From this literature screening the necessity of more structured investigations arises, together with the need to reach a complete comprehension of the multiple interactions existing in antimicrobial packaging, and on their effects on the final properties of the film and on the final shelf-life of the food.

Trying to face and solve these requests, this PhD project aimed at the development of an active packaging system based on nanocellulose and essential oils. The absorption and diffusion of cinnamon, thyme and oregano EOs in nanocellulose films with increasing carboxymethylation degree were investigated in view of active packaging application. Indeed, sorption from the liquid phase was followed by desorption in the gas phase trying to mimic the condition existing in fresh food packages. Moreover, the effect of essential oil incorporation into nanocellulose matrix for shelf-life extension of raspberries was determined as well both through tests on bacterial strains and tests on fresh packed fruit.

The results showed that the absorption and desorption of the oils in these matrices depend on the carboxymethylation degree and on the oil types. In particular, thyme essential oil showed the highest solubility with values of mass uptake close to 22%wt. for the nanocellulose with a surface charge of 1600 μeq/mol, against a value of only 10%wt in the case of oregano, which resulted the oil with lower solubility. Cinnamon also showed very high affinity with the nanocellulose, causing

a partial solubilization of the carboxymethylated films, which prevented a clear evaluation of the mass uptake. The kinetics of the sorption process resulted substantially Fickian, allowing the determination of diffusivity values in the order of 10^{-8} – 10^{-9} cm^2/s. The trend was generally increasing with the carboxymethylation degree. Cinnamon essential oil had the higher diffusivity, followed by thyme and oregano, suggesting that affinity with the nanocellulose and the ability to swell its structure were the main factors affecting the kinetics of the sorption.

For the release in the gas phase two different kinetics were needed to satisfactorily describe the experimental data. In fact, the systems showed a fast release at short times followed by a slower process at long time likely related to the compaction of the fibrous matrix, which was initially swollen by the solved oil. Interestingly, while thyme and cinnamon data could be well described by considering a two separate Fickian process, oregano release was better fitted by considering a parallel exponential approach. Despite their difference, however, both approaches were consistent in determining the relative weight of the fast and slow kinetics.

Then, the optimum technical characteristics of the package and the MIC for each oil were determined through tests on *S. aureus* and *E. coli* bacterial strains. The thyme essential oil was the one having the lower MIC with a value of 0.37 mg/mg of matrix, reaching 100% of antimicrobial effect against Gram-positive *S. aureus* and Gram-negative E. coli. While the oregano reaches this antimicrobial effect with 0.62 mg/mg. When using the MIC quantity directly on raspberries, the oils succeeded in maintaining the general quality parameters of the fruits and to maintain the microbial growth under the accepted limit of 6 log(CFU)/g of fresh weight. Their effect on microbial growth was more evident after 12 days storage, especially for fungi. When increasing the oil concentrations and applying the two best oils (thyme and oregano) and their combination, the positive effect was enhanced, with no significant effect on sensorial appreciation.

This study confirms that the release of EOs from nanocellulose matrices can be controlled by adequately choose the type of nanocellulose. It also suggests that the analysis of mass transport of different active substances in the nanocellulose matrix can give interesting information and useful guidelines in the development of new active packaging solutions. In any case this system has been developed not to be used in direct contact with the food to be preserved, but to take advantage of the volatilized compounds from the essential oils embedded in the matrix. So, in this way, the same system could be implemented in different types of foods, such as fruit,

vegetables or meat products. From the results of this experiment, it is also possible to conclude that active packaging including the thyme and oregano essential oils with a concentration of 1.1 and 1.8 ml of oil/liter of air in the headspace of the package, respectively, is a promising technology to improve shelf-life of raspberries. Further study on different fruit or different type of packaging could be useful to confirm the hypothesis on the effective oil concentration related to the headspace volume.

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Appendix

This section includes the FT-IR plots and the oils diffusion data in the three different nanocellulose matrices which were not included in the main part of the document for conciseness sake. In particular, figures A1-A6 represent the FT-IR analysis of NFC1 and NFC3 prior and after being in contact with cinnamon, thyme and oregano essential oils. While figures A7-A13 show the three essential oils diffusion from NFC1, NFC2 and NFC3 estimated during desorption in gas phase, comparing the experimental data with PEK, single and double Fickian diffusion models.

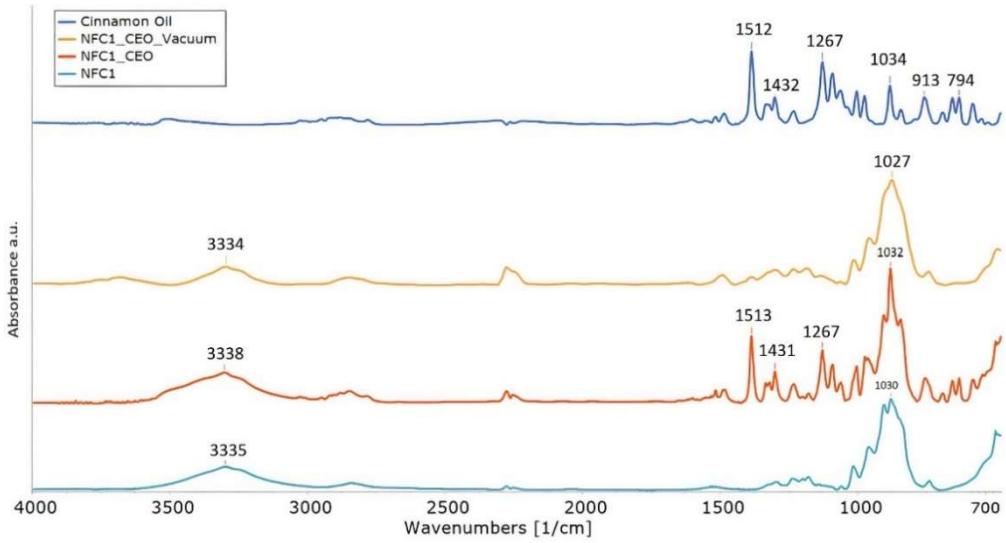

Figure A1. FT-IR analysis of NFC1 prior and after being in contact with the cinnamon essential oil, plus the cinnamon essential oil spectra for comparison.

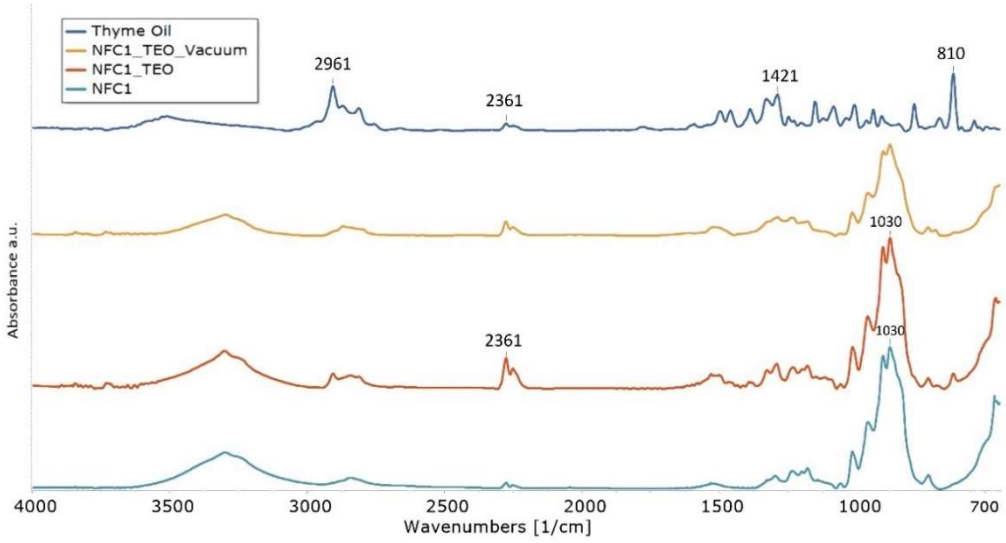

Figure A2. FT-IR analysis of NFC1 prior and after being in contact with the thyme essential oil, plus the Thyme essential oil spectra for comparison.

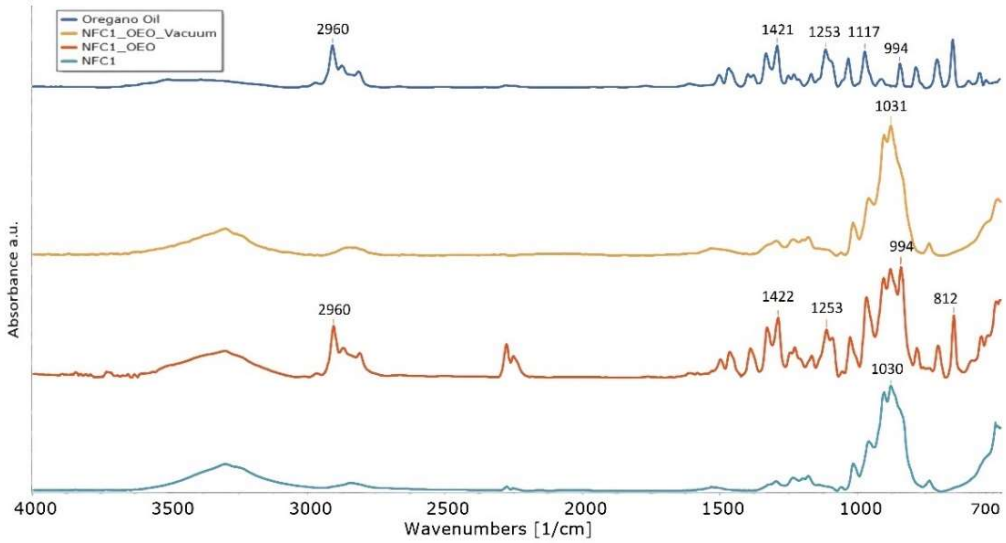

Figure A3. FT-IR analysis of NFC1 prior and after being in contact with the oregano essential oil, plus the Oregano essential oil spectra for comparison.

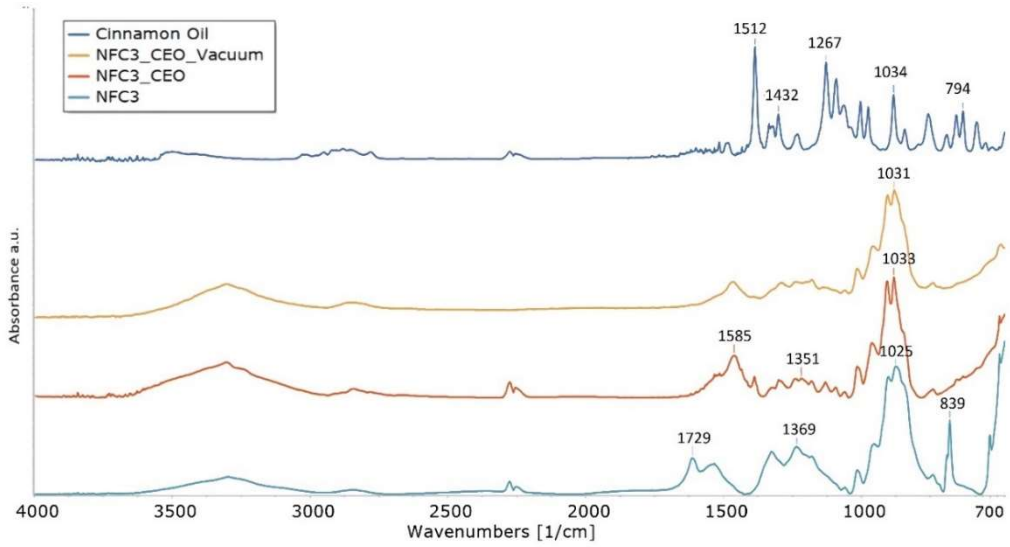

Figure A4. FT-IR analysis of NFC3 prior and after being in contact with the cinnamon essential oil, plus the cinnamon essential oil spectra for comparison.

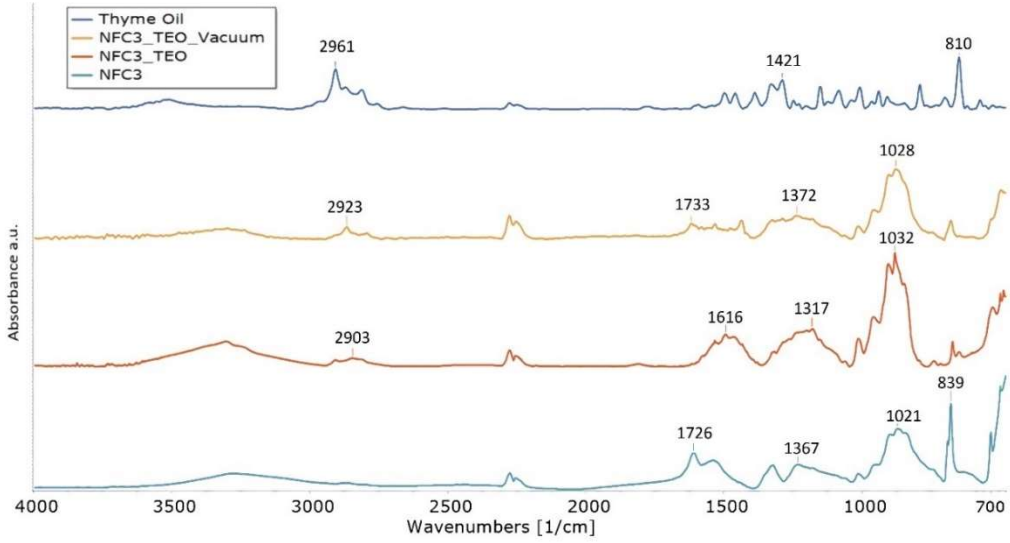

Figure A5. FT-IR analysis of NFC3 prior and after being in contact with the thyme essential oil, plus the Thyme essential oil spectra for comparison.

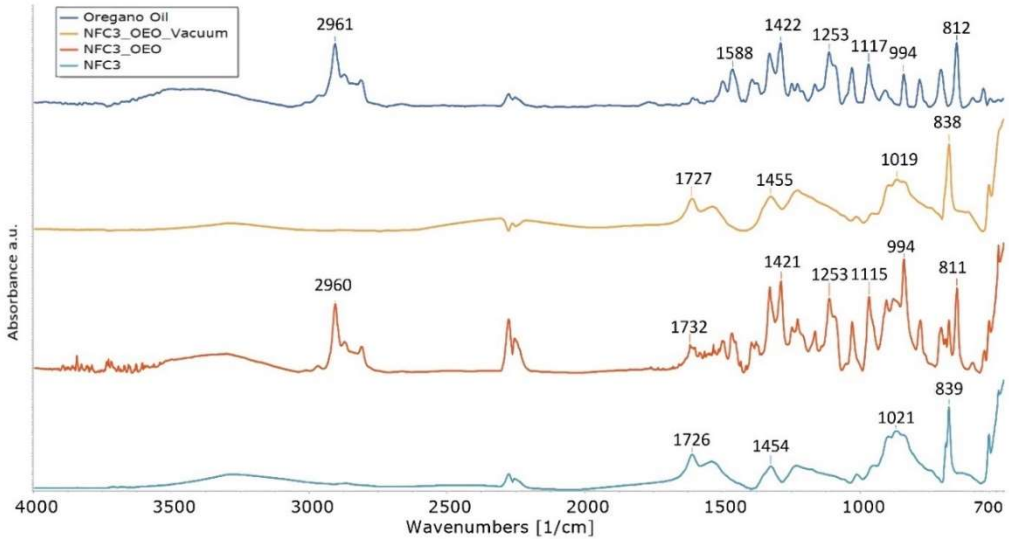

Figure A6. FT-IR analysis of NFC3 prior and after being in contact with the oregano essential oil, plus the Oregano essential oil spectra for comparison.

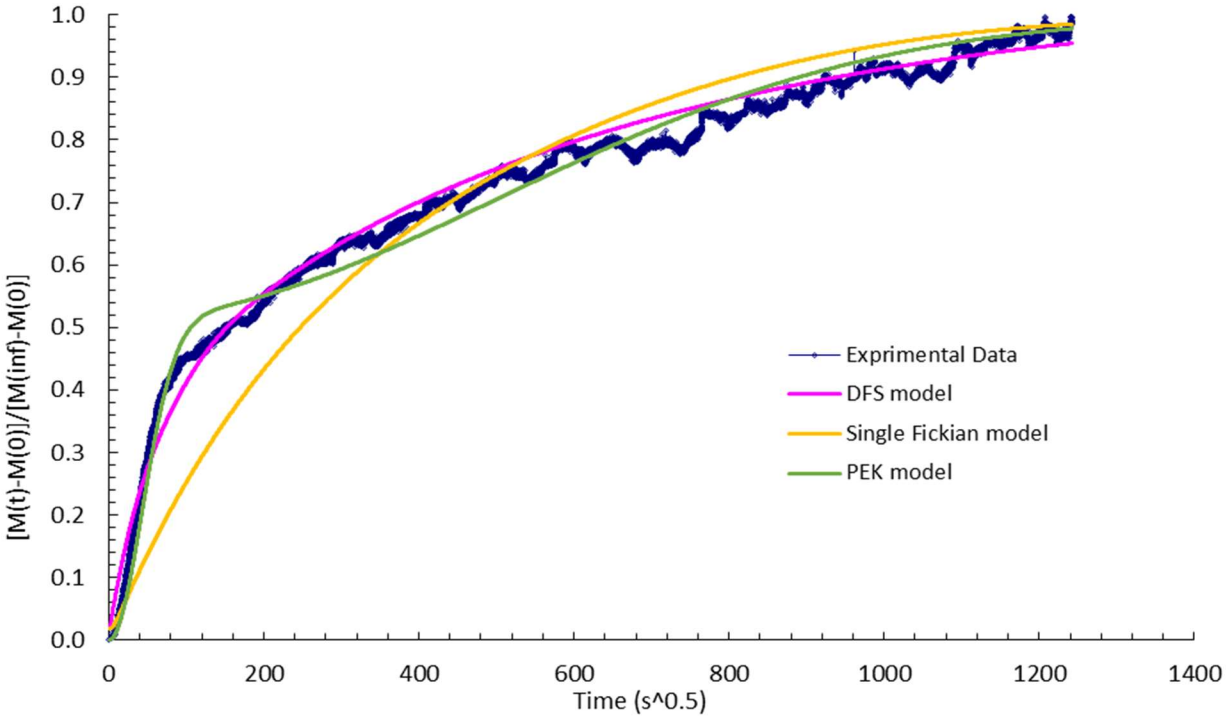

Figure A7. Cinnamon essential oil diffusion from NFC1 estimated during desorption in gas phase

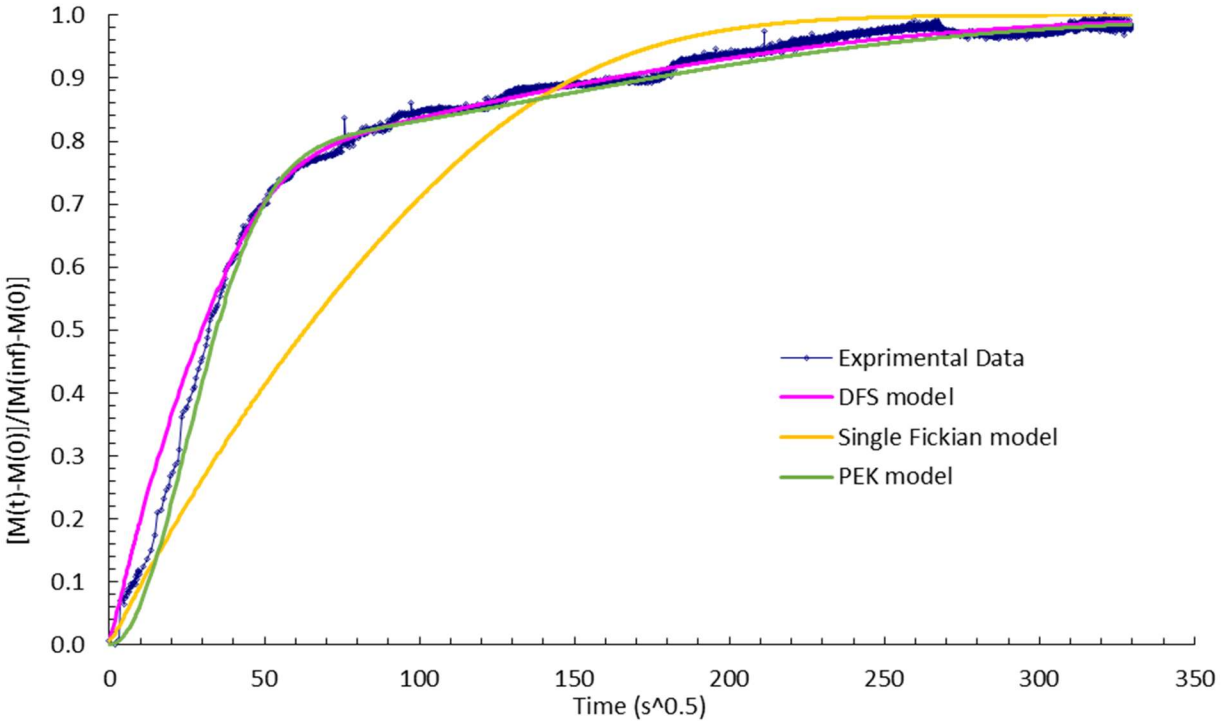

Figure A8. Oregano essential oil diffusion from NFC1 estimated during desorption in gas phase

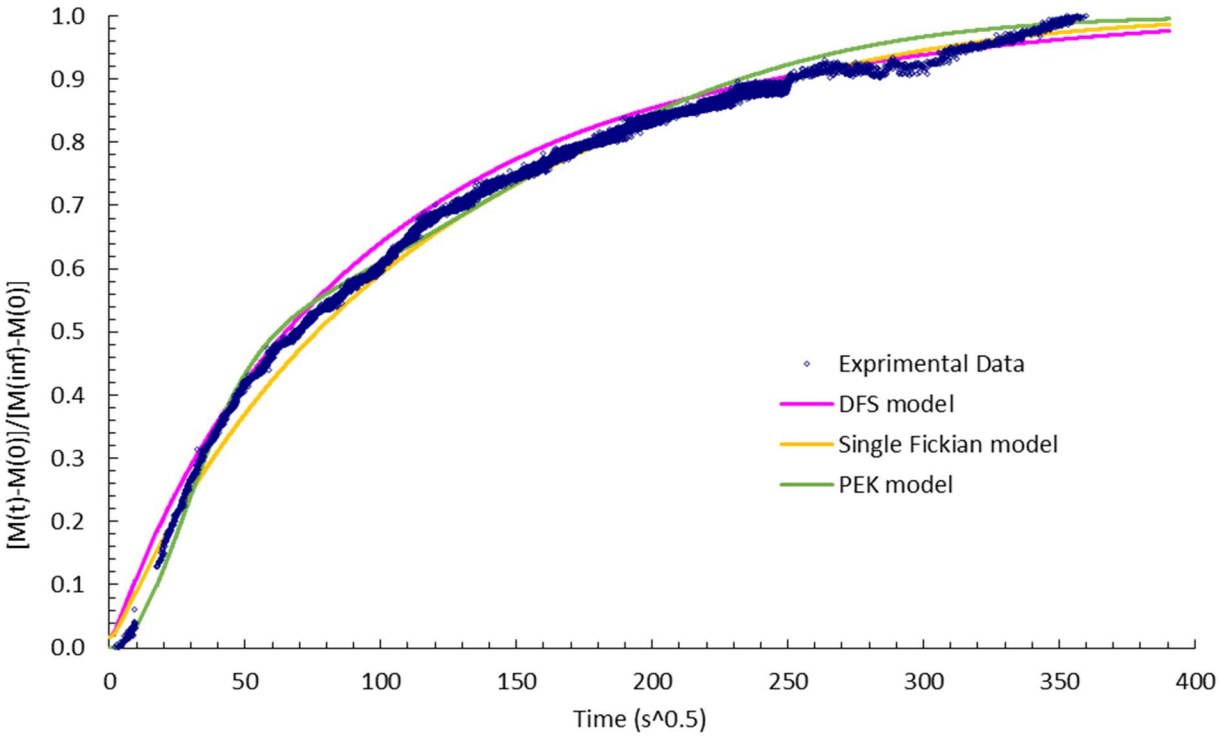

Figure A9. Thyme essential oil diffusion from NFC2 estimated during desorption in gas phase

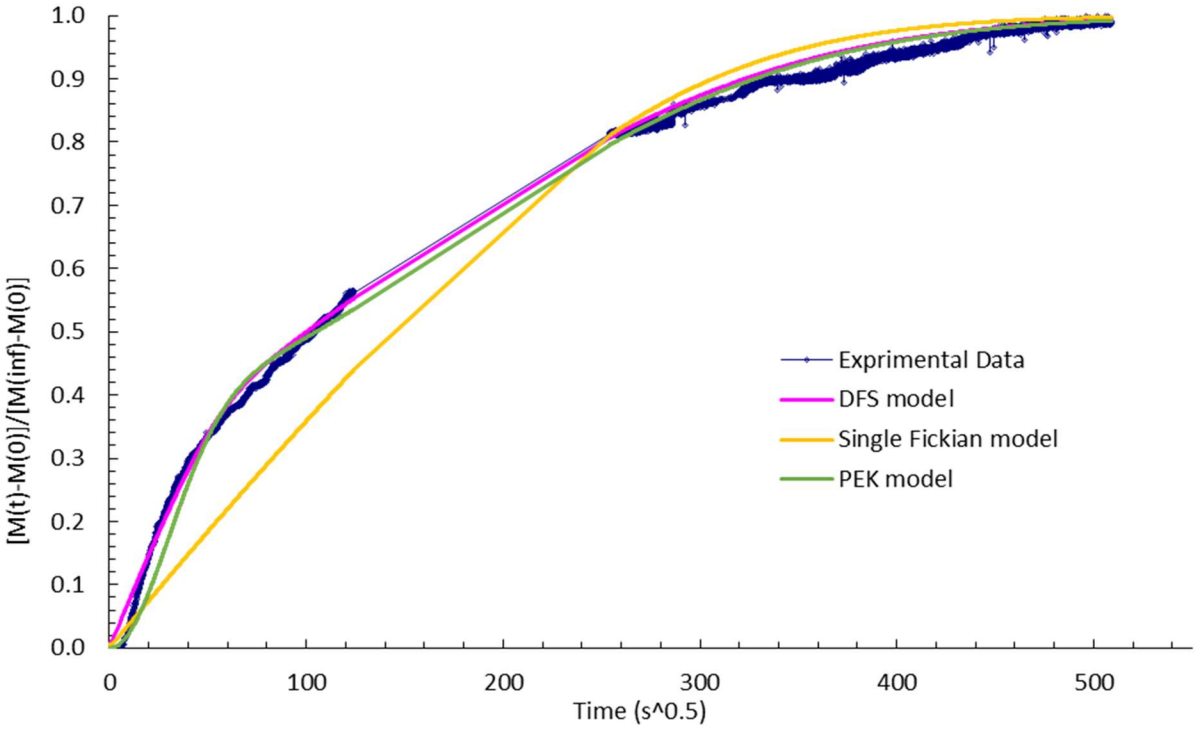

Figure A10. Cinnamon essential oil diffusion from NFC2 estimated during desorption in gas phase

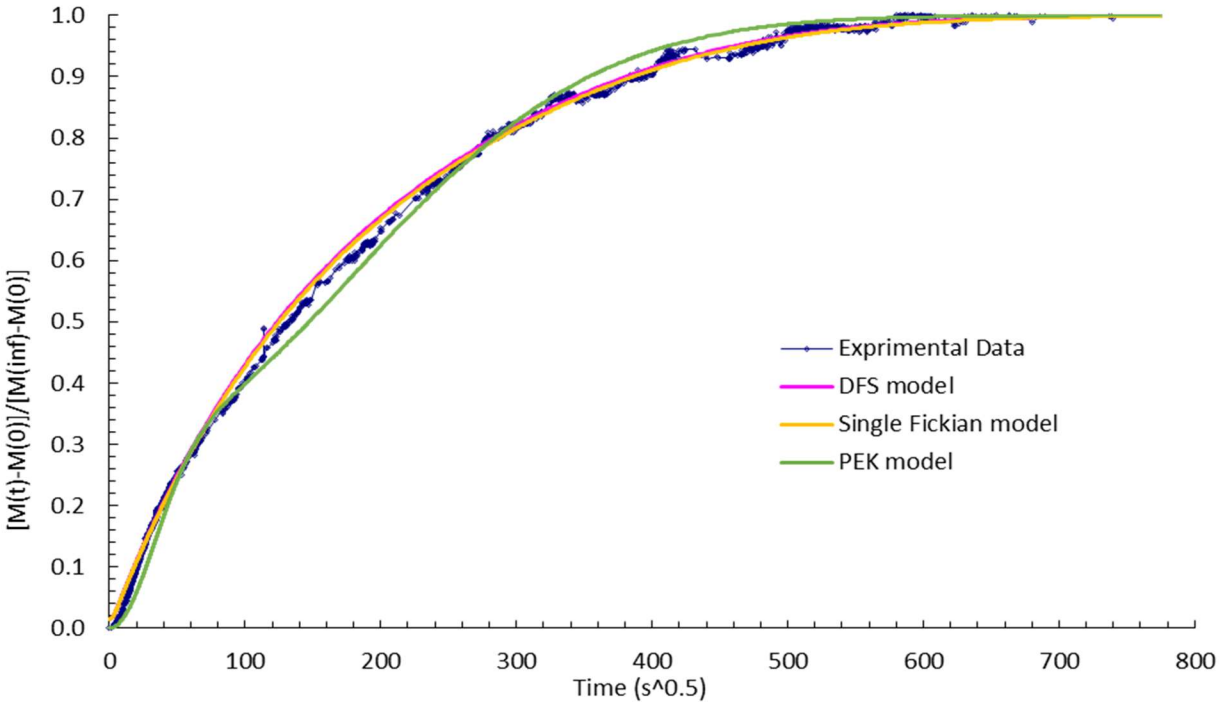

Figure A11. Thyme essential oil diffusion from NFC3 estimated during desorption in gas phase

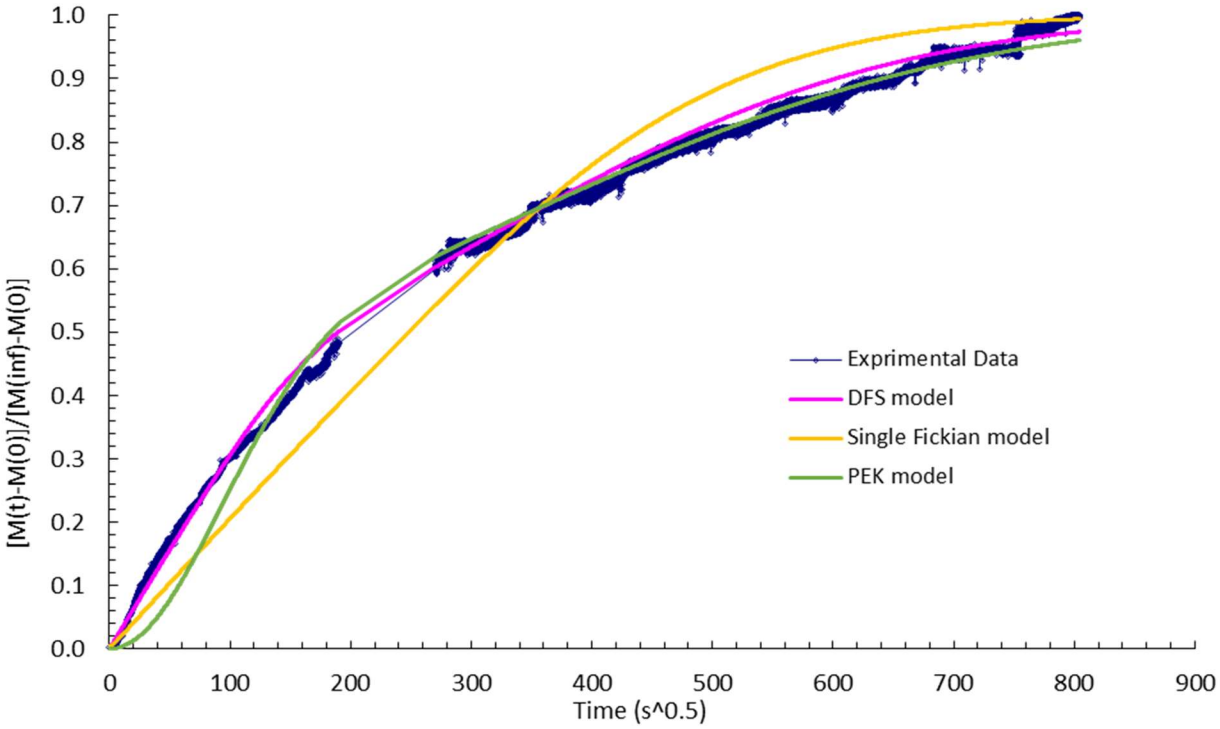

Figure A12. Cinnamon essential oil diffusion from NFC3 estimated during desorption in gas phase

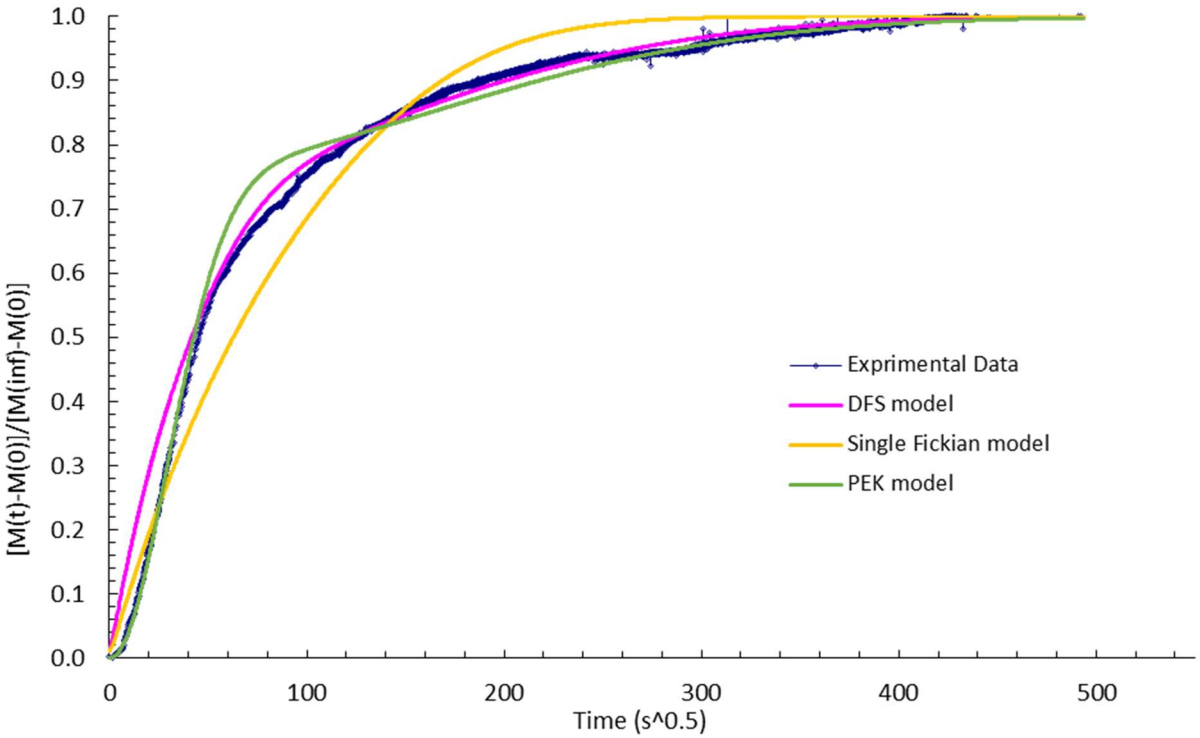

Figure A13. Oregano essential oil diffusion from NFC3 estimated during desorption in gas phase