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Antimicrobial packaging system for minimally processed fruit

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La memoria titulada "**Antimicrobial packaging system for minimally processed fruit**" que presenta Dña. **Marta Inés Lara Lledó** para optar al grado de Doctor por la Universidad Politécnica de Valencia, ha sido realizada en el **Instituto Tecnológico del Embalaje, Transporte y Logística** (ITENE) bajo su dirección y que reúne las condiciones para ser defendida por su autora.

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PREFACE

This report reflects the work undertaken at the Packaging, Transport and Logistics Research Center (ITENE) within the framework of the Project "Easy Fruit" (Active packaging for extending shelf life of peeled and cut fruit). This project (Grant Agreement No 315565) was funded by the European Union 7th Framework Program under the call Research for SMEs, for research and technological development, with the participation of 5 industrial companies, packaging manufacturers and users, and two research centers including ITENE. The main goal of the project was to develop a technology that could be used by the industry to extend the shelf life of peeled and cut fruit through the combination of minimal processing and active packaging solution.

The author's participation in this project, as part of the work undertaken by ITENE, has been focused on the development of antimicrobial active packaging for peeled and cut orange and pineapple. The research performed has given rise to the development of an antimicrobial packaging prototype consisting of an active tray and heat-sealable film containing antimicrobial agents to reduce the deterioration of the fresh cut fruit and to extend its commercial shelf life. The possibilities of the industrial application of this prototype is the reason for a patent application request with EP15382475.0 number. A copy of the requested patent is enclosed in the Annex I of the present Doctoral Thesis.

Once the prototype is fully up-scaled and authorized for use, the commercialization of peeled and cut fruit packed with this packaging is foreseen since there are already companies interested in its possible use.

SISTEMA DE ENVASADO ACTIVO ANTIMICROBIANO PARA FRUTA MINIMAMENTE PROCESADA

RESUMEN

En la presente Tesis Doctoral se han desarrollado materiales de envase activo antimicrobiano, a escala laboratorio y a escala semi-industrial, con el objetivo de reducir la proliferación de la flora natural de la fruta pelada y cortada y extender su vida útil. Se han desarrollado distintos prototipos para su posterior aplicación industrial.

Previo al desarrollo de los materiales de envase, se llevó a cabo una selección de agentes activos más idóneos. Para ello se estudiaron mediante ensayos *in vitro* las propiedades antimicrobianas de agentes activos volátiles, citral, hexanal y linalool y diferentes mezclas de los mismos, frente a distintos microorganismos típicos del deterioro de las frutas, mohos y levaduras, concluyendo que la efectividad de la mezcla de los tres es superior a la suma de la efectividad de los agentes activos de forma individual. Así mismo, también se seleccionaron agentes antimicrobianos no volátiles como el sorbato potásico y benzoato sódico, los cuáles son ampliamente empleados en la industria alimentaria debido principalmente a sus propiedades antifúngicas.

Con los agentes activos seleccionados, se desarrollaron películas monocapa de polipropileno (PP) con distintas concentraciones de la mezcla activa, citral, hexanal y linalool, a escala laboratorio, mediante técnicas de extrusión, y películas bicapa a escala semi-industrial mediante coextrusión. Por otra parte, se desarrollaron bandejas activas a escala semi-industrial mediante termoconformado de láminas obtenidas por coextrusión de compuestos de PP y etilvinilaceteto (EVA) con sorbato potásico o benzoato sódico como agentes antimicrobianos.

Se evaluaron las propiedades mecánicas, barreras y térmicas de los materiales activos desarrollados, así como su sellabilidad y transparencia. En general, las propiedades de los polímeros no se vieron afectadas de manera relevante. Sin embargo, las bandejas activas perdieron su carácter transparente debido a la incorporación de los agentes activos no volátiles.

Se estudió la cinética de liberación de los compuestos activos volátiles y no volátiles a distintas temperaturas, determinando los coeficientes de difusión de los agentes activos

mediante el ajuste a modelos matemáticos de difusión basados en la Segunda Ley de Fick. Entre los agentes volátiles, el hexanal mostró un mayor coeficiente de difusión seguido de citral y linalool. Por otra parte, no hubo apenas diferencia en los coeficientes de difusión del sorbato potásico y benzoato sódico, siendo éstos del mismo orden de magnitud.

Igualmente, se realizaron diferentes experimentos *in vitro* a distintas temperaturas para determinar las propiedades antimicrobianas de los materiales desarrollados. En general, los materiales activos presentaron una elevada capacidad antimicrobiana que se vio potenciada al aumentar la temperatura de exposición.

Una vez evaluadas las características de los materiales desarrollados, se llevaron a cabo ensayos de envasado de piña y naranja pelada y cortada con las películas y la bandeja activas y su combinación (sistema de envase activo). En general, el sistema de envase activo mejoró la conservación de la fruta, entre 4 y 9 días para la naranja y piña, respectivamente, presentando una gran capacidad antimicrobiana y manteniendo los parámetros de calidad de la fruta en niveles estables por un mayor tiempo.

Por último, se estudió la seguridad de estos materiales de acuerdo a la legislación de materiales en contacto con alimentos y la legislación alimentaria europea, concluyendo que los materiales activos desarrollados no presentaron preocupación para la seguridad de los consumidores.

SISTEMA D'ENVASAMENT ACTIU ANTIMICROBIA PER A FRUITA MINIMAMENT PROCESSADA

RESUM

En la present Tesi Doctoral s'han desenvolupat materials d'envasament actiu antimicrobià, a escala de laboratori i a escala semi-industrial amb l'objectiu de reduir la proliferació de la flora natural de la fruita pelada i tallada i estendre la seua vida útil. S'han desenvolupat diferents prototips per a la seua posterior aplicació industrial.

Previ al desenvolupament dels materials actius, s'han seleccionat els agents actius mes idonis estudiant mitjançant assajos *in vitro* les propietats antimicrobianes d'agents actius volàtils, citral, hexanal i linalool i diferents mescles dels mateixos, enfront de diferents microorganismes típics de la deterioració de les fruites -floridures i llevats- concloent que l'efectivitat de la mescla dels tres és superior a la suma de l'efectivitat dels agents actius de forma individual. Així mateix, s'han seleccionat antimicrobians no volàtils, sorbat potàssic i benzoat sòdic, els quals son àmpliament empleats a l'industria alimentaria per les seues propietats antifúngiques.

Amb els agents actius seleccionats, s'han desenvolupat pel·lícules monocapa de polipropilè (PP) amb diferents concentracions de la mescla activa, citral, hexanal i linalool, a escala laboratori, mitjançant tècniques d'extrusió, i pel·lícules bicapa a escala semi-industrial mitjançant coextrusió. D'altra banda, s'han desenvolupat safates actives a escala semi-industrial mitjançant termoconformació de làmines obtingudes per coextrusió de compostos de PP i etil vinil acetat (EVA) amb sorbat potàssic o benzoat sòdic com agents antimicrobians.

S'han avaluat les propietats mecàniques, barrera i tèrmiques dels materials actius desenvolupats, així com la seua sellabilidad i transparència. En general, les propietats dels polímers no es van veure afectades de manera rellevant. No obstant això, les safates actives van perdre el seu caràcter transparent a causa de la incorporació dels agents actius no volàtils.

S'ha estudiat la cinètica d'alliberament dels compostos actius volàtils i no volàtils a diferents temperatures, determinant els coeficients de difusió dels agents actius mitjançant l'ajust a models matemàtics de difusió basats en la Segona Llei de Fick. Entre els agents volàtils, l'

hexanal va mostrar un major coeficient de difusió seguit de citral i linalool. D'altra banda, no va haver-hi a penes diferències en els coeficients de difusió del sorbat potàssic i benzoat sòdic, sent aquests del mateix ordre de magnitud.

Igualment, s'han realitzat diferents experiments *in vitro* a diferents temperatures per determinar les propietats antimicrobianes dels materials desenvolupats. En general, els materials actius presenten una elevada capacitat antimicrobiana que es veu potenciada en augmentar la temperatura d'exposició.

Una vegada avaluades les característiques dels materials desenvolupats s'han efectuat assajos d'envasament de pinya i taronja pelada i tallada amb, les pel·lícules i la safata actives i la seva combinació (sistema d'envàs actiu). En general, el sistema d'envàs actiu va millorar la conservació de la fruita, entre 4 i 9 dies per a la taronja i pinya respectivament, presentant una gran capacitat antimicrobiana i mantenint els paràmetres de qualitat de la fruita en nivells estables per un major temps.

Finalment, s'ha estudiat la seguretat d'aquests materials d'acord a la legislació de materials en contacte amb aliments i la legislació alimentària europea, concloent que els materials actius desenvolupats no presentaren preocupació per a la seguretat dels consumidors.

ANTIMICROBIAL ACTIVE PACKAGING FOR MINIMALLY PROCESSED FRUIT

ABSTRACT

In the present Doctoral Thesis, antimicrobial active packaging materials, at lab and at semi-industrial scale, have been developed with the aim to reduce the natural flora of peeled and cut fruit and extend its shelf life. Packaging prototypes have been developed for their further application.

Prior to developing the active materials, the most suitable active agents were selected. To that end, the antimicrobial properties of the volatile active agents citral, hexanal and linalool and mixtures thereof were evaluated against typical microorganisms related to fruit spoilage, molds and yeast, concluding that the effectiveness of the mixture was higher than the sum of the effectiveness of the individual active agents. Likewise, non-volatile antimicrobial agents such as potassium sorbate and sodium benzoate were selected, which are widely used in the food industry due to their antifungal properties.

With the selected active agents, monolayer polypropylene (PP) films with different concentration of the active mixture citral, hexanal and linalool, at lab scale by means of extrusion, and bilayer films at semi-industrial scale by means of coextrusion were prepared. Besides, active packaging trays were developed at semi-industrial scale by thermoforming active sheets obtained by coextrusion of PP and ethyl vinyl acetate (EVA) compounds containing potassium sorbate and sodium benzoate as active agents.

Mechanical, barrier and thermal properties of the developed active packaging materials, as well as their sealability and transparency were evaluated. In general, the materials properties were not affected in a significant manner. However, active trays decreased in transparency due to the incorporation of non-volatile active agents.

The release kinetics of the volatile and non-volatile active agents were studied at different temperatures, defining their diffusion coefficients by the adjustment to mathematic models based on Second´s Law Fick. Among the volatile active agents, hexanal showed a higher diffusion coefficient, followed by citral and linalool. On the other hand, very small differences were observed between potassium sorbate and sodium benzoate diffusion coefficients, being of the same order of magnitude.

In vitro tests were also performed at different temperatures to evaluate the antimicrobial properties of the developed materials. In general, the active packaging materials showed high antimicrobial properties which were enhanced with the increment of temperature.

Once the properties of the developed materials were evaluated, *in vivo* tests with peeled and cut pineapple and orange were performed by packing these fruits with the active films, active tray and their combination (active packaging system). In general, the active packaging system improved the microbiological preservation of the fruit, between 4 and 9 days for orange and pineapple, respectively, and maintained quality parameters of the fruit at stable levels for longer times.

Lastly, the safety of the active packaging materials was evaluated according to the European food contact materials and food legislation, concluding that these materials were not of any safety concern for the consumers.

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ACRONYMS

ADI: Admissible daily intake

AM: Antimicrobial agent

Bw: Bodyweight

CFU: Colony forming unit

Cmax: Maximum concentration

CMC: Carboxymethyl cellulose

D: Diffusion coefficient

DSC: Differential scanning calorimetry

EDI: Estimated daily intake

EFSA: European Food Safety Agency

EO: Essential oil

EU: European Union

EVA: Ethyl vinyl acetate

EVOH: Ethylene vinyl alcohol

FIC: Fractional inhibition concentration

GC: Gas chromatography

GC-MS: Gas chromatography-Mass

GC-MS-QQQ: Gas chromatography-Mass-Triple Quadrupole

LAB: Lactic acid bacteria

LDPE: Low-density polyethylene

LOD: Limit of detection

LOQ: Limit of quantification

MAP: Modified atmosphere packaging

MEA: Malt extract agar

MIC: Minimum inhibitory concentration

OML: Overall migration limit

OTR: Oxygen transmission rate

ACRONYMS

$P'O_2$: Permeability coefficient

PCL: Polycaprolactone

PET: Polyethylene terephthalate

PHA: Polyhydroxyalkanoates

PLA: Polylactic acid

PP: Polypropylene

PS: Potassium sorbate

PVOH: Polyvinyl alcohol

R^2: Regression coefficient

RC: Reduction concentration

RH: Relative humidity

RMSE: Root mean square error

RSD: Relative standard deviation

RTE: Ready to eat

S: Solubility

SB: Sodium benzoate

SEM: Scanning electron microscopy

SML: Specific migration limit

Tc: Crystallization temperature

Tg: Glass transition temperature

TGA: Thermogravimetic analysis

Ti: Internal transmittance

Tm: Melting temperature

Tmax: Maximum degradation temperature

Tmax_a: Maximum volatilization temperature of active agents

Tmax_p: Maximum degradation temperature of a polymer

Tonset: Onset temperature

ACRONYMS

TSS: Total soluble solids

WHO: World Health Organization

WVTR: Water vapor transmission rate

ΔHc: Crystallization enthalpy

ΔHm: Melting enthalpy

1. INTRODUCTION

1.1. FRUIT AND VEGETABLE CONSUMPTION TRENDS. MINIMALLY PROCESSED FRUIT

Fruits are an important source of many micronutrients and fiber, as well as low molecular carbohydrates (sugars), water and various phytochemicals, vitamins, and polyphenols with positive effects on health. The consumption of fruit and vegetables is important to combat obesity and prevent cardiovascular diseases, cancer and diabetes among other diseases (WHO, 2015).

According to the World Health Organization (WHO), low intake of fruits and vegetables is among the top ten selected risk factors for global mortality. Thus, governments and health organizations have actively been running campaigns to encourage fruit and vegetable consumption. In this regard, the WHO aims at actively promoting an increase in fruit and vegetable intake worldwide, and recommends at least 400 grams per day (WHO, 2003, 2008).

Despite the efforts, the daily consumption of fruits and vegetables is decreasing in Europe. The latest Consumption Monitor published by the European Fresh Produce Association (Freshfel, 2015) showed that the consumption of fresh fruit and vegetables in the European Union (EU) stand at 341.81 g/person/day in 2013. This data represents an increase of 5.6% compared with 2012 but a decrease of 1.9% compared with the average between 2008-2012.

Then, consumption in the EU remains under the recommendation of the WHO to consume a minimum of 400 g of fresh produce per day. Specifically, in 2013, fruit consumption per capita in the EU stood at 188.6 g/person/day. This is 10.1% more than in 2012 but 1.5% less than the average of the years 2008-2012.

In order to assess food intake in Europe, the European Food Safety Authority (EFSA) also compiled national food consumption data based on dietary surveys (EFSA, 2008). These data revealed that the mean of fruit and vegetable intake in Europe is 166 and 220 g per day respectively, implying that the average consumption of fruit and vegetables is 386 g per day.

Statistics indicate that the recommended daily fruit consumption is not met. As for dietary habits in general, a wide range of factors influence fruit consumption. Factors in our physical, social and cultural environment, as well as personal factors such as taste preferences, level of independence and health consciousness have an effect on our consumer habits (EUFIC,

2012). Availability is also a key factor influencing food consumption patterns. Despite statistics on low fruit consumption, there is an increasing consumer demand for fresh, healthy, convenient ready to eat (RTE) products (Dixon and Aldous, 2014).

In order to address consumers' needs and requirements for fruit consumption, fresh cut sector plays an important role, and may help meeting the objective of consuming the recommended daily intake of fruit. Fresh cut or minimally processed fruits are a very convenient way to supply consumers with nutritious, healthy, and appealing food products. The term minimally processed or fresh cut fruits refers to any type of fruit that has been physically altered from its original state (trimmed, peeled, washed, and/or cut), but remains in a fresh, unprocessed state (Olivas and Barbosa-Cánovas, 2005). These products reach the consumer in a RTE form allowing direct consumption without previous preparation or transformation. Then, minimally processed fresh fruit allows consumers eating healthy on the go and saving time on food preparation, becoming an excellent strategy to improve the nutritional quality of consumer's diet.

The United Kingdom (UK) is the largest fresh cut fruits and vegetables market in the European Union (EU), accounting for around a third of total EU consumption. In Germany and Spain, this sector is still in an early stage of development. However, despite the financial crisis, the fresh cut fruits and vegetables market has grown continually in recent years (Oliveira *et al.*, 2015). Despite the continuous growth of fresh cut fruit consumption, its market share is low compare to whole fruit consumption. In 2010, the fresh cut fruit market share was about 1% of total volume of fruit sold in Europe (Rabobank, 2010).

The short shelf life and quality loss of these products is nowadays a serious problem since this rapid expiration makes their marketing and export very difficult. According to the industry, the marketing of fresh cut fruit is limited to 5-7 days compared to 15-20 days of most of the whole products. Therefore, there is a need to increase commercial shelf life of peeled and cut fruit since it limits their commercialization at the national level and their exportation to other European countries.

Within the existing range of fresh cut products, main attention is been paid to orange and pineapple fruit due to the industrial partners interest, involved in the project, to extend the shelf life of these products. Consumer demand for tropical fresh cut products is increasing rapidly in the world market, and fresh cut pineapple is already found in many supermarkets and food service chains (Marrero and Kader, 2006). Fresh cut pineapple fruit is appreciated

for its taste, flavor and juiciness. Moreover, consumers perceive oranges a healthy and natural source of vitamins and other health promoting nutrients, resulting in increasing demand and production (Aschoff *et al.*, 2015). However, peeling of pineapple and orange is the main factor limiting the consumption of these products due to the inconvenience of this operation for consumers. Since convenience is a major factor in consumer's fruit purchase decisions, consumption of pineapples and oranges would increase in a peeled and sliced format.

In the present Doctoral thesis, minimally processed orange and pineapple have been selected for overcoming their quick spoilage and maintain their quality attributes longer.

1.1.1 Deterioration mechanisms of minimally processed fruits

Minimal processing, which includes peeling, shredding, slicing, and dicing cause cell injury in the fruit (Ayala-Zavala *et al.*, 2009). Subcellular compartmentalization is disrupted at the cut surfaces, favoring the contact between substrates and enzymes that are normally separated in different compartments and initiating deterioration reactions that do not occur in the whole fruit (Toivonen and Brummell, 2008). Microbiological, enzymatic, and physicochemical reactions simultaneously take place, causing negative quality changes in the fruit (Artés-Hernández *et al.*, 2007). Then, fresh cut product tissues deteriorate faster than intact fruit and vegetables.

Among the quality attributes that mainly limit the shelf life of peeled and cut fruits are microbial growth and sensory decay (Ayala-Zavala *et al.*, 2009). During minimal processing, the natural protection of fruit is generally removed and they become highly susceptible to microbial attack. In addition, cross-contamination may occur during cutting and shredding operations if sanitation operations are not correctly performed (Hui *et al.*, 2008). The high content of organic acids and sugars present in the fruit tissue can be available after peeling and cutting, becoming a good source of nutrients for bacteria, yeasts and molds growth (Ayala-Zavala *et al.*, 2008). Damaged tissue also becomes more susceptible to the attack of pathogenic microorganisms and contamination by human pathogens (Lamikanra, 2002).

Specifically, shelf life of fresh cut pineapple is limited by changes in color and browning, texture, appearance and off-flavors, juice leakage, increased respiration rates and microbial growth. These parameters are mainly affected by cultivar and maturity stage as well as packaging conditions and storage temperature (Marrero and Kader, 2006; Montero-

Calderón *et al.*, 2008; Soliva-Fortuny and Martin-Belloso, 2003). In the case of oranges, the elimination of the natural protection increases the risk of physiological alterations such as excessive desiccation, accelerated senescence and metabolic changes such as increased respiration rate, which contribute to off-flavors production. In addition, the fruit becomes more susceptible to microbial attack as a consequence of the possible leakage of vesicular juice, and the absence of the protective peel (Pretel *et al.*, 1998).

The natural microbial flora of peeled and cut fruit such as total aerobic bacteria, psychrotrophic bacteria and molds and yeasts are usually found to be below 5 log colony forming units per gram (CFU/g) before its best-before date (Abadias *et al.*, 2008). However, microbial populations can increase rapidly under storage conditions. Studies have revealed that during commercial shelf life, microbial populations change dramatically on fresh cut product (Garg *et al.*, 1990).

Storage at refrigeration temperatures generally favors the growth of psychrotrophic microorganisms (Nguyen-the and Carlin, 1994). Nevertheless, mesophilic microorganisms may continue to grow at low temperature at reduced growth rates (Vescovo et al., 1996). Some fungi (molds and yeasts) found in minimally processed fruits can also grow at refrigeration temperatures, reaching high counts during marketing (Tournas *et al.*, 2006). Abuses of storage temperatures during transport and marketing will also increase the counts of contaminating bacteria, yeasts and molds since these organisms grow at much higher rates at above refrigeration temperatures.

Differences in the microbial evolution could be as a consequence of the washing and decontamination step as well as the handling, cutting, shredding, and slicing, which are potential sources of contamination and could increase the microbial load (Abadias *et al.*, 2008). Moreover, unclean packaging trays and other materials that come in contact with the fruit salads represent an additional source of contamination that could accelerate the spoilage of these products (Tournas *et al.*, 2006). Microbial counts of minimally processed fruits responsible for rejection of these products, due to changes in their sensory quality factors, are in most cases 7-8 log CFU/g. However, exceeding this microbiological limit does not always result in occurrence of visual defects as both microbiological and physiological activity play a role in spoilage of these products (Ragaert *et al.*, 2007).

There is no specific legislation establishing microbiological limits for spoilage microflora in minimally processed fruits and vegetables. The limits of spoilage microorganisms in these

products, which define the quality limits for consumption, are different regarding the information source. For example, in France and Germany microbiological specifications for mesophilic aerobic bacterial populations (total aerobic counts) for these products are 7.5 log CFU/g (Francis *et al.*, 1999). Debevere (1996) proposed 8 log CFU/g as the limiting criteria for RTE fruits and vegetables of aerobic psychrotrophic bacteria, 5 log CFU/g for yeast and 7 log CFU/g for lactic acid bacteria. According to Spanish Royal Decree 3484/2000 laying down hygiene rules for the production, distribution and sale of prepared foods, the quality limit for total aerobic counts on minimally processed vegetable produce for safe consumption are 6 log CFU/g.

1.1.2 Preservation and packaging technologies for minimally processed fruits

In the last two decades, food scientists have attempted to develop new technologies that improve the quality of fresh cut products. Several treatments have been studied to maintain quality and extend shelf life of fresh cut fruit (Patrignani *et al.*, 2015; Rojas-Graü *et al.*, 2009a; Soliva-Fortuny and Martin-Belloso, 2003).

Several chemical compounds have been used at the washing step to reduce bacterial populations on fruit. In particular, the chlorine based chemicals are still the most widely used treatments (Gil *et al.*, 2009; Rico *et al.*, 2007) but at the concentration normally employed (50-200 mg/L), only a reduction between 1 to 2 log CFU/g is achieved (Oliveira *et al.*, 2012).

Modified atmosphere packaging (MAP) and refrigeration are the main tools used to slow down undesirable quality changes and increase the shelf life of fresh cut fruits (Montero-Calderón *et al.*, 2008). In general, low levels of oxygen (O_2) and high levels of carbon dioxide (CO_2) have been employed to reduce the respiration rate of minimally processed fruits with the aim to prolong their shelf life (Corbo *et al.*, 2010). Depleted O_2 and/or enriched CO_2 levels reduce respiration and decrease ethylene production, inhibit or delay enzymatic reactions, alleviate physiological disorders, and preserve the product from quality losses. However, O_2 atmospheres that are too low may trigger anaerobic metabolism in fresh cut fruit resulting in increased fermentation (Solomos, 1997). Besides, it has been postulated that CO_2 dissolution enhances acidity in the cell medium and may be responsible for physiological disorders (Kader *et al.*, 1989). The use of elevated O_2 atmospheres has been also proposed as an alternative to low O_2 atmospheres to inhibit the growth of naturally occurring spoilage

microorganisms, prevent undesired anoxic respiratory processes and maintain the fresh like quality of fresh cut produce (Amanatidou *et al.*, 2000; Van der Steen *et al.*, 2002).

The levels of O_2 and CO_2 required to avoid tissue damage or quality loss are unknown for most fruits and depend on the metabolic characteristic of the specific product (Kader *et al.*, 1989). In addition, the effect of MAP on microorganisms can vary, depending mainly on the storage conditions and the type of packaged product (Oliveira *et al.*, 2015). Marrero and Kader (2006) reported that a reduction of O_2 concentration to 8% or lower and a concentration of 10% CO_2 improved the final appearance of 'Smooth Cayenne' pineapple. Also, elevated CO_2 atmospheres may have delayed microbial growth after 14 days of storage. González-Aguilar *et al.* (2004) reported the beneficial effects of 2-5% CO_2 and 12-15% O_2 for the same cultivar after 14 days. Pretel *et al.* (1998) reported the slower growth rate of bacteria in RTE oranges with 25% CO_2 concentration.

The use of edible coatings is another alternative under study to extend the shelf life of fresh cut products (Alvarez *et al.*, 2013; Tharanathan, 2003). Some polysaccharides such as alginate, pectins and gellan gum have been used as edible coatings to improve the quality of different fresh cut fruit. Edible coatings protect fresh cut fruit from dehydration and water loss (Montero-Calderón *et al.*, 2008; Rojas-Graü *et al.*, 2009a). Edible coatings may also serve as carriers of food additives such as antibrowning and antimicrobial agents (AM), colorants, flavors, nutrients, spices and nutraceuticals (Oms-Oliu *et al.*, 2008; Robles-Sánchez *et al.*, 2013). There are many variables that can influence the effectiveness of edible coatings so that they should be designed for each specific application, which may limit their use and commercialization. In addition, the incorporation of certain antibrowning or antimicrobial agents into edible coatings can yield a organoleptic alteration of the food (Rojas-Graü *et al.*, 2009b).

Freezing fruits has been investigated over several decades to preserve them. However, conventional methods of freezing tend to destroy the turgidity of living cells in fruit tissue since they do not have a fibrous structure that can resist this destructive effect. Additionally, fruits to be frozen should be harvested in a fully ripe stage and are soft in texture (Abd-Elhady, 2014). Fruits have delicate flavors that are easily damaged or changed by freezing. Chemical treatments or additives are often needed to inactivate the deteriorative enzymes in fruits.

The use of irradiation with UV light and pulsed light have also been investigated as physical treatments to sanitize cut fruit surfaces. In recent years, continuous wave ultraviolet light (UV, 200-400 nm) has attracted an increasing interest as a non-thermal method for surface decontamination at post-harvest for preservation of fruits and vegetables (Pataro *et al.*, 2015; Ribeiro *et al.*, 2012; Schenk *et al.*, 2008). Although UV treatments could improve the microbiological quality and safety of foods and increase their shelf life, irradiation can cause changes to the sensory quality of the product, being texture the most affected attribute (FAO/IAEA, 2005). Pulsed light is a non-thermal technology based on the application of intense pulses of short duration to effectively inactivate microorganisms contained either in light-transmitting media or on opaque surfaces (Gómez-López *et al.*, 2007; Pataro *et al.*, 2015). The treatment has been demonstrated to be cost effective and feasible for the microbial inactivation of fresh cut products (Ramos-Villarroel *et al.*, 2012a, 2012b).

Nevertheless, none of these techniques provides a definitive solution for fresh cut fruits, providing only slight increases in their shelf life.

Active packaging is a technology of growing interest to improve the quality and shelf life of food products, and an alternative for packaging fresh cut fruit. Therefore, in this Thesis, the development of an antimicrobial active packaging technology for minimally processed orange and pineapple conservation is proposed.

1.2. ACTIVE PACKAGING

The primary functions of a food package are to contain the product, to protect it against physical and environmental damage and to provide information (Cooksey, 2009). However, in recent decades the concept of active packaging has advanced in science to satisfy the demand for safe, high quality food products.

Active packaging is defined as a system in which the food, the package and the environment positively interact to maintain the safety and quality of the products and to prolong its shelf life (Suppakul *et al.*, 2003a).

According to Regulation (EC) No. 450/2009, active materials and articles are those intended to extend the shelf life or to maintain or improve the condition of packaged food. They are designed to deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food.

Active packaging can be developed using any packaging material, but plastics are the basic materials for the development of this technology since they are widely used for conventional packaging.

Active packaging technology positively employs the mass transport phenomena that take place in polymeric materials such sorption and migration (Catalá and Gavara, 2002). The active packaging could absorb O_2, CO_2, water vapor or food related chemicals from the food or the environment within the packaging surrounding the food (sorption phenomenon); or it could release substances into the food or the environment surrounding the food such as preservatives, antioxidants, and flavorings (migration phenomenon).

According to their function, active packaging can be classified into 3 groups (EC, 2009):

A. Absorbing/scavenging systems:

Absorbing and scavenging systems remove undesired compounds present in the food or in the package headspace such as O_2, CO_2, ethylene, excessive water, and other specific compounds. The absorbing systems are mainly moisture absorber pads used to absorb the drip from packed meat and fish (Brody *et al.*, 2001; Han, 2014). The scavenger systems such as O_2 scavengers are those that scavenge or capture residual O_2 from inside the packaging (from the environment surrounding the foodstuff or from the foodstuff itself). Exposure to O_2 may result in microbiological growth on the food or chemical changes. Ethylene scavengers may be used in sachets or incorporated into a polymer film. Ethylene, a natural plant growth hormone, is the key to the ripening process of fruits and vegetables, being released during respiration and then driving the ripening process itself. The active component is meant to prevent an excess of the gas in order to extend shelf life of the packaged product (Ahvenainen, 2003; Ozdemir and Floros, 2004).

B. Releasing systems:

Releasing systems are usually packaging materials or independent devices that contain releasing substances such as preservatives, antioxidants, flavorings and enzymes. (Burt, 2004; Corrales *et al.*, 2014). These released active substances are intentionally added into or onto the packaged food to fulfill a purpose in the food or the environment surrounding the food and to maintain or extent the shelf life of the packed food.

C. Systems with substances grafted or immobilized on the surface of the packaging:

These are usually packaging materials containing additives or enzymes which are grafted on the surface in contact with food and have a technological effect on the food (Appendini and Hotchkiss, 1997; Muriel-Galet *et al.*, 2013b; Sailaja and Chanda, 2001). These materials deliberately influences the condition of the food without intentional migration. This category of packaging is thus similar to the releasing system with the difference that the active substance is not released into the food but it stays grafted or "immobilized" on the surface of the packaging where it performs its function. Then, any migration into food is non-intentional.

The addition of an independent device, such as a sachet, pad or label containing the active agent is not well accepted by consumers. This has led to the use of packaging materials with antimicrobial or antioxidant agents incorporated in the packaging material itself (Brody *et al.*, 2001).

Any active agent deliberately added to a packaging system to have a technological effect on the food through its release into the food product or by immobilization on the surface packaging wall should be an authorized food preservative according to the Regulation (EC) No. 1333/2008 on food additives.

Of different active packaging technologies developed in the recent decades, active antimicrobial packaging, which aims to control microbial growth in food by incorporating antimicrobial agents in the packaging material, is one that arouses great interest and it is experiencing extensive research and technological applications. Due to the increasing interest and possibilities of antimicrobial active packaging technology, this Doctoral Thesis intends to study its application in fresh cut fruit to improve its shelf life, specifically in pineapple and orange fruit.

1.3. ANTIMICROBIAL ACTIVE PACKAGING

Antimicrobial active packaging is defined as a packaging technology able to kill or inhibit spoilage and pathogenic microorganisms present in food (Han, 2003). The antimicrobial packaging limits or prevents microbial growth by extending the lag period, and/or reducing the growth rate or decreasing microbial counts (Han, 2000). In comparison with direct addition of the active agents into food, antimicrobial packaging can offer slow, continuous

migration of the agent from the packaging material to the food or the headspace of the package so that an adequate concentration of the antimicrobial agent is maintained over the shelf life period of the product (Quintavalla and Vicini, 2002).

As already reported, an antimicrobial packaging system could be in the form of an independent device or the antimicrobials could be incorporated in the wall of the package. In the second case, the antimicrobial agents can be incorporated in a packaging material by: (i) solvent casting; (ii) extrusion or injection; (iii) coating or adsorbing antimicrobials onto polymer surfaces; (iv): immobilization of antimicrobials to polymers by ionic or covalent linkages.

The active materials with antimicrobial properties can be divided into 3 groups according their mechanism of action: (i) materials that allow the antimicrobial agents to migrate into the food acting by direct contact with the food surface; (ii) materials that allow the antimicrobial agents to be released to the headspace of the packaging acting on the food through the vapor phase; (iii) materials that do not release active substances (immobilized antimicrobial agents) and act by direct contact with the food (Corrales *et al.*, 2014).

The steps involved in active agent release from the polymeric matrix comprise diffusion to the surface of the matrix and partition in the interface between the matrix and the food or the headspace. For an active material to be effective, the release should be higher than the minimum inhibitory concentration (MIC) (Han, 2005; Utto, 2008), defined as the lowest concentration required to produce visible inhibition in the growth of microorganisms (Hammer *et al.*, 1999; Lambert *et al.*, 2001).

The diffusion coefficient (D) of the active agent in the polymeric matrix and solubility (S) of the antimicrobials on food are important factors to be considered. The diffusion may be influenced not only by the temperature of exposure but also by interactions between the active agent and the packaging material (Sajilata *et al.*, 2007). Solubility also plays an important role in the effectiveness of the active material. In this regard, if the solubility of the antimicrobial substance in target foods is very high, the release may occur rapidly, decreasing the antimicrobial concentration on the food surface. Conversely, if the solubility is low, the antimicrobial may accumulate on the food surface and migrate slowly through the food matrix (Bastarrachea *et al.*, 2011). Both, the diffusion coefficient of the active agent in the polymeric matrix and solubility of the antimicrobials in the food will affect the antimicrobial effectiveness of the packaging material.

1.3.1. Antimicrobial agents

In general, a large number of synthetic and naturally occurring agents with antimicrobial properties, both of volatile and non-volatile nature, have been tested with the purpose of inhibiting the growth of microorganisms that can lead to deterioration of foodstuffs (Appendini and Hotchkiss, 2002; Corrales *et al.*, 2014; Pereira de Abreu *et al.*, 2012).

Volatile compounds

Some of the common volatile compounds with antimicrobial properties include inorganic gases (i.e SO_2), spices and herb extracts (i.e basil extract or horseradish extract) essential oils (EOs) (i.e oregano EO, thyme EO or cinnamon EO) and their pure compounds (i.e carvacrol, eugenol, thymol or cinnamaldehyde) (Burt, 2004). The advantage of volatile antimicrobials is that they can be released through the headspace, penetrating the bulk matrix of the food (Figure 1.1).

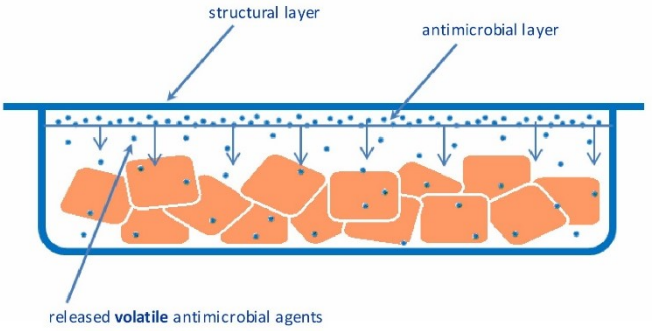


Figure 1.1. Antimicrobial packaging with volatile agents released to the headspace of foodstuff

Antimicrobial vapors or gases are appropriate for applications where there is not suitable contact between the food and the packaging, as it happens for minimally process fruit (Appendini and Hotchkiss, 2002). As reported, the diffusion coefficient of the antimicrobial agents through the packaging matrix and its solubility in the food system will define the concentration of the compound released into the food so that the release should not be slower than microbial growth. Then, a controlled release of the volatile agents is needed to reach the effective concentration during the shelf life of the product. In any case, the active

film should act as a reservoir for the antimicrobial agents that will be gradually released into the headspace of the package.

Non-volatile compounds

Organic acids and their salts, parabens, metals, fungicides, bacteriocins, enzymes, chelating agents or plant extracts have been used as non-volatile antimicrobial agents for packaging applications (Appendini and Hotchkiss, 2002; Corrales *et al.*, 2014; Sung *et al.*, 2013).

Antimicrobial packaging materials must contact the surface of the food if they are non-volatile, so the antimicrobial agents can diffuse (migrate) to the food surface (Figure 1.2). Therefore, surface characteristics and diffusion kinetics become crucial (Appendini and Hotchkiss, 2002). As for volatile compounds, the concentration released to the foodstuff needs to be higher than the MIC during the shelf life of the packed product. In this case, the active agents are available on the packaging surface in contact with the food or have to diffuse through the packaging wall to reach the interface between the packaging and the food.

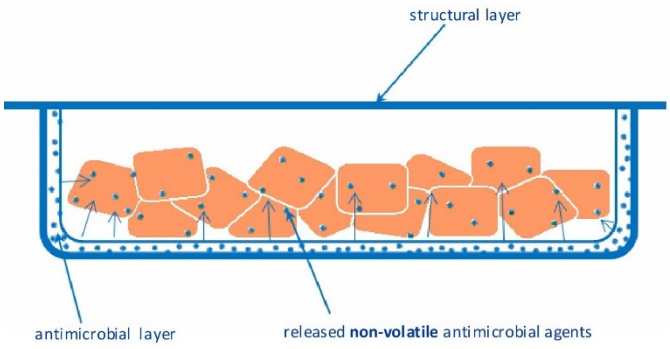


Figure 1.2. Antimicrobial packaging with non-volatile agents released to the foodstuff by direct contact

For good performance of the active packaging, products with high water activity are required in order to support the diffusion of the active compounds through the food matrix.

When non-volatile antimicrobials agents are immobilized on the polymer surface, there is no migration to the food, so that their activity is limited to the contact surface of the food (Figure 1.3). Peptide antimicrobials are among the agents commonly immobilized on film

surfaces, especially bacteriocins such as nisin and pediocin (Mauriello *et al.*, 2004; Scannell *et al.*, 2000) and enzymes such as lysozyme (Appendini and Hotchkiss, 1997; Buonocore *et al.*, 2003; Conte *et al.*, 2007).

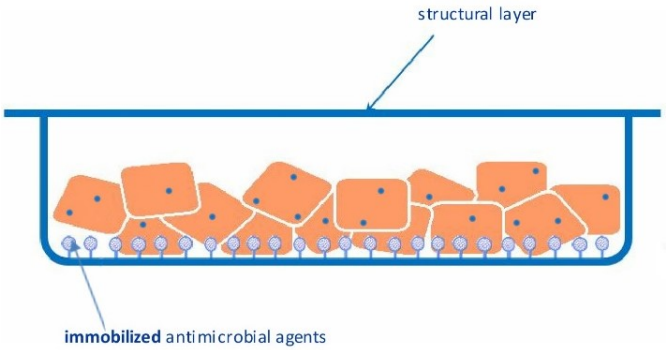


Figure 1.3. Antimicrobial packaging with non-volatile agents immobilized on the packaging surface

1.3.2. Polymers used in antimicrobial active packaging

Various polymers have been studied as possible candidates for the incorporation of antimicrobial agents. Synthetic polymers derived from petroleum have been widely used to develop antimicrobial packaging, including low-density polyethylene (LDPE) (Cran *et al.*, 2010; Suppakul *et al.*, 2008), polyethylene terephthalate (PET) (Junqueira-Gonçalves *et al.*, 2013; Manso *et al.*, 2013), polypropylene (PP) (Lara-Lledó *et al.*, 2013; Mauriello *et al.*, 2004; Ramos *et al.*, 2012) and ethylene vinyl alcohol copolymer (EVOH) (Cerisuelo *et al.*, 2012; Muriel-Galet *et al.*, 2012).

Those polymers are commonly used as conventional food packaging materials due to their versatility and advantageous performance/cost ratio. These polymers are suitable for food applications due to their good mechanical properties, transparency, physical stability, or variable barrier properties.

Both volatile and non-volatile antimicrobial agents can be homogeneously distributed along the whole polymeric structure of the packaging material in the case of monolayer films or in an individual active layer in the case of multilayer materials, which is usually the layer in contact with the foodstuff. Active agents can be also incorporated between passive layers

(Quintavalla and Vicini, 2002). In practice, a matrix of several layers is used to control the rate of release of the active substance.

Antimicrobial agents can be incorporated to the packaging material by different processing techniques. The selection of the most suitable technique will depend on the characteristics of the packaging material, packaging system, antimicrobial agent and food.

In the present Doctoral Thesis, PP homopolymer matrix (Figure 1.4) was selected to incorporate volatile and non-volatile antimicrobial agents by extrusion processing as being the most suitable technique to process this type of polymer. PP homopolymer is translucent, crystalline polymer that offer excellent heat and chemical resistance while maintaining a good balance of stiffness and impact performance. This selection was also made based on the medium temperatures of extrusion which protects antimicrobial agents from volatilization, low O_2 barrier required for minimally processed fruits and thermoforming temperatures needed to obtain final packaging prototypes.

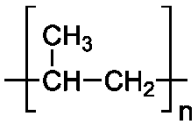


Figure 1.4. PP chemical structure

As polypropylene is a non-polar matrix, ethyl vinyl acetate (EVA) (Figure 1.5) was selected in the present work as a dispersing aid for non-volatile antimicrobials in the PP matrix. In this regard, the polarity of the EVA could have a significant effect on the dispersion of non-volatile compounds with hydrophilic properties (Tang *et al.*, 2002).

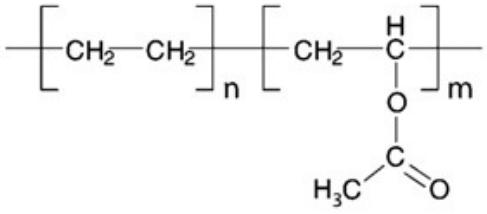


Figure 1.5. EVA chemical structure

In addition, different types of biopolymers such as polysaccharides (starch and cellulose); proteins (wheat gluten, soy protein or gelatin); polylactic acid (PLA) produced from lactic acid obtained by fermentation of sugar cane, or biopolymers that are produced directly by microorganisms such as polyhydroxyalkanoates (PHA) have also been used in the development of active antimicrobial films (Vieira *et al.*, 2011).

Iturriaga *et al.* (2012) incorporated citrus extracts in gelatin and methylcellulose biopolymer matrices, obtaining films with antimicrobial properties and convenient lack of odor and water solubility. Propolis extracts were incorporated in PLA by Mascheroni *et al.* (2010) to develop a preservative releasing system for food packaging. Takala *et al.* (2013) developed polycaprolactone (PCL)/alginate films containing natural extracts from rosemary and Asian and Italian essential oils for controlling the growth of food borne pathogens in fresh cut broccoli. They concluded that these composites might be affected by relative humidity in the packaging, altering the releasing mechanisms of volatile compounds and consequently their antimicrobial activity and film properties.

In general, biopolymers have excellent oxygen barrier properties under dry conditions. However, they present inherently high rigidity, difficulties in processing using conventional equipment and strong water sensitivity due to their hydrophilic character, which leads to plasticization that affects their mechanical and barrier properties (Anderson and Lamsal, 2011; Valdés *et al.*, 2014).

1.4. ANTIMICROBIAL PACKAGING FOR MINIMALLY PROCESSED FRUIT

The incorporation of antimicrobial agents in a food packaging matrix could be a tool to control the microbial population of minimally process fruit, providing food products with higher quality.

Both, synthetic and natural preservatives have been widely incorporated in food packaging materials to control microbial growth. Although most efforts are focused on the use of natural antimicrobial agents, the use of synthetic additives in food are sometimes necessary.

For the selection of antimicrobial agents for active packaging development, factors such as their activity against target microorganisms, their compatibility with the packaging material, their volatility, their stability during processing, their mode of action and their sensory compatibility with the fruit should be taken into account.

The incorporation of antimicrobial agents into polymers can affect their physical properties, mechanical integrity and thermal stability of packaging if the antimicrobial agents used are not compatible with the polymer (Sung *et al.*, 2013). Therefore, the study of antimicrobial packaging materials properties will determine any mechanical, gas barrier, thermal and morphological alterations originated by de addition of the antimicrobial agents (Bastarrachea *et al.*, 2011).

Two different groups of antimicrobials, volatile pure compounds of EOs and solid antimicrobial agents such as organic salts, have been selected in the present Doctoral Thesis to be incorporated into polymers for extending the shelf life of peeled and cut fruits.

1.4.1. Essential oils and their pure compounds

EOs are volatile oily liquids obtained from different plant parts and widely used as food flavors (Burt, 2004). Steam distillation is the most commonly used process for commercial production of EOs. The highly volatile and antimicrobial nature of natural plant EOs or their components make them attractive candidates for active packaging development. Since EOs are rich in volatile terpenoids and phenolic compounds, they have great potential to inhibit a wide spectrum of microorganisms (Cosentino *et al.*, 1999; Sung *et al.*, 2013).

Generally, the active components of plant EOs inhibit microorganisms through disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport and inhibiting protein synthesis (Sung *et al.*, 2013). Many studies indicate their antimicrobial effects are dependent on their ability to act on the cell membrane due to their high hydrophobicity (Patrignani *et al.*, 2015). Indeed, this characteristic allows them to have good partition in the lipids of cell membranes and mitochondria, altering their structures and making them more permeable, leading to the loss of ions and other cell contents (Nazzaro *et al.*, 2013). The chemical structure of the individual EO components affects their precise mode of action and antibacterial activity (Dorman and Deans, 2000).

The antimicrobial activity of EOs and their components have been widely demonstrated *in vitro* against common spoilage and pathogenic microorganisms of fruit (Belletti *et al.*, 2004; Combrinck *et al.*, 2011; Wuryatmo *et al.*, 2014; Wuryatmo *et al.*, 2003; Zheng *et al.*, 2015). Moreover, application of EOs in real systems such as whole and minimally process fruits has shown promising results. Examples of EOs and their compounds widely incorporated into food packaging are oregano EO, lemongrass EO, vanillin EO, hexanal, limonene, linalool,

carvacrol, citral, hexanal, etc. However, their practical application in fruit could be limited due to their strong impact and the organoleptic changes they can cause in food products (Gutierrez *et al.*, 2008b).

Although the desired antimicrobial activity of several EOs against pathogenic and spoilage microorganisms is usually achieved during *in vitro* tests, it has generally been found that a higher concentration is needed to achieve the same effect in foods (Burt, 2004; Shelef *et al.*, 1984). This fact may lead to an organoleptic impact as the use of natural preservatives can alter the taste of food and exceed the flavor threshold acceptable to consumers (Hsieh *et al.*, 2001; Nazer *et al.*, 2005). In this regard, the market application of EOs and their pure compounds in food preservation remains limited due to deterioration in the organoleptic properties of food (Muriel-Galet *et al.*, 2012). Table 1.1 compiles a literature review on the use of EOs and their compounds directly added into fruit or incorporated by means of antimicrobial edible coatings or active packaging materials, able to increase the quality and safety of whole and minimally processed fruits. The impact on the sensory properties of the food product has also been reported.

In general, more evidence was found on the use of EOs and their pure compounds as preservatives for fruits applied by different methods rather than active packaging systems. In all reported cases, great antimicrobial potential was shown against spoilage and pathogenic microflora of the fruit. Despite the fact that exposure of EOs or aroma compounds induced organoleptic changes, the enhancement of sensorial properties was in some cases reported. Therefore, EOs and their pure compounds at concentrations compatible with the sensory features of fruit could significantly prolong the shelf life of minimally processed fruits without altering their sensory properties.

Table 1.1. Antimicrobial effectiveness of EOs and their compounds in whole fruits and minimally processed fruits

Fruit	EO or component	Concentration applied/ incorporation way	Microbial target	Antimicrobial effect	Sensory	Reference
Fresh cut Kiwifruit	Carvacrol and cinnamic acid	1 mM (in dipping solution)	Natural microflora	Treatment with 1 mM of carvacrol or cinnamic acid reduced viable counts on kiwifruit by 4 and 1.5 log CFU/g at 4 °C and 8 °C, respectively	Without adverse sensory consequences	(Roller and Seedhar, 2002)
Fresh cut Honeydew melon	Carvacrol	1 mM (in dipping solution)	Natural microflora	Extended lag phase, but once initiated, growth proceeded at a similar rate in treated and untreated fruit	Without adverse sensory consequences	
Fresh sliced apples	Hexanal	0.15 mmol/100 g (inside packaging)	Natural microflora	Total inhibition of mesophilic bacteria and prolongation of lag phase of psychrotrophic bacteria at 4 °C. Strong inhibition of molds, yeasts, mesophilic and psychrotrophic bacteria at 15 °C	According to a non-structured sensorial analysis, samples could be positively appreciated for flavor and color. Deeper investigations are necessary to assess the organoleptic features	(Lanciotti *et al.*, 1999)
Fresh sliced apples	Hexanal Hexyl acetate 2-(E)- hexenal	150 ppm (hexanal) 150 ppm (hexyl acetate) 20 ppm (2-(E)- hexenal) (in a filter paper disk inside the packaging)	*Salmonella* Enteritidis *Escherichia coli* *Listeria monocytogenes*	Bactericide effect on *Listeria monocytogenes* and significant extensions of lag phase of *E. coli* and *Salmonella* Enteritidis	---	(Lanciotti *et al.*, 2003)
Fresh cut 'Fuji' apples	Oregano EO Lemongrass EO Vanillin EO	0.1-0.5% w/w 1-1.5% w/w 0.3-0.6% w/w (in apple puree alginate edible coatings)	Natural microflora and inoculated *Listeria innocua*	Significant inhibition of psychrophilic bacteria, yeasts and molds. 4 log reduction of *Listeria innocua* with lemongrass (1-1.5% w/w) and oregano (0.5% w/w)	Vanillin containing coatings (0.3% w/w) were the best accepted after 2-weeks storage. Consumers detected a residual aromatic herbal taste of carvacrol.	(Rojas-Graü *et al.*, 2007)

Fruit	EO or component	Concentration applied/ incorporation way	Microbial target	Antimicrobial effect	Sensory	Reference
					Samples containing lemongrass were negatively affected.	
Fresh cut apples	Hexanal Citral Hexanal + Citral Citron EO + Carvacrol Citral+2-(E)-hexenal Citral + Citron EO Hexanal + 2- (E)-hexenal	Alone 250 mg/L Combination 125 mg/L (dipping)	Natural microflora	Citral and hexanal + 2-(E)-hexenal were the most effective to inhibit the yeast growth	---	(Siroli *et al.*, 2015a)
Fruits salad	Citral Citron EO	25-125 ppm (citral) 300-900 ppm (citron EO) (addition in the syrup)	Natural microflora and inoculated *Escherichia coli*, *Salmonella* Enteritidis and *Listeria monocytogenes*	Citral extended lag phase of spoilage flora but exhibited a cytotoxic effect on the fruit. Citron EO caused longer lag phase and reduction of growth rate in exponential phase. Strong inhibition against *Listeria monocytogenes* (about 3 log CFU/g reduction)	---	(Belletti *et al.*, 2008)
Sweet cherries	Eugenol Thymol Menthol	1000 µL (in a gauze inside the packaging)	Natural microflora	2-4 log CFU/g regarding control	---	(Serrano *et al.*, 2005)
Table grapes	Eugenol Thymol	75-150 µL (in a gauze inside the packaging)	Natural microflora	Significant reductions of natural microflora. Slightly more effective for yeast and molds than for mesophilic aerobic bacteria	Higher scores for treated grapes in terms of appearance, firmness, and crunchiness but lower scores regarding	(Valero *et al.*, 2006)

Fruit	EO or component	Concentration applied/ incorporation way	Microbial target	Antimicrobial effect	Sensory	Reference
					sweetness and juiciness compared to control samples. Higher percentage of control samples developed off-flavors and bad odors. Eugenol samples were less accepted than samples treated with thymol	
Strawberries	2-nonanone	16.4 mg/L and 32.80 mg/L (incorporated in a wheat gluten protein active film)	*Botrytis cinerea*	Fruit spoilage started 3 days later with active packaging compared to the control packaging	---	(Cagnon *et al.*, 2013)
Late-maturing peach	Cinnamon EO	0.54 g/m^2 (incorporated in an active label)	Fungal growth	After 12 days of storage at room temperature, percentage of infected fruit in active packaging was 13% vs. 86% in non-active packaging	None of the panellists reported cinnamon flavor in any of the samples	(Montero-Prado *et al.*, 2011)
Fresh cut melon "piel de sapo"	Cinnamon, palmarosa and lemongrass EOs and eugenol, geraniol and citral	0.3 and 0.7% for EO 0.5% for pure compounds (incorporated in alginate-based edible coating)	Natural microflora *Salmonella* Enteritidis	Significant reduction of natural microflora. Significant reduction of *Salmonella* Enteritidis. Effectiveness depending on the EOs or active compound incorporated and their concentrations	Lower acceptation of fresh cut melon in terms of odor and taste when EOs were added. Firmness of the fruit was significantly affected by lemongrass.	(Raybaudi-Massilia *et al.*, 2008)

Fruit	EO or component	Concentration applied/ incorporation way	Microbial target	Antimicrobial effect	Sensory	Reference
Fresh cut pineapple	Lemongrass EO	0.1%, 0.3% and 0.5%, (w/v) incorporated into alginate-based edible coating	Natural microflora	Yeast and molds and total aerobic counts were significantly lower than control samples with 0.3 and 0.5% lemongrass.	0.5% (w/v) lemongrass decreased firmness and sensory scores	(Azarakhsh *et al.*, 2014)
Fresh cut pineapple	Trans-cinnamaldehyde	0.5%, 1% and 2% (w/w) (incorporated into a multilayered edible coating; microencapsulated)	Natural microflora	Total aerobic counts, molds and yeast and psychrotrophic bacteria experiences almost 3 log reduction at the end of storage period compared to the control samples	Control samples were preferred versus coated samples in terms of odor and flavor. Trans-cinnamaldehyde was detected by panelist but coated samples were still acceptable to consumers	(Mantilla *et al.*, 2013)
Peaches	Thyme EO	PET punnets containing sachets with thyme oil (thymol (56.43%), linalool (37.6%) and caryophyllene (9.47%))	Brown rot (*Monilinia* spp.)	Significantly reduced the brown rot incidence to 10% in naturally infected peaches	Panelists preferred fruits packed in commercial punnets containing thyme oil (sachets) in terms of overall appearance, taste, and natural peach flavor	(Cindi *et al.*, 2015)
Oranges	Citral	20, 60 or 150 mL/L (in absorbent pads in a closed system)	*Geotrichum citri-aurantii, Penicillium digitatum, Penicillium italicum*	Oranges treated with citral delayed spoilage by sour rot (*Geotrichum citri-aurantii)* at 5 °C and, to a lesser extent, at room temperature, but not by green and blue mold (*Penicillium digitatum* and *Penicillium italicum* respectively)	---	(Wuryatmo *et al.*, 2014)

In this Doctoral Thesis, the antimicrobial properties of substances such as citral, hexanal, linalool, limonene, orange EO, orange liquid flavoring, mandarin and pineapple essence were tested. After practical experiences, citral, hexanal and linalool are proposed as volatile antimicrobials for the development of a semi-industrial active antimicrobial packaging system for minimally processed fresh cut pineapple and orange.

Citral

The aldehyde citral is present in lemon, orange and bergamot essential oils, often in the form of the stereoisomers, geranial and neral (Figure 1.6 and Figure 1.7, respectively) with citrus scent or lemon odor (Benvenuti *et al.*, 2001; Fisher and Phillips, 2008). Citral has been classified as food flavoring by the European Regulation (EC) No. 1334/2008 (FL No. 05.020).

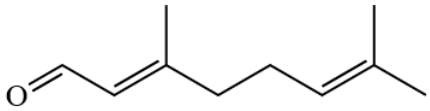


Figure 1.6. Geranial or citral A (trans-citral) chemical structure

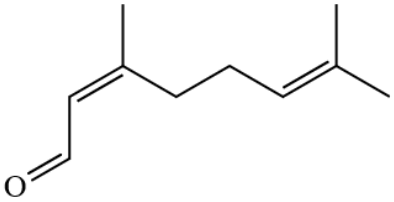


Figure 1.7. Neral or citral B (cis-citral) chemical structure

Antimicrobial activity of citral has been widely investigated. The antibacterial properties of citral against *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus in vitro* and in food systems were demonstrated by Fisher and Phillips (2006). According to Dorman and Deans (2000), cis/trans citral displayed moderate activity against the test microorganisms *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* among others.

Citral was found to be effective against citrus postharvest pathogens such as *Penicillium italicum*, *Penicillium digitatum* and *Geotrichum candidum* in the vapor phase (Wuryatmo et al., 2003). Also, Belletti *et al.* (2004) found this mixture of isomers to have the highest antifungal activity against *Saccharomyces cerevisiae* in broth as compared with other components of citrus essential oil. The antimicrobial effect of citral was also reported in fruit as shown in Table 1.1. Wuryatmo *et al.* (2014) demonstrated that citral delayed the development of sour rot on oranges inoculated with *Geotrichum citri-aurantii* at room temperature and 5 °C, supporting the effect previously observed *in vitro.* Siroli *et al.* (2015a) and Belletti *et al.* (2008) showed the antimicrobial properties of citral on fresh cut apples and fruit salad, reducing the viability of the spoilage microorganisms of the fruit such as lactic acid bacteria (LAB) and yeasts. Belletti *et al.* (2008) also reported strong inhibition of citral against *Listeria monocytogenes* in fruit salad.

Nevertheless, information regarding the antimicrobial activity of citral when incorporated in a film matrix is limited and its application has been focused on minimally processed vegetable salads rather than fresh cut fruits (Muriel-Galet *et al.*, 2013a; Muriel-Galet *et al.*, 2012).

Hexanal

Hexanal, is a six-carbon aldehyde (Figure 1.8) with fruity flavors and a scent which resembles freshly cut grass. Hexanal has been classified as food flavoring by the European Regulation (EC) No. 1334/2008 (FL No. 05.008).

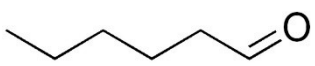


Figure 1.8. Hexanal chemical structure

Sáenz-Garza *et al.* (2013) examined the antifungal properties of microencapsulated hexanal against *Penicillium expansum in vitro* at different temperatures, observing inhibition of conidial germination at 25 °C. It was slightly less effective at 12 °C, probably due to the slower release of hexanal associated with reduced vapor pressure at lower temperature. Almenar *et al.* (2007) encapsulated hexanal into β-cyclodextrins (β-CD) and demonstrated that the fungi tested *in vitro*, *Colletotrichum acutatum*, *Alternaria alternata* and *Botrytis*

cinerea, were susceptible to hexanal vapor. The antimicrobial effect of hexanal was also demonstrated in fruit as shown in Table 1.1. As shown by Lanciotti *et al.* (1999), the addition of hexanal at levels not exceeding 100 ppm in the storage atmosphere of fresh sliced apples had an important effect on their quality by reducing the growth rate of the natural occurring microbial population, mold, yeasts, mesophilic and psychrotrophic bacteria, during storage at 4 and 15 °C. Later on, Lanciotti *et al.* (2003) demonstrated that 150 ppm of hexanal had a significant inhibitory effect against pathogenic microorganisms, showing bactericidal effects on *Listeria monocytogenes* and significant extensions of lag phases of *Escherichia coli* and *Salmonella* Enteritidis. Siroli *et al.* (2015a) also demonstrated the effects of hexanal alone or in combination with citron EO, 2-(E)-hexenal, citral and carvacrol on the shelf life of minimally processed apples packaged in modified atmosphere. The mixtures citral and hexanal + 2-(E)-hexanal were the most effective to inhibit yeast growth compared to the other samples.

However, information on the antimicrobial activity of hexanal incorporated in a polymeric matrix is scarce. Fadida *et al.* (2015) reported the antifungal activity of hexanal covalently attached to chitosan on wheat grain spoilage. After 4 weeks of storage, 80% spoilage was observed in the grain exposed to the active films (no direct contact) whilst 100% spoilage was observed after 2 days for the control samples not exposed to active films.

Linalool

Linalool is an oxygenated monoterpene (Figure 1.9) present in the oil of several plants and fruits, such as citrus, basil and coriander with floral scent and with a touch of spiciness (Fisher and Phillips, 2008; Krist *et al.*, 2008; Suppakul *et al.*, 2003b). Linalool has been classified as food flavoring by the European Regulation (EC) No. 1334/2008 (FL No. 02.013).

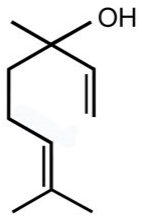


Figure 1.9. Linalool chemical structure

According to the literature, linalool is known to possess a broad spectrum of antimicrobial activity against a variety of pathogenic bacteria such as *Escherichia coli*, *Listeria monocytogene*s, *Staphylococcus aureus* and other spoilage bacteria (Dorman and Deans, 2000; Friedman *et al.*, 2002), but also has antifungal effects on spoilage yeasts and molds such as *Saccharomyces cerevisiae*, *Aspergillus* sp., and *Penicillium* sp. (Lachowicz *et al.*, 1998; Suppakul *et al.*, 2003b).

Linalool is stable at relatively high temperatures and its potential to be incorporated into polymers and used in antimicrobial packaging has been demonstrated. In this perspective, numerous studies have evaluated its antimicrobial activity on food systems such as cheese, but there is limited information on their application on fruits. In this case, Suppakul *et al.* (2008) reported that linalool incorporated into LDPE film exhibited inhibitory activity against the growth of *Staphylococcus aureus*, *Listeria innocua*, *Escherichia coli*, and *Saccharomyces cerevisiae* on culture media and on the surface of Cheddar cheese. Cheddar cheese packaged in LDPE-based film containing linalool also showed significantly lower growth of total aerobic bacteria. Rupika *et al.* (2006) also reported that linalool incorporated into LDPE films had significant inhibitory activity against the growth of *Listeria innocua* and *Escherichia coli* both *in vitro* and on the surface of Cheddar cheese.

Regarding the application of active films containing linalool in fruits, Cindi *et al.* (2015) demonstrate that PET punnets containing thyme oil sachets (thymol (56.43%), linalool (37.6%) and caryophyllene (9.47%)), and sealed with chitosan/bohemite nanocomposite lidding films, significantly reduced the brown rot incidence to 10% in naturally infected peaches stored at 0.5 °C and 90% RH for 7 days.

Although some of these active agents have been incorporated in active packaging materials, these developments have not reached the market yet.

1.4.2. Organic acids and their salts

Some of the most commonly used preservative agents in food are weak organic acids, such as acetic, benzoic, lactic, citric, malic, tartaric, propionic, fumaric, or sorbic acid and their salts. Weak organic acids are naturally occurring preservatives in many fruits (Plumridge *et al.*, 2004) whilst their salts are from laboratory syntheses (Nostrand's, 2005).

Weak acids exist in a pH-dependent equilibrium between the undissociated and dissociated state (Corrales *et al.*, 2014). The principle mode of action is believed to be the transport of undissociated acid into the cell through the plasma membrane which dissociates in a higher pH environment to form protons and anions that cannot return through the plasma membrane (Junqueira-Gonçalves *et al.*, 2013). Intracellular acidification of the cell cytosol resulting from the accumulation of protons inhibits key metabolic activities involved in glycolysis and hence inhibits ATP yields (Plumridge *et al.*, 2004). It is known that maximal antimicrobial activity is obtained at pH values below the acid's dissociation constant (pKa) (Kuplennik *et al.*, 2015).

Among the listed preservatives, potassium sorbate (PS) and sodium benzoate (SB) are the most commonly used preservatives in fruits. The preservatives are often added as the salt of the acid because salts are more soluble in aqueous solution (Suhr and Nielsen, 2004).

Therefore, in this Doctoral Thesis, potassium sorbate and sodium benzoate are proposed as non-volatile antimicrobials for the development of an antimicrobial active packaging for minimally processed fresh cut orange and pineapple.

Potassium sorbate

Potassium sorbate is the salt of sorbic acid, a polyunsaturated fatty acid used to inhibit molds and yeasts and some bacterial strains in various foodstuff (Francis *et al*., 2005), although it inhibits bacteria to a lesser degree (Figure 1.10) (Sofos and Busta, 1981).

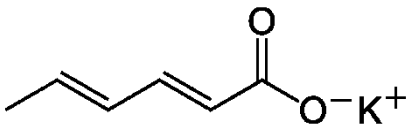


Figure 1.10. Potassium sorbate chemical structure

Potassium sorbate is a white crystalline powder with greater solubility in water than sorbic acid. It is effective at pH up to 6.5 and its pKa is 4.75 (Sofos and Busta, 1981). As pH increases, less acid is undissociated, so the antimicrobial activity diminishes (Schmidl and Labuza, 2000).

Potassium sorbate has been classified as food additive (E-202) under European Regulation (EC) No. 1333/2008 on food additives. This additive is suitable to be used in beverages, syrups, fruit juices, jellies, jams, salads, pickles, dairy products, cheeses, dried fruits and vegetables, fruits and vegetables in vinegar, oil or brine, confectionary, bakery products, processed meat, processed fish and fish products, soups, sauces, flavored drinks and wine.

Sodium benzoate

Sodium benzoate is the salt of benzoic acid, and works well in acidic media to inhibit yeasts, molds and bacterial growth (Figure 1.11) (Pylypiw Jr and Grether, 2000).

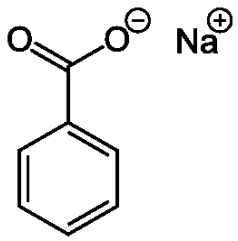


Figure 1.11. Sodium benzoate chemical structure

As for potassium sorbate, as pH increases the antimicrobial activity of sodium benzoate diminishes. In this case it is effective at pH up to 4-4.5 and its dissociation constant is 4.21 (Sofos and Busta, 1981). According to European Regulation (EC) No. 1333/2008 on food additives, sodium benzoate has been classified as food additive (E-211) to be used in fruit and vegetables in vinegar, oil, or brine, jam, jellies and marmalades and similar products, processed meat, processed fish and fishery products, sauces, fruit juices, flavored drinks and wine.

The antimicrobial activity of potassium sorbate and sodium benzoate has been evaluated *in vitro* against common spoilage microorganisms of fruits. Heydaryinia *et al.* (2011) demonstrated the effect of potassium sorbate and sodium benzoate on *Aspergillus niger*. In another study, Fagundes *et al.* (2013) showed the antimicrobial effect of potassium sorbate and sodium benzoate *in vitro* against *Botritis cinerea* and *Alternaria alternate* as fungal pathogens of cherry tomato fruit. Stanojevic *et al.* (2009) also demonstrated the antimicrobial potential of these preservatives against typical fruit spoilage molds that included *Aspergillus flavus*, *Fusarium oxysporum*, *Trichoderma harsianum* and *Penicillium*

italicum. Karaca *et al.* (2014) showed significant inhibitory effects of potassium sorbate and sodium benzoate on the growth of *Monilinia fructicola,* an important mold causing postharvest disease of stone fruits.

When specified by the Regulation (EC) No. 1333/2008, potassium sorbate and sodium benzoate can be used in combination. Synergistic effects of potassium sorbate and sodium benzoate have been reported. Buranapim and Areekul (2011) demonstrated that the combination of these preservatives increased their effectiveness. The combination of potassium sorbate and sodium benzoate at 600 and 400 ppm, respectively, completely inhibited *Penicillium citrinum*. However, there are many factors such as water activity, pH and temperature of storage that can affect this effect (Schmidl and Labuza, 2000). For example, the synergism between benzoic acid and sorbic acid was pH dependent with respect to the growth rate of the yeast *Zygosaccharomyces bailii.*

Potassium sorbate and sodium benzoate are generally applied through dipping or spraying onto the food matrix. However, this procedure could result in a potential loss of their antimicrobial activity due to a possible reaction with food components, dilution, or quick consumption (Appendini and Hotchkiss, 2002). Therefore, their incorporation into the packaging material could result in longer term protective effect. In addition, these antimicrobials are traditional food preservatives that have been widely used in the food industry. Their high thermal stability and minimal tendency to cause sensory alteration make them attractive antimicrobial agents for active packaging.

In this perspective, potassium sorbate, sodium benzoate or their combination have been successfully incorporated into various packaging materials to extend the shelf life of different foodstuff. Potassium sorbate incorporated into starch films inhibited the growth of *Salmonella* Typhimurium and *Escherichia coli* and extended the shelf life of poultry (Baron and Summer, 1993). Cellulose films containing potassium sorbate prevented the growth of *Staphylococcus* spp., mesophilic and psychotropic bacteria in pastry dough (Silveira *et al.*, 2007). Han and Floros (1997) found that potassium sorbate (1% w/w) incorporated in LDPE films not only extended the lag time of a bakery yeast but also reduced the growth rate and its maximum growth. Villarruel *et al.* (2015) demonstrated the effectiveness of carboxymethyl cellulose (CMC) and polyvinyl alcohol (PVOH) blends containing 0.1% (w/w) of sodium benzoate against *Penicillium* spp. and *Candida* spp. with potential applications to food.

The use of potassium sorbate and sodium benzoate in fruits directly added into the product or incorporated by means of antimicrobial edible coatings or active packaging materials has also been reported. Table 1.2 compiles a literature review on the use of potassium sorbate and sodium benzoate in fruits.

In general, potassium sorbate and sodium benzoate significantly inhibited the growth of spoilage flora in minimally processed fruit. As observed for the volatile antimicrobials, the application of potassium sorbate and/or sodium benzoate by dipping or as a coating were more often reported than active packaging applications. Sensory alteration of fruit was not reported for these antimicrobials. In all the reported cases, direct contact with the food is a requisite to ensure effective antimicrobial activity on the food surface.

As reported for active packaging materials containing volatile active agents, active packaging materials containing potassium sorbate or sodium benzoate are not yet commercialized.

Table 1.2. Antimicrobial effectiveness of potassium sorbate and sodium benzoate and their combination in whole and minimally processed fruits

Fruit	Active agent	Concentration applied/ incorporation way	Microbial target	Antimicrobial effect	Sensory	Reference
Strawberries	Potassium sorbate	0.2 g/L (potato starch coating)	Natural microflora	Yeast and mold counts significantly decreased compared with untreated samples extending strawberry storage life from 14 days to 28 days in coated strawberries	---	(Garcia *et al.*, 1998)
Citrus (oranges and lemons)	Potassium sorbate Sodium benzoate mixture	0.2 M individually, 0.1 M +0.1 M in combination (by dipping)	*Penicillium digitatum* *Penicillium italicum*	Potassium sorbate and sodium benzoate reduced green mold *Penicillium digitatum* by 70-80% after 7 days of storage at 20 °C	---	(Palou *et al.*, 2002)
Berries	Potassium sorbate	2.5 and 4.0% (Incorporated by extrusion in recycled PET and thermoformed into clamshells)	*Botrytis cinerea*	2 log CFU/g reduction	---	(Junqueira-Gonçalves *et al.*, 2013; Junqueira-Gonçalves *et al.*, 2014)
Apples, cucumbers, and tomatoes	Potassium sorbate	0.1% (w/v) (incorporated in polysaccharide edible coatings)	*Penicillium expansum, Cladosporium herbarum* and *Aspergillus niger* from apples. *Penicillium oxalicum* and *Cladosporium cucumerinum* from cucumbers. *Penicillium expansium* and *Cladosporium fulvum f*rom tomatoes	1.5-2.9 log CFU/g reduction for apples 1.1-1.5 log CFU/g for cucumbers 1.3-1.5 log CFU/g for tomatoes	---	(Mehyar *et al.*, 2011)

Fruit	Active agent	Concentration applied/ incorporation way	Microbial target	Antimicrobial effect	Sensory	Reference
Sliced apple	Potassium sorbate Sodium benzoate	0.1-1.5 % sodium benzoate or potassium sorbate (incorporated in a cellulose-based edible coating)	Natural microflora	Significant CFU/g reduction compared to control coating during 4 weeks of storage	---	(Baldwin *et al.*, 1996)
Peaches and and plums	Potassium sorbate	10% (coating material on wrapping paper for individual packing of fruit)	*Candida pelliculosa* *Kloeckera apis*	Strong significant decrease in *Candida pelliculosa and Kloeckera apis*	---	(Rudra *et al.*, 2013)
'Clemenules' mandarins	Potassium sorbate Sodium benzoate and their mixture	2% PS 2.5% SB 2 + 0.5% SB + PS (in hydroxypropyl methylcellulose lipid edible composite coatings)	*Penicillium digitatum* *Penicillium italicum*	SB + PS-based coating was the most effective to reduce disease severity	No alteration of flavour or fruit appearance	(Valencia-Chamorro *et al.*, 2011)

1.5. LEGISLATIVE FRAMEWORK ON ACTIVE FOOD PACKAGING MATERIALS

The framework Regulation (EC) No. 1935/2004 establishes general principles for all the materials intended to come into contact with food. The principle underlying this Regulation is that any material or article intended to come into contact directly or indirectly with food shall be manufactured in compliance with good manufacturing practices so that, under normal or foreseeable conditions of use, they do not transfer their constituents to food in quantities which could: (a) endanger human health; or (b) bring about an unacceptable change in the composition of the food; or (c) bring about a deterioration in the organoleptic characteristics thereof. This Regulation applies to all materials and articles intended to be in contact with foodstuff, including active and intelligent food contact materials and articles.

The framework Regulation establishes general principles for each packaging material but it does not describe how they must be accomplished depending on its nature. It is the Regulation (EC) No. 450/2009 which lays down the specific provisions for active and intelligent materials and articles in addition to the general requirements established in Regulation (EC) No. 1935/2004 for their safe use.

Unlike conventional food contact materials, active packaging materials and articles, which are designed to actively maintain or improve the condition of the food, are not inert by their design. According to the active packaging Regulation, only substances which are included in the 'Community or positive list' of authorized substances may be used in components of active materials and articles. For new active substances, an application for their authorization shall be submitted. The Community list should include the identity, conditions of use, restrictions and/or specifications of use of the substance or of a combination of substances and, where necessary, of the component or of the material or of the article in which they are added to or incorporated into. The list has not been published yet.

For the active substances which are intended to be released into food or are immobilized on the packaging surface in contact with food should only be used under the conditions set out in Food Law Regulation. In this case, preservatives that have a technological effect on the food prolonging their shelf life and/or protecting them against growth of pathogenic microorganisms should be listed in the Regulation (EC) No. 1333/2008 on food additives for their intended use. New food additives or an extension of use are subject to an authorization procedure laid down in Regulation (EC) No. 1331/2008 that establishes a common

authorization procedure for food additives, food enzymes and food flavorings. Then, EFSA performs a risk assessment of the substance and publishes a Scientific Opinion about its use. Finally, it is the European Commission who approves the use and the applicable restrictions for these substances.

Regarding the passive parts in which the active substances are incorporated, they should be covered by the specific Community or national provisions applicable to those materials and articles. For example, if an active substance is incorporated in a polymeric matrix, the polymer have to comply with the Regulation (EU) No. 10/2011 on plastic materials and articles intended to come into contact with food. In this way, Articles 11 and 12 of plastic Regulation specify the applicable restrictions regarding specific migration limits (SML) and overall migration limits (OML), respectively. The SML is the maximum permitted amount of a given substance released from a material or article into food or food simulants, and those limits are established in the Union list of the Regulation. On the other hand, the OML is the maximum permitted amount of non-volatile substances released from a material or article into food simulants. Plastic materials and articles shall not transfer their constituents into food simulants in quantities exceeding 10 mg of total constituents released per dm^2 of food contact surface (mg/dm^2). However, according to Regulation (EC) No. 450/2009 on active and intelligent materials, the overall migration from active releasing materials can exceed the overall migration limits as long as the levels transferred to the food comply with restrictions in the existing food law (e.g. as authorized food additives). As a result, the transfer of these active substances should not be included in the calculation of the overall migration limit.

Active materials and articles shall be adequately labeled to indicate that the materials or articles have an active function, indicating also the non-edible components whenever they are perceived as edible as it could occur for external active devices.

Regarding the active substances incorporated in active packaging materials to extend the shelf life of minimally processed fruit, they must be approved as food additives for their use in Group 4, fruits and vegetables, category 04.1.2 (peeled, cut and shredded fruit and vegetables) under Regulation (EC) No. 1333/2008 on food additives.

Therefore, in order to incorporate citral, hexanal and linalool in a packaging material to be used with minimally processes fruit order as it is proposed in the present Doctoral Thesis, it

will be necessary to submit a new food additive application for their use as preservatives for peeled, cut and shredded fruit and vegetables under Regulation (EC) No. 1333/2008.

In addition, the solid antimicrobials, potassium sorbate and sodium benzoate, proposed in the present Doctoral Thesis for the development of an active packaging material for minimally processed fruit, are already approved as food additives by Regulation (EC) No. 1333/2008. However, for its use as preservatives for minimally process fruits, it will be necessary to submit an application for extension of use.

2. OBJECTIVES

2.1. GENERAL OBJECTIVE

The main objective of the present Doctoral Thesis is the development of an active antimicrobial packaging system, which involve the characterization of its functional properties and its ability to extend the shelf life of minimally processed pineapple and orange fruit. The active packaging system is composed of an active PP-based film containing volatile antimicrobials and an active PP:EVA-based tray with non-volatile antimicrobials with industrial application.

2.2. SPECIFIC OBJECTIVES

To achieve the main objective, the specific objectives set are the following:

- To select the antimicrobial agents based on the study of their antimicrobial properties *in vitro* and their synergistic effect.
- To develop a PP-based antimicrobial bilayer film at a semi-industrial scale containing an equal mixture or citral, hexanal and linalool by compounding followed by cast film coextrusion.
- To develop a PP:EVA-based antimicrobial bilayer tray at a semi-industrial scale containing a mixture of potassium sorbate and sodium benzoate by compounding followed by sheet coextrusion and thermoforming.
- To evaluate the effect of the antimicrobial agents on the most relevant engineering properties of the developed film and tray.
- To study the release kinetics of the active agents from the film and the tray.
- To evaluate the antimicrobial properties of the film and tray *in vitro* against typical fruit spoilage microorganisms.
- To study the potential of the active film, active tray and their combination to keep the quality parameters of peeled and cut pineapple and orange for a longer period of time.
- To evaluate the food safety of the film and tray in food and food simulants, according to the current European legislation on food and food contact materials.

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Chemicals

- Citral (purity ≥ 96%). Sigma-Aldrich (Madrid, Spain).
- Hexanal (purity ≥ 98%). Sigma-Aldrich (Madrid, Spain).
- Linalool (purity ≥ 97%). Sigma-Aldrich (Madrid, Spain).
- Limonene (purity ≥ 97%). Sigma-Aldrich (Madrid, Spain).
- Orange liquid flavoring (80-90% limonene). Lucta (Barcelona, Spain).
- Orange essential oil (90-95% limonene, 0.2-0.5 linalool, < 0.05 % citronellol and < 0.05% geraniol). Guinama (Valencia, Spain).
- Mandarin essence (85-90% limonene, 0.05-0.1% linalool). Guinama (Valencia, Spain).
- Pineapple essence. Guinama (Valencia, Spain).
- Potassium sorbate powder (purity ≥ 99%). Coralim Ingredients and Colours (Valencia, Spain).
- Sodium benzoate powder (purity ≥ 99%). Coralim Ingredients and Colours (Valencia, Spain).
- Calcium chloride (analysis grade). Scharlab (Barcelona, Spain).
- Acetic acid (HPLC grade). Scharlab (Barcelona, Spain).
- MilliQ water. Millipore (Madrid, Spain).
- Ammonium acetate (HPLC grade). Scharlab (Barcelona, Spain).
- Acetonitrile (HPLC grade). Scharlab (Barcelona, Spain).
- Methanol multisolvent (HPLC grade). Scharlab (Barcelona, Spain).
- Acetone (GC grade). VWR (Leuven, Belgium).

3.1.2. Gases

- High purity helium (> 99.999%).
- Nitrogen (≥ 99.99%).
- Oxygen (≥ 99.5%).
- Carbon dioxide (≥ 99.5%).
- Dry nitrogen.

Gases were supplied by Abelló Linde (Valencia, Spain).

3.1.3. Culture media

- Malt extract agar (MEA).
- Potato dextrose broth (PDB).
- Peptone water.
- Plate Count Agar (PCA).
- Sabouraud agar.
- Oxytetracicline supplement for Sabouraud agar.

Culture media and supplement were supplied by Scharlab (Barcelona, Spain).

3.1.4. Microorganisms

- *Saccharomyces cerevisiae* CECT 13084, *Aspergillus niger* CECT 2807, *Penicillium aurantiogriseum* CECT 2264. Colección Española de Cultivos tipo, CECT (Valencia, Spain).

3.1.5. Polymers

- Polypropylene CAPILENE G70 TF. Carmel Olefins Ltd., (Haifa, Israel).
- Ethylene-vinyl acetate PA540. REPSOL (Madrid, Spain).

3.1.6. Others

- Petri dishes. Gosselin (Barcelona, Spain).
- Parafilm®. Pechiney Plastic Packaging (Chicago, IL, USA).
- Fluoroelastomer tube ISO Versinic. Saint Gobain (Charny, France).
- Nylon filters 0.22 µm. Scharlab (Barcelona, Spain).
- Butyl/Teflon® septum. National Scientific (Langerwehe, Germany).
- Self-adhesive septum 15 mm diameter. PBI-Dansensor (Barcelona, Spain)
- Peeled and cut orange and pineapple (2 kg tray). Cooperativa Benaguasil (Valencia, Spain).

3.2. METHODS

3.2.1. Antimicrobial efficacy test in vapor phase

3.2.1.1. Culture preparation

Saccharomyces cerevisiae, *Aspergillus niger* and *Penicillium aurantiogriseum* were kept on Malt Extract Agar (MEA) at 4 °C and streaked on agar plates on a weekly basis in fresh media for further experiments. The yeast stock culture was prepared by transferring a single colony of *Saccharomyces cerevisiae* to 50 mL of Potato Dextrose Broth (PDB) and incubated overnight at 25 °C reaching a 7 log CFU/mL inoculum. The mold stock cultures were prepared by adding 3-4 mL of sterile peptone water to a sporulated MEA plate, collecting spores and counting them with a Neubauer chamber from Marienfield Superior (Lauda-Königshofen, Germany) adjusting the concentration to 10^6 spores/mL.

3.2.1.2. Volatile active agents vapor phase inhibition test

The antimicrobial properties of citral, hexanal, linalool, limonene, orange EO, orange liquid flavoring, mandarin and pineapple essence were tested *in vitro* vapor phase.

Fifty µL of yeast and mold inoculum were cultured on MEA agar Petri dishes. Sterilized square pieces of paper (2x2 cm) were placed on the lid of the Petri dish and impregnated

individually with 10, 20 and 30 µL of each active agent. According to the initial antimicrobial effectiveness results, 1, 2.5, 5, 7.5 and 15 µL of citral, hexanal and linalool, were also tested.

Petri dishes were sealed with parafilm® to reduce volatiles losses. Inoculated agar plates without active agents were included as reference control. Plates were incubated at 25 °C for 5 days. After the incubation period, qualitative analysis was carried out by visual inspection, measuring the inhibition halo of the microbial growth. From this test, the reduction concentration (RC), the minimum inhibitory concentration (MIC), and the maximum concentration (Cmax) of the antimicrobials were also evaluated. The RC is defined as the lowest concentration resulting in a visible microorganism growth reduction. The MIC is defined as the lowest concentration showing absence of visible growth (inhibition halo) and the Cmax is the concentration of the antimicrobial that yielded the highest inhibition area (90 mm) (Goñi et al., 2009). Tests were performed in triplicate.

After the exposure to the active agents for 5 days at 25 °C, the fungistatic or fungicidal effect was evaluated. To that, agar plates that showed no visual growth were incubated for an extra 48 h at 25 °C without the presence of active agents, replacing the lid containing the active paper by an empty Petri dish lid. The antimicrobials were considered fungistatic when growth appeared during the additional incubation period while they were considered fungicidal if no additional growth was detected. Tests were performed in triplicate.

In addition, the three most effective active agents evaluated, citral, hexanal and linalool, were mixed in equal volumes (1:1:1 mL). One, 2.5, 5, 7.5, 10, 15, 20 and 30 µL of the mixture were tested in vapor phase against *Saccharomyces cerevisiae*, *Aspergillus niger* and *Penicillium aurantiogriseum* and incubated for 5 days at 25 °C. After the incubation period, the diameter of the resulting inhibition zone was measured, identifying the MIC and Cmax concentrations. Tests were performed in triplicate.

In order to quantitatively assess the synergistic, additive or antagonistic effects of the antimicrobial combination, the FIC index (fractional inhibition concentration) of the mixture of citral, hexanal and linalool was calculated as the sum of the FIC for each individual compound in the selected mixture. The FIC index was calculated in terms Cmax (µL/Petri dish) according to the Equation 3.1.

$$FIC = \frac{Cmax\ citral\ mix}{Cmax\ citral\ alone} + \frac{Cmax\ hexanal\ mix}{Cmax\ hexanal\ alone} + \frac{Cmax\ linalool\ mix}{Cmax\ linalool\ alone} \quad \textbf{Equation 3.1}$$

According to Pei *et al.* (2009), FIC values below 1 were considered synergistic, values equal to 1 were considered additive, values between 1-2 were set as indifferent and values above 2 were considered antagonistic.

3.2.2. Organoleptic compatibility of volatile active agents with minimally processed fruit

In order to evaluate the organoleptic compatibility of the selected volatile agents, a PP tray (170x127x28 mm) was filled with 0.150 ± 15 g of cut and peeled pineapple or orange and the active agents were added to a paper disk stacked to the inner side of a PP film. The trays were sealed with the PP film containing the impregnated paper disk by using a semiautomatic tray-sealing machine, Smart 300 from ULMA (Oñati, Spain), under air conditions. The packed fruit was stored for 24 h at 4 ± 2 °C before testing.

The concentrations tested were proportionally converted from the Cmax obtained for individual citral, hexanal, linalool and their mixture (1:1:1 volume ratio) in the Petri dish described in section 3.2.1.2. This conversion was performed taking into account the free headspace of the Petri dish (0.039 L) and the free headspace of the tray (0.3 L) subtracting the agar and the fruit and volume, respectively.

A blind test was performed by 10 consumers that evaluated the organoleptic compatibility of the volatile active agents with the fruit in terms of flavor (smell and taste). Ratings were based on a 4-point hedonic scale where 4 was very compatible, 3 was compatible, 2 was poorly compatible and 1 was not compatible.

3.2.3. Thermal stability of active agents

Thermogravimetric analysis of the selected active agents was carried out using a TGA Q-5000IR from TA Instruments (New Castle; USA). Approximately, 5 to 15 mg of active agents, were weighed in a platinum pan. Samples were heated from 25 °C to 900 °C at a heating rate of 20 °C/min. The flow rate of dry nitrogen was 25 mL/min.

3.2.4. Active materials processing

Active materials, films and trays, were processed at laboratory and at semi-industrial scale. Monolayer active films containing the selected volatile active agents were developed at lab scale by one-step extrusion. Bilayer active films containing the same volatile agents were developed at semi-industrial scale by compounding followed by cast film coextrusion. Preliminary monolayer materials containing solid active agents were developed at lab scale by micro-compounding. Active trays containing solid active agents were also developed at semi-industrial in 3 steps: compounding, bilayer sheet by coextrusion and a final thermoforming step. Figure 3.1 shows a scheme of the active packaging materials developed at lab and semi-industrial scale and the nomenclature of the materials used along the present work.

3.2.4.1. Monolayer active films at lab scale with volatile active agents

A twin screw extruder, Brabender DSE 20/40D (Duisburg, Germany), equipped with a gravimetric main feeder (Maguire MGF 4-ST, Aston, USA) was used to process two monolayer active films of PP at lab scale containing different concentrations of an active mixture of citral:hexanal:linalool (1:1:1; volume ratio) and 100 µm thickness. PP control film was also processed in the same way.

The main feeder was set to deliver 1 kg of PP. Incorporation of active agents was carried out in the last section of the extruder by means of a piston pump (Lewa M3, Leonberg, Germany).

The volumetric pump flow was adjusted to 2.3 and 2.8 mL/min to provide film with 10 and 12% nominal active agent content on a weight basis to polymer (% w/w), respectively. This procedure was carried out by weighing the pumped mixture per minute once the pump reached a steady state. A fluoroelastomer tube was used for transferring the liquid active agents to the extruder.

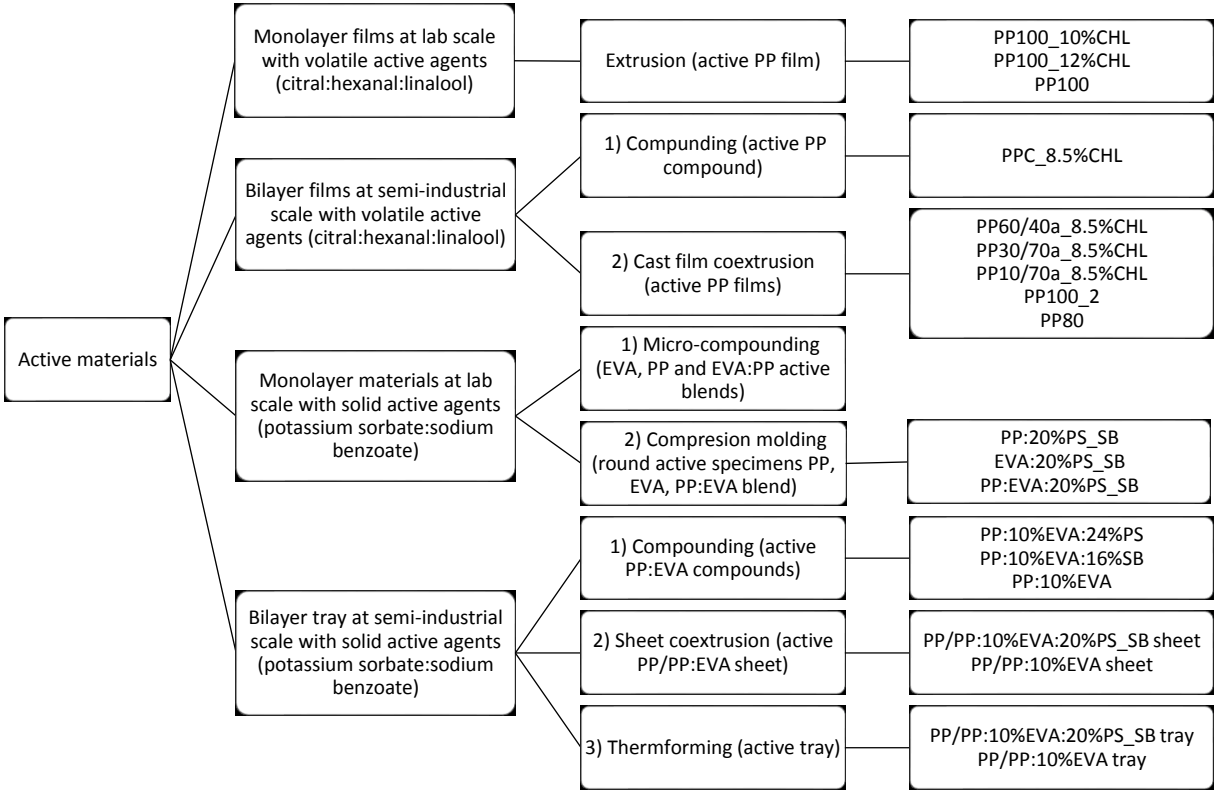


Figure 3.1. Active packaging materials developed at lab and at semi-industrial scale

Figure 3.2 shows the parts of a twin extruder including the main hopper for the polymer feeding, the screw, the active agents feeding port and the active film.

Screw speed was fixed at 50 rpm. Temperature profile along the six barrel zones from hopper to die was set as indicated in Table 3.1 for control and active film processing.

The active packaging materials were processed through a variable thickness flat sheet die. A nominal die gap of 200 µm was adjusted, and final film thickness was controlled by means of take-off unit speed (m/min).

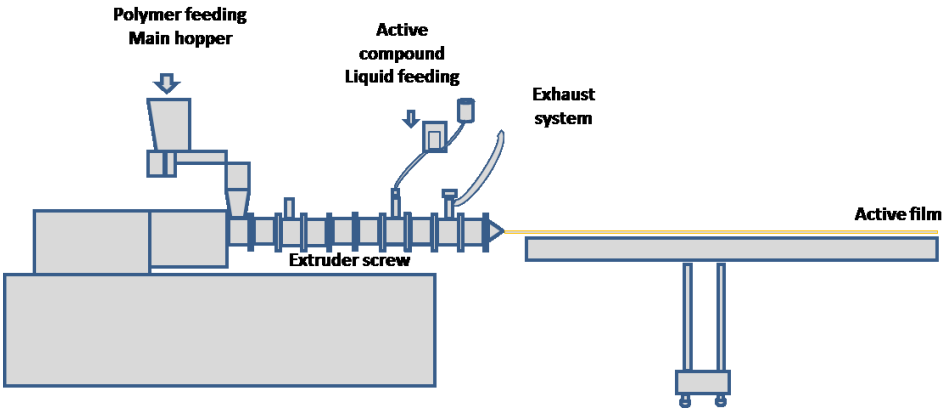


Figure 3.2. Twin extruder scheme

Table 3.1. Temperature profile set for the processing of control and active PP films with citral, hexanal and linalool mixture along the twin extruder barrel

Zone	1	2	3	4	5	6
Temperature (°C)	180	185	185	185	190	190

Zone 1 (hopper, PP feeding)
Zone 4 (active agents feeding)
Zone 6 (die)

3.2.4.2. Bilayer active films at semi-industrial scale with volatile active agents

As a first step, a polymeric compound of PP with 8.5% (w/w) of citral, hexanal and linalool in an equal composition (1:1:1) was processed using a compounding line based on a co-rotating twin screw extruder (Coperion Zsk 26 Mc, Stuttgart, Germany). The twin screw extruder had

a 26 mm diameter screw and a length-diameter ratio (L/D) of 40 and was equipped with a gravimetric main feeder for polymeric pellets (Brabender DDW-MST FW40, Duisburg, Germany) and a liquid gravimetric feeder (Brabender FDDW-MD2 DZP-6, Duisburg, Germany) to incorporate the liquid active mixture. A water bath, drying unit and pelletizer were coupled to the twin screw extruder for the compound processing as shown in Figure 3.3.

For the processing of polymeric active compounds, a screw speed of 400 rpm was selected. Temperature profile along the 10 barrel zones from hopper to die was set as indicated in Table 3.2.

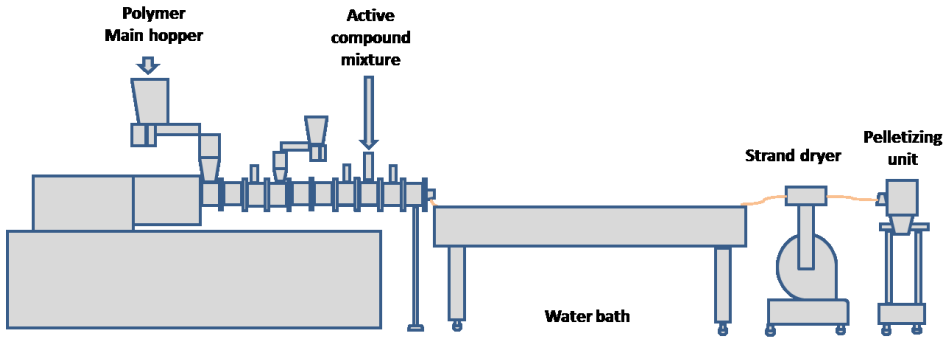


Figure 3.3. Semi-industrial scale compounder scheme

Table 3.2. Temperature profile set for the processing of PP compound with citral, hexanal and linalool mixture along the barrel of the co-rotating twin screw extruder

Zone	1	2	3	4	5	6	7	8	9	10
Temperature (°C)	170	175	180	185	185	185	185	185	185	185

Zone 1 (hopper, PP feeding)
Zone 5 (liquid feeding)
Zone 10 (Die)

The polymeric compound initially processed, which contained 8.5% (w/w; nominal concentration) of the citral, hexanal and linalool (1:1:1) mixture, was used as raw material to feed the line that supplied the active layer. Two PP bilayer structures based on 100 µm films, with two different thicknesses of active layer, (40 and 70 µm), and a PP film based on 80 µm thickness, with 70 µm of active layer, were processed using a semi-industrial scale co-

extrusion line. Two control films of 80 and 100 µm with no antimicrobials were also processed as reference materials.

The bilayer active films were processed at a semi-industrial scale using a MF-EXB 600 Coextrusion line from Dr. Collin, Ebersberg, Germany. This co-extrusion line was based on three single screw extruders (E30P, 25 L/D) connected to a feed-block and a variable thickness flexible lip die. The feed-block can provide up to five layers (ABCBA), as well as different multilayer rearrangements (ABC, BCB, ACA), bilayer (AC, BC), or monolayer structures. In this case, the feed-block was modified to provide a two-layer structure (BC). For film processing, the extruders were fed with solid pellets through the hopper. Blank PP was used to feed extruder B and the active compound was used to feed extruder C.

Figure 3.4 describes the coextrusion line scheme which shows the two extruders used to obtain the bilayer films. The material was transferred, melted and pushed towards the end of the equipment as the screw rotated.

Width of the films was adjusted to 480 mm by controlling the air gap between the die and chill roll using an air knife. Overall thickness was adjusted through coextrusion line take off speed. Thickness deviations on cross section of the film were adjusted by means of die screws during processing. Trimming of edges was carried out online using edge trimmers to adjust the reel to 27 cm, as it is the required width for tray sealing equipment.

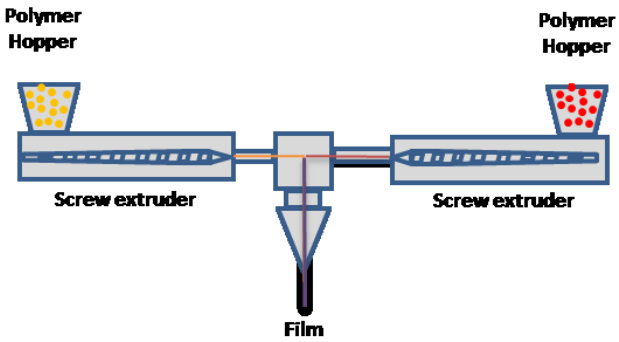


Figure 3.4. Co-extrusion line scheme

Processing temperatures of control and active films along the extrusion line are indicated in Table 3.3. Table 3.4 compiles the screw speed and take-off of control and active materials along the extrusion process.

Table 3.3. Temperature of extruders and die zone for active film processing in the semi-industrial scale co-extrusion line

Equipment zone	Extruder B (°C)	Extruder C (°C)	(°C)
Inlet	40	40	-
Cylinder 1	190	190	-
Cylinder 2	190	190	-
Cylinder 3	200	200	-
Cylinder 4	200	200	-
Adapter to feed-block	200	200	-
Co-extrusion feed-block	-	-	200
Die temperature	-	-	210
Lip temperature	-	-	210

Table 3.4. Processing parameters of control and active bilayer films developed by cast co-extrusion

Sample	Screw speed Extruder B (rpm)	Screw speed Extruder C (rpm)	Take-off (m/min)
PP60/40a_8.5%CHL	160	66	6.3
PP30/70a_8.5%CHL	80	127	6.3
PP10/70a_8.5%CHL	30	127	5.3
PP100_2	-	160	6.3
PP80	-	145	6.3

Extruder B: raw PP feeding

Extruder C: PP active compound feeding

3.2.4.3. Monolayer active materials at lab scale with solid active agents for tray development

A preliminary lab scale extrusion process was carried out to evaluate the processability and dispersion of the active agents potassium sorbate and sodium benzoate in different polymeric matrixes (PP, EVA and PP:EVA blends).

A conical twin screw mini-extruder micro-compounder (Xplore® MC 15, 15 mL from Xplore Instruments, Geleen, The Netherlands) was used to incorporate potassium sorbate and sodium benzoate mixture in a ratio 60:40 (w/w) and at 20% (w/w) concentration in the

polymer. The selection of the ratio was based on the literature (Buranapim and Areekul, 2011).

The strand obtained from the micro-compounder was processed by compression molding by using a manual hydraulic press (Atlas 15 Ton from Specac, Orpington, England) with a heated accessory (High temperature film maker, Specac, Orpington, England). Round specimens were obtained by applying one Ton pressure to a fixed weight of polymer (0.1 g) at 180 °C for 2 min using a spacer ring between plates of 75 µm. A specific adapter was used to cool down this heated accessory to ambient temperature, coupled to a water refrigerator, with a cooling speed of 40 °C/min.

Figure 3.5 and Figure 3.6 show the extrusion and compression molding process, respectively to obtain the final test samples.

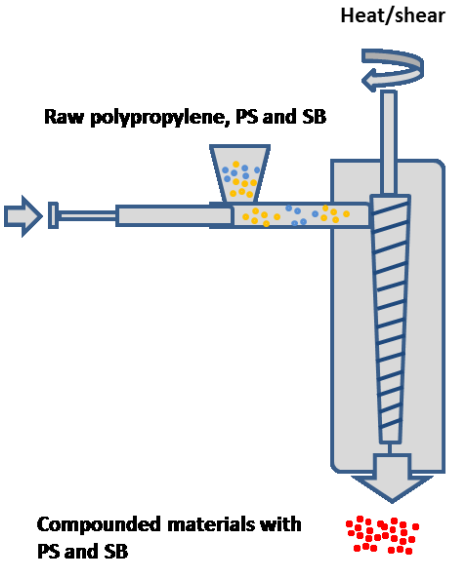


Figure 3.5. Micro-compounding of active materials containing solid antimicrobial agents (hot plate press)

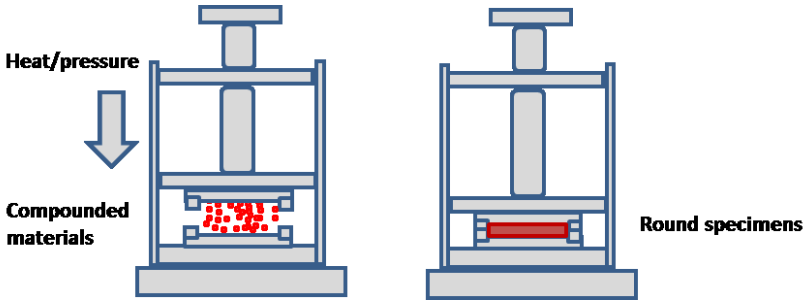


Figure 3.6. Compression molding of active materials containing solid antimicrobial agents

The extrusion processing conditions were selected for each polymer based on the technical data sheets supplied by polymer producers. The extrusion conditions such as temperature profile, screw speed, residence time and average melt temperature for each polymer or blend are shown in Table 3.5. Residence time was selected considering slightly higher residence time than those applicable at pilot/industrial scale.

Table 3.5. Processing conditions of the mini-extruder equipment set for PS:SB round specimens

Sample	Temperature profile (°C)	Screw speed (rpm)	Residence time (min)	Average melt temperature (°C)
PP-based materials	210/220/230	100	2	218
EVA-based materials	90/90/100	100	2	98
PP:EVA blends	210/220/230	100	2	207

3.2.4.4. Active tray at semi-industrial scale with solid active agents

Active trays were obtained through two extrusion steps followed by a final thermoforming step. As a first step, two different master batches with potassium sorbate or sodium benzoate were formulated and processed. The first blend consisted of PP and 10% EVA (w/w) containing 24% of potassium sorbate (w/w). A second blend was made of PP and 10% EVA (w/w) containing 16% of sodium benzoate (w/w). Control PP compounds with 10% EVA (w/w) were processed as reference materials.

These master batches were processed using a co-rotating twin screw extruder (Zsk 26Mc), equipped with a gravimetric main feeder for PP dosing (DDW-MST FW40, Brabender; Duisburg, Germany), a small gravimetric feeder for EVA dosing (Maguire MGF-8-41; Aston,

USA), and a powder gravimetric feeder for potassium sorbate and sodium benzoate feeding (DDW MD2 DSR 28-10 from Brabender, Duisburg, Germany).

Feeding was set to provide 10 kg/h for the different master batches, and processing speed was set at 500 rpm. Temperature profile along the barrel for the processing of PP:EVA master batches containing potassium sorbate or sodium benzoate are compiled in Table 3.6. This profile was applied for processing control and active compounds. The semi-industrial scale compounder scheme was already described in Figure 3.3.

Table 3.6. Temperature profile along the barrel of the co-rotating twin screw extruder set for the processing of active PP:EVA master batches containing PS or SB

Zone	1	2	3	4	5	6	7	8	9	10
Temperature (°C)	170	175	180	185	185	185	185	185	185	185

Zone 1 (hopper, PP feeding)
Zone 5 (liquid feeding)
Zone 10 (Die)

A bilayer sheet of 680 µm (nominal layer) distributed in an external layer of PP (580 µm) and an internal active layer of PP:EVA (100 µm) containing potassium sorbate and sodium benzoate were processed using a co-extrusion line, MF-EXB 600 from Dr. Collin, as described for the processing of active films in section 3.2.4.2. A bilayer control sheet with 580 µm PP external layer and 100 µm of blank PP:EVA layer was also processed as control material.

Two extruders were operated to obtain an AC structure. PP was fed using the extruder A and the two master batches (PP:10%EVA:24%PS and PP:10%EVA:16%SB) were fed through extruder C.

Processing temperatures of control and active sheet set for extruder A and C and for the die zone are indicated in Table 3.7. Table 3.8 summarizes the details of the processing parameters for control and active sheets.

The sheet was trimmed to remove edges and provide a uniform sheet of 450 mm width. Thermoforming of the sheet was carried out using a vacuum thermoforming machine (FORMECH 450, Harpenden, United Kingdom).

The sheet with approximately 680 µm nominal thicknesses was clamped in a frame and inserted into the heating chamber, previously preheated to obtain an active tray with dimensions 170x127x28 mm.

Table 3.7. Extrusion temperatures of extruders and die zone for control and active sheet processing in the semi-industrial scale co-extrusion line

Extruder zone	Extruder A (°C)	Extruder C (°C)	Die zone (°C)	
Inlet	45	45	**Co-extrusion feed-block**	210
Cylinder 1	185	185	**Die zone**	210
Cylinder 2	195	195	**Die lip**	230
Cylinder 3	200	200		
Cylinder 4	210	210		
Adapter to die	210	210		

Extruder A: raw PP feeding

Extruder C: PP active compound feeding

Table 3.8. Processing parameters of control and active bilayer sheets developed by co-extrusion

Reference	Screw speed Extruder A (rpm)	Screw speed Extruder C (rpm)	Take-off (m/min)
PP/PP:10%EVA sheet	150	50	0.8
PP/PP:10%EVA:20%PS_SB sheet	150	43	1

The thermoforming mold (Figure 3.7) was an aluminum male mold drilled for vacuum flow with uniformly distributed thermoforming holes (100 µm diameter). Thermoforming parameters were 43 s and 140 °C. Figure 3.8 shows the final appearance of control and active thermoformed trays.

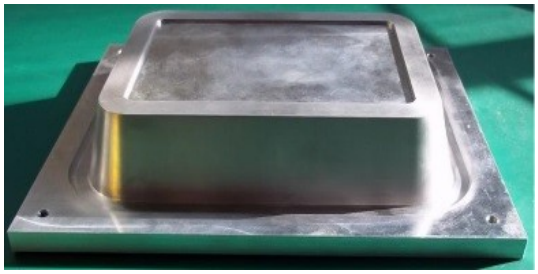


Figure 3.7. Thermoforming tray mold

Figure 3.8. Control tray (left); Active tray (right)

3.2.5. Active material characterization

3.2.5.1. Thickness and weight measurements

The thickness of the films was measured using a micrometer (MiniTest 7200 FH from ElektroPhysic, Cologne, Germany) with a sensitivity of ± 2 μm. The mean thickness and standard deviation were calculated from measurements taken at 10 different locations of each film.

Film and active sheet weight were determined by weighting 5 pieces of 10x10 cm of each film and tray bottom using a KERN 440-45N balance (KERN & Sohn GmbH (Ballingen, Germany).

3.2.5.2. Quantification of active agents in the packaging materials

3.2.5.2.1. Quantification of volatile active agents

The volatile active substances (citral, hexanal and linalool) incorporated in the active compound and active films were recovered by solvent extraction and quantified by chromatographic techniques. Active compound (0.5 g) was extracted with 10 mL of acetone in a 20 mL headspace vial. The vial was closed using a butyl/Teflon® septum and shaken at 100 rpm for 24 h at 25 ± 2 °C. A second extraction was carried out with 10 mL of fresh acetone and incubated as described for the first extraction.

For the film quantification, 6x10 cm of each film were extracted in acetone as already described for the active compounds. First and second extractions were also performed.

Separation and quantification of the active agents were performed by gas chromatography (GC), using a 7890A gas chromatograph (GC) coupled to a Triple Quad 7000C Mass detector (Agilent Technologies, Santa Clara, CA; USA). An HP-5MS column (30 m x 0.317 mm ID, 0.25µm) from Agilent Technologies was used. The volume of injection was 1 µL. The temperature program of the GC oven was set initially at 45 °C (held for 1 min) followed by a ramp of 4 °C/min to 160 °C and then a ramp of 20 °C/min to 280 °C (held for 1 min). The GC injection temperature was set at 280 °C working in split mode (split ratio 1:20). The GC-MS transfer line was set at 280 °C. High purity helium was used as carrier gas at 20 mL/min flow rate. Mass spectrum in the electron mode was generated at 70 eV and the ion source temperature was 250 °C. The quadrupole mass filter was operated at 150 °C. The MS detector was set in SIM mode at m/z of 69 for citral, 44 for hexanal and 71 for linalool.

Stock standard solutions of 1000 mg/kg were prepared by accurately weighing the required amount of each standard of citral, hexanal and linalool and dissolving them in acetone. Calibration curves were obtained by adequate dilution of the stock standard solutions. Five standard solutions of different concentration were prepared in acetone to estimate the calibration curve. Each standard solution was injected in triplicate.

The calibration curve was obtained by linear regression analysis of the chromatographic peak area versus concentration of each active agent. Slope and intercepts were estimated for each calibration curve and used to estimate the concentration of the active agents in the samples by interpolating in the linear model obtained by least squares.

The analytical method was validated in terms of linearity, repeatability, limit of detection (LOD) and limit of quantification (LOQ):

- Linearity was evaluated considering the regression coefficients (R^2) of the linear fit by least squares.
- Repeatability was evaluated by analyzing five replicates of a standard solution of 10 mg/kg of each active agent injected within the same day and batch of analysis. It was estimated as the relative standard deviation (RSD) of the five chromatographic peak areas obtained and calculated as: 100 x (standard deviation / mean value of the 5 peak area values).
- Limits of detection (LOD) and quantification (LOQ) were calculated based on the injection of ten standard solutions at levels of 1 mg/kg of each active agent. LOD

and LOQ were calculated from the standard deviation of the area obtained for each active agent in the ten injections. In particular, LOD was determined as three times the standard deviation divided by the slope of the calibration curve while LOQ was determined as ten times the standard deviation divided by the slope.

3.2.5.2.2. Quantification of solid active agents

The quantification of potassium sorbate and sodium benzoate in the active compounds and active tray was carried out by determining the ash content in the compounds and in the packaging material as described in UNE-EN ISO 3451-1:2008 standard, "Plastics - Determination of ash - Part 1: General methods (ISO 3451-1:2008)" by using a Nabertherm muffle furnace (Lilienthal, Germany).

Fifteen grams of active compound and whole trays (20 g approximately) were heated from 25 °C to 950 °C at 8 °C/min and held at 950 °C for 30 min. To correlate the ash content of the active compounds and tray with the amount of organic salts, 5 g of the potassium sorbate or sodium benzoate and 5 g of the mixture (PS:SB; 3:2) also experienced the same procedure. The evaluation of the potassium sorbate and sodium benzoate in the compound was obtained by relating the ash content of the corresponding salts to the ash of the compound. For the tray evaluation, the ash content of the active mixture was related to the ash content of the trays. Samples were analyzed in triplicate.

3.2.5.3. Thermal properties

3.2.5.3.1. Thermogravimetric analysis (TGA)

TGA analysis of active films were performed as described in section 3.2.3 for thermal stability assessment of volatile compounds.

3.2.5.3.2. Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) analysis of the packaging materials was performed by using a TA DSC Q-2000 instrument (New Castle, USA) under inert nitrogen atmosphere. Five to 10 mg of film and tray samples were accurately weighted in a Tzero aluminum pan hermetically sealed with a Tzero lid from TA instruments.

Films were analyzed using the following thermal program: fast cooling to -80 °C and holding for 4 min, then heating from -80 °C to 200 °C at a rate of 20 °C/min, cooling from 200 °C to -80 °C at a rate of 10 °C/min followed by a second heat treatment from -80 °C to 200 °C at a rate of 10°/min.

Trays were analyzed by applying a thermal ramp of initial heating from 0 °C to 200 °C at 10 °C/min, cooling from 200 °C to 0 °C at 10 °C/min and a second heating from 0 °C to 200 °C at 10 °C/min.

Melting temperature (T_m), crystallization temperature (T_c), melting enthalpy (ΔH_m) and crystallization enthalpy (ΔH_c) of control and active films and trays were evaluated with the Universal Analysis software 2000 from TA instruments (New Castle, USA). The percentage of crystallinity was calculated according to Equation 3.2 (Persico *et al.*, 2009; Ramos *et al.*, 2012):

$$\% \, crystallinity = \Delta H_m / \, (W * \Delta H_m^0 \, * \, 100) \qquad \textbf{Equation 3.2}$$

where W is the PP weight fraction in the sample and ΔH_m^0 is the theoretical latent heat of fusion for 100% crystalline PP set in 165 J/g (Zhu, 2002).

3.2.5.4. Visual appearance

Changes in the visual appearance of the films was evaluated by photographic inspection placing a logo behind the control and active films using an EOS 500D Camera from Canon coupled with a flash (Speedlite 430EX II, Canon, Tokyo, Japan).

3.2.5.5. Mechanical properties

Tensile tests were carried out by using a Universal Testing machine (Testometric M-350, Lancashire, United Kingdom) equipped with a 1000 N load cell and manual screw action grips. Samples were cut in 10 mm width and 150 mm length according to standard ISO 527-1:2012 Plastics-Determination of tensile properties - Part 1: General principles. Grips had an initial separation of 100 mm and crosshead speed was set to 300 mm/min.

Tensile strength (force per unit area applied when the film is broken), elongation at break (percentage of change in the film length when the film is broken after a certain level of force)

and Young Modulus or elastic modulus (force per unit area necessary to increase the length of a film sample to a specific extent) of the films were evaluated. Results were the average of ten measurements ± standard deviation.

Compression tests of the trays were carried out by using the same Universal Testing machine equipped with flat plates with a preload of 1 N, based on the standard ASTM D 642 "Test method for determining compressive resistance of shipping containers". This test was performed to evaluate the behavior of the tray to compressive forces that simulate the stacking of the trays during storage. The speed compression was set at 15 mm/min and the test was stopped at 15 mm of plate separation to avoid load cell damage. Maximum force and deformation at the point of maximum load were evaluated. Results were the average of ten measurements ± standard deviation.

3.2.5.6. Sealing properties

The heat sealability between active and control monolayer films developed at lab scale was evaluated by using a Gradient Laboratory Heat Sealer RDM HSE-3 from MOCON (Minneapolis, USA). Temperatures tested ranged from 125 °C to 175 °C. Dwell time and pressure were set at 0.5 sec and 31.4 psi, respectively. Correct sealing was judged when the two sealed materials could not be manually separated.

The heat sealability of materials developed at semi-industrial scale were evaluated using a semi-automatic tray sealing machine Smart 300 (Ulma, Oñati, Spain). The temperatures tested ranged from 150 to 200 °C and the sealing time was set at 3.5 sec. Optimum sealing was determined when the film could not be manually separated from the tray.

3.2.5.7. Optical properties

Surface reflectance spectra of the control and active films and trays developed at semi-industrial scale was determined from 400 to 700 nm with a spectrocolorimeter (CM-2600d, Minolta Co., Tokyo, Japan) on both a white and a black background.

Transparency was determined by applying the Kubelka–Munk theory (Hutchings, 1999) by means of the internal transmittance (Ti) of the packaging materials according to Equation 3.3. In this equation, R_0 is the reflectance of the film on an ideal black background. Parameters a and b were calculated in Equation 3.4 and Equation 3.5, respectively where R

is the reflectance of the sample layer backed by an ideal white background and Rg is a known reflectance on a white background. Measurements were carried out in triplicate for each sample.

$$Ti = \sqrt{(a - R_0)^2 - b^2} \qquad \textbf{Equation 3.3}$$

$$a = \frac{1}{2}\left(R + \frac{R_0 - R + R_g}{R_0 \cdot R_g}\right) \qquad \textbf{Equation 3.4}$$

$$b = (a^2 - 1)^{1/2} \qquad \textbf{Equation 3.5}$$

3.2.5.8. Structural properties

The microstructure of bilayer films and trays developed at semi-industrial scale was analyzed by using light microscopy. For cross-sectional detail, samples were cut transversally into 70 µm layers using a microtome (RM 2145 from Leica, Wetzlar, Germany) and mounted onto slides. For top visualization, samples were cut longitudinally into 2x1 cm sections.

Light microscopy was performed using a DMLM light microscope from Leica (Wetzlar, Germany) in transmission mode with Leica 10x/0.25 N PLAN objectives. Micrographs of random areas of the samples were acquired at 2088x1550 pixel resolution.

3.2.5.9. Barrier properties

3.2.5.9.1. Oxygen transmission rate

Oxygen transmission rate (OTR) of bilayer control and active films developed at semi-industrial scale was carried out using an OX-TRAN equipment from MOCON (Model 2/21 ST, Minneapolis, USA). The specimen was pre-conditioned for 1 h. The test was performed according to the standard ASTM D3985 "Standard test method for oxygen gas transmission rate through plastic film and sheeting using a coulometric sensor" at 23 ± 0.5 °C, 0 ± 0.5% relative humidity (RH) and 100% oxygen permeant concentration. The area of the sample was 100 cm^2. Measurements were carried out in quadruplicate for each sample.

OTR of control and active trays were carried out using also an OX-TRAN 2/21 ST. Trays were hermetically sealed to an aluminum base, which was crossed by two sealed tubes that circulated the gas carrier carrying permeating oxygen molecules into the tub to the measuring sensor. The exposed area was the complete tray (170x127x28 mm). The test was

performed based on the standard ASTM F1307 "Standard test method for oxygen transmission rate through dry packages using a coulometric sensor" at 23 ± 2 °C, 50 ± 5% RH and 21% oxygen permeant concentration (ambient air). Measurements were carried out in quadruplicate for each sample.

3.2.5.9.2. Water vapor transmission rate

Water vapor transmission rate (WVTR) of control and active bilayer films developed at semi-industrial was performed according to the standard ASTM E96 "Standard test methods for water vapor transmission of materials". Active films were attached with foil tape to a perforated jar lid in such a manner that the diameter of the perforation (1.92 cm) defined the area of the film exposed to the water vapor. Three glass jars of 30 mL volume, containing about 15 g of calcium chloride previously dried for 4 h at 240 °C and two reference containers (jars with sample and without calcium chloride), were tightly closed to the mounted lids and placed in a cabinet at 5 ± 2 °C and 100% of RH. Samples were continually weighed until constant weight was reached.

Water vapor transmission rate of control and active trays (170x127x28 mm) were carried out using a PERMATRAN-W instrument 3/33 Plus from MOCON (Minneapolis, USA). This instrument sets the standard for water vapor transmission rate testing of finished packages. Trays were hermetically sealed to an aluminum base which was crossed by two copper tubes that lead the carrier gas that collected the water vapor molecules permeating the tray to the measuring sensor. The test was performed at 23 ± 2 °C and a difference in RH (ΔRH) of 90 ± 5%.

3.2.6. Evaluation of active agents release kinetics from active packaging materials

3.2.6.1. Release kinetics of volatile active agents from multilayer active film

The release kinetics of volatile active agents from the bilayer active film developed at semi-industrial scale were studied at 4 ± 2 °C and 25 ± 2 °C.

For the release test carried out at refrigeration temperature, 4 ± 2 °C and 90 ± 5% RH, the active film was cut into small pieces (3x4 cm, 0.25 ± 0.02 g) and were weighed in a KERN 440-45N balance. The film pieces were placed on a glass tray with the active side up and

introduced inside a chamber with controlled RH and temperature. The RH was adjusted by using a humidified air flow (500 mL/min) through a chamber of 216 L. Air was humidified by bubbling air in a tray containing water. Temperature and RH were registered using a hygrometer (HR EBI 20-TH1 from Ebro Logger, Ingolstadt, Germany). Figure 3.9 depicts a schematic representation of the system developed to evaluate the release of the active agents to the atmosphere. Three pieces of film were removed from the chamber each time at different intervals for 38 days, and the remaining citral, hexanal and linalool were determined by solvent extraction followed by gas chromatography as described in section 3.2.5.2.1.

The release kinetics of active volatile active agents from the active film was also studied at 25 ± 2 °C and 50 ± 5% RH. The film was cut into pieces (6x10 cm) and were weighed and placed on a tray with the active side up in a laboratory bench. Temperature and RH were registered using a hygrometer. Two pieces of film were taken each time at different intervals for 28 days, and the remaining active agents, citral, hexanal and linalool were determined by solvent extraction followed by gas chromatography as described in section 3.2.5.2.1.

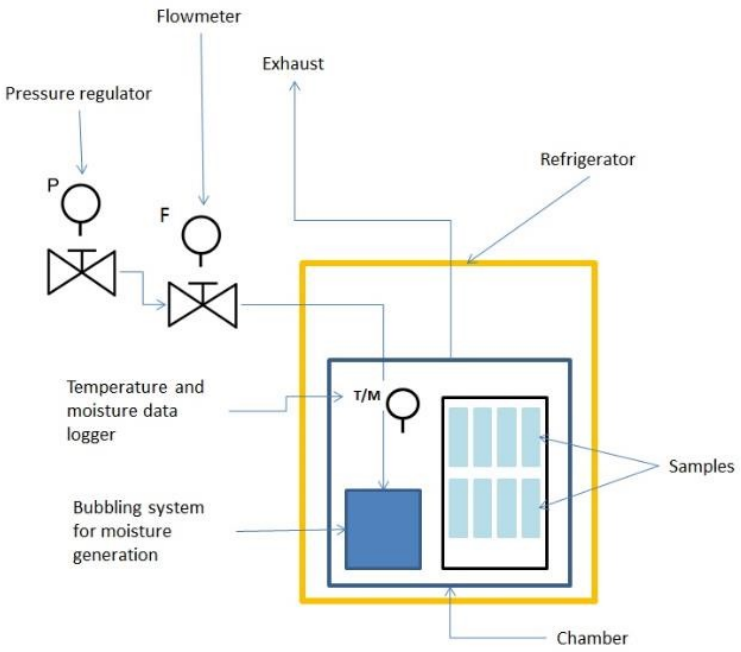


Figure 3.9. Schematic representation of the system developed to evaluate the release kinetics of volatile agents from active films

3.2.6.2. Release kinetics of non-volatile agents from monolayer and bilayer active materials

Active materials were cut in circles of 2.5 cm diameter. Samples were weighed accurately and the samples thickness measured with a micrometer (MiniTest 7200 FH from ElektroPhysic, Cologne, Germany). The releasing test was carried out in a closed Erlenmeyer with 50 mL of an aqueous solution of 3% (w/v) acetic acid continuously shaken on an orbital shaker (IKA KS 130 Basic, IKA, Staufen, Germany), and placed inside an incubator at 4 ± 2 °C. Three pieces of monolayer samples were immersed in the food simulant. For the active trays, samples from the tray bottom were taken and one piece was immersed in the food simulant. The test was performed in duplicate. In order to follow the release kinetics of potassium sorbate and sodium benzoate from the active materials, aliquots of 2 mL were withdrawn from the solution medium at various time intervals over 10 days for monolayer materials and 36 days for the active tray. The withdrawn volume was refilled each time with 2 mL of new 3% (w/v) acetic acid.

To quantify the amount of potassium sorbate and sodium benzoate released, aliquots were passed through 0.22 µm nylon filters and analyzed by High performance liquid chromatography (HPLC) from Waters equipped with a separation module (model 2659) and a photodiode array detector module (model 2998). The chromatographic column used was an Atlantis T3 (3 µm, 4.6x150 mm) from Waters (Barcelona, Spain). The method was based on that of Pylypiw Jr and Grether (2000). The mobile phase was acetate buffer (pH 4.3):acetonitrile (85:15) (v/v) with a flow rate of 0.8 mL/min at 25 °C. Peaks were detected by measurement of absorbance at 227 nm for sodium benzoate and 260 nm for potassium sorbate.

Stock standard solutions of 1000 mg/kg were prepared by accurately weighing the required amount of each standard of sodium benzoate and potassium sorbate and dissolving them in methanol. Calibration curves for each analyte were obtained by adequate dilution of the stock standard solutions. Five standard solutions of different concentration were prepared in methanol to estimate the calibration curve. Each standard solution was injected in triplicate.

The calibration curve and the validation of the method in terms of linearity, repeatability, limit of detection (LOD) and limit of quantification (LOQ) was carried out as described in

section 3.2.5.2.1. Specifically, LOD and LOQ were calculated based on the injection of ten standard solutions at levels of 0.4 mg/kg of each active agent.

3.2.6.3. Mathematical modeling of the active agents from the packaging materials

The diffusion of the active agents was modeled based on Fick's Second Law:

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} \qquad \textbf{Equation 3.6}$$

In particular, the diffusion coefficients (D) of the active agents released from the active materials were estimated by adjusting the experimental data obtained as described above to a mathematical solution of Fick's Second Law.

It should be considered that the release of active agents was determined by quantifying the amount of active agent remaining in the packaging material over time or by quantifying the amount of active agent in the media (food simulant) over time in contact with the packaging material. For the first case, Equation 3.7 was used to estimate the D values while Equation 3.8 was used for the second case:

$$\frac{m^t_{packaging\ material} - m^{eq}_{packaging\ material}}{m^0_{packaging\ material} - m^{eq}_{packaging\ material}} = \sum_{n=1}^{\infty} \frac{2\alpha\ (1+\alpha)}{1+\alpha+\alpha^2 q_n^2} \exp\left[\frac{-4Dq_n^2 t}{L^2_{packaging\ material}}\right] \qquad \textbf{Equation 3.7}$$

$$\frac{m^t_{simulant} - m^0_{simulant}}{m^{eq}_{simulant} - m^0_{simulant}} = 1 - \sum_{n=1}^{\infty} \frac{2\alpha\ (1+\alpha)}{1+\alpha+\alpha^2 q_n^2} \exp\left[\frac{-4Dq_n^2 t}{L^2_{packaging\ material}}\right] \qquad \textbf{Equation 3.8}$$

where in Equation 3.7, $m^0_{packaging\_material}$, $m^{eq}_{packaging\_material}$, $m^t_{packaging\_material}$, are the quantities of active substance at initial, final (or equilibrium) and at any time, t, in the packaging material, respectively. In Equation 3.8, $m^0_{simulant}$, $m^{eq}_{simulant}$, $m^t_{simulant}$, are the quantity of active substance at initial, final (or equilibrium) and at any time, t, in the food simulant, respectively.

Other parameters present in both equations are the thickness of the packaging materials releasing the active substances ($L_{packaging\_material}$); the diffusion coefficient (to be estimated)

for each substance through the packaging material (*D*); the time (*t*) and the non-zero positive roots (q_n values) of the following equation:

$$tan(q_n) = -\alpha \cdot q_n \qquad \textbf{Equation 3.9}$$

where, α is calculated from the percentage of substance migrated from the active material at equilibrium or after infinite time as shown in Equation 3.10:

$$\alpha = \frac{\left[\frac{migration\ (\%)}{100}\right]}{1 - \left[\frac{migration\ (\%)}{100}\right]} \qquad \textbf{Equation 3.10}$$

The application of Equation 3.7 and Equation 3.8 supposes the assumption of the following hypotheses and conditions:

1) A constant diffusivity is assumed whatever the active agent concentration in the material and in the external media.

2) The rate at which the diffusing substance leaves the material is constantly equal to the rate at which it enters the external media.

3) The external media is well stirred, then the concentration in the external media depends only on time.

4) In the case of monolayer materials, the release of the active agents is unidirectional towards both sides of the material. In this case, L is considered the real thickness of the active layer. In the case of bilayer materials, the release of the active agents is unidirectional towards one side of the material. In this case, L is considered as double the real thickness.

5) The external media cannot be considered of limited volume considering the experimental design for the study of the release of the active agents from the packaging materials (3.2.6.1 and 3.2.6.2). Complete migration is expected after infinite time. In the case of the volatile active agents, their concentration is considered as null because the air flow blown through the chamber would enable

a purge of the air at each time, t. In the case of non-volatiles, the food simulant is continuously renewed with new simulant, which forces the complete migration by displacing the system towards the release. Therefore, in both situations, the equilibrium conditions are continuously perturbed and not really achieved. This can be solved if α values are made equal to 99, which corresponds to 99% migration being expected at the end of the release tests.

Equation 3.7 and Equation 3.8 need to be solved to estimate *D*. In this regard, it is quite usual in the practice to simplify these equations based on several approaches and to take a few q_n values that are tabulated for some α values. In this Thesis, Equation 3.7 to Equation 3.10 were used as shown by developing some routines made in house using MATLAB software (Version 5.3.0.10183 (R11), ©The Mathwoks Inc., Natick, MA).

The routines developed are able to calculate as many q_n values as desired for any α value. In this case, the number of q roots were fixed at 15. It was verified that the introduction of more q roots did not improve the D estimations.

D was estimated by an iterative process that finds the *D* value that gives the best fit between the observed (experimental) and modelled kinetic data. The fit was determined as root mean square error (RMSE), which was calculated as follows in Equation 3.11:

$$RMSE = \sqrt{\frac{(\hat{y} - y)^2}{(N - p)}} \qquad \textbf{Equation 3.11}$$

where y and $\hat{y}$ are the experimental and predicted fractional release of the active agents respectively, as expressed in Equation 3.7 and Equation 3.8. N is the number of measurements, and p the number of identified parameters.

3.2.7. Evaluation of antimicrobial properties of active materials *in vitro*

3.2.7.1. Antimicrobial effectiveness of active films with volatile active agents

The antimicrobial effectiveness of active films was evaluated in vapor phase diffusion at 4 ± 2 °C and 25 ± 2 °C. MEA Petri dishes were plated with 50 µL of *Saccharomyces cerevisiae*, *Aspergillus niger* or *Penicillium aurantiogriseum* inoculum prepared as described in section

3.2.1.1. Pieces of monolayer active films (5x5 cm) were stacked on the lid of the Petri dish which were were sealed with Parafilm®

For the test carried out at 25 °C, the inoculated plates with yeast and molds were incubated for 7 and 10 days, respectively. The test was performed in triplicate for the yeast and in duplicate for the molds. The inoculated plates exposed at 4 °C were incubated for 12 days and the test was performed in triplicate. Inoculated Petri dishes exposed to control film in the absence of active agents were also evaluated as control samples.

During the exposure period, plates incubated at 25 °C were evaluated by visual inspection in order to quantify the degree of microbial growth. After the exposure period at 4 °C, the agar was collected and re-plated according to the method described by Lara-Lledó *et al.* (2013). For this purpose, the inoculated agar was recovered with a sterile spatula and homogenized with 50 mL of peptone water in 178×308 mm stomacher bags (Bag Filter®, Interscience, St-Nom, France) for 2 min using a Stomacher (Bagmixer® 400W, Interscience, St-Nom, France). Appropriate serial dilutions were carried out in sterile 0.1% (w/v) peptone water and plated in duplicate on MEA plates using an Automatic spiral plater (easySpiral Pro®, Interscience, Saint Nom, France). Then, the plates were incubated for 3 days at 25 °C and the colonies were counted with digital colony counter S from J. P. Selecta (Barcelona, Spain).

3.2.7.2. Antimicrobial effectiveness of preliminary monolayer active materials for active tray development

The antimicrobial activity of the EVA:20%PS_SB and PP:20%PS_SB monolayer materials developed at lab scale was evaluated against the target microorganisms by direct contact in liquid media at 25 ± 2 °C.

One disk of 2.38 cm diameter was introduced into the tubes containing 5 mL of PDB and 50 µL of *Saccharomyces cerevisiae*, *Aspergillus niger* or *Penicillium aurantiogriseum* inoculum. The tubes were incubated for 3 days at 25 °C. The area of exposure of the specimens corresponded to 2.5 and 2.2 mg/mL of PS:SB mixture for PP and EVA specimens, respectively, and represented the maximum concentration that could be released into the liquid media. PP and EVA specimens without active agents were also evaluated as reference materials. A positive control with only microorganisms was also added to the test. After the

exposure period, appropriate serial dilutions were carried out in sterile 0.1% (w/v) peptone water and plated and incubated as described in section 3.2.7.1. The test was performed in triplicate.

3.2.7.3. Antimicrobial effectiveness of active tray with solid antimicrobial agents

The antimicrobial effectiveness of the active trays against the target microorganisms was evaluated in liquid media. The active and the control trays were UV exposed for 1 min using a UV chamber ELC 500 from Edmund optics (New Jersey, USA) and filled with 100 mL of PDB. The broth was inoculated with 1 mL of the inoculum suspension of *Saccharomyces cerevisiae*, *Aspergillus niger* or *Penicillium aurantiogriseum* prepared as described in section 3.2.1.1. The trays were sealed with a PP control film in a semi-automatic tray sealing machine and incubated at 25 ± 2 °C for 7 days or at 4 ± 2 °C for 12 days. Samples were evaluated in triplicate.

After the incubation period, the trays were opened and the inoculated broth was poured in a stomacher bag and mixed for 2 min using a Stomacher to properly mix the mycelial growth. Appropriate serial dilutions were carried out in sterile 0.1% (w/v) peptone water and plated in duplicate on MEA plates using an Automatic spiral plater. Then, the plates were incubated at 25 ± 2 °C for 3 days and the colonies were counted. The test was performed in triplicate.

A gravimetric method was also performed after the incubation period at 4 ± 2 °C based on the method described by Córdova-López *et al.* (1996). The inoculated broth exposed to active and control trays was filtered with a filter paper previously desiccated at 65 °C for 1 h in an incubator. The filtered broth was placed in a Petri dish previously conditioned and the samples were weighted. Samples were covered with aluminum foil with few perforations and incubated at 65 °C for 24 h. After that, samples were conditioned in a desiccator for 0.5 h and weighed. Differences between samples before and after drying were considered the microbial mass able to grow in contact with control and active trays.

3.2.8. Shelf life of minimally processed orange and pineapple

3.2.8.1. Fruit samples, packaging process and storage conditions

Disinfected, peeled and cut pieces of pineapple (Gold Madura or MD2 cultivar) and orange (Navel, Lane-late or Navelina cultivar) were supplied in 2 kg trays by Cooperativa Benaguasil

(Benaguasil, Spain). The fruit was re-packed in the semi-industrial active and control packaging materials, developed in the present research, using a semi-automatic tray sealing machine (Smart 300) under aerobic conditions. Trays were previously UV exposed for 1 min in a UV chamber and then filled with 150 ± 15 g of peeled and cut orange or pineapple pieces. Filled trays were sealed with the multilayer films developed.

All the films were micro-perforated with two holes of 250 µm to allow gas exchange with the environment and avoid anaerobic conditions of the fruit. Packed samples were stored at 4 ± 2 °C for the initial 7 days followed by 8 ± 2 °C for the last days of storage to simulate abuse temperatures that can occur along the supply chain.

3.2.8.2. Antimicrobial activity of films on the microflora of minimally processed fruit

The microbial evolution of the packed fruit was evaluated during storage at 4/8 °C. Packed fruit was removed from the packages and blended with 150 mL of 0.1% peptone water (w/v) for 60 sec in a Stomacher. Additional dilutions were made in 0.1% peptone (w/v). Then, 50 µL of the undiluted homogenate and the subsequent dilutions were spread in duplicate in agar plates by using am Automatic spiral plater. PCA was used for enumerating total mesophilic aerobic counts and psychrotrophic bacteria whilst supplemented Sabouraud agar with oxytetracycline was used for molds and yeasts counting. PCA was incubated at 30 ± 2 °C for 48 h and at 4 ± 2 °C for 10 days for mesophilic bacteria and psychrotrophic bacteria, respectively, and supplemented Sabouraud agar was incubated at 25 ± 2 °C for 3 days. Samples were evaluated in triplicate during storage of the fruit (from 0 to 12 days) at different sampling days.

3.2.8.3. Evolution of active agent concentrations in the packaging materials

The remaining active agents in the active packaging materials was evaluated during the storage of the packed fruit. Volatile active agents, citral, hexanal and linalool, remaining in the active lid were evaluated at day 0 (packaging day), and days 3, 7, 10 and 12. The potassium sorbate and sodium benzoate remaining in the active tray were measured at day 0, 6 and 12. Each sample was analyzed in triplicate.

The concentration of citral, hexanal and linalool remaining in the active film was evaluated by solvent extraction with acetone followed by GC-MS analysis as described in 3.2.5.2.1. The

total amount of potassium sorbate and sodium benzoate was evaluated by muffle furnace as described in section 3.2.5.2.2.

3.2.8.4. Evolution of headspace composition

The O_2 and CO_2 concentration (% v/v) in the headspace of packed pineapple and orange samples was monitored frequently during storage using a portable gas analyzer (Check Mate II from PBI Dansensor, Barcelona, Spain). A given volume of the headspace was withdrawn by injecting a needle through a self-adhesive septum attached to the top film. Five replicates were evaluated during storage of the fruit (from 0 to 12 days) at different sampling days.

3.2.8.5. Juice leakage

Juice leakage from pineapple and orange pieces packed with control and active packaging materials was measured by making a 0.5 cm hole in the packaging lid and placing the packaging in an upright position on top of a cylinder (60 mL). Accumulated liquid was recovered for 5 min. The juice was weighed using a balance. Samples were tested in triplicate during storage of the fruit (from 0 to 12 days) at different sampling days.

3.2.8.6. pH

The pH of the pineapple and orange packed with control and active packaging materials was evaluated during storage of the fruit (from 0 to 12 days) at different sampling days. Fruit pieces were crushed and homogenized with a mixer (Hand Blender BAPI 750 from Taurus, Barcelona, Spain). Measurements were made using a pH meter (Basic 20+ from Crison, Barcelona, Spain). Samples were evaluated in triplicate during storage of the fruit (from 0 to 12 days) at different sampling days.

3.2.8.7. Total soluble solids (TSS)

Changes in TSS of pineapple and orange packed with control and active packaging materials were evaluated during storage of the fruit. To that, orange and pineapple pieces were smashed and homogenized using a mixer (Hand Blender BAPI 750 from Taurus, Barcelona, Spain). The released juice was directly measured in a digital refractometer (PAL-3, Atago, Tokyo, Japan). Samples were evaluated in triplicate during storage of the fruit (from 0 to 12 days) at different sampling days.

3.2.8.8. Sensory evaluation

A sensory evaluation test was carried out by a panel of 5 male and 5 female consumers aged between 27 and 36 years. The panelists scored the color, general appearance and odor perception of peeled and cut pineapple and orange packed in control and active packaging materials during storage of the fruit from 0 to 12 days. Ratings were based on a 5-point hedonic scale where 5 was very good, 4 was good, 3 was fair (limit of marketability), 2 was poor and 1 was bad (unusable).

A visual comparison between the fruit packed in control and active packaging materials was performed during storage by photography using an EOS 500D camera from Canon coupled with a Canon Speedlite 430EX II flash (Tokyo, Japan).

3.2.9. Food contact materials compliance

3.2.9.1. Overall migration tests in food simulants

The overall migration of the control and active packaging materials into food simulants was evaluated under standardised testing conditions according to the European Regulation (EU) No. 10/2011, which establishes specific requirements for plastic materials and articles intended to come into contact with food. Specifically, the overall migration test was carried out with food simulant B (3% (w/v) acetic acid) and food simulant C (20% (v/v) ethanol) during 10 days of contact at 20 ± 2 °C (OM1) in an incubator (FED 240 from Binder, Tuttlingen, Germany).

The overall migration of the control and active films at semi-industrial scale were performed by migration cell according to the European Standard EN 1186-5:2002, part 5 “Test methods for overall migration into aqueous food simulants by cell”. Active and control films (1.76 dm^2) were mounted on a migration cell (MC 60 from Fabes, Munich, Germany) (Figure 3.10) where the active layer was in contact with 90 mL of food simulant. Three samples and two blank simulants were evaluated for each material.

The overall migration of the control and active trays was performed by total immersion in 150 mL glass tubes from Afora (Barcelona, Spain) (Figure 3.10), according the European Standard EN 1186-3:2002, part 3 “Test methods for overall migration into aqueous food simulants by total immersion”. Packaging material (1 dm^2) was submerged into 100 mL of

food simulant and the glass tube was tightly closed. Three samples and two blank simulants were evaluated for each material.

Figure 3.10. Cell migration (Left picture); Tube migration (right picture)

After the contact period, the tested samples were removed and the food simulants were poured into a DURAN migration capsules (Scharlab, Barcelona, Spain) and evaporated at 180 °C in an orbital Rotatherm from Selecta (Barcelona, Spain) until constant weight was reached. The overall migration was calculated according Equation 3.12:

$$M = \frac{(m_{\ sample} - m_{\ blank})\ x\ 1000}{S} \quad \textbf{Equation 3.12}$$

where m_{sample} is the dry residue (g) after simulant evaporation in contact with the materials; m_{blank} is the dry residue from blank simulant and S (dm^2) is the area of exposure of the material.

After the overall migration test of active tray, the dry residue resulting from overall migration was redissolved in 100 mL of the corresponding food simulant, B or C, and analyzed by HPLC-UV as described in section 3.2.6.2.

3.2.9.2. Specific migration test in food simulants

The specific migration of the active agents incorporated in the active packaging materials was evaluated into food simulants and under standardised testing conditions according to

the plastic Regulation (EU) No. 10/2011 and the European Standard EN 13130-1:2005 on test methods for the specific migration of substances from plastics to foods and food simulants. The specific migration of the films was performed by cell migration as described in section 3.2.9.1. The specific migration of the active tray was performed by total immersion as also described in section 3.2.9.1. In this case, 1 dm^2 of tray was used.

The specific migration tests of citral, hexanal and linalool incorporated in the active films developed at semi-industrial scale were carried out in food simulant C (ethanol 20% (v/v)) for 10 days of contact at 20 ± 2 °C. The specific migration of potassium sorbate and sodium benzoate incorporated in the active tray was carried in food simulant B (3% (w/v) acetic acid) and C (20% (v/v) ethanol) for 10 days of contact at 20 ± 2 °C.

After the contact period, the tested materials were removed and the food simulants in contact with the film were analysed by GC-MS while the simulants in contact with the tray were analyzed by HPLC-UV as described in section 3.2.5.2.1 and section 3.2.6.2, respectively.

For the expression of migration test results, according to Article 17 of the Regulation (EU) No. 10/2011, the value of migration was expressed in mg/kg applying a surface to volume ratio of 6 dm^2 per kg of food.

The exposure of active agents from the migration and the fruit storage test was calculated according to Equation 3.13 and expressed in mg/kg body weight (bw)/day considering an average of 60 kg bw/person.

$$Exposure = migration\left(\frac{mg\ active}{kg\ simulant\ or\ fruit}\right) x\ fruit\ consumption\left(\frac{kg\ fruit}{person}\right) x\ \left(\frac{person}{60\ kg\ bw}\right) \quad \textbf{Equation 3.13}$$

3.2.10. Statistical analysis

Statistical analyses of the results were performed with Statgraphics Plus version 2.0 (Warrenton, Virginia, USA). One-way analysis of variance (ANOVA) was carried out. Differences between means were evaluated on the basis of confidence intervals using Tukey's test at $p \leq 0.05$ significance level.

4. RESULTS AND DISCUSSION

4.1. EVALUATION AND SELECTION OF ANTIMICROBIAL SUBSTANCES FOR ACTIVE MATERIAL DEVELOPMENT

4.1.1. Selection of active agents

An initial selection of antimicrobial agents was carried out in order to select the most promising ones to be further incorporated in the active packaging materials. This selection was performed according to the literature data (presented in the introduction of this Thesis) in terms of antimicrobial activity in fruit products, origin (given preference to natural or those that have been already used in food), organoleptic compatibility with the fruit product, processability and also the current European legislation on food.

The selected antimicrobials included the following volatile (flavoring) and non-volatile (food preservative) agents:

- Volatiles: citral (FL No. 05.020), hexanal (FL No. 05.008), linalool (FL No. 02.013), limonene (FL No. 01.001), orange essential EO (90-95% limonene, 0.2-0.5 linalool, < 0.05 % citronellol and < 0.05% geraniol) orange liquid flavoring (80-90% limonene), mandarin (85-90% limonene, 0.05-0.1% linalool) and pineapple essence.
- Non-volatiles: potassium sorbate (E-202) and sodium benzoate (E-211).

4.1.2. Study of antimicrobial effectiveness of the volatile active agents in the vapor phase

The evaluation of the antimicrobial activity of the volatile selected agents was carried out against some species of yeast and molds such as *Saccharomyces cerevisiae*, *Penicillium aurantiogriseum* and *Aspergillus niger* in the vapor phase. Microbial cultures were prepared as described in 3.2.1.1. To this end, a range between 1 to 30 µL of the selected antimicrobials were individually added to a paper disk which was placed in the lid of MEA Petri dishes and incubated at 25 ± 2 °C over a period of 5 days as described in 3.2.1.2. Inoculated Petri dishes without antimicrobials and non-inoculated Petri dishes with paper disks without antimicrobials were included as positive and negative controls, respectively.

Table 4.1 shows the antimicrobial effect of the selected antimicrobials in the vapor phase evaluated by measuring the diameter of the inhibition halo (mm). From this test, the

reduction concentration (RC) (lowest concentration resulting in a visible growth reduction density), the minimum inhibitory concentration (MIC) (lowest concentration showing an inhibition halo) and the maximum concentration of the antimicrobials (Cmax) (concentration that yielded the highest inhibition area) were evaluated in terms of µL active agent/Petri dish.

The evaluated active agents differed significantly in their antimicrobial activity and a general dose-dependent effect was observed. In general, citral, hexanal and linalool showed strong activity against *Saccharomyces cerevisiae*, *Penicillium aurantiogriseum* and *Aspergillus niger.*

The MIC of citral against the 3 target microorganisms corresponded to the lowest concentration tested (1 µL/Petri dish). The MIC observed for hexanal against *Saccharomyces cerevisiae* and *Penicillium aurantiogriseum* was also the lowest concentration tested for this antimicrobial (1 µL/Petri dish). However, a higher MIC of hexanal, 2.5 µL/Petri dish, was observed for *Aspergillus niger* with a RC of 1 µL/Petri dish.

Compared to citral and hexanal, linalool exerted lower antimicrobial activity with a MIC of 5 µL/Petri dish for *Saccharomyces cerevisiae* and 7.5 µL/Petri dish for *Penicillium aurantiogriseum* and *Aspergillus niger.* For molds, an RC was also set at 5 µL/Petri dish of linalool.

Regarding Cmax values, and in agreement with the pattern observed for the MIC values, the lowest amount of citral, hexanal and linalool that yielded the highest inhibition area (90 mm) increased in the following order: citral (5 µL/Petri dish) < hexanal (5-10 µL/Petri dish) < linalool (7.5-15 µL/Petri dish). According to these values, citral showed the same effectiveness against the three microorganisms, while hexanal was more effective against molds. In the case of linalool, the highest effect was observed against *Aspergillus niger*.

Inhibition halos were observed for the three amounts of limonene and orange essential oil tested against *Saccharomyces cerevisiae*, establishing 10 µL/Petri dish as the MIC.

Moreover, a visible density reduction in the growth of *Penicillium aurantiogriseum and Aspergillus niger* took place in presence of orange liquid flavoring for the amounts tested.

Table 4.1. ***In vitro*** **antimicrobial effect of volatile agents in the vapor phase after 5 days at 25 °C**

Active agent	µL/Petri dish	Inhibition halo (mm ± SD)		
		Saccharomyces cerevisiae	***Penicillium aurantiogriseum***	***Aspergillus niger***
Control	---	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Citral	1	25.0 ± 2.8 (MIC)	34.3 ± 1.6 (MIC)	19.8 ± 3.3 (MIC)
	2.5	84.5 ± 0.9	46.7 ± 3.5	37.0 ± 12.0
	5	90.0 ± 0.0 (Cmax)	90.0 ± 0.0 (Cmax)	90.0 ± 0.0 (Cmax)
	7.5	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0
	10	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0
	15	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0
	20	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0
	30	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0
Hexanal	1	26.7 ± 8.8 (MIC)	25.7 ± 4.5 (MIC)	21.2 ± 1.0 (RC, growth reduction halo)
	2.5	39.8 ± 1.1	46.5 ± 6.6	29.5 ± 1.3 (MIC)
	5	53.8 ± 2.5	90.0 ± 0.0 (Cmax)	90.0 ± 0.0 (Cmax)
	7.5	61.7 ± 8.5	90.0 ± 0.0	90.0 ± 0.0
	10	90.0 ± 0.0 (Cmax)	90.0 ± 0.0	90.0 ± 0.0
	15	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0
	20	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0
	30	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0
Linalool	1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	2.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	5	18.3 ± 2.9 (MIC)	Growth reduction (RC)	Growth reduction (RC)
	7.5	20.7 ± 0.8	25.8 ± 0.2 (MIC)	90.0 ± 0.0 (MIC; Cmax)
	10	35.0 ± 1.5	28.8 ± 2.4	90.0 ± 0.0

Active agent	µL/Petri dish	Inhibition halo (mm ± SD)		
		Saccharomyces cerevisiae	*Penicillium aurantiogriseum*	*Aspergillus niger*
	15	90.0 ± 0.0 (Cmax)	90.0 ± 0.0 (Cmax)	90.0 ± 0.0
	20	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0
	30	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0
Limonene	10	22.5 ± 2.3 (MIC)	0.0 ± 0.0	0.0 ± 0.0
	20	27.1 ± 0.5	0.0 ± 0.0	0.0 ± 0.0
	30	29.0 ± 7.4	0.0 ± 0.0	Growth reduction (RC)
Orange essential oil	10	10.6 ± 2.8 (MIC)	0.0 ± 0.0	Growth reduction (RC)
	20	18.2 ± 4.3	0.0 ± 0.0	Growth reduction
	30	16.7 ± 7.7	0.0 ± 0.0	Growth reduction
Orange liquid flavoring	10	0.0 ± 0.0	Growth reduction (RC)	Growth reduction (RC)
	20	0.0 ± 0.0	Growth reduction	Growth reduction
	30	0.0 ± 0.0	Growth reduction	Growth reduction
Mandarin essence	10	0.0 ± 0.0	0.0 ± 0.0	Growth reduction (RC)
	20	0.0 ± 0.0	0.0 ± 0.0	Growth reduction
	30	0.0 ± 0.0	0.0 ± 0.0	Growth reduction
Pineapple essence	10	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	30	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Retraction in the growth of *Aspergillus niger* was also observed for orange essential oil and mandarin essence. For pineapple essence, no effect was observed against the target microorganisms.

After exposing the microorganisms to the antimicrobials, the plates with total inhibition (citral, hexanal and linalool) were re-incubated in the absence of the antimicrobial agents for two extra days at 25 °C. The antimicrobial effect was considered fungistatic if microbial growth was observed during the additional incubation period or fungicidal if no additional growth was detected (Balaguer et al., 2013). Results of the fungistatic/fungicidal activity of the active films are shown in Table 4.2.

As it can be observed, a general fungicidal effect was observed for citral, hexanal and linalool against *Saccharomyces cerevisiae* and *Penicillium aurantiogriseum.* On the contrary, only citral and hexanal at the highest concentrations (i.e. 20 and 30 µL) showed fungicidal activity against *Aspergillus niger,* whilst this microorganism was able to grow in the case of linalool showing fungistatic activity.

Antifungal properties of citral in the vapor phase was found by Wuryatmo *et al.* (2003) against some citrus postharvest pathogens such as *Penicillim italicum*, *Penicillium digitatum* and *Geotrichum candidum.* In this study, 15 µL of citral directly added to the lid of the inoculated Petri dish complete inhibited the growth of the three tested fungi while 6 µL of citral exerted almost complete inhibition. This effect was directly comparable with the inhibition observed whereby 5 µL of citral was the minimum amount (Cmax) that completely inhibited the growth of *Saccharomyces cerevisiae*, *Penicillium aurantiogriseum* and *Aspergillus niger* under similar exposure conditions in the present research.

Also, Belletti *et al.* (2004) found this mixture of isomers to have the highest antifungal activity as compared with other components of citrus essential oil against *Saccharomyces cerevisiae* in the vapor phase. Wuryatmo et al. (2003) observed the fungicidal activity of 15 µL/Petri dish of citral on the growth of *Penicillium digitatum* while a fungistatic effect was observed for *Penicillum italicum*. Therefore, the fungicidal/fungistatic activity of citral and its isomers differed among the type of fungi and the concentration tested. These results are in accordance to what was observed in the present research, where 10 µL of citral showed fungistatic activity against *Saccharomyces cerevisiae* and *Aspegillus niger* while the same amount of citral showed fungicidal activity against *Penicillium aurantiogriseum*.

Table 4.2. Fungistatic/fungicidal activity of citral, hexanal and linalool against the target microorganisms after 2 days at 25 °C

Active agent	µL/Petri dish	*Saccharomyces cerevisiae*	*Penicillium aurantiogriseum*	*Aspergillus niger*
Citral	10	Fungistatic	Fungicidal	Fungistatic
	20	Fungicidal	Fungicidal	Fungicidal
	30	Fungicidal	Fungicidal	Fungicidal
Hexanal	10	Fungicidal	Fungicidal	Fungistatic
	20	Fungicidal	Fungicidal	Fungicidal
	30	Fungicidal	Fungicidal	Fungicidal
Linalool	10	-	-	Fungistatic
	20	Fungicidal	Fungicidal	Fungistatic
	30	Fungicidal	Fungicidal	Fungistatic

(-) samples not evaluated.

Hexanal has a well-known antifungal capacity. Sáenz-Garza *et al.* (2013) examined the antifungal properties of microencapsulated hexanal against *Penicillium expansum* in laboratory media at different temperatures, observing inhibition of conidial germination at 25 °C and slightly less effective at 12 °C, probably due to the slower release associated with reduced vapor pressure at the lower temperature. Almenar *et al*. (2007) also encapsulated hexanal into β-cyclodextrins (β-CD) and demonstrated that hexanal vapor completely inhibited growth of *Colletotrichum acutatum*, *Alternaria alternata* and *Botrytis cinerea* at concentrations of 1.1, 2.3 and 1.3 µL/L air, respectively at 23 °C. These authors also observed at the concentration tested that hexanal had fungistatic effects on all fungi tested, and fungicidal activity was observed only for *Colletotrichum acutatum.*

The antimicrobial activity of linalool in vapor phase against numerous microorganisms has been already reported in several studies (Fisher and Phillips, 2006; Sato *et al.*, 2007). According to Nakahara *et al.* (2003), linalool exerted antifungal activity in the vapor phase against different strains of *Aspergillus and Penicillium* at 27 °C, showing potent activity with MIC ranging from 28 to 56 ppm (mg/L in air). In this case, the MIC was defined as the lowest concentration of volatile compounds which inhibited colony formation of test fungi by 50%. In addition, Kordali *et al.* (2008) observed potent fungicidal effects of linalool against most of the tested fungal species in accordance with the fungicidal tendencies observed in the present study for this antimicrobial.

The rest of the orange-based antimicrobials tested in the present study, whose main compound was largely limonene, showed lower or no inhibition of the growth of the yeast and molds. These results are in line with the low antifungal effect observed for pure limonene tested. In accordance with these findings, Caccioni *et al.* (1998) also observed a weaker antifungal activity of orange essential oil in the vapor phase against *Penicillium digitatum* and *Penicillium italicum* with respect to other citrus essential oils. In contrast to the effect found in the present and previous studies in the vapor phase, limonene showed antifungal and antibacterial activity against many species of microorganisms such as *Trichoderma viride*, *Cladosporium herbarum* and *Aspergillus flavus* in liquid media or in direct contact with agar (Mourey and Canillac, 2002; Ozturk and Ercisli, 2006; Singh *et al.*, 2010).

In general, when microorganisms were exposed to the vapor of essential oils, the inhibitory effect was different from those found by direct contact (Edwards-Jones *et al.*, 2004). Tyagi *et al.* (2013) observed that inhibition resulting from the exposure to mentha oil vapors in the disk volatilization method was significantly larger than the same concentration of essential oil in liquid phase measured by well diffusion. These differences may be dependent on the physicochemical properties of the antimicrobial agents, the culture media, the microorganism and type of contact between the microorganism and the antimicrobial agent. In vapor, the antimicrobial efficiency depends on the volatility of each compound while in liquid or agar it depends on the diffusion and solubility of the active compounds into the culture medium (Goñi *et al.*, 2009; Kurek *et al.*, 2013). The antimicrobial effect in direct contact is mostly attributed to the activity of the more hydrophilic (water-soluble) components. However, during vapor phase an equilibrium is achieved among the volatile compounds released in the headspace, both hydrophilic and hydrophobic, so that part of the most hydrophilic ones are absorbed at the agar surface (Goñi *et al.*, 2009; Kalemba and Kunicka, 2003).

4.1.2.1. Study of the synergistic effect derived from the combination of volatile active agents

The use of volatile natural preservatives can alter the organoleptic properties of the food system and exceed the flavor threshold acceptable by consumers. A way to reduce the

sensory impact is to combine different EOs or their pure components to obtain possible additive or synergistic effects (Bassolé and Juliani, 2012).

Synergy occurs when the effect of the combined substances is higher than the sum of the individual effects (Burt, 2004), which could provide effective antimicrobial action below the acceptable sensory threshold but above the required MIC values. An additive effect is observed when the combined effect is equal to the sum of the individual effects. On the contrary, antagonism is observed when the effect of the active agents is less when they are applied together than when individually applied (Bassolé and Juliani, 2012).

To identify the most promising combination that exerted the highest antimicrobial activity, a range of volumes between 1 and 30 μL/Petri dish of citral, hexanal and linalool mixture (C:H:L) (equal volume), were tested against *Saccharomyces cerevisiae, Penicillium aurantiogriseum* and *Aspergillus niger* in the vapor phase at 25 °C for 5 days as described in 3.2.1.2. Results of inhibition test (halos) are shown in Table 4.3 as the mean of three replicates ± standard deviation. The MIC and Cmax values of the mixtures were also identified.

As it can be observed in Table 4.3, the lowest amount of the mixture that produced a clear inhibition halo in *Saccharomyces cerevisiae*, *Penicillium aurantiogriseum* and *Aspergillus niger* growth was 1 μL/Petri dish (MIC). The amount that generated total inhibition for the three microorganisms was 5 μL/Petri dish (Cmax).

Table 4.3. Antimicrobial effect of citral, hexanal and linalool combinations (1:1:1) in the vapor phase at 25 °C for 5 days

C:H:L mixture	Inhibition halo (mm ± SD)		
(μL/Petri dish)	***Saccharomyces cerevisiae***	***Penicillium aurantiogriseum***	***Aspergillus niger***
1	23.2 ± 0.1 (MIC)	26.5 ± 4.6 (MIC)	23.7 ± 1.5 (MIC)
2.5	33.8 ± 2.8	37.7 ± 6.3	35.7 ± 3.7
5	90.0 ± 0.0 (Cmax)	90.0 ± 0.0 (Cmax)	90.0 ± 0.0 (Cmax)
7.5	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0
10	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0
15	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0
20	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0
30	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0

The FIC index (fractional inhibitory concentration) was used to numerically assess the antimicrobial effect of the combination of individual substances. This index is defined as the concentration that produces a given effect when used in combination with another agent divided by the concentration that has the same effect when used alone (Hall *et al.*, 1983).

In general, MIC or Cmax are used as the reference concentration to calculate the FIC index (Delaquis *et al.*, 2002; Goñi *et al.*, 2009). In this case, the FIC index for the combination of citral, hexanal and linalool was calculated using the Cmax for the individual agents (Table 4.1) and their combination (Table 4.3) according to the Equation 3.1 (section 3.2.1.2). Cmax was considered since the MIC values were not accurately determined (lower amounts of the individual agents in the ternary mixture should be tested).

The FIC values obtained for the individual agents in the mixture and the sum of the individual FIC values for each microorganism are shown in Table 4.4. In general, FIC values were below 1 indicating a synergistic antimicrobial effect of the ternary mixture. In particular, the synergistic effect was clearly observed in the case of *Saccharomyces cerevisiae* and *Penicillium aurantiogriseum* while it was in the border of synergistic-additive effect in the case of *Aspergillus niger*.

Table 4.4. FIC index of the combination citral, hexanal and linalool (1:1:1) for 3 days at 25 °C considering (Cmax) as reference

	Individual FIC (V in mixture (µL)/ V alone (µL))			C:H:L	
Microorganism	**Citral**	**Hexanal**	**Linalool**	**FIC**	**Effect**
Saccharomyces cerevisiae	0.33	0.17	0.11	0.61	<1 Synergistic
Penicillium aurantiogriseum	0.33	0.33	0.11	0.78	<1 Synergistic
Aspergillus niger	0.33	0.33	0.22	0.89	<1 Synergistic

The antimicrobial effect of the combination of citral, hexanal and linalool has not been previously reported in the literature, but there are some studies demonstrating the synergistic effect between other EOs or their components. Several authors reported the synergistic effect between thymol and carvacrol against bacteria and fungi *in vitro* (Cosentino *et al*., 1999; Guarda *et al*., 2011). Delaquis *et al.* (2002) found that the combination of cilantro, coriander, dill and eucalyptus EOs, resulted in additive, synergistic or antagonistic effects subject to the microorganisms and the fraction tested. The combination of clove and rosemary exerted all three effects, depending on the

corresponding microorganism (Fu *et al.*, 2007). The potential of the combination of cinnamon EO and clove EO (1:1; v/v) as antibacterial agents in the vapor phase was demonstrated by Goñi *et al.* (2009). However, due to the absence of standard screening tests for the synergistic effect of EOs and their pure compounds, and difficulties in evaluating the real effective concentration or amount released that prevent microbial growth, it is difficult to compare results between researchers (Balaguer *et al.*, 2013; Bassolé and Juliani, 2012; Kalemba and Kunicka, 2003; Kloucek *et al.*, 2012).

4.1.3. Study of the organoleptic compatibility of the volatile antimicrobial agents with the fruit

Organoleptic alteration of the food due to the addition of volatile active agents through the packaging could affect the consumer's acceptability. In order to minimize sensory impact of the fruit packed with active packaging materials, the selected antimicrobials citral, hexanal and linalool were evaluated regarding their sensory compatibility with peeled and cut orange and pineapple.

The maximum Cmax obtained for citral, hexanal, linalool (µL/Petri dish), individually tested and in the mixture (C:H:L) (section 4.1.2), were proportionally added to a paper disk and exposed to packed orange and pineapple for 24 h at 4 ± 2 °C before testing. The compatibility between fruit and individual active agents and their ternary mixture were evaluated by 10 consumers in terms of flavor (smell and taste). Ratings were based on a 4-point hedonic scale. Table 4.5 summarizes the amount of active agents added to the paper disk and the results of the compatibility tests expressed as the mean of ten answers ± standard deviation. According to the compatibility test, the combination of citral, hexanal and linalool a was best accepted by the consumers with scores higher than 3 (compatible with fruit).

Specifically, for orange, the smell attribute of the mixture was scored significantly higher, with values above 3, compared with the rest of individual agents. Although there were no significant differences for the smell attribute between the mixture and citral, the score was higher for the mixture. Taste was scored significantly higher for the mixture. In the case of pineapple, no significant differences were perceived by the consumers in terms of smell compatibility although the scores were higher for the active mixture. The taste of the mixture in pineapple fruit was also scored as compatible, being this compatibility significantly higher than the individual citral, hexanal and linalool.

Table 4.5. Organoleptic compatibility of citral, hexanal and linalool and their mixture with peeled and cut orange and pineapple

Active agents	Concentration tested (µL/tray)	Orange		Pineapple	
		Smell	Taste	Smell	Taste
Citral	38	2.9 ± 0.6 [a]	2.6 ± 0.7 [a]	2.1 ± 0.7 [a]	1.7 ± 0.8 [a]
Hexanal	77	2.0 ± 0.8 [b]	1.9 ± 0.7 [ab]	2.8 ± 0.8 [a]	2.3 ± 0.9 [a]
Linalool	115	1.7 ± 0.7 [b]	1.6 ± 0.7 [b]	2.4 ± 1.1 [a]	2.0 ± 0.8 [a]
Citral in C:H:L mixture	13				
Hexanal in C:H:L mixture	13	3.4 ± 0.8 [a]	3.5 ± 0.7 [c]	3.0 ± 0.8 [a]	3.3 ± 0.7 [b]
Linalool in C:H:L mixture	13				

Values within the same column with different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis). 4 = very compatible; 3 = compatible; 2 = poorly compatible; 1 = not compatible.

4.1.4. Study of the thermal stability of the antimicrobial agents

The antimicrobial activity of the active substances may be lost during the manufacturing process of the active packaging materials as well as during storage and distribution. In this research, the packaging materials were manufactured with PP resins by means of melting extrusion. During this process, the active agents are subjected to high pressure and high temperatures (T > 180 °C) inside the extruder. This could affect the chemical stability of the active agents, leading to their degradation and loss through evaporation. All these processes would reduce the final concentration and antimicrobial activity of the active packaging materials.

Thermal stability of the selected active agents (citral, hexanal, linalool, potassium sorbate and sodium benzoate) was determined by means of TGA as described in section 3.2.3. TGA measures the loss of weight of a given sample as a function of the temperature. Figure 4.1 shows the percentage of weight loss for the volatile active agents (citral, linalool and hexanal) as a function of temperature as well as the derivative of this curve which shows the degradation rate in %/min. As can be observed in Figure 4.1, the three active agents showed a clear loss of weight as a function of temperature. Differences among the three active agents were observed. In this regard, the derivative curve identifies the temperature at which the maximum degradation/volatilization rate occurs (T_{max}). In this case, hexanal is the least thermally stable with a maximum temperature of volatilization of 58 °C followed by linalool and citral with a T_{max} of 136 and 155 °C, respectively.

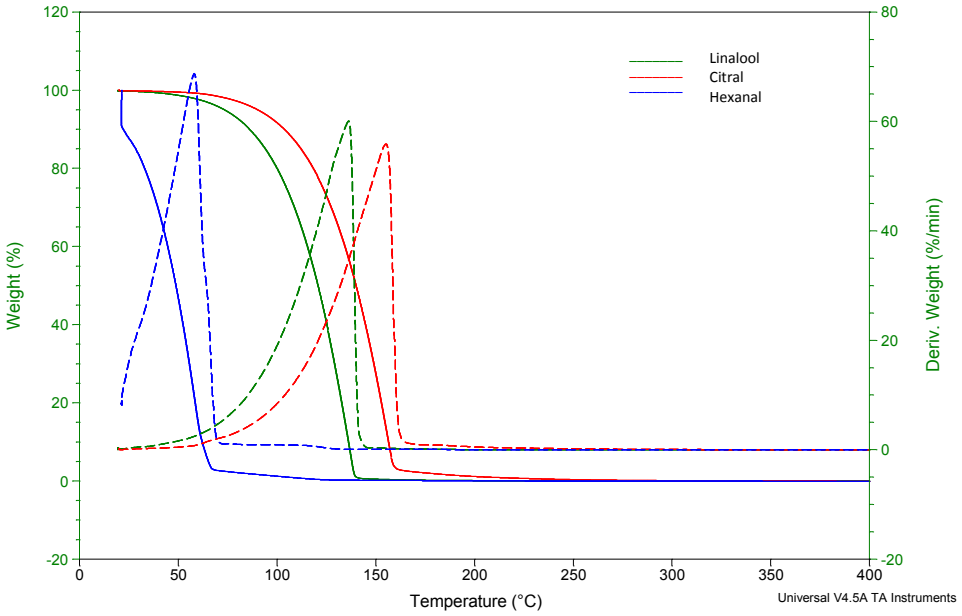


Figure 4.1. TGA curves of citral, hexanal and linalool
(—): weight loss curve (%); (---): derivative curve (%/min)

From the TGA curves, it cannot be concluded if the loss of weight is only due to the volatilization of the pure compounds or if degradation also occurs. Some degradation products could be generated being responsible for the subsequent loss of weight by volatilization. In this case, the thermal stability of the active compounds seems to be related to their vapor pressure (Patrignani *et al.*, 2013) being hexanal (11.3 mmHg; 25 °C) > linalool (0.16 mmHg; 25 °C) > citral (0.091 mmHg; 25 °C) (Pubchem, 2015).

Considering the maximum volatilization temperatures of citral, hexanal and linalool, they are below the processing temperatures of conventional polymers such as PP (around 180-190 °C) selected in the present Doctoral thesis. However, previous experience showed that polymeric matrices protect in some way the active agents from volatilization during the extrusion process (Lara-Lledó *et al.*, 2013; Suppakul *et al.*, 2008). Therefore, the results obtained by means of TGA should be confirmed after materials processing (section 4.2.1.2).

The thermal stability of potassium sorbate and sodium benzoate was also evaluated by TGA. Figure 4.2 shows the percent weight loss and derivative weight versus time (%/min) for sodium benzoate.

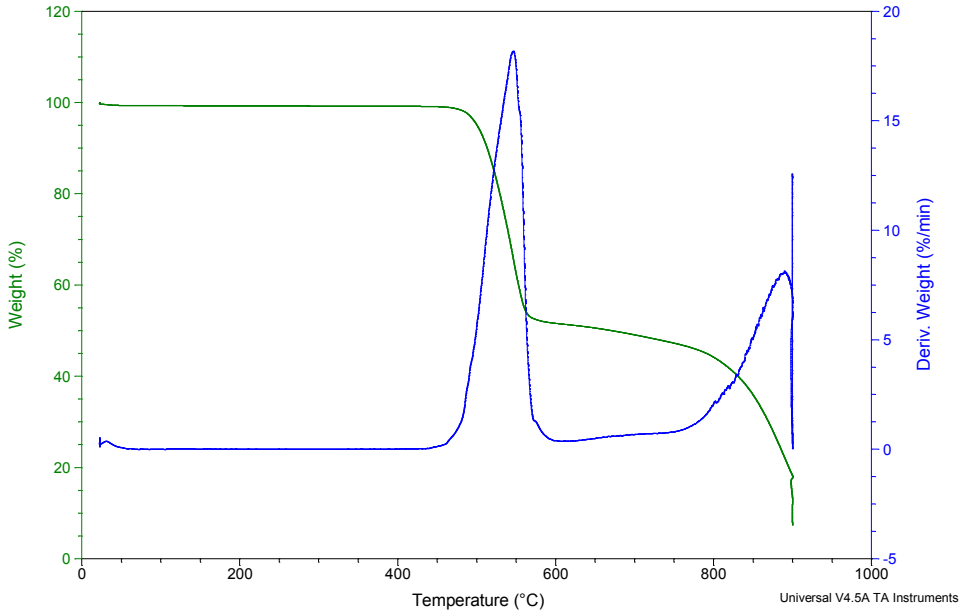


Figure 4.2. TGA curves of sodium benzoate

The thermogravimetric curves of potassium sorbate could not be obtained since it presented electrochemical interference with the equipment, probably due to the presence of metallic traces (results not shown).

The results obtained in Figure 4.2 showed that the T_{max} of sodium benzoate (554 °C) was substantially higher than the values obtained for the volatiles. However, as mentioned before, TGA detects loss of weight due to volatilization but it does not provide information about chemical degradations unless volatile compounds are generated.

The melting point of potassium sorbate is around 270 °C (Pubchem, 2015), higher than the processing temperature of the PP material. Therefore, the high stability of both additives, potassium sorbate and sodium benzoate makes these additives suitable to be incorporated by extrusion in polymeric materials such as PP. In any case, the high stability of potassium sorbate and sodium benzoate should be checked in real processing trials with the polymeric resins (see section 4.2.3.4).

4.1.5. Conclusions for the selection of antimicrobial substances for active material development

4.1.5.1. Volatile antimicrobial agents

Of the tested volatile antimicrobials, citral, hexanal and linalool seemed to be the most promising antimicrobials to be incorporated into a packaging matrix for fruit applications due to their significant inhibitory effect against reference yeasts and molds typical of those causing spoilage on fruits. Synergistic effects were observed for the ternary mixture (C:H:L) in comparison with the individual agents. Therefore, the effect of the combined substances was higher than the sum of the individual effects of citral, hexanal and linalool.

The combination of citral, hexanal and linalool was the best accepted by consumers in terms of organoleptic compatibility with the fruit. Taking advantage of this effect, lower concentrations would be needed to achieve the same antifungal effect in fruits than for the individual compounds. Their affordable cost also played an important role in deciding to move forward with their industrial application (citral: 9.7 €/kg; hexanal: 17 €/kg; linalool: 8.2 €/kg).

Despite the high volatility of the citral, hexanal and linalool, the incorporation of these compounds into a polymeric matrix such as PP should be feasible as shown by previous studies that reported how the polymeric matrix could protect somehow the active agents from volatilization.

All these results, lead to the conclusion that the mixture of citral, hexanal and linalool could be considered suitable for further active film packaging development for peeled and cut orange and pineapple for industrial applications.

Of the reported studies, the mixture evaluated in the present study had not been previously described, giving an innovative character to its further development as active packaging.

4.1.5.2. Solid antimicrobial agents

Potassium sorbate, sodium benzoate and their combination have been demonstrated to possess strong antifungal properties in food as described in the literature review in the introduction of the present Doctoral Thesis. They are widely used for food applications. Synergistic effects have been reported for their combination. These active agents are also

categorized as not causing alteration of the organoleptic properties of the food and are attractive because of their limited cost (potassium sorbate: 3.3 €/kg; sodium benzoate: 1.2 €/kg).

In addition, the thermal properties of sodium benzoate and potassium sorbate indicated they may be processed at high temperatures by means of extrusion. Thus, the mixture of potassium sorbate and sodium benzoate could be considered desirable for active packaging tray development for peeled and cut pineapple and orange at a semi-industrial scale.

4.2. ACTIVE MATERIALS CHARACTERIZATION

Active packaging materials, films and trays, were obtained at lab and at semi-industrial scale under the conditions described in 3.2.4. These materials were characterized in terms of active agents remaining after processing, thermal, mechanical, optical [transparency] properties, sealability, microstructural characteristics, and oxygen and water vapor barrier characteristics. Figure 4.3 shows the analysis performed on the active packaging materials developed in the present Doctoral Thesis. Sample nomenclature was also included in the diagram.

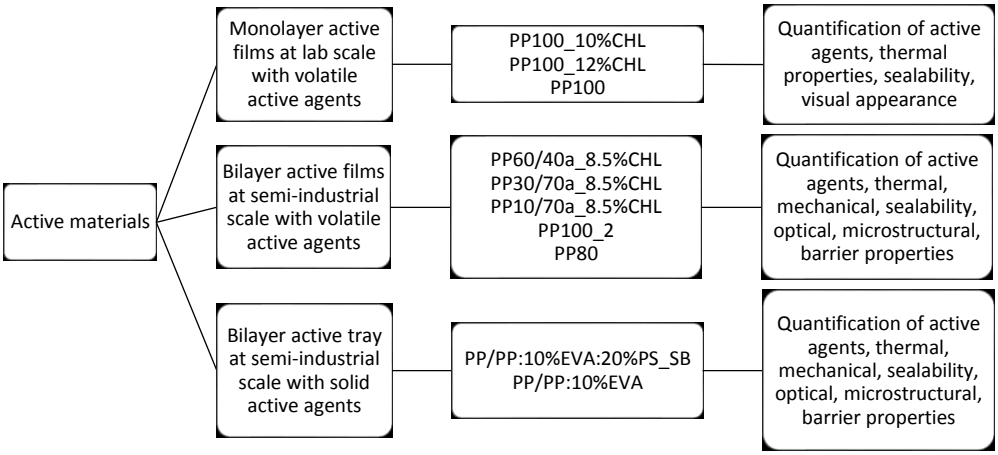


Figure 4.3. Characterization of active packaging materials

4.2.1. Monolayer active films at lab scale

Two monolayer PP films with 10 and 12 % (w/w) of citral, hexanal and linalool mixture (1:1:1) with 100 µm nominal thickness were obtained by one step extrusion with a twin screw extruder as described in the experimental section 3.2.4.1. A PP film without active agents (PP100) was also identified as a control film. Table 4.6 compiles the description of control and active films developed at lab scale in terms of nominal concentration of active agents, thickness, weight and width. Films were stored at -18 °C for further studies to preserve their active properties.

Table 4.6. Description of monolayer active and control films developed at lab scale

Film	Reference	Nominal concentration active layer (%) (w/w)	Total thickness ± SD (µm)	Weight ± SD (g/m²)	Width ± SD (mm)
1	**PP100_10%CHL**	10	92 ± 4	88 ± 3	70 ± 10
2	**PP100_12%CHL**	12	97 ± 2	91 ± 1	70 ± 10
3	**PP100**	-	100 ± 3	86 ± 2	75 ± 10

4.2.1.1. Quantification of active agents

The concentration of the active agents in the films was evaluated by extraction with acetone followed by GC-MS analysis as described in experimental section 3.2.5.2. Extraction was carried out in one-step since successive extraction steps did not showed more than 1.5% of additional recovery.

The chromatographic analysis of citral, hexanal and linalool showed good separation performance and resolution as presented in Figure 4.4.

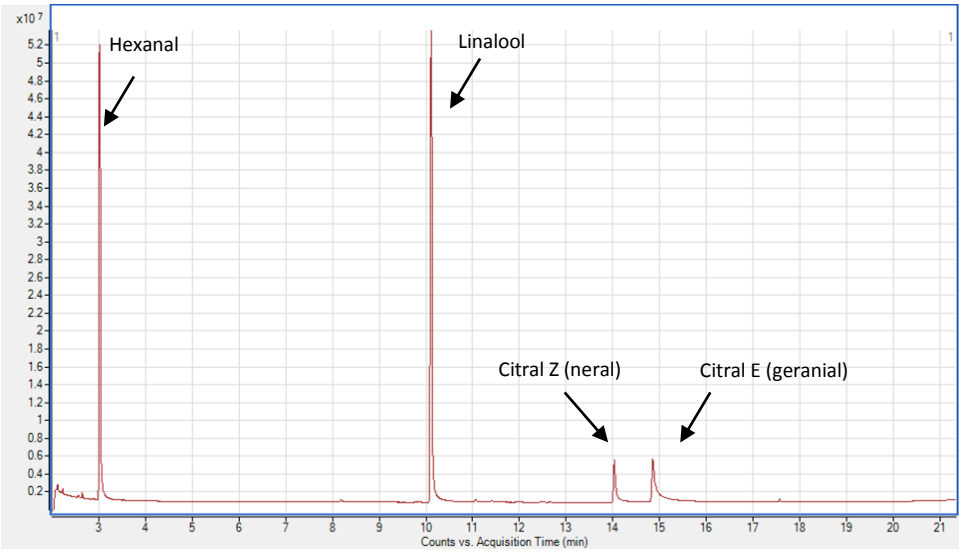


Figure 4.4. Chromatograms (GC-MS) of hexanal, linalool and citral (neral and geranial) in acetone solution

Table 4.7 shows the retention time and molecular weight of the three active agents. Citral was characterized to be the sum of cis-citral or citral Z (neral) and trans-citral or citral E (geranial).

The analytical parameters of the method (slope, intercept, linearity, repeatability, limit of detection (LOD) and limit of quantification (LOQ)) where calculated as described in experimental section 3.2.5.2.1 and results are shown in Table 4.8. The regression coefficients (R^2) obtained were higher than 0.99 for all the calibration curves. The relative standard deviations (RSD) for all the cases were below 10%. LODs were in the range of 0.26-0.89 mg/kg, while LOQs were in the range of 0.88-2.98 mg/kg. These results were considered acceptable for the determination of linalool, hexanal and citral by GC-MS.

Table 4.7. Retention time and molecular weight of hexanal, linalool and citral

Active agent	Retention time (min)	Molecular weight (g/mol)
Hexanal	2.93	100.1588
Linalool	9.98	154.2493
Citral Z (neral)	13.88	152.2334
Citral E (geranial)	14.71	152.2334

Table 4.8. Analytical parameters of GC-MS-QQQ method for hexanal, linalool and citral

Active agent	Calibration range (mg/kg)	Slope ± SD	Intercept ± SD	Linearity (R^2)	Repeatability (RSD, %)	LOD (mg/kg)	LOQ (mg/kg)
Hexanal	10-140	205663 ± 4541	1256811 ± 377176	0.9980	4.31	0.89	2.98
Linalool	10-170	349848 ± 11588	-2880521 ± 1170509	0.9945	3.72	0.29	0.95
Citral Z	5-50	145876 ± 5288	-530249 ± 150062	0.9935	7.15	0.26	0.88
Citral E	10-150	144042 ± 4748	-1399973 ± 416120	0.9935	6.51	0.64	2.13

The concentration of each volatile active agent in the films after the extrusion process is shown in Table 4.9. Reported values are the mean of three replicates ± standard deviation. As can be observed from the quantification by GC in Table 4.9, volatile losses occurred in the extrusion process ranged between 29 and 34% of the nominal concentration added to the films.

Table 4.9. Volatile active agent concentrations in monolayer active films

Sample	Active agent (%) (w/w)			Total active agents ± SD (%) (w/w)	Total active agents ± SD (g active/m² film)
	Citral	Hexanal	Linalool		
PP100_10%CHL	3.54 ± 0.02	1.27 ± 0.03	1.80 ± 0.09	6.60 ± 0.10	5.80 ± 0.09
PP100_12%CHL	4.51 ± 0.13	1.70 ± 0.05	2.28 ± 0.12	8.50 ± 0.18	7.74 ± 0.16

Moreover, different ratio of volatiles remained in the film after processing (C:H:L; 1:0.4:0.5). This means that during processing, the losses of active agents were not equivalent. This fact could be mainly explained based on the differences in their vapor pressure which provide them with different volatility behavior (Muriel-Galet *et al.*, 2013a). Specifically, hexanal experienced higher losses due to its higher volatility, followed by linalool and citral.

By using conventional processing methods, different researchers found significant higher losses of volatile antimicrobial agents during active film processing. The extrusion blowing of LDPE-based film at 160 °C developed by Suppakul *et al.* (2006) resulted in a loss of 65% of linalool and methylchavicol (w/w). Ramos *et al.* (2012) also reported losses between 56 and 75% of carvacrol and thymol incorporated in PP films by melt blending followed by compression molding in a hot press at 190 °C. However, the losses of active volatiles such as oregano, thyme and cinnamon essential oil, carvacrol, thymol and citral incorporated in a PP matrix by using a twin screw extruder were similar to the losses observed in the present research, in the range of 21-43% (w/w) (Lara-Lledó *et al.*, 2013). Differences in the retention of volatile antimicrobial agents is dependent on the processing method and processing conditions, but also on the materials and additives used for film production (Cran *et al.*, 2010; Guarda *et al.*, 2011; Suppakul *et al.*, 2011b).

4.2.1.2. Thermal properties of active films

Thermogravimetric analysis was used to evaluate the thermal stability of the monolayer active films PP100_10%CHL and PP100_12%CHL versus the thermal stability of the control PP film (PP100) as described in experimental section 3.2.5.3. Figure 4.5 shows the percent weight loss curve vs. temperature and their derivative weight curve (%/min) of control and active films. From the first loss of weight step at 200 °C, information on the total content of the three volatiles (citral, hexanal and linalool) in the active films and their maximum temperature of volatilization (T_{max_a}) is shown. A second loss of weight was observed

between 400 and 500 °C and was related to the initial decomposition temperature of the polymeric matrix at 1% of weight loss ($T_{1\%}$) and its temperature at maximum decomposition rate (T_{max_p}). These values extracted from Figure 4.5 are indicated in Table 4.10 and expressed as the mean of three replicates ± standard deviation.

PP100_10%CHL and PP100_12%CHL active films showed a first degradation step at low temperatures, with a T_{max_a} of 118 and 113 °C, respectively as shown in Table 4.10. This first step is associated with the evaporation of active agents and it provides the total active agent concentrations remaining in the film after processing. Lower volatilization temperature was observed for the film containing higher amounts of volatiles (PP100_12%CHL). The higher the content, the sooner the volatilization occurred. At temperatures up to 200 °C, citral, hexanal and linalool were totally volatilized.

The volatilization temperatures observed are in agreement with work done by Ramos *et al.* (2012) who found that PP extruded films containing carvacrol showed a first step degradation at 115 °C. As reported in previous experiences, a single degradation step was observed for the control film PP100 (Navarro *et al.*, 2003) with T_{max_p} of 474 °C.

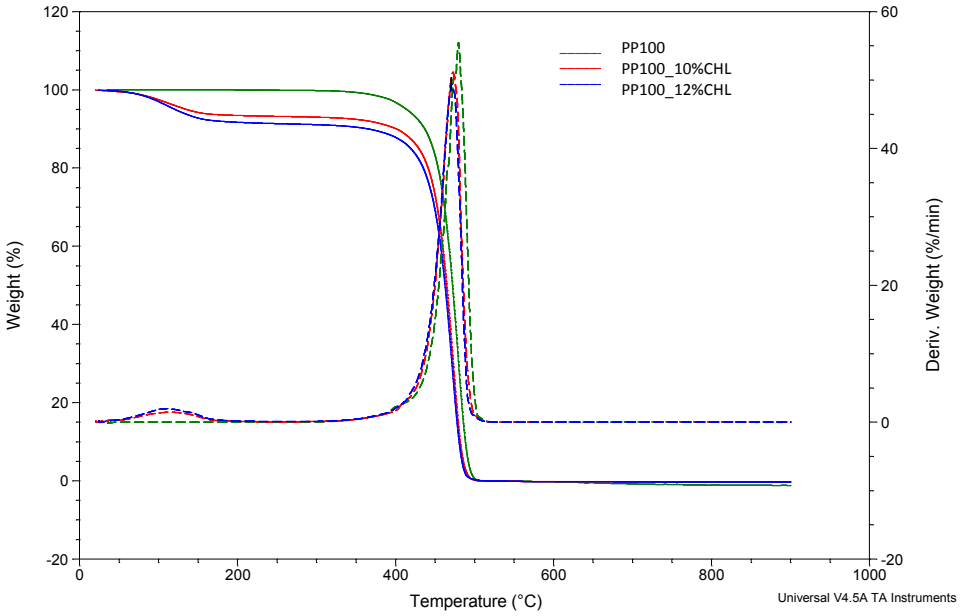


Figure 4.5. TGA and derivative curves of monolayer PP-based films
(—): weight loss curve (%); (---): derivative curve (%/min)

Table 4.10. Thermal properties of monolayer control and active PP films from TGA curves

	First weight loss (active agent volatilization)		Second weight loss (polymer degradation)	
Sample	**Active agents ± SD (%)**	**T_{max_a} ± SD (°C)**	**$T_{1\%}$ ± SD (°C)**	**T_{max_p} ± SD (°C)**
PP100	-	-	374 ± 6 [a]	474 ± 4 [a]
PP100_10%CHL	6.4 ± 0.2 [a]	118 ± 1 [a]	351 ± 1 [b]	473 ± 1 [a]
PP100_12%CHL	8.2 ± 0.1 [b]	113 ± 2 [b]	337 ± 1 [c]	472 ± 2 [a]

Values within the same column with different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis).

The percent active agent remaining in the films after processing were estimated by TGA as 6.4 and 8.2% (w/w) for PP100_10%CHL and PP100_12%CHL, respectively (Table 4.10). The total content of the volatiles examined by TGA were slightly lower than results of quantification obtained by GC-MS, 6.60 and 8.50% for PP100_10%CHL and PP100_12%CHL, respectively.

No significant differences were observed for the T_{max_p} at the second loss of weight between control and active films concluding that the active agents did not have an effect on the maximum degradation temperature of the polymers. This behavior was also supported by Ramos *et al.* (2012) who did not find differences in degradation temperature of PP when thymol or carvacrol were incorporated by melt blending.

4.2.1.3. Sealability of active films

A preliminary heat sealing test was performed to evaluate the effect of the incorporation of citral, hexanal and linalool mixture on the films sealability as described in section 3.2.5.6. The sealing performance between active and control films was evaluated by using a laboratory heat sealer. According to previous knowledge, the sealing temperature was adjusted to provide adequate sealing areas, with a dwell time and pressure set to 0.5 sec and 31.4 psi, respectively.

The integrity of the sealed film system was evaluated qualitatively. Optimum sealing was considered when the two films could not be manually separated and no deformations of the sealed area such as burns were found. Table 4.11 shows the range of temperature tested and the optimum sealing temperature selected for the tested materials. Films were heat sealable in a range of 130-165 °C.

As observed in Table 4.11, the temperature needed for sealing control films was in the range of 130-135 °C. Higher sealing temperature was needed for sealing active with control films, in the range of 140-145 °C. The sealing temperature between active films was found to be even higher (160-165 °C) but still within the normal sealing range of temperature for PP (Hashimoto *et al.*, 2006).

As the amount of antimicrobial agents increased, seal strength was significantly decreased and higher sealing temperature was required. It seems that volatile compounds decreased the melting susceptibility of the films, reducing their ability to properly melt and perform the chain diffusion and entanglement with the material (Dobiáš *et al.*, 2000). As reported by Appendini and Hotchkiss (2002), incorporation of antimicrobials in polymers may alter the heat sealing strength properties of the plastics.

Table 4.11. Sealing temperature for PP-based monolayer films

	Sealing temperature (°C)										
Sample	**125**	**130**	**135**	**140**	**145**	**150**	**155**	**160**	**165**	**170**	**175**
PP100//PP100	×	✓	☑	-	-	-	-	-	-	-	-
PP100//PP100_10%CHL	×	×	×	✓	☑	-	-	-	-	-	-
PP100//PP100_12%CHL	×	×	×	✓	☑	-	-	-	-	-	-
PP100_10%CHL // PP100_10%CHL	×	×	×	×	×	×	×	✓	☑	-	-
PP100_12%CHL // PP100_12%CHL	×	×	×	×	×	×	✓	☑	-	-	-

(×): not sealing; (✓): acceptable sealing; (☑): optimum sealing; (--): not tested.

4.2.1.4. Visual appearance of the films

Changes in the visual appearance of the films caused by the addition of the antimicrobial mixture were evaluated by photographic inspection. Visual transparency of the films was performed placing a logo behind the films.

Figure 4.6 shows the visual aspect of the control PP100 and active films PP100_10%CHL and PP100_12%CHL. As the active content increased, the film changed from transparent (control film) towards whitish and translucent. This behavior was much pronounced for the films containing higher concentrations of the active mixture. Changes in the transparency of the films were also reported by Dias *et al.* (2013) who found that extruded LDPE films with lemon

aroma or lemon EO in the range of 5-10% showed a more opaque, yellow coloration and therefore less transparent with respect to films without active agents.

This effect could reduce the acceptance of the films for their use in the food industry, especially in the fresh cut fruit sector, as transparency is one of the main requirements of the packaging materials (FAO, 1995). This effect was mitigated with a faster cooling rate of the active materials processing at semi-industrial scale (section 4.2.2.6).

Figure 4.6. Visual appearance of active films: (A) PP100; (B) PP100_10%CHL; (C) PP100_12%CHL

4.2.2. Bilayer active films at a semi-industrial scale

Bilayer active films were developed at semi-industrial scale under the conditions set in section 3.2.4.2. Two processing steps were needed. The first one was a compounding process to mix PP and the active agents to obtain pellets with 8.5% (w/w) active mixture (citral, hexanal and linalool; 1:1:1). The second one consisted of transforming the compound into the films by means of a cast film coextrusion process.

Table 4.12 compiles the description of control and active films developed at a semi-industrial scale in terms of thickness, weight and width. Films were stored at -18 °C to prevent losses of active agents until further studied.

The three active films had a nominal concentration in the active layer (a) of 8.5% (w/w) of the ternary mixture citral, hexanal and linalool.

Films PP60/40a_8.5%CHL and PP30/70a_8.5%CHL and their corresponding film control PP100_2 were fully characterized in terms of active agents concentration, thermal, mechanical, optical and barrier properties, sealability, and microstructural characteristics. Basic characterization was done for the film PP10/70a_8.5%CHL and its corresponding

control PP80 since the only difference with the film PP30/70a_8.5%CHL was a reduction of the structural layer, keeping the thickness of the active layer.

Table 4.12. Description of bilayer active films developed at pilot scale

Reference	Total thickness ± SD (µm)	Structural layer (µm)	Active layer (µm)	Weight ± SD (g/m²)	Width ± SD (mm)
PP60/40a_8.5%CHL	100 ± 5	60	40	97 ± 4	270 ± 5
PP30/70a_8.5%CHL	100 ± 6	30	70	97 ± 1	270 ± 5
PP10/70a_8.5%CHL	80 ± 3	10	70	75 ± 3	270 ± 5
PP100_2	100 ± 5	100	-	92 ± 0	270 ± 5
PP80	80 ± 3	80	-	74 ± 2	270 ± 5

4.2.2.1. Quantification of active compounds

The content of citral, hexanal and linalool in the polymeric compound was determined by extraction with acetone followed by GC-MS as described in 3.2.5.2.1. Validation of the analytical procedure was described in same section.

The concentration of citral, hexanal and linalool (%, w/w) quantified in each extraction are presented in Table 4.13. The total percentage of the volatile active agents in the compound was estimated in 8.05%. Considering the amount of the active mixture remaining in the polymeric compound and their nominal concentration (8.5%), 5% of losses during the compounding process were considered.

Table 4.13. Active agent concentrations in PP compound

	Active agent (%) ± SD (w/w)		
Sample	**Citral**	**Hexanal**	**Linalool**
First extraction PPC_ 8.5%CHL	2.62 ± 0.05	2.39 ± 0.17	2.49 ± 0.09
Second extraction PPC_ 8.5%CHL	0.31 ± 0.07	0.12 ± 0.11	0.12 ± 0.30
Total active agent (%) (w/w)	2.93 ± 0.09	2.51 ± 0.20	2.61 ± 0.31

The losses obtained during this process were significantly lower than the losses obtained with the lab scale monolayer films (29-34%). This might have been due to differences between the performance of the semi-industrial scale processes versus laboratory scale, specially due to processing time. At the laboratory scale, estimated residence time for active

agents at processing temperatures was around 90 sec. At the semi-industrial scale, residence time was around 15 sec, minimizing the potential thermal degradation and volatilization of active agents, as corroborated in Table 4.13.

4.2.2.2. Quantification of active films

The content of citral, hexanal and linalool in the bilayer active films was determined by one step extraction with acetone followed by GC-MS analysis as described in section 3.2.5.2.1. The extraction procedure was carried out in one step since successive extraction steps did not lead to more than 1.5% of additional recovery. The final concentrations of the active films are presented in Table 4.14.

According to the quantification results by GC-MS, no losses of the active volatiles were produced during the cast film co-extrusion step as the shear applied was lower when compared to the twin screw extruder used for the processing at laboratory scale. Considering the two steps processing yield, the incorporation of the active agents in the active films was set in 97% (w/w).

The ratio of volatiles after film processing remained close to the nominal ratio (1:1:1), being citral the major active agent, followed by linalool and hexanal. This effect could be related to the differences in the vapor pressure of the volatiles. This behavior was much pronounced for lab scale materials where higher losses happened for hexanal and linalool due to higher residence time that can lead to an increase in losses for active agents with higher volatility.

Table 4.14. Active agent concentrations in bilayer films

	Active agent (%) ± SD (w/w)			Total active agents ± SD (%) (w/w)	Total active Agents ± SD (g active/m² film)
Sample	**Citral**	**Hexanal**	**Linalool**		
PP60/40a_8.5%CHL	1.17 ± 0.00	1.08 ± 0.03	1.10 ± 0.01	3.35 ± 0.03	3.25 ± 0.03
PP30/70a_8.5%CHL	2.01 ± 0.13	1.96 ± 0.06	1.85 ± 0.11	5.82 ± 0.18	5.67 ± 0.17
PP10/70a_8.5%CHL	2.49 ± 0.01	2.28 ± 0.21	2.38 ± 0.01	7.15 ± 0.21	5.36 ± 0.20

4.2.2.3. Thermal properties

TGA and DSC tests were carried out to evaluate the influence of the volatile active agents on the thermal stability of the active bilayer films (PP60/40a_8.5%CHL and PP30/70a_8.5%CHL) compared to the thermal stability of control film (PP100_2).

TGA was performed by using the methodology describe in section 3.2.3. Figure 4.7 shows the percent weight loss curves vs. temperature and their derivative weight curves (%/min) of control and bilayer active films.

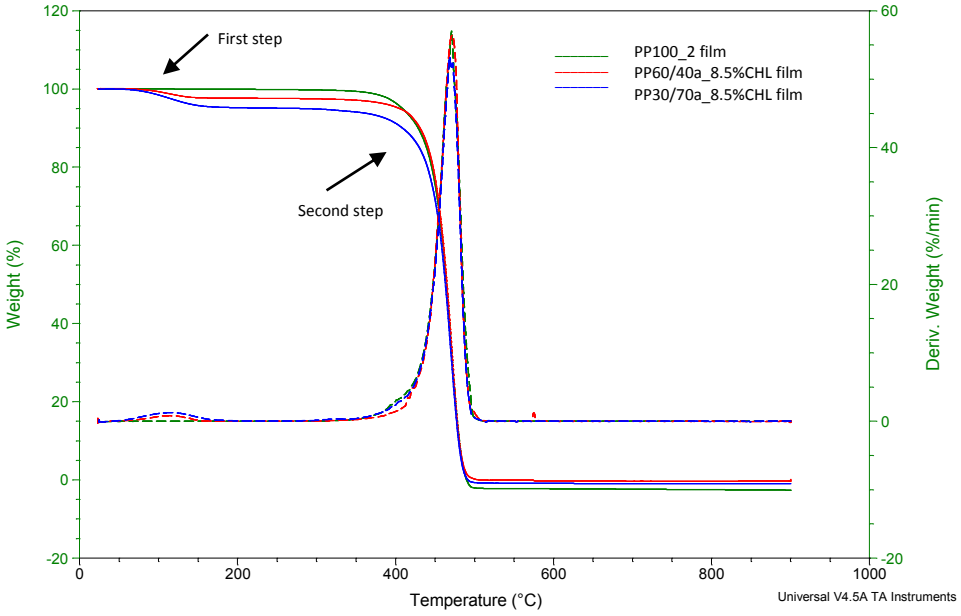


Figure 4.7. TGA and derivative curves of control and active bilayer PP-based films (—): weight loss curve (%); (---): derivative curve (%/min)

As described in section 4.2.1.2, thermogravimetrical characterization could be useful to estimate the total content of the active mixture in the films and their maximum volatilization temperature (T_{max_a}). In this way, from the first weight loss of the thermograms, the qualitative amount of active content and the T_{max_a} were identified.

The initial decomposition temperature of the polymeric matrix at 1% of weight loss ($T_{1\%}$) and its temperature at maximum decomposition rate (T_{max_p}) were calculated from the second

weight loss step of the thermogram. These values extracted from Figure 4.7 are indicated in Table 4.15 expressed as the mean of three replicates ± standard deviation.

A single degradation step was observed for the PP bilayer control film with T_{max} of 474 °C. This result corroborated the information reported in section 4.2.1.2 for monolayer PP film.

In the case of active materials, no significant differences in the volatilization temperature of the active agents were observed in the first step of the thermogram despite their concentration (T_{max_a}; 137 °C and 136 °C, respectively).

Table 4.15. Thermal properties of control and active bilayer PP-based films from TGA curves

	First weight loss (active agents volatilization)		Second weight loss (polymer degradation)	
Sample	**Active agent ± SD (%)**	**T_{max_a} ± SD (°C)**	**$T_{1\%}$ ± SD (°C)**	**T_{max_p} ± SD (°C)**
PP100_2	-	-	374 ± 6 [a]	474 ± 4 [a]
PP60/40a_8.5%CHL	2.8 ± 0.1 [a]	137 ± 7 [a]	365 ± 5 [a]	472 ± 4 [a]
PP30/70a_8.5%CHL	5.3 ± 0.2 [b]	136 ± 5 [a]	367 ± 2 [a]	471 ± 1 [a]

Values within the same column with different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis).

From this first step, the percentage of active agents was estimated to be 2.8 and 5.3% for PP60/40a_8.5%CHL and PP30/70a_8.5%CHL films, respectively, as described in Table 4.15. As is shown in section 4.2.2.2, the global content of volatiles determined by extraction and GC-MS analysis was slightly higher than the concentration obtained by TGA.

A second step corresponding with the thermal degradation of the polymer is shown in Figure 4.7. Likewise with monolayer active materials, differences were not observed in the maximum degradation temperature of the polymer between active and control films. Therefore, it can be concluded that active films maintained their thermal performance after the two processing steps.

Thermal properties of the active films were also evaluated by DSC. Thermal transitions extracted from the melting and crystallization curves were investigated by using the methodology described in section 3.2.5.3.2. The main thermal transitions, which give information on the melting behavior of the polymer, are melting temperature (T_m), melting enthalpy (ΔH_m), crystallization temperature (Tc), crystallization enthalpy (ΔH_c) and their onsets. By using theoretical calculations, it is possible to obtain material crystallinity.

It is well described by Gill *et al.* (1993) that the glass transition temperature of isotactic PP is found at -14 °C (T_g) and its T_m which depend on the polymer is between 170-220 °C (Ramakumar *et al.*, 2001).

In this work, a first heating was performed in order to analyze the thermal history of PP films. Thermal history of a polymeric material can be a useful record of the processing conditions used to obtain the material representing its real status from a thermal point of view (Scheirs, 2000). Figure 4.8 shows the DSC thermogram obtained for control and bilayer active films corresponding to the first heating of the polymer.

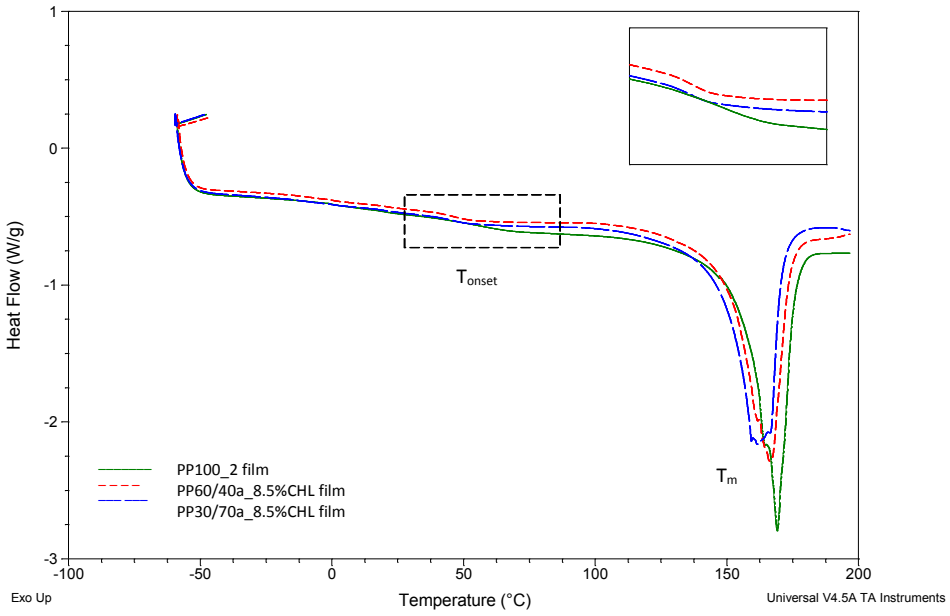


Figure 4.8. DSC curves of control and active bilayer PP-based films during the first heating

The information obtained from the first heating (Figure 4.8) such as onset temperature of the melting peak (T_{onset}), T_m, ΔH_m and crystallinity of the films is summarized in Table 4.16. The crystallinity degree of the polymers was calculated from the first heating curve based on the melting enthalpy ratio of the films to the heat of fusion of 100% crystalline PP (ΔH_m^0) as described in Equation 3.2 (section 3.2.5.3.2), correcting the enthalpy according the weight of the PP in the film. The ΔH_m^0 for 100% crystalline PP was set in 165 J/g (Zhu, 2002). Reported values are means of three replicates ± standard deviation.

Table 4.16. DSC results for the control and active bilayer PP-based films during first heating

Sample	T_{onset} ± SD (°C)	T_m ± SD (°C)	ΔH_m ± SD (J/g)	Crystallinity ± SD (%)
PP100_2	53 ± 2 [a]	170 ± 2 [a]	100.50 ± 2.88 [a]	62 ± 2 [a]
PP60/40a_8.5%CHL	40 ± 0 [b]	167 ± 1 [ab]	98.57 ± 3.20 [a]	62 ± 2 [a]
PP30/70a_8.5%CHL	41 ± 1 [a]	164 ± 3 [b]	100.25 ± 2.23 [a]	65 ± 2 [a]

Values within the same column with different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis).

As can be observed in Figure 4.8, all thermograms showed an initial slight step (zoom area) corresponding to the onset temperature of the melting peak that was anticipated by the addition of the active agents. The onset temperature was 53 °C for the control PP film and 40-41 °C for the active films as indicated in Table 4.16.

The same behavior observed in the first melting step was corroborated with the melting temperatures of the melting peak (T_m) for both active films. In particular, as the active content increased, the melting temperature significantly decreased from 170 °C for control PP to 164 °C for the film with the highest active concentration. The melting peak shape of the active films was also affected by the presence of active agents as can be observed in Figure 4.8. A reduction in the melting temperature could be explained by some plasticizing effect caused by the addition of the antimicrobials to the polymer matrix due to the fact the active agents interrupted crystallization of the polymer chain (Persico *et al.*, 2009; Ramos *et al.*, 2012; Torres *et al.*, 2014). The plasticizing effect observed in the study of thermal history of the material was corroborated by mechanical properties evaluation as further described in section 4.2.2.4. No significant differences were found for the melting enthalpy among films as Table 4.16 shows. For that reason, crystallinity of the PP matrix was not affected by the addition of volatile active agents with values between 62-65%, probably because of nucleating agents present in the raw PP.

From the analysis of the first heating, it could be concluded that the presence of volatile antimicrobials caused a slight effect on the thermal properties of the films. Concretely, the melting temperature decreased as the active content increased. However, crystallinity of the films remained unaltered with the addition of active agents.

To further investigate the crystallization and melting behavior of developed materials, a cooling and second heating step were evaluated. Results extracted from these thermograms

represent the intrinsic thermal properties of the films. Related cooling and heating curves are represented by Figure 4.9 and Figure 4.10, respectively.

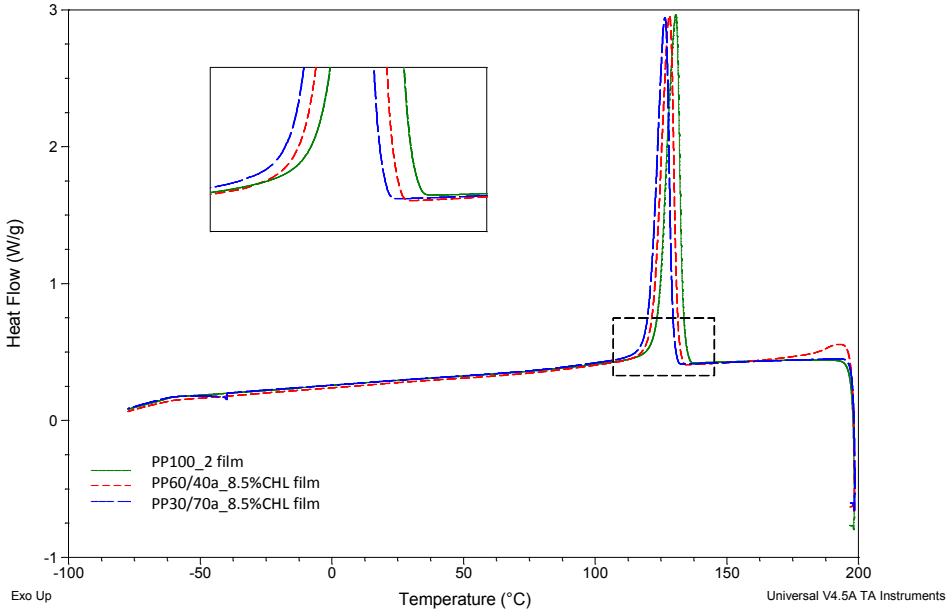


Figure 4.9. DSC curves of control and active bilayer PP-based during cooling

The cooling step corresponds to the exothermic peak and gives information about the onset (T_{onset}), crystallization temperature (T_c) and its associated crystallization enthalpy (ΔH_c) summarized in Table 4.17. Reported values are means of three replicates ± standard deviation.

A reduction in the crystallization temperature was found in the cooling step as the concentration of the antimicrobials increased. This effect could be related to the plasticizing effect previously described in the thermal history results (first heating). The higher the active agent concentrations, the later the crystallization occurs during the cooling step. In this way, the high content of additives within the matrix may reduce the ability of the polymer chains to crystallize (Muriel-Galet *et al.*, 2015).

The endothermic peak observed in Figure 4.10 corresponds to the second heating of the polymers and gives information on the melting temperature, melting enthalpy and crystallinity as described for the first heating. The second heating gives information on the

intrinsic properties of the material. The information obtained from second heating is also summarized in Table 4.17.

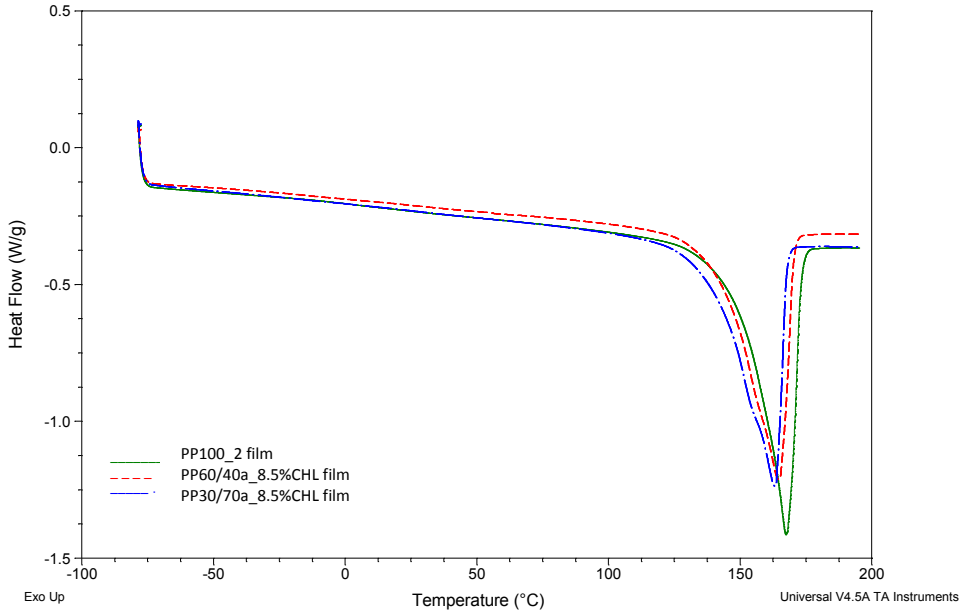


Figure 4.10. DSC curves of control and active bilayer PP-based films during the second heating

Table 4.17. DSC results for the control and active bilayer PP-based films during cooling and the second heating

	Cooling			Second heating		
Sample	**T_{onset} ± SD (°C)**	**T_c ± SD (°C)**	**ΔH_c ± SD (J/g)**	**T_m ± SD (°C)**	**ΔH_m ± SD (J/g)**	**% crystallinity ± SD**
PP100_2	136 ± 0 ^{a}	131 ± 0 ^{a}	104.10 ± 1.25 ^{a}	167 ± 0 ^{a}	115.50 ± 3.08^{a}	71 ± 2 ^{a}
PP60/40a_ 8.5%CHL	133 ± 0 ^{b}	128 ± 0 ^{b}	99.88 ± 2.06 ^{ab}	164 ± 1 ^{b}	109.77 ± 4.05^{ab}	69 ± 3 ^{a}
PP30/70a_ 8.5%CHL	131 ± 1 ^{c}	126 ± 1 ^{c}	96.97 ± 1.69 ^{b}	163 ± 0 ^{b}	105.40 ± 3.99^{b}	68 ± 3 ^{a}

Values within the same column with different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis).

The information extracted from the second heating showed a significant reduction in the melting temperature as the amount of active compounds increased. As the amount of

antimicrobials increased, lower melting energy was needed to melt the polymer and this effect was reflected by the slight reduction of the crystallization of the polymer which decreased from 71% for the PP control film to 69 and 68% for PP60/40a_8.5%CHL and PP30/70a_8.5%CHL films, respectively. However, this reduction was not considered significant.

The thermal behavior of synthetic polymer materials containing volatile antimicrobials has been further investigated by several authors. Results found by Suppakul *et al.* (2006) for LDPE-based films containing linalool or methylchavicol were in partial agreement with the results found in the present research, where no significant differences in the melting temperature and crystallinity degree between control and active films for the second heating were shown. Persico *et al.* (2009), Ramos *et al.* (2012) and Sung *et al.* (2014) also found that melting temperature did not change with the addition of antimicrobial agents in synthetic polymers, but the addition of antimicrobials changed the crystallinity of the films studied in the second heating. Specifically, Ramos *et al.* (2012) concluded that the crystallinity of PP films decreased significantly with the addition of thymol or carvacrol.

In principle, the presence of these active agents could be acting as plasticizers, interfering with crystalline chain growth of the polymer. Indeed, the plasticizers position themselves between polymer molecules and interfere with the polymer-polymer interactions. Plasticizers also increase the free volume of polymer structures or the molecular mobility of polymer molecules (Sothornvit and Krochta, 2000).

4.2.2.4. Mechanical properties

Tensile tests were performed in order to study the effect on the mechanical properties of the incorporation of citral, hexanal and linalool in the PP films. Different parameters such as Young's or elastic modulus, stress at peak and elongation at break were studied in control and bilayer active films as described in section 3.2.5.5. Table 4.18 shows the results obtained as the average of 10 replicates ± standard deviation.

The addition of volatiles to the PP matrix resulted in a modification of the mechanical properties of the film. A significant decrease of the elastic modulus was found in the presence of active agents, as they became more elastic materials. The reduction was concentration dependent, since Young's Modulus decreased as the active agent

concentrations increased. This value is not critical as the target application of these materials is a lid film without requirements for high mechanical properties.

The stress at peak decreased with the presence of active agents, reducing their tensile resistance. In addition, a significant increase of the elongation at break was also observed for the materials containing volatile active agents, showing higher elasticity before the break point. The changes observed in the mechanical properties of the active films could be explained by some plasticizing effect caused by the addition of volatile additives as previously described.

These results are in agreement with those of Ramos *et al.* (2012) who also observed a decrease in the elastic modulus and a certain increase of elongation at yield of PP matrixes, explaining this behavior by a plasticizing effect caused by the addition of volatile carvacrol and thymol. Persico *et al.* (2009) also observed a marked decrease in the elastic modulus and tensile strength due to the plasticizing effect of carvacrol in LDPE films. Bastarrachea *et al.* (2011) also reported that even small quantities of antimicrobials can change the tensile properties of the films when they interact with the polymeric matrix.

This plasticizing effect caused by the addition of active agents to the polymer could be considered as positive behavior since they improve flexibility and processability of the polymeric materials (Guilbert *et al.*, 1996).

Table 4.18. Mechanical properties of PP-based films according to ISO 527

Sample	Young's Modulus ± SD (GPa)	Stress at peak ± SD (MPa)	Elongation at break ± SD (%)
PP100_2	0.63 ± 0.10 [a]	67.47 ± 3.29 [a]	940.48 ± 36.46 [a]
PP60/40a_8.5%CHL	0.36 ± 0.09 [b]	63.34 ± 2.16 [b]	1002.65 ± 40.73 [b]
PP30/70a_8.5%CHL	0.31 ± 0.03 [c]	60.45 ± 3.83 [b]	1095.60 ± 52.90 [c]

Values within the same column with a different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis).

4.2.2.5. Sealing properties

The heat-sealability of the control and active films was evaluated using a semiautomatic tray-sealing machine as described in section 3.2.5.6. Sealing trials were performed using a

commercial injection-molded PP tray as a reference (PP tray). The sealing time was set at 2.5 sec, considered as a fixed value.

Optimum sealing was determined when the film could not be manually separated from the tray and no deformation of the sealed area such as burns were found. Table 4.19 shows the range of temperature tested and the qualitative optimum sealing temperature selected for the tested materials.

Table 4.19. Sealing temperature for control and active PP bilayer films

	Sealing temperature (°C)										
Sample	**150**	**155**	**160**	**165**	**170**	**175**	**180**	**185**	**190**	**195**	**200**
PP100_2//PP tray	×	×	×	×	×	✓	✓	✓	☑	☑	-
PP60/40a_8.5%CHL // PP tray	×	×	×	×	×	×	×	✓	✓	✓	☑
PP30/70a_8.5%CHL // PP tray	×	×	×	×	×	×	×	✓	✓	✓	☑

(×): not sealing; (✓): acceptable sealing; (☑): optimum sealing; (-): not tested.

As can be observed in Table 4.19, higher temperature was needed for sealing active films with control trays (200 °C), compared to the sealing temperature of the control film with the control tray (190-195 °C). However, the sealing temperatures for the active films were still within the conventionally range used on industrial packaging lines. Both control and active films were satisfactorily heat-sealed to PP trays.

In general, and as previously mentioned in section 4.2.1.3, seal strength usually decreases when additives are incorporated into polymers. The use of additives that can lead to a blooming surface could decrease sealing performance, reducing heat seal strength and affecting the initiator sealing temperature (Gregory, 2008; Scheirs, 2000).

4.2.2.6. Optical properties

The effect of the antimicrobial agents on the transparency of the films was determined by means of the internal transmittance (Ti) of the visible spectrum from 400 to 700 nm by applying the Kubelka–Munk theory as described in section 3.2.5.7.

Figure 4.11 shows the internal transmittance vs. wavelength curves for the control and active bilayer films.

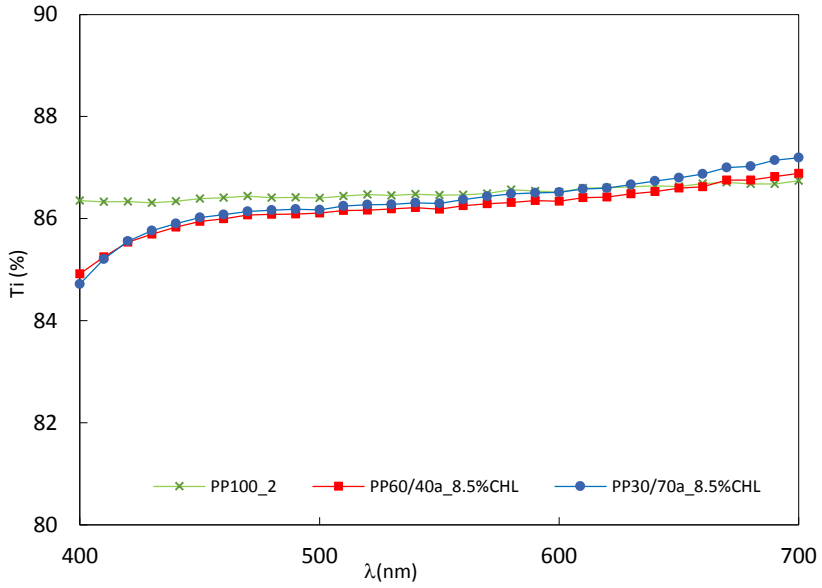


Figure 4.11. Spectral distribution of internal transmittance (Ti) of control and active bilayer PP based films

High values of Ti are related to more transparent films. On the contrary, lower values of Ti are correlated with the opacity of the materials. In this case, control and active PP-based films showed high transparency. In fact, no significant differences were found among films, regardless the concentration of active agents, except for the Ti between 400 and 500 nm (violet/blue region) where a slight difference was observed between control and active films. This difference was probably due to a slight yellowish appearance of the active films (yellow complementary color of blue/violet), hardly detected by the human eye. Therefore, it could be concluded that the addition of volatiles did not affect the transparency of the PP matrix.

These results are in agreement with the work performed by Suppakul *et al.* (2006) who observed slight differences in transparency between HDPE-based films and HDPE-based films fortified with carvacrol and methylchavicol. In general, the optical properties of the films depend on the number and type of crystalline aggregates among other factors (Pritchard, 1964). Nucleating agents are usually included in PP to promote the transparency of the polymeric material; its mechanism is based on the formation of multiple nucleation sites, leading to small crystalline regions. According to the technical data sheet, Raw G70 TF PP has nucleating agents. In this regard, no differences in crystallinity percentage were

observed between active and control films, and this effect could have contributed to the observation of no differences in internal transmittance between control and active materials.

4.2.2.7. Microstructural evaluation

The microstructure of the control PP100_2 and the active PP-based films, PP60/40a_8.5%CHL and PP30/70a_8.5%CHL, was analyzed by light microscopy as described in section 3.2.5.8. Top visualization of the control and active films is shown in Figure 4.12.

Top view bright field light micrographs showed a homogeneous structure with no apparent differences between control and active films. Thus, the addition of the active agents to the PP matrix did not affect the structure of the films at a microscopic level, confirming that active agents were homogeneously distributed.

Although the micrographs did not show differences when active agents were added to the films, the presence of cavities could be visually observed in the active films as the thickness of the active layer increased. These cavities might have been caused by the evaporation of the active agents which caused bubble formation in the active layer due to their high vapor pressure during extrusion processing (Sung *et al.*, 2014).

Ramos *et al.* (2012) observed using scanning electron microscopy (SEM) some porosity in the active PP-based films containing carvacrol or thymol at different concentrations due to the presence of active agents. This porosity was more evident when the content of volatile compounds increased.

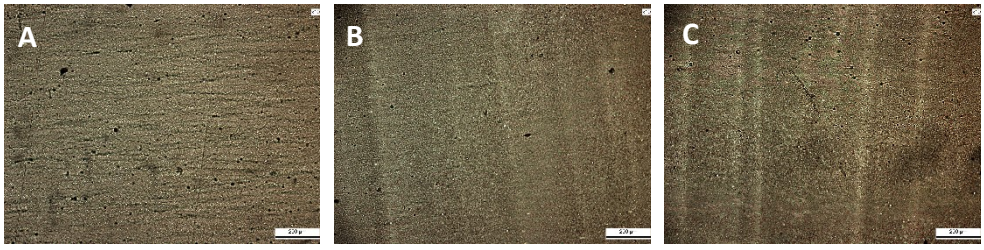


Figure 4.12. Top microstructure of PP-based bilayer films
A: PP100_2 film; B: PP60/40a_8.5%CHL film; C: PP30/70a_8.5%CHL film (10x)

4.2.2.8. Barrier properties

4.2.2.8.1. Oxygen transmission rate

Barrier properties to oxygen of control and active films were studied using an oxygen transmission rate monitoring system according to ASTM D3985 standard at 23 ± 0.5 °C, 0 ± 0.5% RH and 100% oxygen permeant concentration as described in 3.2.5.9. According to the standard, only OTR values were reported (Table 4.20) since the permeability coefficient ($P'O_2$) is meaningful only for homogeneous materials. The reported values were the mean of four replicates ± standard deviation.

An increase in the OTR of the film is not considered an undesirable effect in the final packaging film for the present application since two micro holes are made per film in the final package to avoid anaerobic conditions around the fruit. Anyhow, this test was performed in case barrier needs could change according to the food application.

As can be observed in Table 4.20, the OTR of the films became significantly higher as the concentration of active agents increased. An increase of two and three fold in the OTR were observed for PP60/40a_8.5%CHL and PP30/70a_8.5%CHL films, respectively, compared to the control film.

Table 4.20. OTR of control and active bilayer films at 23 °C, 0% RH and 100% oxygen permeant concentration

Sample	OTR ± SD (cc/m²/day)
PP100_2	464.42 ± 11.46 [a]
PP60/40a_8.5%CHL	1012.45 ± 18.14 [b]
PP30/70a_8.5%CHL	1367.71 ± 23.18 [c]

Values within the same column with a different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis).

These results were corroborated by the presence of some small cavities in the active film surface layer that increased with the content of active agents as described in section 4.2.2.7. These cavities might stimulate the passage of oxygen molecules, increasing the permeability of the material (Sung *et al.*, 2014).

These results are in agreement with findings of Ramos *et al.* (2012), who showed an increase in the OTR of extruded PP films containing volatile additives due to an increase in the

porosity of the surface of the materials caused by the partial evaporation of these volatiles from the polymeric matrix during processing, and by a reduction of the crystallinity of the films. The plasticizing effect of the active agents previously reported could have caused interference with the polymer-polymer interactions and increased the free volume of polymer structures or the molecular mobility of polymer molecules (Sothornvit and Krochta, 2000), decreasing the oxygen barrier (Yuniarto *et al.*, 2014).

4.2.2.8.2. Water vapor transmission rate

Barrier properties to water vapor of control and active films were studied by a gravimetric method based on the ASTM E 96 as described in 3.2.5.9. WVTR results are presented in Table 4.21 as the mean of three replicates ± standard deviation. As for oxygen barrier evaluation, only WVTR is given since the material under evaluation is a bilayer with different layer composition.

Despite the high standard deviation of the measurements, in general, barrier properties of the PP matrix to water were reduced in presence of active agents and this effect was dependent on the amount of active agents in the film. An increase of 3.6 and 4.4 fold of WVTR was observed for the film PP60/40a_8.5%CHL and PP30/70a_8.5%CHL, respectively compared with the control film PP100_2.

Usually the EOs and their pure compounds are hydrophobic molecules. Specifically, citral, hexanal and linalool are poorly or non-soluble in water. Therefore, a reduction in WVTR of the films was expected as the concentration of these antimicrobials increased. However, the presence of small cavities in the films due to evaporation of the antimicrobials during extrusion could lead to an increase of the WVTR.

Contrary to the effect observed in the present research, Suppakul *et al.* (2006) found a reduction in WVTR on basil extract-EVA/LDPE film. In this case, methylchavicol reduced the WVTR greater than linalool due to its higher hydrophobicity.

As reported for OTR results, an increase in the WVTR of the film is not considered an undesirable effect in the final packaging film since two micro holes are made per film in the final package, altering the intrinsic barrier properties of the films. In any case, this test was carried out to know their barrier properties for further food packaging applications.

Table 4.21. WVTR of control and active bilayer PP- based films at 5 °C and 100% RH

Sample	WVTR ± SD (g/m²/day)
PP100_2	0.26 ± 0.25 [a]
PP60/40a_8.5%CHL	0.93 ± 0.65 [b]
PP30/70a_8.5%CHL	1.15 ± 0.82 [b]

Values within the same column with a different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis).

4.2.3. Semi-industrial scale trays

As a preliminary step to the development of active trays, PP, EVA and PP:EVA blends containing 20% of a mixture of potassium sorbate and sodium benzoate (w/w) (ratio 60:40) were processed. As PP is a hydrophobic matrix, EVA was mixed with PP to improve the dispersion of solid active agents with hydrophilic properties. Circular specimens were obtained at lab scale by micro-compounding followed by compression molding under the conditions described in section 3.2.4.3.

Table 4.22 summarizes the description, in terms of composition and thickness of PP, EVA and PP:EVA blends specimens obtained as preliminary active materials at lab scale.

Table 4.22. Composition and thickness of PP, EVA and PP:EVA specimens containing PS and SB

Sample	Composition (%) (w/w)	Thickness ± SD (µm)
PP:10%EVA:20%PS_SB	70_10_20	73 ± 4
PP:20%EVA:20%PS_SB	60_20_20	78 ± 5
PP:30%EVA:20%PS_SB	50_30_20	75 ± 6
PP:20%PS_SB	80_20	80 ± 4
EVA:20%PS_SB	80_20	70 ± 3

These preliminary materials were developed as a first approach to evaluate their processability and the release kinetics (section 4.3) of potassium sorbate and sodium benzoate as well as their antimicrobial properties against the selected microorganisms (section 4.4).

Some processing issues were found with EVA at the processing temperature of PP since the melting temperature of EVA is far below that of PP (108 °C vs 218 °C). As a result, a formulation of PP and 10% of EVA was selected as the best processing blend to facilitate the

dispersion of the potassium sorbate and sodium benzoate (PP:10%EVA:20PS_SB). This matrix was selected as starting point for the formulation of the tray materials at semi-industrial scale.

The active and control trays was processed by three steps: compounding, sheet coextrusion and thermoforming as described in section 3.2.4.4.

The bilayer active sheet and tray was composed of two structures: one external structural layer of neat 100% PP and an internal layer based on a blend of 70% PP, 10% EVA and 20% of potassium sorbate and sodium benzoate (60:40) (PP/PP:10%EVA:20%PS_SB). Nominal thickness of the bilayer sheet before thermoforming was set at 580 µm for the structural layer and 100 µm for the active layer. The weight of the unitary thermoformed trays was 20 ± 0.5 g with 170x127x28 mm dimensions. The control bilayer sheet and tray was composed of one layer of 100% PP as external layer and PP with 10% EVA as the inner layer (PP/PP:10%EVA). The control tray possessed the same thickness and dimensions as the active tray.

Trays were stored for further studies in high barrier bags at room temperature in order to preserve them from ambient moisture.

The active tray and its corresponding control tray were fully characterized in terms of microstructural characteristics, quantification of active agents, sealability, thermal, mechanical, optical and barrier properties.

4.2.3.1. Microstructural evaluation

The microstructures of the active sheet (before thermoforming) and active tray containing potassium sorbate and sodium benzoate were analyzed by light microscopy as described in 3.2.5.8. Cross-sectional details of the active sheet and tray are shown in Figure 4.13 and Figure 4.14, respectively. Specifically, Figure 4.14A represents the cross section of the tray bottom and Figure 4.14B represents the cross section of the tray wall.

The co-extrusion process yielded sheets with a bilayer structure of 670 ± 20 µm thickness distributed in two layers as shown in Figure 4.13. The total thickness was composed of an active layer 100 ± 5 µm thick (PP:10%EVA:20%PS_SB) and a structural PP layer 570 ± 15 µm thick. The total thickness of the thermoformed trays decreased compared to the sheet before thermoforming as a result of the thermoforming process, showing different

reductions in thickness along the tray section. Because of the stretching of the material during thermoforming, the male mold usually provides a thicker bottom and thinner walls. In detail, the thickness at the bottom was 660 ± 20 µm, which was reduced only 1.5% compared to the non-thermoformed sheet. On the other hand, the thickness of the wall was substantially reduced up to 380 ± 20 µm (up to 43% of the sheet thickness).

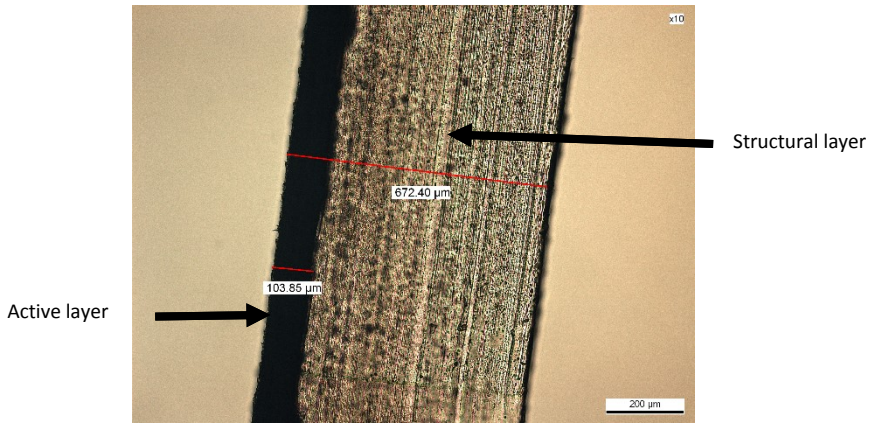


Figure 4.13. Cross-section of bilayer active sheet (PP/PP:10%EVA:20%PS_SB sheet) (10x)

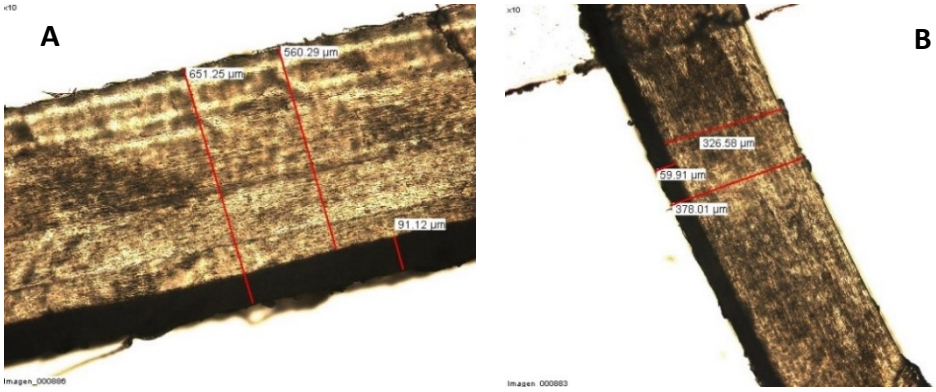


Figure 4.14. Cross-sections of bilayer active tray (PP/PP:10%EVA:20%PS_SB tray) (10x)
(A): Bottom section; (B): Wall section

A top visualization of the trays was also performed by light microscopy for control and active trays as shown in Figure 4.15. The control tray was comprised by a homogeneous matrix as observed in Figure 4.15A. However, when potassium sorbate and sodium benzoate were added to the active layer, heterogeneous agglomerates were clearly observed along the sample (Figure 4.15B). This fact should be taken into account for further developments because the agglomerates could act as weak points both for mechanical and barrier properties (Sung *et al.*, 2013).

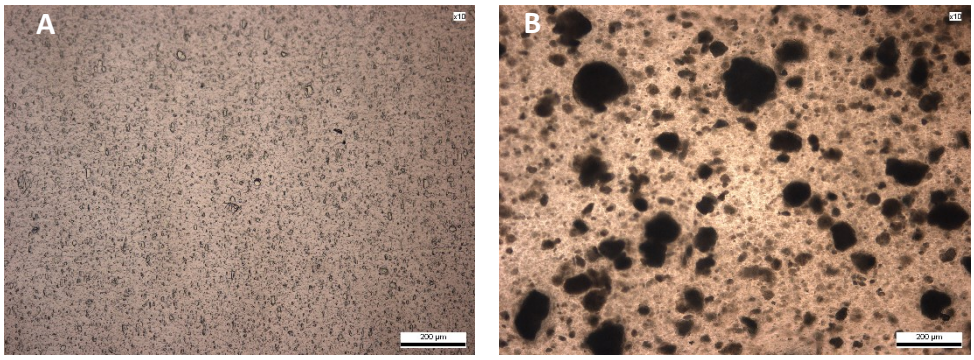


Figure 4.15. Top microstructure of thermoformed trays (10x)
(A): Control tray PP/PP:10%EVA; (B): Active tray (PP/PP:10%EVA:20%PS_SB)

4.2.3.2. Quantification of active compounds

Two polymeric compounds composed of 90% PP and 10% EVA containing a nominal concentration of 24% of potassium sorbate (w/w) or 16% sodium benzoate were successfully processed by a twin screw extruder as described in the materials and methods, section 3.2.4.4.

The quantification of potassium sorbate and sodium benzoate was carried out by determining the residual ash of the compounded materials using a muffle furnace as described in 3.2.5.2.2. The residual ash of each active agent and the polymeric compound control (90% PP and 10% EVA) were also determined. In this perspective, the concentration of potassium sorbate and sodium benzoate in the active compounds were calculated relative to the ash content of the salts (inorganic part) as reported in Table 4.23.

The ashes obtained for control compound were subtracted from the active compounds ashes (0.13 ± 0.01%). Reported values are the mean of three replicates ± SD.

Considering the results obtained, the percentage of potassium sorbate and sodium benzoate in the active compounds were 24.91 and 15.73% (w/w), respectively. Taking into account a nominal concentration of the active compounds of 24% potassium sorbate (w/w) and 16% sodium benzoate (w/w), it could be assumed that both salts suffered no degradation in the compounding process and therefore, no losses at this stage were considered.

Table 4.23. Content of ash (%) obtained for potassium sorbate and sodium benzoate, control and active compounds containing PS or SB

Sample	Ash ± SD (%)
Potassium sorbate	35.66 ± 0.17
Sodium benzoate	22.92 ± 0.23
PP:10%EVA	0.13 ± 0.01
PP:10%EVA:24%PS	9.01 ± 0.40
PP:10%EVA:16%SB	3.73 ± 0.09

4.2.3.3. Quantification of potassium sorbate and sodium in the active trays

The content of potassium sorbate and sodium benzoate in the final trays was evaluated from the ash content determined with a muffle furnace. Prior to this, a mixture of 60% of potassium sorbate and 40% of sodium benzoate was subjected to this method, and results were considered as reference. The content of ash obtained for the blank PP:10%EVA compound (0.13 ± 0.01%) was subtracted from the ash of the active tray to finally estimate the percent of active salts in the tray as indicated in Table 4.24. Reported values are the mean of three replicates ± SD.

Table 4.24. Content of ash (%) obtained for the mixture potassium sorbate and sodium benzoate and the active tray

Sample	Ash ± SD (%)
PS:SB (60:40)	32.98 ± 0.29
PP/PP:10%EVA:20%PS_SB tray	0.98 ± 0.12

Considering the results obtained, the percentage of the total active agents in the tray was estimated as 2.98 ± 0.37% (w/w). The content in the active layer was calculated considering that the active layer thickness was 100 µm average along the tray profile, while the total tray thickness average was 660 µm. In this regard, the percentage of potassium sorbate and sodium benzoate mixture expressed in terms of active layer was estimated as 19.67% (w/w). This result is in agreement with the nominal concentration of active mixture added to the active layer (20%). Therefore, losses of potassium sorbate and sodium benzoate were not considered along the processing steps: compounding, sheet coextrusion and thermoforming.

4.2.3.4. Thermal properties

TGA and DSC tests were carried out to evaluate the influence of potassium sorbate and sodium benzoate mixture on the thermal stability of the active tray compared to the control tray as described in 3.2.5.3. Figure 4.16 shows the percent weight loss curves vs. temperature and their derivative weight curves (%/min) of control and active trays.

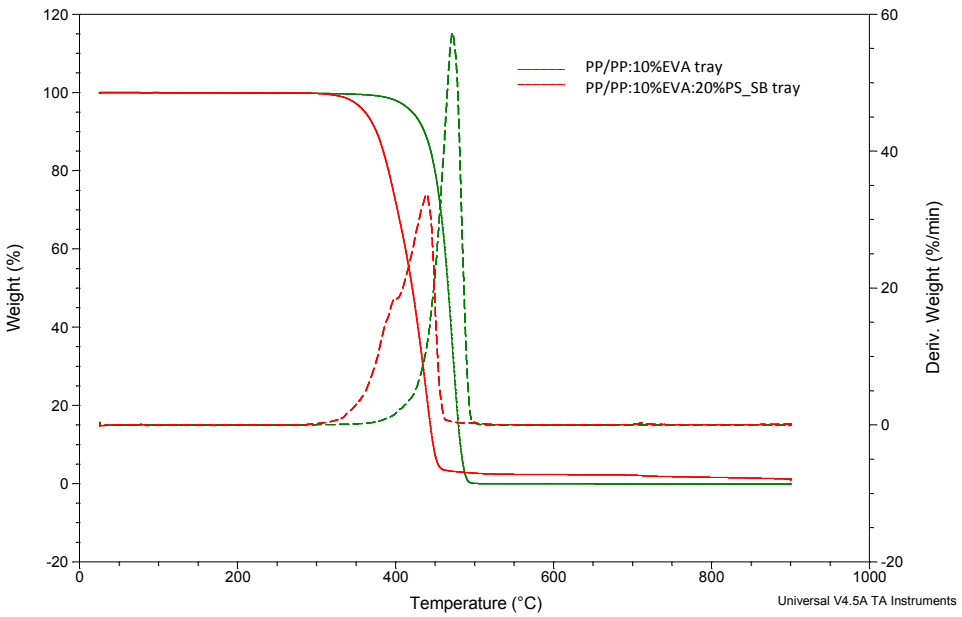


Figure 4.16. TGA and derivative curves of control and active trays
(—): weight loss curve (%); (---): derivative curve (%/min)

As can be observed in Figure 4.16, only one weight loss step was observed for active and control trays. However, in the case of the active tray, the degradation temperature (derivative curve) of the active salts and the polymeric compound (PP:EVA) was overlapped. As previously discussed in section 4.1.4, the maximum degradation temperature for sodium benzoate was around 500 °C, corresponding to the maximum degradation temperatures of the polymer. This effect was clearly observed in the derivative curve of the active tray by the existence of a shoulder where the polymer and the salts are being degraded within the same range of temperature.

In order to estimate the effect of the active agents on the thermal stability of the polymer, the initial decomposition temperature of the polymeric matrix at 1% of weight loss ($T_{1\%}$) and its temperature at the maximum decomposition rate (T_{max_p}) were calculated. These values, taken from Figure 4.16, are indicated in Table 4.25 and were expressed as the mean of three replicates ± standard deviation.

Table 4.25. Thermal properties of control and active trays obtained from TGA curves

	Second weight loss (polymer degradation)	
Sample	**$T_{1\%}$ ± SD (°C)**	**T_{max_p} ± SD (°C)**
PP/PP:10%EVA tray	382 ± 0 [a]	471 ± 0 [a]
PP/PP:10%EVA:20%PS_SB tray	334 ± 3 [b]	437 ± 3 [b]

Values within the same column with different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis).

The degradation temperature of the polymer was clearly affected by the incorporation of the active agents in the polymeric matrix. A significant decrease in the $T_{1\%}$ and T_{max_p} of the polymer occurred in the presence of potassium sorbate and sodium benzoate. This reduction in thermal stability of the active trays could be explained by the presence of active salts that stimulate the scission of polymer chains, accelerating the thermal degradation of the samples. A slight reduction of thermal stability of LLDPE/EVA blends with potassium sorbate was also observed by Kuplennik *et al.* (2015).

Processing temperatures during extrusion steps were around 200 °C, whereas thermoforming temperature, which tempers the sheets below the melting temperature, was around 150 °C. Both ranges are far below degradation temperature of the polymer, and

therefore this reduction in thermal stability did not affect the suitability of active trays for food packaging applications.

The thermal properties of the trays were also evaluated by DSC. The melting and crystallization behaviors were investigated by using the methodology described in section 3.2.5.3.

A first evaluation of the thermal history of the tray was extracted from the first heating scan corresponding to Figure 4.17. First heating thermograms showed a single endothermic peak corresponding to the melting temperature (T_m) of PP. No peak of EVA polymer was identified due to the small percentage present in the blend. Melting enthalpy (ΔH_m) values were extracted from the thermogram.

The degree of crystallinity was calculated as described in Equation 3.2 (3.2.5.3.2) correcting the enthalpy according the weight of the PP in the blend. The heat of fusion for 100% crystalline PP was set at 165 J/g (Zhu, 2002).

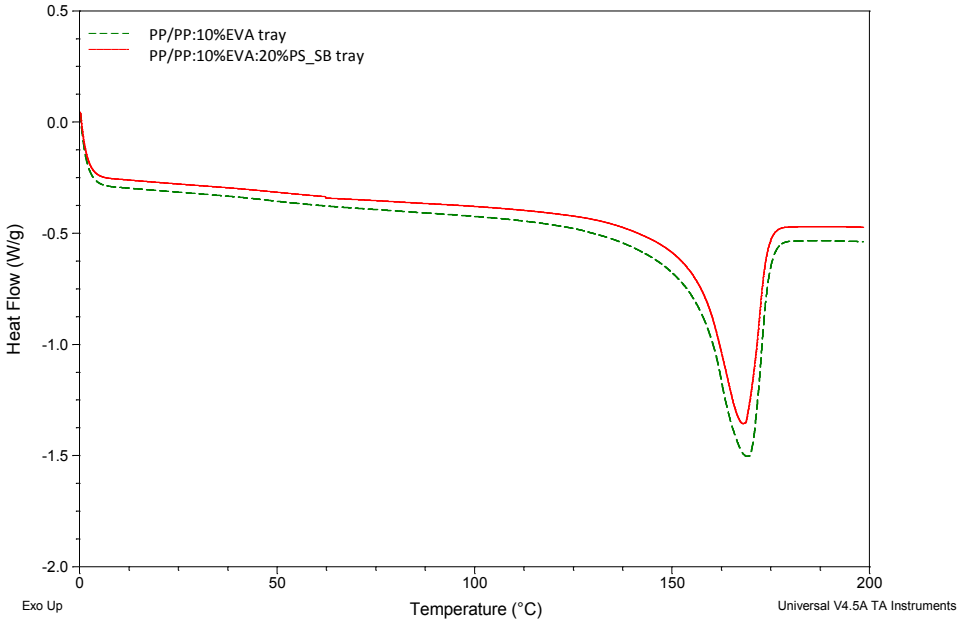


Figure 4.17. DSC curves of control and active trays during the first heating

The information obtained from the first heating is summarized in Table 4.26 and reported values are the average of three replicates ± standard deviation.

Table 4.26. DSC results for the control and active trays during the first heating

Sample	T_m ± SD (°C)	ΔH_m PP ± SD (J/g)	Crystallinity PP (%) ± SD
PP/PP:10%EVA tray	169 ± 1 [a]	103.67 ± 1.22 [a]	65 ± 1 [a]
PP/PP:10%EVA:20%PS_SB tray	168 ± 1 [a]	100.82 ± 5.63 [a]	65 ± 4 [a]

Values within the same column with different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis).

As indicated in Table 4.26, the melting temperature of the composite materials did not change with the addition of solid active antimicrobials. In addition, no significant differences in the melting enthalpy of the PP were observed between active and control trays. In this case, the incorporation of potassium sorbate and sodium benzoate did not affect the crystallinity of the PP with values of 65%.

To further investigate the crystallization process and melting behavior under controlled conditions which represent the intrinsic thermal properties of the trays, a cooling and second heating step were evaluated.

Figure 4.18 represents the cooling and heating curves. The cooling step corresponds to the exothermic peak observed and gives information on the onset (T_{onset}) and crystallization temperature (T_c) and crystallization enthalpy (ΔH_c) summarized in Table 4.27. The endothermic peak corresponds to the second heating of the polymer and gives information on the intrinsic properties of the material. The melting temperature (T_m), melting enthalpy (ΔH_m) and crystallinity from the second heating are also summarized in Table 4.27. Reported values are the average of three replicates ± standard deviation.

As observed for the first heating, no differences in crystallization temperature and crystallization enthalpy were found between active and control trays. In addition, the same melting temperatures and melting enthalpies were observed in the second heating, with no differences in crystallinity.

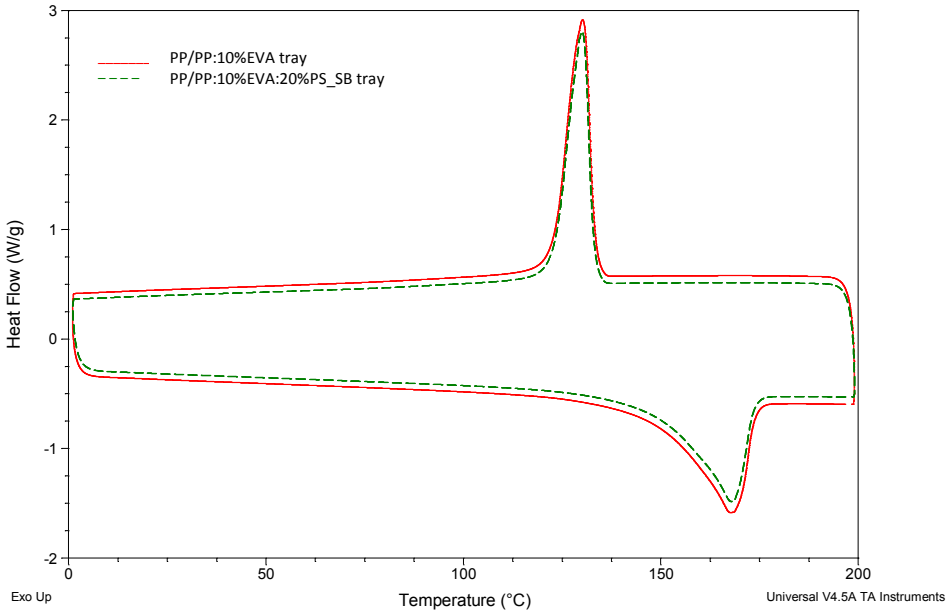


Figure 4.18. DSC curves of control and active trays during cooling and second heating

Table 4.27. DSC results for the control and active trays during cooling and second heating

	Cooling			Second heating		
Sample	**T_{onset} ± SD (°C)**	**T_c ± SD (°C)**	**ΔH_c ± SD (J/g)**	**T_m ± SD (°C)**	**ΔH_m ± SD (J/g)**	**% Crystallinity ± SD**
PP/PP:10%EVA tray	135 ± 0 [a]	130 ± 0 [a]	103.67 ± 1.66 [a]	168 ± 0 [a]	110.50 ± 0.58 [a]	69 ± 1 [a]
PP/PP:10%EVA: 20%PS_SB tray	134 ± 1 [a]	130 ± 0 [a]	103.43 ± 1.60 [a]	168 ± 1 [a]	106.37 ± 1.01 [a]	69 ± 1 [a]

Values within the same column with different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis).

Contrary to this effect, Junqueira-Gonçalves *et al.* (2013) reported a nucleating effect of potassium sorbate when incorporated in a PET matrix, increasing the crystallinity of the polymer from 33 to 43% (evaluated in the second heating). In this perspective, it is well known that the presence of solid additives in the polymer matrix can favor the nucleation/crystallization effect (Dong *et al.*, 2008; Urushihara *et al.*, 2006). Nucleating agents are generally inorganic materials with a small average particle size around 3 µm and a high melting point (Liang, 2002). However, the additives used for the development of the

active tray were food grade with an average particle size > 300 µm. Therefore, a nucleating effect was not found in the present work. This behavior could also possibly be related to a low degree of visual dispersion of the salts in the polymer that generated low levels of nucleating-interactions (Caveda, 2012).

4.2.3.5. Sealing properties

Sealing between control and active trays and control films was evaluated using a semiautomatic tray-sealing machine as described in section 3.2.5.6. The heat-sealability between active film and active tray was also evaluated. The sealing integrity was qualitatively evaluated. Table 4.28 shows the results of sealability at the different temperatures tested.

As can be observed in Table 4.28, higher temperature was needed for sealing active trays with control films (180-190 °C), compared to the sealing temperature with control trays (170 °C). In addition, even higher temperatures were needed for sealing active trays with active films as previously reported. However, the sealing temperatures were still within the intervals accepted by the industry (200 °C).

As was expected, the incorporation of solid particles affected the heat-seal strength of the material. Large particles are affecting material caulkability not allowing the polymer chains to properly distribute and interact with the film to provide a homogeneous sealing area. In detail, caulkability refers to the ability of the sealant material to flow, filling in gaps around folds, wrinkles or product contaminants.

These results were in agreement with those of Pires *et al.* (2014) who observed that the sealing properties of LDPE films were affected by potassium sorbate incorporated into the film, acting as an impurity in the sealed area.

Table 4.28. Sealing parameters for control and active trays with control films

	Sealing temperature (°C)						
Sample	**140**	**150**	**160**	**170**	**180**	**190**	**200**
PP100_2 film// PP/PP:10%EVA tray	×	×	×	☑	☒	☒	☒
PP100_2 film// PP/PP:10%EVA:20%%PS_SB tray	×	×	×	✓	☑	☑	☒
PP10/70a_8.5%CHL film// PP/PP:10%EVA:20%%PS_SB tray	×	×	×	×	×	✓	☑

(×): not sealing; (✓): acceptable sealing; (☑): optimum sealing;

4.2.3.6. Mechanical properties

The compressive forces that trays suffer from stacking along the supply chain were evaluated by compression tests as described in 3.2.5.5. Maximum force and deflection (deformation of tray walls) at the point of maximum load were evaluated in the compression test. Table 4.29 shows the resistance and deformation values obtained from compression tests for control and active trays. Reported values are the average of ten replicates ± standard deviation.

The presence of potassium sorbate and sodium benzoate mixture in the polymeric tray did not significantly alter the resistance of the tray to the external load applied. However, in the case of the deformation parameter, trays with active substances showed higher deformation compared to the control tray for the same load. This could be explained by the presence agglomerates distributed in the matrix that led to a heterogeneous material, decreasing the material resistance to deflection.

From Table 4.29 it was calculated that trays will resist a maximum load of around 58 kg. Considering conventional distribution along the supply chain, packed trays will not be exposed to such loading forces during commercialization, experiencing usual loads of around one to two kg (5-10 trays). These usual loads may lead to small deflections of around 1 mm or less. Therefore, the addition of active agents to the polymeric tray will not affect their performance along the food distribution chain.

The effect of the addition of organic salts to PET clamshells on the mechanical compression properties was also evaluated by Junqueira-Gonçalves *et al.* (2013). These authors demonstrated that the addition of potassium sorbate at 2.5% (w/w) did not significantly affect the packaging resistance, but 4% (w/w) affected resistance and deformation of the package.

Table 4.29. Resistance to compression test of control and active trays

Sample	Force at peak ± SD (N)	Deflection at peak ± SD (mm)
PP/PP:10%EVA tray	577.8 ± 41.4 [a]	6.28 ± 0.8 [a]
PP/PP:10%EVA:20%%PS_SB tray	583.5 ± 24.1 [a]	8.15 ± 0.8 [b]

Values within the same column with a different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis).

4.2.3.7. Optical properties

The transparency of the trays was determined by means of internal transmittance (Ti) monitored from 400 to 700 nm with a spectrocolorimeter by applying the Kubelka–Munk theory (Hutchings, 1999) as described in section 3.2.5.7.

Figure 4.19 shows the internal transmittance vs. wavelength of control trays and those containing the active agents.

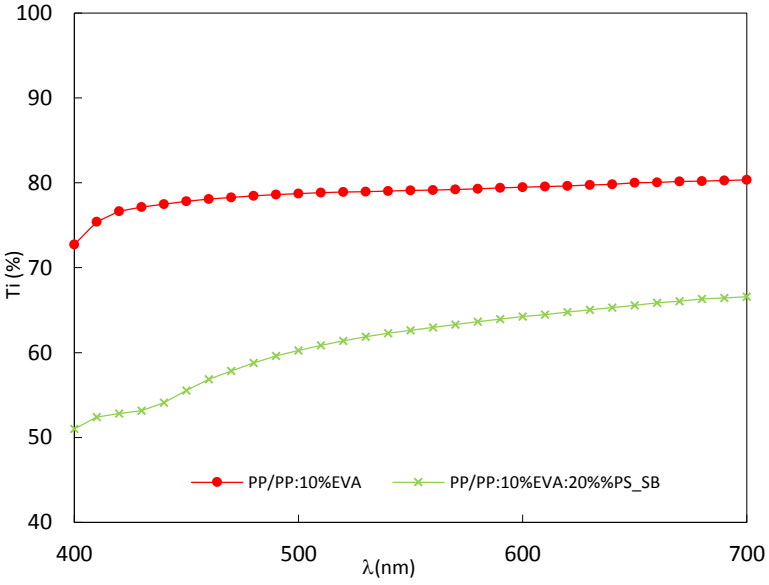


Figure 4.19. Spectral distribution curves of internal transmittance (Ti) of control and active trays

As already discussed in section 4.2.2.6, high values of Ti are directly related to higher transparency. In this way, control trays were significantly less opaque than active trays since potassium sorbate and sodium benzoate remain as agglomerates in the polymeric matrix. At a specified wavelength of 600 nm, Ti significantly decreased from 79.5 ± 0.1% to 64.2 ± 0.3% as a consequence of active agent addition.

These results are in agreement with studies performed with other polymers such as LDPE and PET. Han and Floros (1997) reported that the transparency of LDPE films decreased as potassium sorbate content increased. According to Junqueira-Gonçalves *et al.* (2013), when

potassium sorbate was added, a yellowish colour was observed depending on the concentration, lowering significantly the transparency of the packaging material.

4.2.3.8. Barrier properties

4.2.3.8.1. Oxygen transmission rate

Barrier properties to oxygen of control and active trays were studied using an oxygen transmission rate system based to ASTM F1307 standard as described in 3.2.5.9. According to the standard, permeability is meaningful only for homogenous materials. Therefore, only OTR values were evaluated and results are displayed in Table 4.30 as the mean of four replicates ± standard deviation.

OTR results were not affected by the effect of active substances. These preservatives are distributed throughout the active layer and the thicker structural layer could have counteracted the possible increase in OTR caused by the active layer. This result was corroborated by the thermal analysis of the polymeric tray (section 4.2.3.4) where no changes in percent crystallinity occurred with the addition of potassium sorbate and sodium benzoate.

Table 4.30. OTR of control and active trays at 23 °C, 50% RH and 21% oxygen permeant concentration

Sample	OTR ± SD (cc/package/day)
PP/PP:10%EVA	0.865 ± 0.058 [a]
PP/PP:10%EVA:PS_SB	0.802 ± 0.063 [a]

Values within the same column with a different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis).

4.2.3.8.2. Water Vapor transmission rate

Barrier properties to water vapor of control and active trays were studied using a water vapor transmission rate system as described in 3.2.5.9. Water vapor transmission rate results are shown in Table 4.31 as the mean of four replicates ± standard deviation.

WVTR of the active tray was not significantly affected by the addition of hydrophilic molecules such as potassium sorbate and sodium benzoate. Therefore, the effect observed

might have been due the structural layer that counteracted the increase of the WVTR of the active layer.

Table 4.31. WVTR results of control and active trays at 23 °C and ΔRH 90-0%

Sample	WVTR ± SD (g/package/day)
PP/PP:10%EVA	0.0058 ± 0.0010^{a}
PP/PP:10%EVA:PS_SB	0.0066 ± 0.0016^{a}

Values within the same column with a different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis).

In active packaging materials, WVTR is dependent on the hydrophilic and hydrophobic ratio between the polymeric matrix and the antimicrobial agents. The hydrophilic materials tend to increase water vapor transmission (Sung *et al.*, 2013). In this way, Vartiainen *et al.* (2003) reported that the addition of 5% EDTA with hydrophilic characteristics to a monolayer LDPE film increased the water vapor permeability. This effect was also observed by Vásconez *et al.* (2009) in monolayer biopolymer films where the addition of potassium sorbate into chitosan-tapioca films increased film water vapor permeability. The addition of potassium sorbate with a hydrophilic hydrogen bond could have increased the hydrophilic:hydrophobic ratio of the film.

4.2.4. Conclusions on the active materials characterization

In general, the active materials developed in the present Doctoral Thesis showed good potential for their use as food packaging materials.

Specifically, bilayer active films and trays developed at the semi-industrial scale showed a high level of incorporation of volatile and non-volatile active agents, respectively.

Thermal properties of the active films both at lab and semi-industrial scales were not significantly affected by the addition of volatile agents, maintaining their thermal performance after processing. The addition of active volatiles did not affect the percent crystallinity of the semi-industrial bilayer active films, but conferred on the polymeric matrix some plasticizing effect, making the films more flexible and improving their process ability.

On the contrary, the degradation temperature of the active tray was reduced by the incorporation of solid antimicrobial in the polymeric matrix but this degradation did not

affect the suitability of active trays for food packaging applications. The addition of solid active agents did not affect the percent crystallinity of the tray material.

The effect on mechanical properties of the films and trays was not critical for their envisaged application, and they were able to sustain their integrity under the influence of various stresses that could occur during the packaging, transport and storage of the packed fruit.

Sealing of the polymeric materials was affected by the addition of volatile and non-volatile active agents, since higher temperatures were usually needed to achieve the same sealing as for the control materials. However, the sealing temperatures needed were still within those conventionally applied on industrial packaging lines.

The incorporation of volatile active agents in the polymeric films at a semi-industrial scale did not affect their transparency. In the case of active trays, the incorporation of potassium sorbate and sodium benzoate to the PP:EVA matrix significantly decreased their transparency. This effect might be mitigated by reducing the particle size of the additives used. The addition of a white food contact colorant (titanium dioxide) could also be considered for improving the tray appearance.

Another important parameter to consider when choosing polymeric materials for food packaging is their oxygen and water vapor barrier characteristics. In this case, only low barrier requirements were needed since micro-perforations would be made to the films during fruit packaging process to avoid anaerobic conditions inside the packed fruit. In any case, the addition of volatile active agents reduced the oxygen barrier properties of the active films. WVTR also increased, probably due to the presence of small cavities in the films caused by the evaporation of the active agents in the extrusion step. The addition of solid active agents to the active tray did not significantly affect its barrier properties since the structural layer was probably counteracting the increases in OTR and WVTR caused by the addition of hydrophilic additives.

4.3. ACTIVE PACKAGING MATERIALS RELEASE KINETICS

The antimicrobial activity of a given preservative is dose-dependent. In this regard, the release rate of the active agents from the packaging material is critical in order to maintain the content of a given preservative above the minimum inhibitory concentration during the desired food shelf life (Chalier *et al.*, 2009).

The extent of the release of a given active agent from a packaging material is usually expressed by the diffusion coefficient (D) which gives information to evaluate the performance of the active packaging material in contact with a food product.

The following sections describe the results of the release kinetic studies carried out with active film and tray at 4 °C and 25 °C.

4.3.1. Release of volatile active agents from semi-industrial active films

4.3.1.1. Release kinetics at 4 °C

The study of the volatiles citral, hexanal and linalool release from the bilayer PP-based film PP30/70a_8.5%CHL was carried out at 4 ± 2 °C and 90 ± 5% RH as described in section 3.2.6.1. The pieces of film were removed from the chamber at different time intervals to quantify the content of the active agents remaining in the materials. The quantification was carried out by solvent extraction with acetone followed by GC-MS analysis as described in section 3.2.5.2.1.

Figure 4.20, Figure 4.21 and Figure 4.22 show the content of hexanal, citral and linalool remaining in the film expressed as mg of active agent per dm^2 of packaging material over time at 4 °C, respectively. Results are the mean of three replicates ± standard deviation.

As can be observed in Figure 4.20, hexanal showed a clear release from the packaging film over time. The content of hexanal remaining in the packaging materials decreased fast during the first 10 days of the test and reached almost a complete release after 35 days. Conversely, citral and linalool did not show a significant release at 4 °C over 38 days.

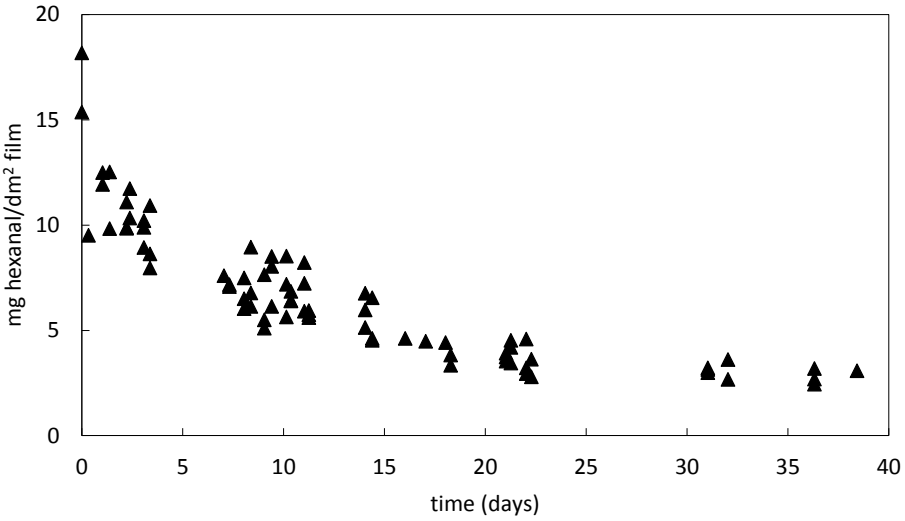


Figure 4.20. Release kinetics of hexanal at 4 °C and 90% RH from PP30/70a_8.5%CHL active film

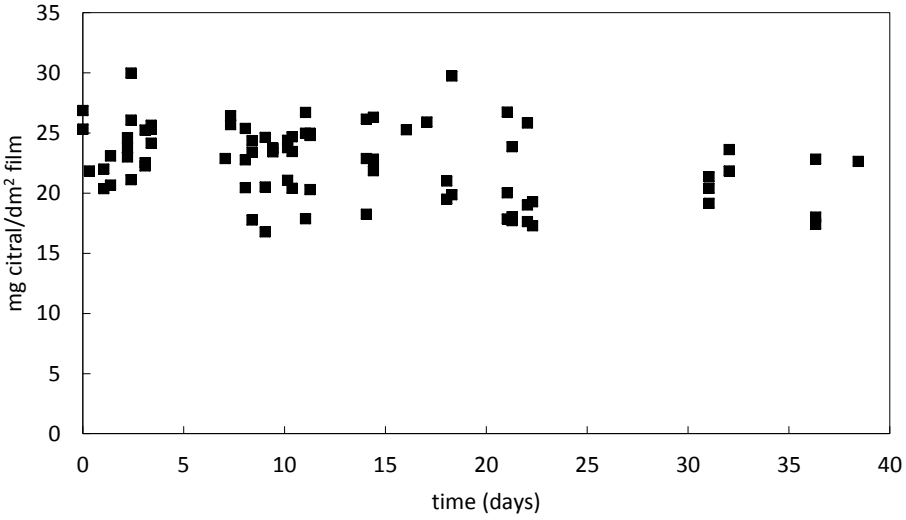


Figure 4.21. Release kinetics of citral at 4 °C and 90% RH from PP30/70a_8.5%CHL active film

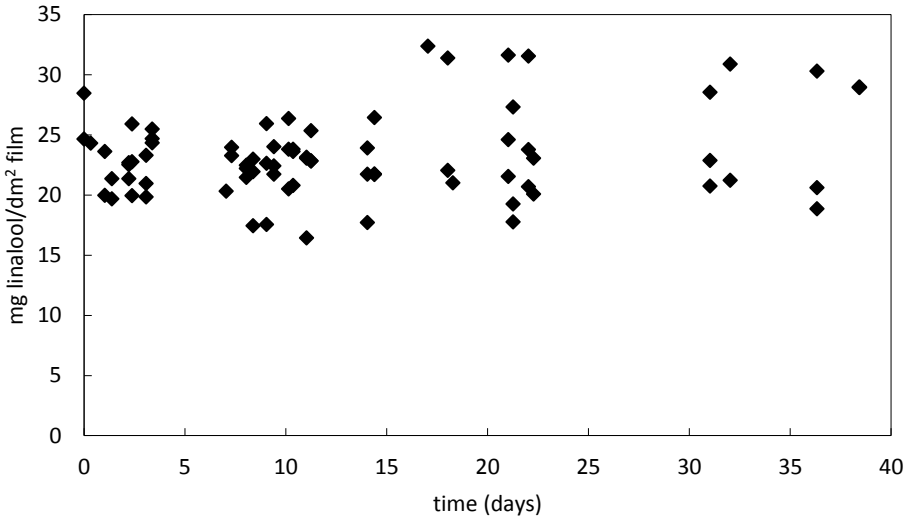


Figure 4.22. Release kinetics of linalool at 4 °C and 90% RH from PP30/70a_8.5%CHL active film

The diffusion coefficient of hexanal was estimated by adjusting Equation 3.7 to the experimental data. For this purpose, the experimental data $m^0_{packaging\_material}$, $m^{eq}_{packaging\_material}$ and $m^t_{packaging\_material}$ which are the quantity of the active substance at initial, final and at any time in the packaging material, respectively, were used as indicated in Equation 3.7 to obtain a new variable which ranged between 1 (at initial time) and 0 (when total release occurred). This variable was defined as the ratio between the amount of active substance that still has to migrate from the packaging material and the total amount released at the final time.

Other input variables needed in Equation 3.7 to estimate a D value were:

- the α value that was set at 99 since the experimental set-up was designed to promote the total release of the active agents after infinite time (> 99% of migration);
- L (active layer thickness) was considered twice the real active layer thickness (140 μm instead of the real 70 μm), since the active packaging material is a co-extruded bilayer film composed of an active layer and a structural layer. Further, it was assumed that the active packaging film released the active substances in one

direction while Equation 3.7 considers a release towards both sides of the packaging material. This can be mathematically solved by doubling the real active layer thickness in Equation 3.7.

All these input variables were introduced into the MATLAB routines in order to estimate the diffusion coefficients. Figure 4.23 shows two plates (a and b) related to the estimation of the diffusion coefficient for the hexanal released from PP30/70a_8.5%CHL active film. The MATLAB routine calculates the root mean square error (RMSE) associated with a range of several D values between 10^{-14} to 10^{-3} cm^2/s. In this regard, Figure 4.23a shows the percent RMSE associated with the different D values. As can be observed in this figure, a minimum RMSE was obtained for a specific D value, which is considered the best D value fitting the experimental release kinetic curve. Figure 4.23b shows the kinetic curve estimated with the model based on the best D value and how this curve adjusts to the experimental data. In particular, the symbol (o) reflects the experimental values whilst the solid line represents the kinetic curve obtained with the best D value.

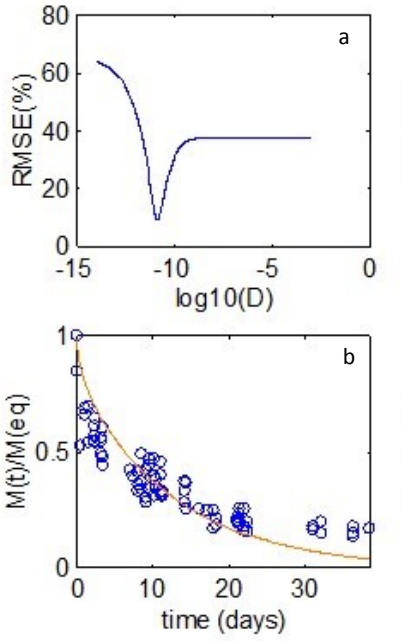


Figure 4.23. Release of hexanal from PP30/70a_8.5%CHL film at 4 °C
(a) RMSE associated with the different D values tested; (b) fitting the kinetic curve obtained with the best D value (providing the lowest RMSE) of the experimental data

The fitting of the release kinetics of hexanal from the active packaging film to Equation 3.7 (RMSE), which describes the diffusion from a plane sheet, was 9.5%. The D value obtained for hexanal was 1.31 x 10^{-11} cm^2/s. The diffusion coefficient of linalool and citral was not calculated as no release was observed at 4 °C.

4.3.1.2. Release kinetics at 25 °C

The release of the volatiles citral, hexanal and linalool from the PP-based active film (PP30/70a_8.5%CHL) at 25 ± 2 °C and 50 ± 5% RH was carried out as described in section 3.2.6.1. Films were removed from a bench at different time intervals to quantify the content of the active agents remaining in the materials following the same procedure as described in section 4.3.1.1.

Figure 4.24, Figure 4.25 and Figure 4.26 show the remaining content of hexanal, citral and linalool, respectively, expressed as mg of active agent per dm^2 of film over time at 25 °C.

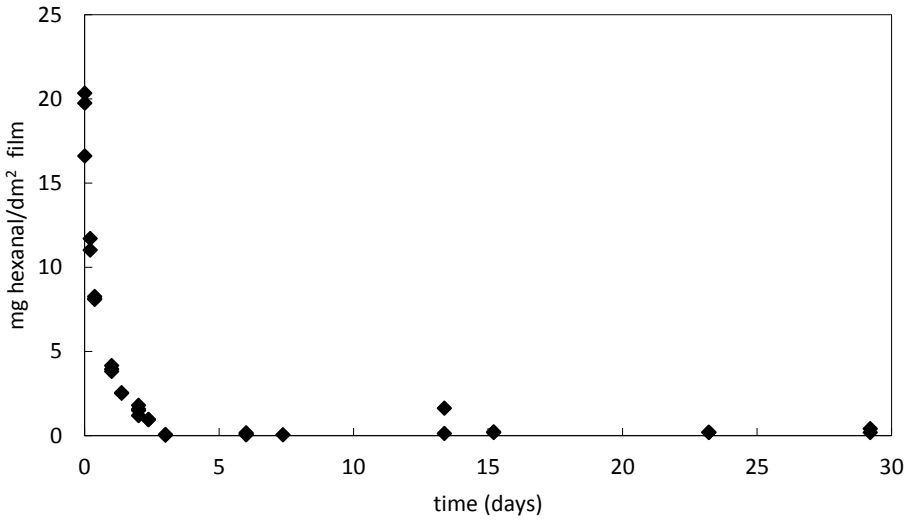


Figure 4.24. Release kinetics of hexanal at 25 °C and 50% RH from PP30/70a_8.5%CHL active film

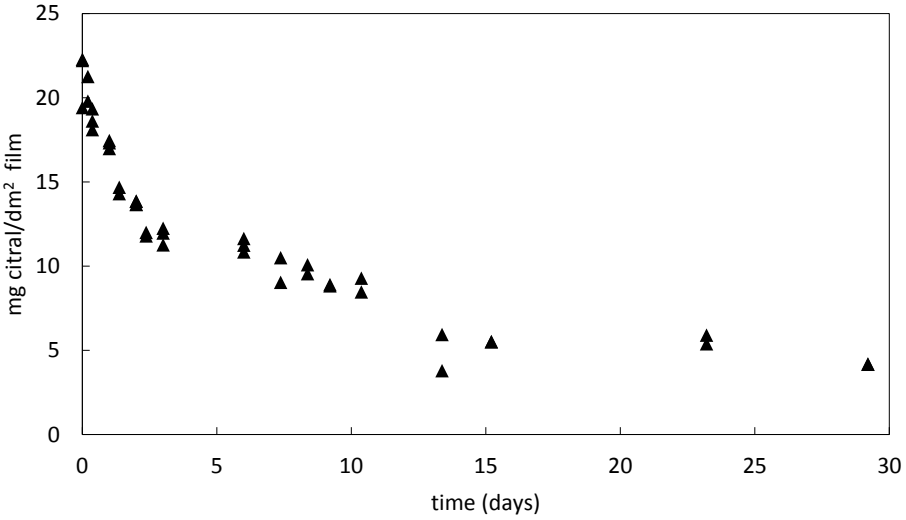


Figure 4.25. Release kinetics of citral at 25 °C and 50 % RH from PP30/70a_8.5%CHL active film

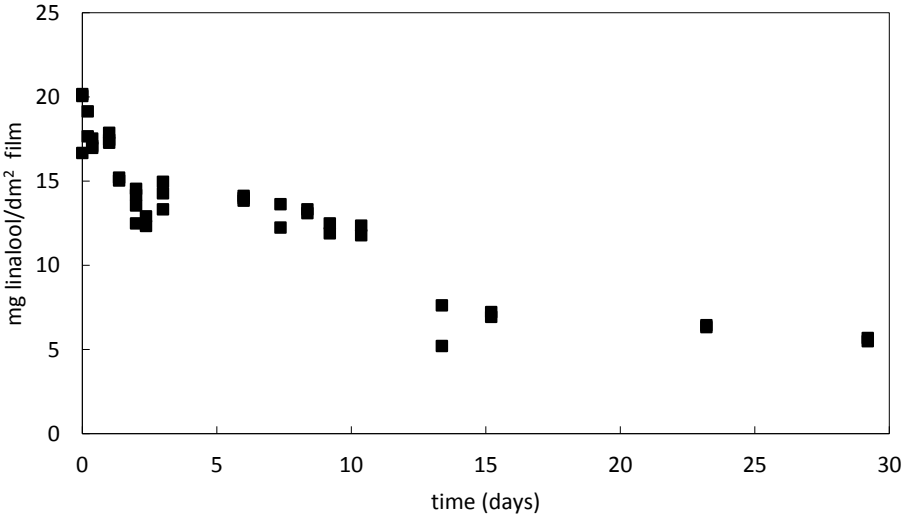


Figure 4.26. Release kinetics of linalool at 25 °C and 50% RH from PP30/70a_8.5%CHL active film

In this case, the three active agents showed a clear release over time from the packaging film. Hexanal reached a complete release after 3 days of test while there was still some content of citral and linalool remaining in the packaging film at day 28 of the test. Hexanal was released faster than citral and linalool. The release kinetics of linalool and citral were similar, showing a faster release up to 15 days of the experiment and slowing down significantly until the end of the test.

The diffusion coefficients of citral, hexanal and linalool were estimated by adjusting Equation 3.7 to the experimental data. The experimental data $m^{0}_{packaging\_material}$, $m^{eq}_{packaging\_material}$ and $m^{t}_{packaging\_material}$ along with α value (= 99) and thickness (L= 140 μm) of the active layer were used to estimate D values.

Figure 4.27 shows the plots related to the estimation of the diffusion coefficient for hexanal, citral and linalool, from the active PP30/70a_8.5%CHL film at 25 °C. As can be observed in Figure 4.27a, a minimum RMSE was obtained for a given D value in all the cases. On the other hand, Figure 4.27b shows the kinetic curves estimated with the model in Equation 3.7 (solid line) based on the best D value and how this curve adjusts to the experimental data (symbol "o"). The fit of the release kinetics at 25 °C of the three active agents from the active film to Equation 3.7 was quite good based on the RMSE values, below 10% in all cases.

Table 4.32 shows the diffusion coefficient obtained for hexanal, and citral and linalool, and their associated RMSE. Hexanal showed the highest D value followed by citral and linalool. These results mean that hexanal is released faster than citral and linalool, and citral is released faster than linalool.

The differences in the release kinetics among the active substances at the same temperature (25 °C) could be explained considering the different molecular size of the substances. In this regard, the molar volume of hexanal is much lower than the molar volume of citral and linalool (hexanal: 124.9 mL/mol < citral: 177.8 mL/mol < linalool: 179.7 mL/mol) (CSID, 2015). The smaller molecular size of the molecules within a polymeric matrix, the faster diffusion occurs (Begley *et al.*, 2005; Földes, 1994).

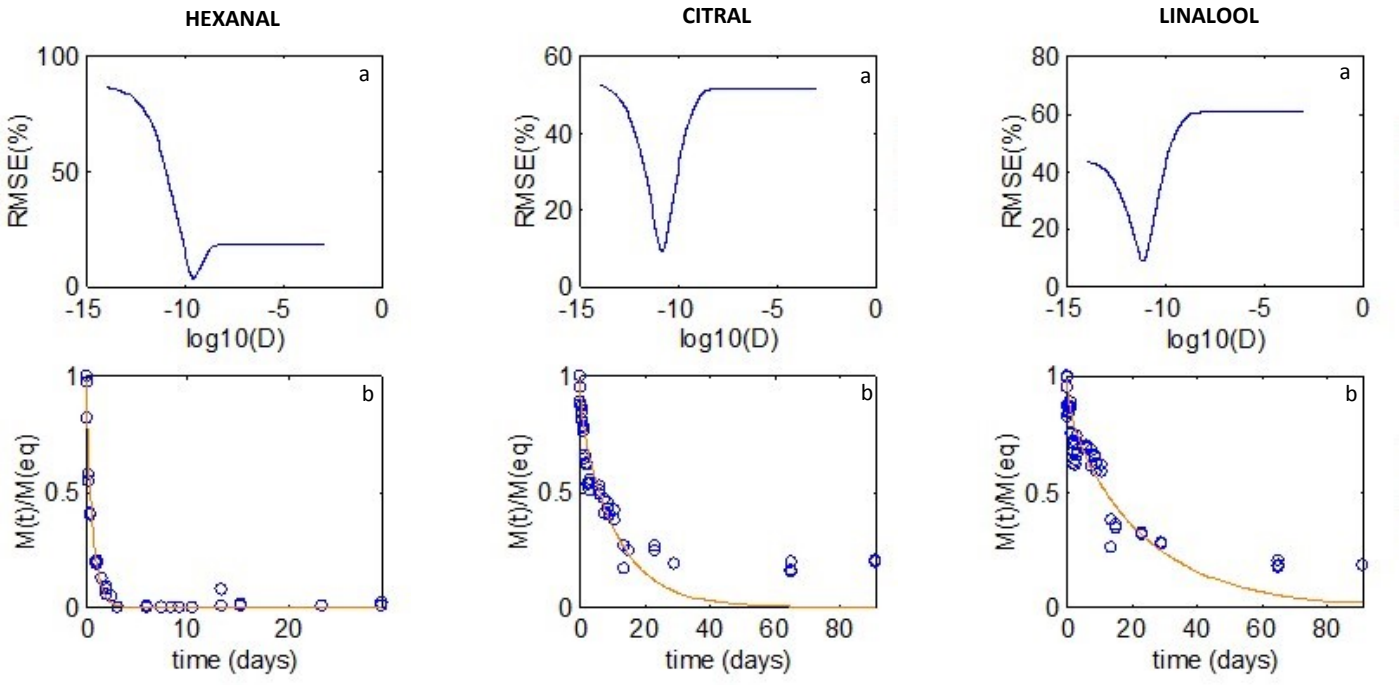


Figure 4.27. Release of hexanal, citral and linalool from PP30/70a_8.5%CHL active film at 25 °C
(a) RMSE associated with the different D values tested; (b) fitting the kinetic curve obtained with the best D value (providing the lowest RMSE) of the experimental data

Table 4.32. Diffusion coefficient (D) and RSME corresponding to the fit of the kinetic curves of released hexanal, citral and linalool to Equation 3.7 (PP30/70a_8.5%CHL active film at 25 °C)

	Hexanal	Citral	Linalool
D (cm^2/s)	2.61×10^{-10}	1.42×10^{-11}	7.38×10^{-12}
RMSE (%)	3.8	7.7	8.1

On the other hand, volatilization from the film surface depends on the vapor pressure of the active agents. At 25 °C, the vapor pressure of the active agents decreases in the following order as already described in section 4.1: hexanal > linalool> citral. Although no experimental vapor pressure values were found at 4 °C, they were estimated using the MPBPWIN software developed by the U.S. Environmental Protection Agency (v1.43 US EPA, 2010). The vapor pressure values at 4 °C were 2.44 mm Hg for hexanal and 0.01 mm Hg for linalool and citral. These values explain the different behavior observed between active agents at 4 °C where just release of hexanal occurred.

Considering the results obtained at both temperatures, 4 and 25 °C, citral, hexanal and linalool showed different release rates from the active packaging film. At 4 °C, only hexanal was released from the packaging material whilst linalool and citral remained inside the packaging material. At 25 °C, the three active agents were released from the packaging material but different release rates were observed as indicated by the estimated D values in Table 4.32. An increase of temperature from 4 °C to 25 °C caused the diffusion coefficient of hexanal to increase 20-fold.

The impact of the temperature on the release rate of active agents from packaging materials has been described in the literature. For instance, an increase of the temperature clearly caused faster release of linalool and methylchavicol from LDPE matrices into isooctane (Suppakul *et al.*, 2011a). The diffusion coefficient of linalool calculated by these authors increased from 4.2×10^{-09} cm^2/s to 2.5×10^{-08} cm^2/s (6-fold) from 4 to 25 °C and from 3.5×10^{-09} cm^2/s to 1.10×10^{-08} cm^2/s (3-fold) for methylchavicol, indicating that the migration of linalool was more sensitive to temperature than methylchavicol.

In general, higher D values were reported in the literature for the diffusion coefficient of the active agents in food simulants. Ramos *et al.* (2014) reported that the diffusion coefficient of carvacrol and thymol, incorporated in PP active films by melt blending followed by compression molding, ranged from 1 to 2×10^{-10} cm^2/s in 10% ethanol, 3% acetic acid and

95% ethanol at 40 °C. The diffusion coefficient calculated for thymol and carvacrol from migration into iso-octane was 4 and 6 times higher, respectively, due to the higher affinity of the non-polar matrix to the non-polar simulant. Cran *et a*l. (2010) also studied the diffusion of linalool incorporated in LDPE films containing EVA into food simulants iso-octane, 95% and 15% ethanol at 25 °C. The diffusion coefficients decreased in the following order: iso-octane (4.14 x 10^{-9} cm^2/s), > 95% ethanol (6.7 x 10^{-10} cm^2/s) > 15% ethanol (4.5 x 10^{-10} cm^2/s). This effect reflected the increasing order of solvent polarity and the decreasing order of affinity of the simulants to the polymeric substrate.

Those results are not directly comparable with the release kinetic results modelled from PP-based films toward the atmosphere carried out in the present research. In this regard, the study of active agents moving toward the atmosphere better represents the current application of the active film with volatile active agents being released to the headspace of packed food.

4.3.2. Release of non-volatiles active agents from monolayer materials at 4 °C

The study of the release of the non-volatile potassium sorbate and sodium benzoate from PP and EVA monolayer materials was carried out in 3% (w/v) acetic acid at 4 °C as described in section 3.2.6.2. The amount of potassium sorbate and sodium benzoate released from the materials to the food simulant over time was carried out through the analysis of the 3% (w/v) acetic acid by HPLC-UV as also described in 3.2.6.2.

The analytical parameters of the method (slope, intercept, linearity, repeatability, limit of detection (LOD) and limit of quantification (LOQ)) are shown in Table 4.33. Linear ranges were calculated with five calibration points, each one in triplicate. The regression coefficients (R^2) obtained were higher than 0.99 for all the calibration curves. Repeatability was evaluated by analyzing five replicates of a standard solution of 10 mg/kg of each active agent. The relative standard deviation (RSD) for all the cases was below 10%. Limits of detection (LOD) and quantification (LOQ) were also calculated based on the injection of 10 standard solutions at levels of 0.4 mg/kg of each active agent. LOD and LOQ were calculated from the standard deviation of the area obtained for each active agent in the ten injections.

Table 4.33. Analytical parameters of HPLC-UV method for potassium sorbate and sodium benzoate determination

Active agent	Calibration range (mg/Kg)	Slope ± SD	Intercept ± SD	Linearity (R^2)	Repeatability (RSD, %)	LOD (mg/Kg)	LOQ (mg/Kg)
PS	2-200	90379 ± 333	-29842 ± 28179	0.9999	2.42	0.08	0.25
SB	2-200	35552 ± 110	-19570 ± 9415	0.9999	4.96	0.17	0.58

In particular, LOD was determined as three times the standard deviation divided by the slope of the calibration curve, while LOQ was determined as 10 times the standard deviation divided by the slope. LOD was in the range of 0.08-0.17 mg/kg, while LOQ were in the range of 0.25-0.58 mg/kg. These results were considered acceptable for the determination of potassium sorbate and sodium benzoate by HPLC-UV.

Figure 4.28 to Figure 4.31 show the potassium sorbate and sodium benzoate released into food simulant from PP or EVA materials expressed as mg of active agent per dm^2 of packaging material over time at 4 °C, respectively.

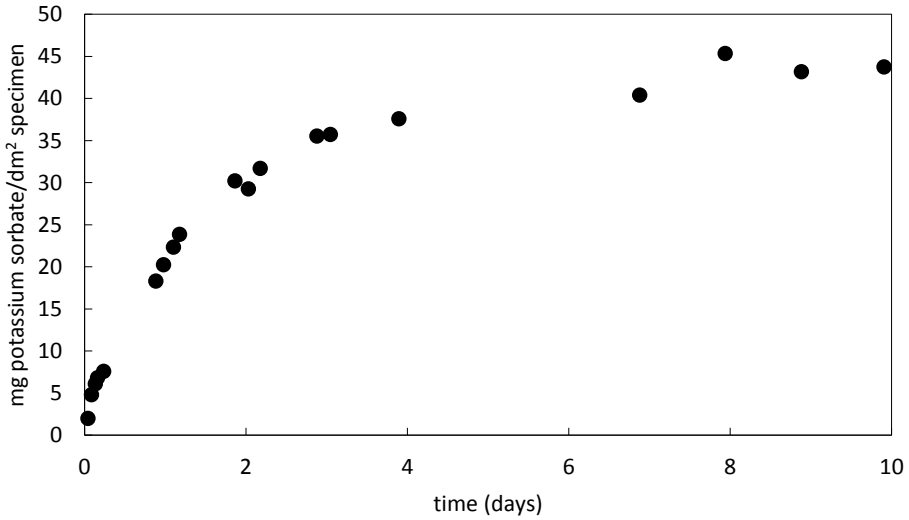


Figure 4.28. Release kinetics of potassium sorbate from PP:PS_SB (80_20) at 4 °C

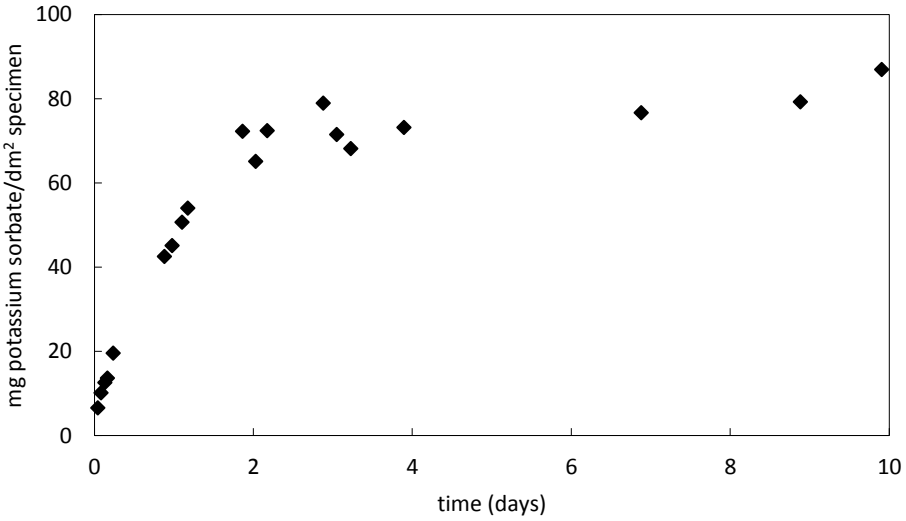


Figure 4.29. Release kinetics of potassium sorbate from EVA:PS_SB (80_20) at 4 °C

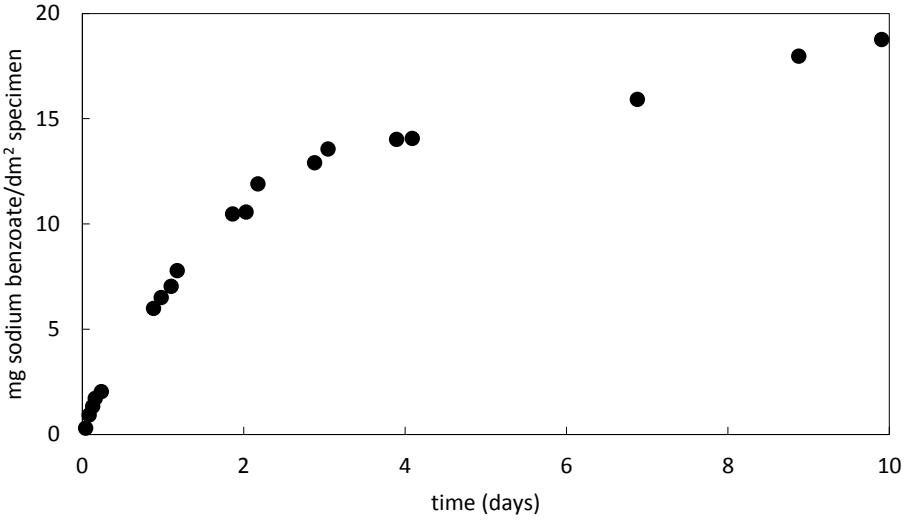


Figure 4.30. Release kinetics of sodium benzoate from PP:PS_SB (80_20) at 4 °C

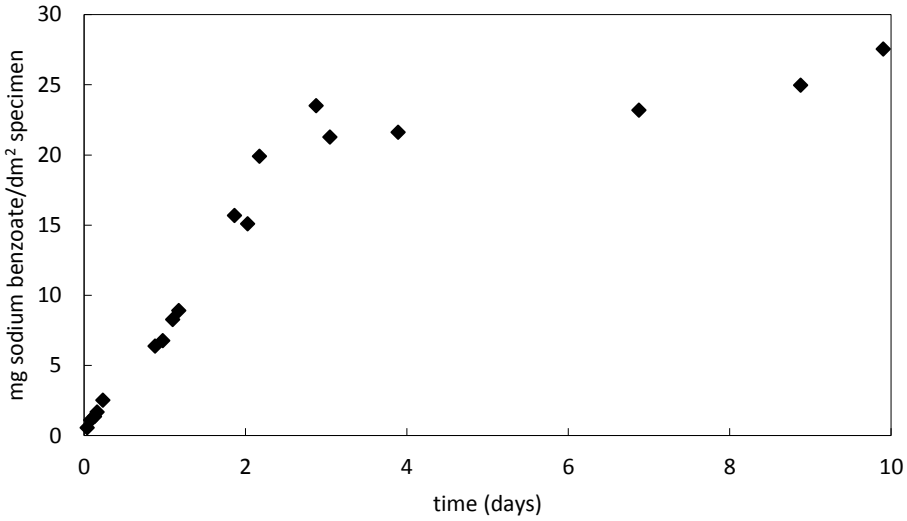


Figure 4.31. Release kinetics of sodium benzoate from EVA:PS_SB (80_20) at 4 °C

As can be observed in these figures, potassium sorbate and sodium benzoate showed a clear release from the two polymeric matrixes (PP and EVA) into the food simulant (3% (w/v) acetic acid) over time. Moreover, in all the systems, the content of active agents released from the packaging materials increased faster during the first 4 days and almost reached a complete release after 10 days in contact with the acetic acid.

As Figure 4.28 and Figure 4.29 show, after 10 days, the maximum amount of potassium sorbate released from PP and EVA films was 44.1 and 80.9 mg/dm^2, respectively. The maximum amount of sodium benzoate released from PP and EVA films (Figure 4.30 and Figure 4.31) was 17.6 and 23.7 mg/dm^2, respectively.

The diffusion coefficients of potassium sorbate and sodium benzoate were estimated by adjusting Equation 3.8 to the experimental data. For that, the experimental data, i.e. $m^0_{simulant}$, $m^{eq}_{simulant}$ and $m^t_{simulant}$ which are the quantity of the active substance at initial, final and at any time in the food simulant, respectively, were used in Equation 3.8 to obtain a new variable which ranged between 0 (at initial time) and 1 (at the time at which a total release occured). This variable is defined as the ratio between the amount of active substance released from the packaging material to the total amount released at the final time.

Other input variables needed in Equation 3.8 to estimate the D value were:

- the α value that was set at 99 since the experimental set-up was designed to promote the total release of the active agent after infinite time (> 99% of migration);
- L (active layer thickness) which represents the real active layer (80 and 72 μm for PP and EVA materials, respectively). In this case, the active packaging system released the active substances towards both sides of the packaging material.

As described in section 4.3.1, these input variables were introduced into MATLAB routines in order to estimate the diffusion coefficient of potassium sorbate and sodium benzoate for PP and EVA materials.

Figure 4.32 show the different figures related to the estimation of the diffusion coefficient for potassium sorbate and sodium benzoate released from PP and EVA materials. As can be observed in Figure 4.32a, a minimum RMSE was obtained for a given D value in all the cases. On the other hand, Figure 4.32b shows the kinetic curves estimated with the model in Equation 3.8 (solid line) based on the best D value and how this curve adjusts to the experimental data (symbol "o").

Table 4.34 compiles the diffusion coefficients obtained for potassium sorbate and sodium benzoate in PP and EVA materials and the associated RMSE. The fit of the release kinetics of the two active agents from PP and EVA material at 4 °C to Equation 3.8 was quite good based on the RMSE values. These values were below 10% in all the cases except for sodium benzoate released from EVA material. No large differences were observed in the diffusion coefficients of potassium sorbate and sodium benzoate regarding the type of material. Specifically, the diffusion coefficient of potassium sorbate was 1.3 fold higher in EVA than in PP. In addition, the sodium benzoate diffusion coefficient was the same for both polymeric materials.

Table 4.34. Diffusion coefficients (D) and RMSE corresponding to the fit of the kinetic curves of PS and SB released from PP and EVA materials at 4°C to Equation 3.8

	PP:PS_SB (80_20)		EVA:PS_SB (80_20)	
	Potassium sorbate	**Sodium benzoate**	**Potassium sorbate**	**Sodium benzoate**
D (cm^2/s)	3.33×10^{-11}	2.58×10^{-11}	4.23×10^{-11}	2.55×10^{-11}
RMSE (%)	3.2	5.5	5.6	12.7

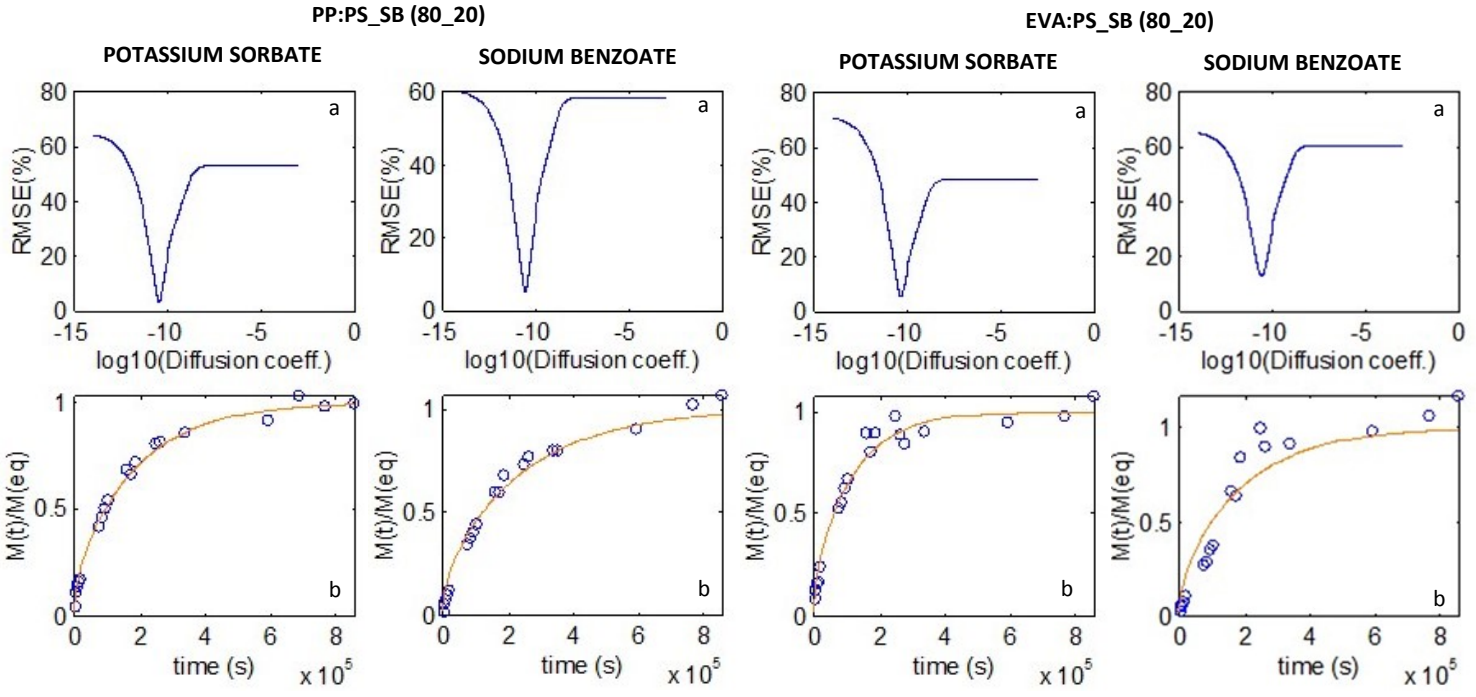


Figure 4.32. Results obtained from the release of potassium sorbate and sodium benzoate from PP:PS_SB (80_20) and EVA:PS_SB (80_20) at 4 °C (a) RMSE associated with the different D values tested and (b) fitting the kinetic curve obtained to the best D value (providing the lowest RMSE) of the experimental data

Although PP and EVA have different chemical structure and they differ in the degree of crystallinity and density (which could have a direct impact on the diffusion of the molecules dispersed within the polymeric matrixes), no significant differences were found. Comparing both active agents, small differences were observed for both packaging materials. In this regard, D values for potassium sorbate were 1.3 and 1.6 times higher than the D values for sodium benzoate for PP and EVA materials, respectively.

From these results, it could be concluded that the polymeric matrix did not significantly affect the release kinetics of potassium sorbate and sodium benzoate, with the release rate of potassium sorbate being slightly higher than the release rate of sodium benzoate within the same polymeric matrix.

4.3.3. Release of non-volatiles active agents from semi-industrial trays at 4 °C

The study of the release of the non-volatile potassium sorbate and sodium benzoate from bilayer trays were carried out in 3% (w/v) acetic acid at 4 °C as described in section 3.2.6.2. The amount of potassium sorbate and sodium benzoate released from the active materials to the food simulant over time was carried out through the analysis of the 3% (w/v) acetic acid by HPLC-UV as described in section 3.2.6.2.

Figure 4.33 and Figure 4.34 show the content of potassium sorbate and sodium benzoate, respectively, expressed as mg of active agent per dm^2 of the active tray released over time at 4 °C. Results are the mean of two replicates ± standard deviation.

As can be observed in these figures, both active agents showed a clear release from the active trays over time. Whilst the kinetic curve of sodium benzoate seemed to reach a plateau after 22 days, the kinetic curve of potassium sorbate reached that point after 28 days. After 36 days of test, the maximum amount of potassium sorbate and sodium benzoate released from active trays was 35.7 and 27.6 mg/dm^2, respectively.

The diffusion coefficient of both active agents was estimated by adjusting Equation 3.8 to the experimental data as indicated in the previous section (4.3.2) for the PP and EVA monolayer specimens. In this case, L (active layer thickness) was considered to be double than the real active layer. The reason was that the active tray is composed of a PP structural layer and an active PP:EVA layer.

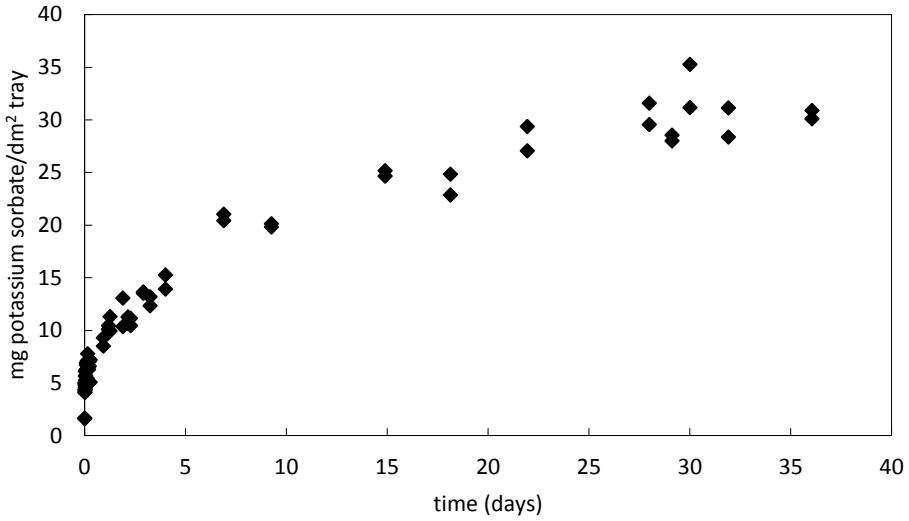


Figure 4.33. Release kinetics of potassium sorbate from PP/PP:10%EVA:20%PS_SB trays at 4 °C

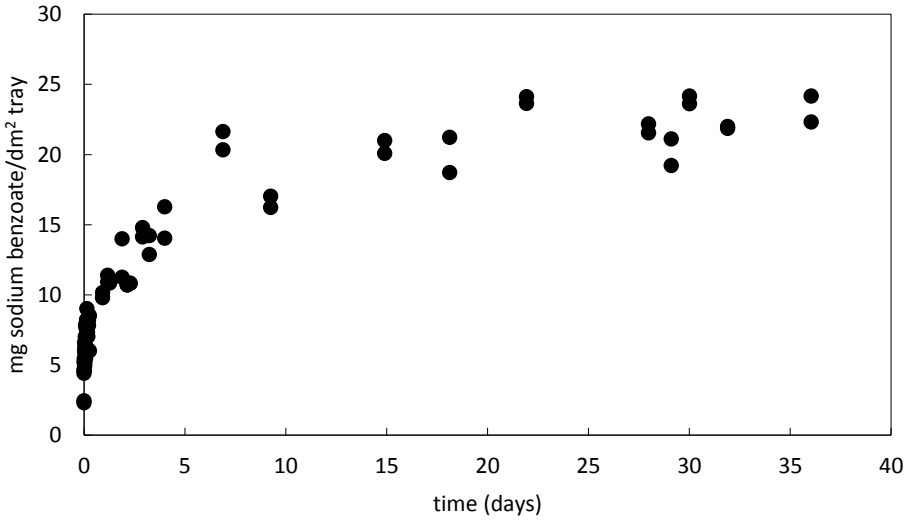


Figure 4.34. Release kinetics of sodium benzoate from PP/PP:10%EVA:20%PS_SB trays at 4 °C

The active packaging material released substances in one direction, while in Equation 3.8 release towards both sides of the packaging material is considered. This was mathematically

solved by doubling the actual active layer thickness in Equation 3.8 to 200 μm instead of 100 μm (the actual thickness).

All these input variables were introduced into MATLAB routines in order to estimate the diffusion coefficients of potassium sorbate and sodium benzoate. Figure 4.35 contain data related to the estimation of the diffusion coefficients for potassium sorbate and sodium benzoate released from the active tray.

As can be observed in Figure 4.35a, a minimum RMSE was obtained for a given D value in all cases. On the other hand, Figure 4.35b shows the kinetic curve estimated with the model in Equation 3.8 (solid line) based on the best D value and how this curve adjusts to the experimental data (symbol "o").

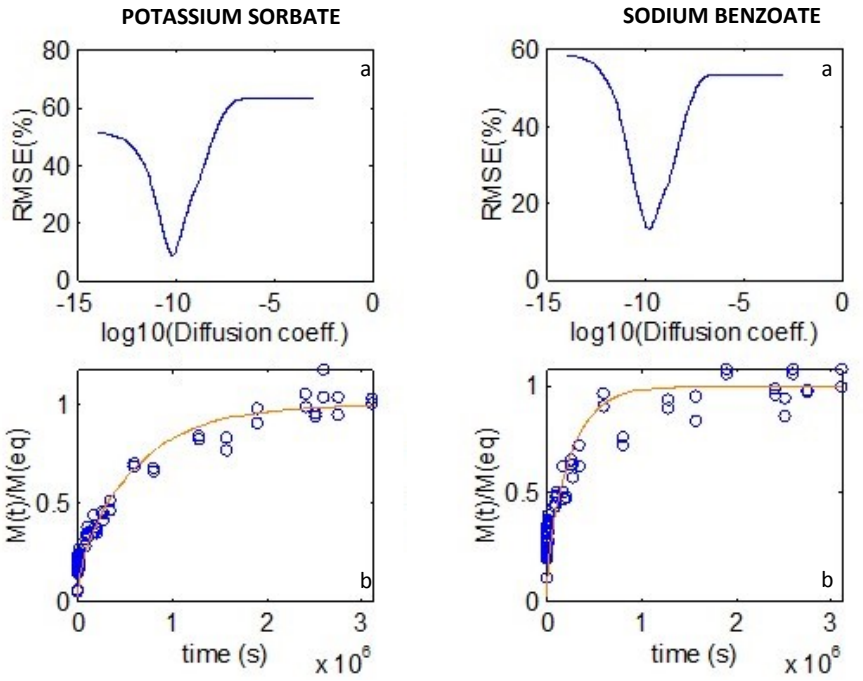


Figure 4.35. Results obtained from the release of potassium sorbate and sodium benzoate from the PP/PP:10%EVA:20%PS_SB tray at 4 °C

(a) RMSE associated with the different D values tested and (b) fitting the kinetic curve obtained to the best D value (providing the lowest RMSE) of the experimental data

Table 4.35 shows the diffusion coefficient obtained for potassium sorbate and sodium benzoate released from the PP/PP:10%EVA:20%PS_SB tray and the associated RMSE.

The fitting of the release kinetics at 4 °C of the two active agents from the active trays to Equation 3.8 (describing the diffusion from a plane sheet) was quite good based on the RMSE values around 10%. No large differences were found in the D values. Potassium sorbate showed a D value 4 times higher than sodium benzoate.

In comparison with the D values of potassium sorbate and sodium benzoate obtained for the compression-molded materials based on 100%PP or 100%EVA, the D value obtained for the active agents released from the active tray were in the same order of magnitude. As for PP and EVA specimens, the D value for sodium benzoate was slightly lower than for potassium sorbate.

Table 4.35. Diffusion coefficient (D) and RMSE corresponding to the fitting of the kinetic curves of PS and SB released from PP/PP:10%EVA:20%PS_SB trays at 4°C to Equation 3.8

	Potassium sorbate	Sodium benzoate
D (cm^2/s)	6.19×10^{-11}	1.52×10^{-11}
RMSE (%)	9.03	13.4

4.3.4. Conclusions on the active materials release kinetics

The release kinetics of volatile active agents from bilayer semi-industrial active films into the atmosphere was carried out at refrigeration and room temperature. At 4° C, only hexanal was released with a diffusion coefficient of 1.31×10^{-11} cm^2/s. However, an increase of temperature from 4 to 25 °C had a significant effect on the diffusion coefficient of hexanal which increased up to 2.61×10^{-10} cm^2/s (20 fold). At 25 °C, citral and linalool were released from the active film with D values of 1.42×10^{-11} and 7.38×10^{-12} cm^2/s, respectively. In this perspective, hexanal showed the highest D value followed by citral and linalool.

Preliminary active materials (PP and EVA) containing potassium sorbate and sodium benzoate, developed at lab scale, showed a clear release of the active agents into food simulant 3% (w/v) acetic acid at 4 °C over time. No large differences were found in the D values of potassium sorbate and sodium benzoate between materials. In addition, the D

values of potassium sorbate and sodium benzoate were in the same order of magnitude within each material.

Active trays developed at the semi-industrial scale also showed a clear release of potassium sorbate and sodium benzoate into a food simulant 3% (w/v) acetic acid at 4 °C over time. The D value obtained for potassium sorbate and sodium benzoate from the active tray were in the same order of magnitude, 6.19×10^{-11} and 1.52×10^{-11} cm^2/s, respectively, being the D value of potassium sorbate slightly higher than sodium benzoate.

4.4. ANTIMICROBIAL PROPERTIES OF ACTIVE MATERIALS

4.4.1. Antimicrobial properties of lab scale monolayer films with volatile active agents

4.4.1.1. Antimicrobial effect *in vitro* at 25 °C

The antimicrobial activity of the monolayer films, PP100_10%CHL and PP100_12%CHL, containing citral, hexanal and linalool was evaluated by vapor phase diffusion at 25 °C against *Saccharomyces cerevisiae, Aspergillus niger and Penicillium aurantiogriseum* as described in experimental section 3.2.7.1. PP film without active agents (PP100) was evaluated as reference material. The growth of the microorganisms in the inoculated plates was evaluated by visual inspection during the exposure period.

Figure 4.36, Figure 4.37 and Figure 4.38 show the inhibition halos of the active films against *Saccharomyces cerevisiae*, *Penicillium aurantiogriseum* and *Aspergillus niger,* respectively over the exposure period.

Figure 4.36. Antimicrobial activity of monolayer active films in the vapor phase against *Saccharomyces cerevisiae* at 25 °C

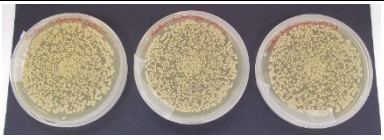
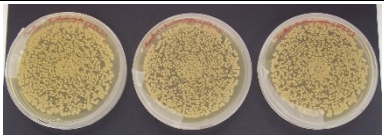
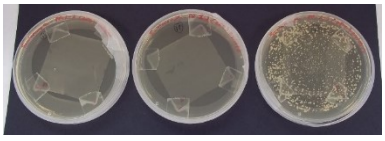
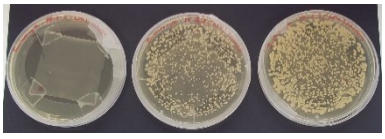
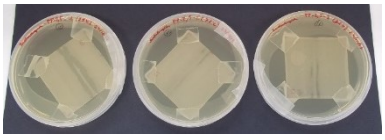
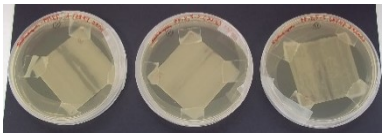
Sample	4 days-25 °C	7 days-25 °C
PP100		
PP100_10%CHL		
PP100_12%CHL		

Figure 4.37. Antimicrobial activity of monolayer active films in the vapor phase against *Penicillium aurantiogriseum* at 25 °C

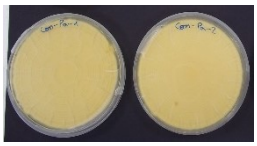
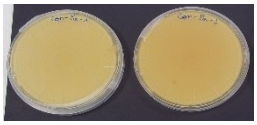
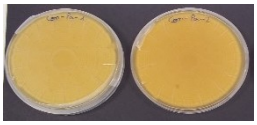
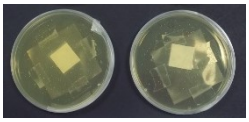
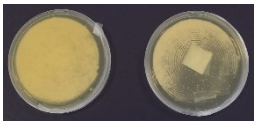
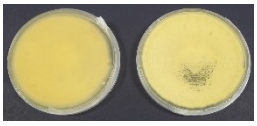
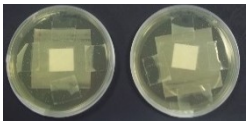
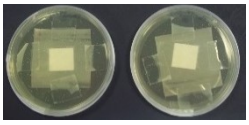
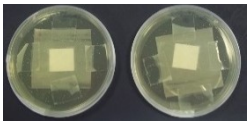
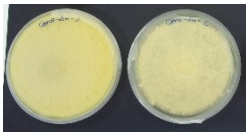
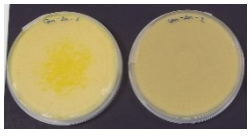
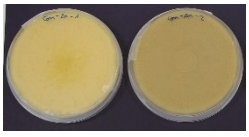
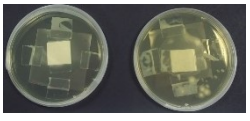
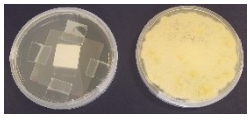
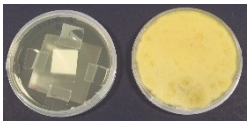
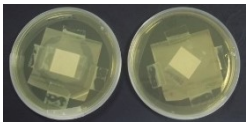
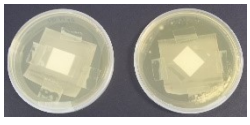
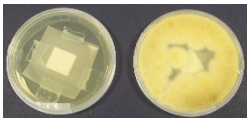
Sample	3 days-25 °C	5 days-25 °C	10 days-25 °C
PP100			
PP100_10%CHL			
PP100_12%CHL			

Figure 4.38. Antimicrobial activity of monolayer active films in the vapor phase against *Aspergillus niger* at 25 °C

Sample	3 days-25 °C	5 days-25 °C	10 days-25 °C
PP100			
PP100_10%CHL			
PP100_12%CHL			

The three microorganisms showed sensitivity toward the active films whilst no inhibition was observed for the samples exposed to the film control. Active films showed a dose-related inhibitory effect, with PP100_12%CHL causing the largest zones of growth inhibition.

Complete inhibition of *Saccharomyces cerevisiae* was observed over time for the PP100_12%CHL film. However, only a delay in the growth of *Saccharomyces cerevisiae* occurred when exposed to the film with lower concentration of active agents, PP100_10%CHL, which allowed only partial visual growth after 7 days of exposure. As reported by Tserennadmid *et al.* (2011), the delay in the microbial growth observed could be caused by a lengthening of the lag phase due to the presence of the volatile active agents.

PP100_12%CHL film showed complete inhibition of *Penicillium aurantiogriseum* which was sustained during the exposure period. On the contrary, partial growth was observed from day 5 when exposed to PP100_10%CHL film.

No growth of *Aspergillus niger* was observed after 3 days of exposure to the active films. From day 5 of exposure, partial growth was observed, being more pronounced for the film with lower concentration. As reported by several authors (Angelini *et al.*, 2006; Wuryatmo *et al.*, 2003), the degree of the growth of microorganisms could be related to the concentration of the active agents tested.

4.4.1.2. Antimicrobial effect *in vitro* at 4 °C

In this study, the effect of active films against microbial growth at refrigeration conditions was analyzed. Monolayer films were exposed to *Saccharomyces cerevisiae, Aspergillus niger* and *Penicillium aurantiogriseum* in the vapor phase for 12 days at 4 °C as described in the experimental section 3.2.7.1. After the exposure period, the agar was collected, homogenized and re-plated for quantitative analysis.

This methodology was applied due to the low mycelial formation and the difficulty to observe an inhibition zone at 4 °C. As reported by Bertolini and Tian (1996), the growth rate, sporulation and spore germination of the fungus are reduced as the temperature is lowered, delaying the time at which sporulation appears.

Table 4.36 shows the microbial growth during the exposure period and the reduction with respect to their positive control. Results are the mean of three replicates agar ± standard deviation. A significant antimicrobial effect was observed at refrigeration temperature. In general, the film with higher concentration, PP100_12%CHL, showed stronger antimicrobial activity against the three microorganisms tested. By day 6 of storage, significant reductions were observed for *Saccharomyces cerevisiae* growth compared to the control. By day 12, the

antimicrobial effect of the film on *Saccharomyces cerevisiae* was strengthened and significant growth reduction was observed for *Penicillium aurantiogriseum*. However, the growth of *Aspergillus niger* in the control plates was probably affected by the low temperature and no clear effect of active films was observed after 12 days of storage. Although PP100_12%CHL showed the highest antimicrobial effect, PP100_10%CHL still showed some significant activity against *Saccharomyces cerevisiae* by day 6 and 12 of storage, but no significant reductions of *Penicillium aurantiogriseum* or *Aspergillus niger* were found.

Even though the comparison between tests at 4 and 25 °C was difficult due to variations in the method of analysis and the sampling days, similar trends in microbial growth were found between temperatures. The growth of *Saccharomyces cerevisiae* followed the same trend for both temperatures, showing the highest reduction for the film with higher antimicrobial concentration. In the case of *Penicillium aurantiogriseum,* higher concentrations of active agents were needed to achieve a significant delay in its growth at the end of the storage period for both temperatures. *Aspergillus niger* showed small or no differences in growth between active films at both exposure temperatures by the end of the exposure period.

Table 4.36. Antimicrobial activity of monolayer active films in the vapor phase at 4 °C

	log CFU/g ± SD	log reduction	log CFU/g ± SD	log reduction
Saccharomyces cerevisiae	**Day 6**		**Day 12**	
PP100	2.69 ± 0.16 [a]		3.30 ± 0.08 [a]	
PP100_10%CHL	2.26 ± 0.24 [b]	0.43	2.04 ± 0.20 [b]	1.26 *
PP100_12%CHL	< DL	≥ 0.84 *	< DL	≥ 1.45*
Penicillium aurantiogriseum	**Day 6**		**Day 12**	
PP100	3.42 ± 0.05 [a]		3.72 ± 0.09 [a]	
PP100_10%CHL	3.94 ± 0.14 [b]	-	3.80 ± 0.23 [a]	-
PP100_12%CHL	3.72 ± 0.19 [c]	-	2.65 ± 0.81 [b]	1.07 *
Aspergillus niger	**Day 6**		**Day 12**	
PP100	3.59 ± 0.04 [a]		2.00 ± 0.28	
PP100_10%CHL	3.57 ± 0.16 [a]	0.02	< DL [a]	≥ 0.15
PP100_12%CHL	3.22 ± 0.33 [b]	0.37	< DL [a]	≥ 0.15

Values within the same column with a different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis). DL=1.85 log CFU/g. (-) No reduction. (*) Significant log CFU/g reduction (any reduction less than 0.5 log is not considered biologically meaningful, whether statistically significant or not).

Temperature played an important role in the antimicrobial activity of the active films containing volatile agents. It is well-established that the diffusion coefficient of the active substances increases with the temperature (Baner *et al.*, 1996; Muriel-Galet *et al.*, 2015; Suppakul *et al.*, 2011a). This effect was also observed in the release test carried out in section 4.3.1, where the release of citral, hexanal and linalool was enhanced with an increase of temperature (higher D values). Moreover, the vapor pressure of the active volatiles also increases with the temperature thus promoting volatility from the packaging surface (Gardini *et al.*, 1997). In fact, it has been reported that the inhibitory effectiveness of volatile molecules is dependent and positively affected by their vapor pressure rather than by their whole concentration in the active system (Gardini *et al.*, 1997; Lanciotti *et al.*, 1999). It should be also considered that an increase of temperature also reduces the growth of the microorganisms, counteracting the increase of the released active substances (Corbo *et al.*, 2000). All these parameters make difficult to predict the real concentration or effective amount released that could prevent fungal growth.

4.4.2. Antimicrobial properties of semi-industrial scale bilayer films with volatile active agents

4.4.2.1. Antimicrobial effect *in vitro* at 4 °C

The antimicrobial activity of the bilayer active films was evaluated in vapor phase diffusion tests at 4 °C for 12 days against *Penicillium aurantiogriseum*, *Aspergillus niger* and *Saccharomyces cerevisiae* as described in experimental section 3.2.7.1. A PP film without active agents was evaluated as reference material. After the incubation period, the agar was collected, homogenized and re-plated for quantitative analysis as described in experimental section 3.2.7.1.

Table 4.37 shows the microbial growth of *Saccharomyces cerevisiae*, *Penicillium aurantiogriseum* and *Aspergillus niger* after 12 days at 4 °C and the log reduction with respect to their control. Results are the mean of three replicates ± standard deviation.

As presented in Table 4.37, the antimicrobial effectiveness of the films was directly related to the concentration of the active agents as it was for the monolayer lab scale films.

Table 4.37. Antimicrobial activity of bilayer active films in the vapor phase at 4 °C after 12 days

Saccharomyces cerevisiae	**log CFU/g ± SD**	**log reduction**
PP100_2	4.82 ± 0.03 [a]	
PP60/40a_8.5%CHL	4.16 ± 0.05 [b]	0.66*
PP30/70a_8.5%CHL	4.01 ± 0.16 [b]	0.81*
Penicillium aurantiogriseum	**log CFU/g ± SD**	**log reduction**
PP100_2	4.03 ± 0.03 [a]	
PP60/40a_8.5%CHL	3.39 ± 0.20 [b]	0.64*
PP30/70a_8.5%CHL	2.71 ± 0.16 [c]	1.32*
Aspergillus niger	**log CFU/g ± SD**	**log reduction**
PP100_2	3.53 ± 0.05 [a]	
PP60/40a_8.5%CHL	3.08 ± 0.06 [b]	0.45
PP30/70a_8.5%CHL	2.10 ± 0.13 [c]	1.44*

Values within the same column with a different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis). DL=1.85 log CFU/g. (*) Significant log CFU/g reduction (any reduction less than 0.5 log is not considered biologically meaningful, whether statistically significant or not).

The film with the highest active layer thickness (PP30/70a_8.5%CHL) and consequently with the highest amount of active agents, showed the highest activity against the target microorganisms with reductions above 1 log CFU/g for *Penicillium aurantiogriseum* and *Aspergillus niger*. The film PP60/40a_8.5%CHL seemed to be approaching a minimum effective concentration since a log reduction around 0.5 log CFU/g was observed. In this regard, the release kinetics study carried out with the bilayer films at 4 °C (see section 4.3.1.1) showed a clear release of hexanal which could have been primarily responsible for the microbial reduction observed in this *in vitro* study at refrigeration temperature for all the materials. Although release tests were performed only with bilayer films, the polymeric matrix was the same for monolayer and bilayer films, and therefore no large differences in the diffusion coefficients of active agents could be expected.

Antimicrobial results demonstrated that the release of active substances from the different active films, monolayers and bilayers, was enough to cause effective inhibition of the growth of the microorganisms. In this respect, films with the lower concentration of active agents (bilayer materials at semi-industrial scale) would be preferred since they would lead to lower organoleptic alteration of the food and lower economic impact in the final packaging cost.

In general, these results demonstrated the powerful utility of the antimicrobial PP-based films as antimicrobial packaging materials for refrigerated foodstuff. However, the antimicrobial activity is usually different in food systems, requiring higher concentration to

achieve the same effect as in the *in vitro* tests (Balaguer *et al.*, 2013). It has been demonstrated that the efficacy of natural antimicrobials added to the food could be reduced by certain food components (Gutierrez *et al.*, 2008a). In this way, it is supposed that high levels of fat and/or protein in foodstuffs protect bacteria from the action of EOs and their main components (Shelef *et al.*, 1984; Tassou *et al.*, 1995). Carbohydrates in foods do not appear to protect bacteria from their antimicrobial effects (Shelef *et al.*, 1984).

Therefore, specific foodstuff should be tested to demonstrate the actual antimicrobial activity of active materials under real conditions taking into account inherent parameters of the food such as water activity, pH, nutrient content, but also extrinsic parameters such as temperature of storage, atmosphere and type of microorganism (Pitt and Hocking, 2009). Hence, in the present Doctoral Thesis, the development of active packaging films at a semi-industrial scale has been validated as being practical for use with minimally processed pineapple and orange. Further work will determine the actual shelf life extension of these foodstuffs. In this regard, active films with 70 µm active layer thickness containing 8.5%CHL (nominal concentration) should be kept as a minimum active layer requirement for antimicrobial activity to be tested in real foodstuff systems.

4.4.3. Antimicrobial properties of monolayer active materials with non-volatile active agents for active tray development

4.4.3.1. Antimicrobial effect *in vitro* at 25 °C

The antimicrobial activity of PP and EVA specimens containing 20% of potassium sorbate and sodium benzoate mixture (w/w) was evaluated against *Saccharomyces cerevisiae*, *Aspergillus niger* and *Penicillium aurantiogriseum* for 3 days at 25 °C in PDB media as described in 3.2.7.2. PP and EVA specimens without active agents were also evaluated as reference materials. A positive control with only microorganisms was also added to the test.

Table 4.38 shows the viability of each microorganism exposed to the active specimens and the microbial reduction caused by each material compared to reference material. Results are the mean of three replicates ± standard deviation.

EVA and PP specimens containing potassium sorbate and sodium benzoate showed significant growth reduction of *Saccharomyces cerevisiae*, *Penicillium aurantiogriseum* and *Aspergillus niger* under the test conditions.

Table 4.38. Antimicrobial activity of PP and EVA active specimens containing potassium sorbate and sodium benzoate in PDB at 25 °C for 3 days

Saccharomyces cerevisiae	**log CFU/g ± SD**	**log reduction**
Positive control	6.44 ± 0.05 [a]	
EVA	6.26 ± 0.06 [b]	
PP	6.38 ± 0.05 [ab]	
EVA:20%PS_SB	5.68 ± 0.10 [c]	0.76*
PP:20%PS_SB	5.48 ± 0.09 [c]	0.96*
Penicillium aurantiogriseum	**log CFU/g ± SD**	**log reduction**
Positive control	4.85 ± 0.03 [a]	
EVA	5.14 ± 0.02 [ab]	
PP	5.20 ± 0.03 [b]	
EVA:20%PS_SB	4.16 ± 0.13 [c]	0.69*
PP:20%PS_SB	4.18 ± 0.09 [c]	0.67*
Aspergillus niger	**log CFU/g ± SD**	**log reduction**
Positive control	4.43 ± 0.01 [a]	
EVA	4.96 ± 0.09 [a]	
PP	4.71 ± 0.04 [a]	
EVA:20%PS_SB	3.39 ± 0.12 [b]	1.04*
PP:20%PS_SB	3.36 ± 0.35 [b]	1.07*

Values within the same column with a different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis). DL=1.3 log CFU/g. (*) Significant log CFU/g reduction (any reduction less than 0.5 log is not considered biologically meaningful, whether statistically significant or not).

This effect was slightly higher for *Aspergillus niger* with values around 1 log CFU/mL reduction. Moreover, no significant differences were observed between the effect of the active PP and EVA specimens within microorganisms. The reference materials, PP and EVA specimens without antimicrobials did not cause any microbial growth reduction. These results suggested that the release of the active agents from the packaging materials generated the antimicrobial activity.

These results are in agreement with Kuplennik *et al.* (2015) who evaluated the antimicrobial effect of compression molded LDPE and LDPE:EVA (70:30) specimens containing 5% potassium sorbate in liquid media at pH 4.2 and at 30 °C, getting reductions around 1.4 log for both materials against *Saccharomyces cerevisiae*.

For the potassium sorbate and sodium benzoate agents, the maximal antimicrobial activity is obtained at pH values below the pKa where the salts are in the acid form. In this regard, Kuplennik *et al.* (2015) showed the pH dependent effect of potassium sorbate where at a pH lower than its pKa, the MIC decreased from 1250 mg/L (neutral pH) to 156 mg/L (pH 4.2). In

the present work, the pH of the media (pH 5.6) was above the pKa of potassium sorbate and sodium benzoate (4.69 and 4.21, respectively). Therefore, higher microbial reductions could be expected at the lower pH values of orange and pineapple fruits (4 and 3.3, respectively) as further described in section 4.5.

4.4.4. Antimicrobial properties of semi-industrial trays with non-volatile active agents

4.4.4.1. Antimicrobial effect *in vitro* at 25 °C

The antimicrobial properties of the active tray PP/PP:10%EVA:20%PS_SB were evaluated by means of *in vitro* tests in PDB against *Saccharomyces cerevisiae, Penicillium aurantiogriseum* and *Aspergillus niger* for 7 days at 25 ± 2 °C as described in 3.2.7.3.

Table 4.39 shows the viability of each microorganism exposed to the control and active tray and the microbial reduction reached in comparison with the control material. Results are the mean of three replicates ± standard deviation.

Additionally, Figure 4.39, Figure 4.40 and Figure 4.41 visually illustrate the microbial growth in the active and control trays.

Table 4.39. Antimicrobial activity of active trays containing potassium sorbate and sodium benzoate in PDB at 25 °C for 7 days

Saccharomyces cerevisiae	**log CFU/g ± SD**	**log reduction**
PP/PP:10%EVA tray	6.35 ± 0.07 [a]	
PP/PP:10%EVA:20%PS_SB tray	4.89 ± 0.25 [b]	1.46*
Penicillium aurantiogriseum	**log CFU/g ± SD**	**log reduction**
PP/PP:10%EVA tray	6.45 ± 0.05 [a]	
PP/PP:10%EVA:20%PS_SB tray	2.88 ± 0.09 [b]	3.57*
Aspergillus niger	**log CFU/g ± SD**	**log reduction**
PP/PP:10%EVA tray	6.18 ± 0.07 [a]	
PP/PP:10%EVA:20%PS_SB tray	< DL	≥ 4.88 *

Values within the same column with a different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis). DL=1.3 log CFU/g. (*) Significant log CFU/g reduction (any reduction less than 0.5 log is not considered biologically meaningful, whether statistically significant or not).

Figure 4.39. Growth of *Saccharomyces cerevisiae*. A: Control tray; B: Active tray

Figure 4.40. Growth of *Penicillium aurantiogriseum*. A: Control tray; B: Active tray

Figure 4.41. Growth of *Aspergillus niger*. A: Control tray; B: Active tray

From the visual analysis, a clear inhibition of the growth of *Penicillium aurantiogriseum* and *Aspergillus niger* generated by active trays after 7 days of exposure was observed. Slight visual differences between control and active trays were observed for *Saccharomyces cerevisiae* growth. Considering the quantitative results, it was concluded that the active trays

were strongly inhibitory to the studied molds *Penicillium aurantiogriseum* and *Aspergillus niger* with 3.57 and 4.88 log CFU/mL reduction, respectively. Significant inhibition of *Saccharomyces cerevisiae* was also caused by the active trays (1.46 log CFU/mL), but this effect was lower compared with the strong inhibitory effect observed for the molds. These results suggest that the release of potassium sorbate and sodium benzoate from the packaging materials generated the antimicrobial activity.

The antimicrobial effect of antimicrobial LDPE films containing 1% potassium sorbate was also studied by Han and Floros (1997) who found that this antimicrobial lowered the growth rate and maximum growth of *Saccharomyces cerevisiae* and lengthened the lag period before microbial growth became apparent. On the contrary, Devlieghere *et al.* (2000), confirmed that EVA/LLDPE film impregnated with 5% (w/w) potassium sorbate exerted a small inhibitory effect on the growth of *Candida* spp., *Pichia* spp., *Trichosporon* spp, and *Penicillium* spp due to the very limited migration of potassium sorbate in distilled water. This effect was explained by the incompatibility of the polar salt with the non-polar LLDPE. However, in the present work, substantial antimicrobial effects of the active trays made with PP and EVA blends and containing potassium sorbate and sodium benzoate was confirmed against molds and yeast as already discussed.

4.4.4.2. Antimicrobial effect *in vitro* at 4 °C

The antimicrobial properties of the active tray were also evaluated by means of *in vitro* tests in PDB against *Saccharomyces cerevisiae*, *Penicillium aurantiogriseum* and *Aspergillus niger* for 12 days at 4 ± 2 °C as described in 3.2.7.3. Table 4.40 shows the viability of each microorganisms exposed to the active tray and the microbial reduction reached in comparison with the control tray at refrigeration temperature. Results are the mean of three replicates ± standard deviation.

The active trays exerted a significant reduction against *Saccharomyces cerevisiae* with around 1 log reduction. However, no significant effect was observed for the studied molds *Penicillium aurantiogriseum* and *Aspergillus niger*.

As no reduction was observed for *Penicillium aurantiogriseum,* an additional gravimetric test based on the method described by Córdova-López *et al.* (1996), described in experimental section 3.2.7.3, was carried out due to the possible difficulties in measuring molds growth in liquid media. To that, the inoculated broth exposed to active and control trays was filtered

and dried, quantifying the microbial mass growth in contact with control and active trays after the exposure period.

Table 4.40. Antimicrobial activity of active trays containing potassium sorbate and sodium benzoate in PDB at 4 °C for 12 days

Saccharomyces cerevisiae	**log CFU/g ± SD**	**log reduction**
PP/PP:10%EVA tray	6.07 ± 0.12 [a]	
PP/PP:10%EVA:20%PS_SB tray	5.15 ± 0.10 [b]	0.92*
Penicillium aurantiogriseum	**log CFU/g ± SD**	**log reduction**
PP/PP:10%EVA tray	2.20 ± 0.39 [a]	
PP/PP:10%EVA:20%PS_SB tray	2.35 ± 0.14 [a]	-
Aspergillus niger	**log CFU/g ± SD**	**log reduction**
PP/PP:10%EVA tray	3.29 ± 0.39 [a]	
PP/PP:10%EVA:20%PS_SB tray	3.07 ± 0.06 [a]	0.22

Values within the same column with a different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis). DL=1.3 log CFU/g. (-) No reduction. (*) Significant log CFU/g reduction (any reduction less than 0.5 log is not considered biologically meaningful, whether statistically significant or not).

Results from the gravimetric test showed a reduction of 47% (w/w) of biomass growth of *Penicillium aurantiogriseum* exposed to the active tray compared to the biomass quantified in the control tray. Once again, these results suggest that the release of the active agents from the packaging materials generated the antimicrobial activity at 4 °C. Indeed, these results were supported by the release of both salts into 3% (w/v) acetic acid at 4 °C as previously described.

In comparison with results at 25 °C, the microbial reduction was much lower under refrigeration conditions, although some significant reduction was still observed. Any interruption of the cold chain, which may occur during the shelf life of the product, can lead to an increase in the release rate and the amount released of solid active agents at higher temperature.

4.4.5. Conclusions on the antimicrobial properties *in vitro* of the active materials

Active films, monolayer and bilayer, showed antimicrobial properties against *Saccharomyces cerevisiae*, *Penicillium aurantiogriseum* and *Aspergillus niger* during *in vitro* vapor diffusion tests at 4 and 25 °C with a dose-dependent effect. The antimicrobial effect was enhance by the increase of temperature, and this effect was directly related with the increase of

diffusion coefficients of the volatile active agents, citral, hexanal and linalool, corroborated in section 4.3.1.

The active trays containing potassium sorbate and sodium benzoate showed great inhibitory effect toward *Saccharomyces cerevisiae*, *Penicillium aurantiogriseum* and *Aspergillus niger* in broth at 25 °C. At 4 °C, the microbial reduction caused by the active tray was much lower, although some significant reduction was still observed for the target microorganisms.

4.5. SHELF LIFE OF FRESH CUT FRUITS PACKED WITH ACTIVE PACKAGING MATERIALS DEVELOPED AT A SEMI-INDUSTRIAL SCALE

The active films and trays developed in this research were validated in peeled and cut pineapple and orange fruit to extend their shelf lives.

The effect of the active materials on the food spoilage flora and their effect on the evolution of headspace composition and physiochemical properties such as total soluble solids (TSS), pH and juice leakage of cut and peeled orange and pineapple was evaluated. The effect of active agents release on the sensory characteristics of the fruit was also studied.

Table 4.41 summarizes the specific tests carried out for the fruit packaged with active films or tray and their combination (active system).

Table 4.41. Tests performed in peeled and cut orange and pineapple for the validation of active films and tray developed at semi-industrial scale and their combination

Samples	Microbial counts	Headspace gas composition	Juice leakage	pH	TSS	Sensory evaluation
PP60/40a_8.5%CHL film	✓	✓	-	-	-	-
PP30/70a_8.5%CHL film	✓	✓	-	-	-	-
PP10/70a_8.5%CHL film	✓	✓	✓	✓	✓	✓
PP/PP:10%EVA:20%PS_SB tray	✓	✓	-	-	-	✓
PP10/70a_8.5%CHL film + PP/PP:10%EVA:20%PS_SB tray	✓	✓	✓	✓	✓	✓

(✓) Performed test. (-) Non-performed test.

4.5.1. Validation of bilayer active films at a semi-industrial scale containing volatile active agents

Peeled and cut pineapple and orange were packaged separately with control (PP100_2 and P80) or active films (PP60/40a_8.5%CHL, PP30/70a_8.5%CHL and PP10/70a_8.5%CHL) under aerobic conditions as described in 3.2.8.1. Films were micro-perforated with two 250 µm holes to allow the gas exchange with the environment and to avoid anaerobic conditions. Control tray (PP tray) was used as a holder.

Packed samples were stored at 4 °C for 7 days followed by other 5 days at 8 °C in order to simulate possible abuse temperatures that can occur along the supply chain. Microbial counts, gas composition of the headspace, juice leakage, pH, TSS and sensory properties were evaluated in packed fruit over time.

Lane-Late (orange) and Gold Madura (pineapple) cultivars were supplied for the validation of PP60/40a_8.5%CHL and PP30/70a_8.5%CHL films. Navel (orange) and MD2 (pineapple) cultivars were supplied for the validation of PP10/70a_8.5%CHL film. Different cultivars were used because the tests were conducted in different seasons.

4.5.1.1. Antimicrobial activity of active films on peeled and cut fruit

Peeled and cut pineapple and orange were subjected to microbiological analyses during storage at 4 °C and then at 8 °C. The microflora of the fruit packed with active films and control films with no active agents was evaluated by day 0, 3, 7 and 12 in terms of total aerobic counts, molds and yeast and psychrotrophic bacteria. Day 0 was defined as the microbial load of the fruit before packaging. For PP10/70a_8.5%CHL film, day 5 and 10 were also analyzed.

Table 4.42 shows the microbial population of the two pineapple cultivars during storage. Reported results are the mean of three replicates ± standard deviation. In general, the microbial numbers on pineapple packed with active films were significantly lower than on fruit packed with control films.

Particularly, the total aerobic numbers for the Gold Madura cultivar packed with active films (PP60/40a_8.5%CHL and PP30/70a_8.5%CHL) slightly changed at 4 °C from day 0 (packaging day) up to day 7, as described in Table 4.42. However, total aerobic numbers of the same cultivar packed with control film (P100_2) increased 1 log during the same period. After 7 days, temperature of storage was shifted from 4 to 8 °C and an increase of total aerobic numbers up to 7 log CFU/g at day 12 was quantified for pineapple packed with control and PP60/40a_8.5%CHL active film. Nevertheless, some antimicrobial effect remained at day 12 for the film with higher antimicrobial concentration (PP30/70a_8.5%CHL), with microbial numbers around 6 log CFU/g.

Table 4.42. Microbial population of peeled and cut pineapple packed with active and control films stored at 4 °C for 7 days followed by 5 days at 8 °C

		Gold Madura cultivar			MD2 cultivar	
		P100_2 (control)	PP60/40a_8.5%CHL	PP30/70a_8.5%CHL	P80 (control)	PP10/70a_8.5%CHL
Day	**Temperature (°C)**	**Total aerobic numbers (log CFU/g)**			**Total aerobic numbers (log CFU/g)**	
0	4		4.33 ± 0.11		4.84 ± 0.58	
3	4	4.15 ± 0.18 ^{a}	3.71 ± 0.14 ^{b}	3.89 ± 0.13 ^{ab}	5.36 ± 0.01 ^{a}	5.43 ± 0.02 ^{a}
5	4	-	-	-	5.46 ± 0.20 ^{a}	4.48 ± 0.15 ^{b*}
7	4	5.41 ± 0.38 ^{a}	4.49 ± 0.23 ^{b*}	4.05 ± 0.17 ^{b*}	5.99 ± 0.21 ^{a}	4.73 ± 0.12 ^{b*}
10	8	-	-	-	6.44 ± 0.14 ^{a}	6.07 ± 0.39 ^{a}
12	8	7.06 ± 0.03 ^{a}	7.04 ± 0.04 ^{a}	6.09 ± 0.05 ^{b*}	6.69 ± 0.41 ^{a}	6.32 ± 0.11 ^{a}
Day	**Temperature (°C)**	**Molds and yeasts (log CFU/g)**			**Molds and yeasts (log CFU/g)**	
0	4		4.12 ± 0.24		4.29 ± 0.12	
3	4	5.47 ± 0.17 ^{a}	4.17 ± 0.18 ^{b*}	4.25 ± 0.39 ^{b*}	6.01 ± 0.23 ^{a}	5.00 ± 0.29 ^{b*}
5	4	-	-	-	6.55 ± 0.44 ^{a}	5.33 ± 0.43 ^{b*}
7	4	7.35 ± 0.19 ^{a}	5.82 ± 0.04 ^{b*}	5.94 ± 0.12 ^{b*}	8.06 ± 0.10 ^{a}	6.06 ± 0.06 ^{b*}
10	8	-	-	-	8.34 ± 0.05 ^{a}	8.28 ± 0.01 ^{a}
12	8	8.57 ± 0.11 ^{a}	8.10 ± 0.05 ^{b}	7.87 ± 0.19 ^{b*}	8.53 ± 0.03 ^{a}	8.34 ± 0.01 ^{b}
Day	**Temperature (°C)**	**Psychrotrophic bacteria (log CFU/g)**			**Psychrotrophic bacteria (log CFU/g)**	
0	4		4.27 ± 0.24		2.76 ± 0.08	
3	4	5.34 ± 0.11 ^{a}	3.91 ± 0.11 ^{b*}	4.14 ± 0.51 ^{b*}	5.97 ± 0.20 ^{a}	4.87 ± 0.24 ^{b*}
5	4	-	-	-	6.67 ± 0.53 ^{a}	4.69 ± 0.50 ^{b*}
7	4	6.73 ± 0.08 ^{a}	5.85 ± 0.05 ^{b*}	5.11 ± 0.28 ^{c*}	7.77 ± 0.01 ^{a}	6.05 ± 0.04 ^{b*}
10	8	-	-	-	8.23 ± 0.03 ^{a}	8.22 ± 0.09 ^{a}
12	8	>6.83	>6.83	>6.83	8.40 ± 0.16 ^{a}	8.25 ± 0.03 ^{a}

Values among same day of analysis within the same row with a different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis). Statistical analyses were individually performed for each cultivar. DL=1.6 log CFU/g. (-) Test not performed. (*) Significant log reduction (any reduction less than 0.5 log is not considered biologically meaningful, whether statistically significant or not).

As for the Gold Madura cultivar, the active film (PP10/70a_8.5%CHL) kept total aerobic microorganisms of MD2 pineapple cultivar to the initial levels at 4 °C with a significant reduction of 1.26 log CFU/g compared to the control film after 7 days of storage. The increase of temperature after 7 days of storage promoted an increase in the growth of total aerobic microorganisms on pineapple packed with both, control and the active film, above 6 log CFU/g, showing no significant differences in growth between them.

The molds and yeasts population quantified in pineapple Gold Madura cultivar packed with control film (P100_2) overcame 7 log CFU/g after 7 days of storage at 4 °C. Conversely, the increase of molds and yeasts population was less pronounced for pineapple packed with PP60/40a_8.5%CHL and PP30/70a_8.5%CHL active films, with 1.53 and 1.41 log CFU/g reductions, respectively, compared with the film control after 7 days of storage. The shift of temperature from 4 to 8 °C caused a sharp increase of molds and yeasts numbers for pineapple packed with control and active films at day 12 of storage. However, some significant reductions regarding the film control were quantified in 0.7 log CFU/g for PP30/70a_8.5%CHL film after 12 days.

In addition, the molds and yeasts from the pineapple MD2 cultivar packed with control film (P80_2) sharply increased from 4.29 to 8.06 log CFU/g at 7 days of storage at 4 °C. Conversely, same cultivar packed with the 10/70a_8.5%CHL film experienced a significant reduction of 2 log compared to the control film at 7 days of storage. After the change of temperature, the growth of molds and yeast sharply increased for pineapple packed with active film, reaching similar numbers as the control film.

Psychrotrophic bacteria from Gold Madura pineapple packed with control film (P100_2) increased from 4.27 to 6.73 log CFU/g after 7 days of storage at 4 °C. Significant reductions of 0.88 and 1.62 log CFU/g after 7 days of storage were reported for 60/40a_8.5%CHL and PP30/70a_8.5%CHL films, respectively.

For the MD2 cultivar, psychrotrophic bacteria evolved from 2.76 to 7.77 log CFU/g at day 7. The growth of psychrotrophic bacteria in MD2 pineapple was slowed by the PP10/70a_8.5%CHL film with significant reductions of 1.10, 1.98 and 1.72 log CFU/g at 3, 5 and 7 days of storage at 4 °C, respectively. However, the increased temperature caused rapid growth of these microorganisms in the fruit packed with control and active films, without significant differences in effectiveness between them.

In general, the maximum microbial load reduction was observed for all films and cultivars at day 7 before the shift of temperature occurred and for the films with higher antimicrobial concentration. Between cultivars, the highest reductions were reported for the MD2 cultivar at 7 days of storage.

Reduction of the film activity occurred in general from day 7 to day 12 (at 8 °C), although some residual effectiveness could be still observed. An increment in temperature from 4 to 8 °C promoted microbial growth and accelerated the spoilage metabolism of the fruit (Mossel et al., 1995). This effect was not counteracted by the increase in their vapor pressure, from 4 to 8 °C, and the increase in the release rate of the active agents. Thus, it is possible that exhaustion of the content of active agents in the films from day 7 could have occurred.

In this test, the content of active agents released from the packaging films was not evaluated and a possible exhaustion was not corroborated. If so, a modification of the formulation of the active layer should be carried out. However, at this point, it was decided not to carry out any change in the active composition of the film until the evaluation of the final active packaging system (active film and active tray) was completed.

The microbiological limits of cut fruits and vegetables considered safe for consumption are different regarding the information source as previously described in the introduction. The quality limits for microbial populations on minimally fresh processed fruit set in the present study were 6 log CFU/g for aerobic bacteria, yeasts and molds (growth is mainly represented by yeasts) and psychrotrophic bacteria. In this case, only pineapple packed with active film would be suitable for consumption up to day 7 (commercial estimated best-before date), whilst pineapple packed with the control film already exceeded the quality limits by day 7. In order to more accurately estimate the microbial quality limit of fruit packed with active films, the sampling frequency should be increased. At day 12, all the packaging films failed to keep the microbial counts under the quality limits, but these results will be confirmed when the complete active system (active film and active tray) is evaluated.

These results were in accordance with other authors where the antimicrobial effectiveness of volatile active agents in peeled and cut pineapple was demonstrated. In this regard, Mantilla *et al.* (2013) demonstrated the antimicrobial effectiveness of multilayered edible coating with a microencapsulated antimicrobial complex (beta-cyclodextrin and trans-cinnamaldehyde), extending their microbial quality up to 15 days at 4 °C, with a 2-3 log

reduction regarding the control coating. The coating also helped preserving color, texture, and pH of the fruit. In this case, the coating affected the fruit's flavor. Azarakhsh *et al.* (2014) also showed the antimicrobial effects of an alginate-based coating with 0.3 and 0.5% (w/v) lemongrass on fresh cut pineapple. However, the incorporation of 0.5% (w/v) lemongrass in the coating formulation significantly decreased the firmness and sensory scores (taste, texture and overall acceptability) of fresh cut pineapples.

Table 4.43 shows the evolution of microbial population of the two orange cultivars during storage test. Reported results are the mean of three replicates ± standard deviation. As reported for pineapple cultivars, microbial counts of orange packed with active films were significantly lower than orange packed with control films.

Total aerobic bacteria for Lane-late cultivar packed with control (P100_2) and active films (PP60/40a_8.5%CHL and PP30/70a_8.5%CHL) gradually increased from packaging day up to day 7 with numbers below 5 log CFU/g. A significant reduction of 0.56 log CFU/g was observed for Lane-Late cultivar packed with the film with higher antimicrobial concentration (PP30/70a_8.5%CHL) compared to the control film at day 7. After 7 days of storage, temperature changed from 4 to 8 °C and a substantial increase of total aerobic counts was reported in orange with numbers above 6 log CFU/g for control and active films. Despite the increase, PP60/40a_8.5%CHL and PP30/70a_8.5%CHL films produced significant microbial growth inhibition compared to control film, with a reduction of about 1 log CFU/g.

Total aerobic counts enumerated from Navel cultivar and packed with control (PP80) and active film (PP10/70a_8.5%CHL) similarly evolved after 7 days of storage at 4 °C with numbers above 5 log CFU/g and without significant differences between films. Same behavior was observed after temperature change, with numbers above 6 log CFU/g for control and active films.

Molds and yeasts counts from Lane-Late cultivar packed with control film (P100_2) sharply increased from 2.89 to 6.55 log CFU/g after 7 days of storage at 4 °C. The increase of molds and yeasts populations was less pronounced for orange packed with PP60/40a_8.5%CHL and PP30/70a_8.5%CHL films with numbers below 6 log CFU/g, which corresponded to significant reductions of 0.62 and 0.86 log CFU/g, respectively compared to the film control.

Table 4.43. Microbial population of peeled and cut orange packed with active and control films stored at 4 °C for 7 days followed by 5 days at 8 °C

		Lane-Late cultivar			Navel cultivar	
		P100_2 (control)	PP60/40a_8.5%CHL	PP30/70a_8.5%CHL	P80 (control)	PP10/70a_8.5%CHL
Day	**Temperature (°C)**	**Total aerobic numbers (log CFU/g)**			**Total aerobic numbers (log CFU/g)**	
0	4		3.12 ± 0.07		4.49 ± 0.75	
3	4	3.59 ± 0.04 ^{a}	3.31 ± 0.01 ^{a}	3.34 ± 0.26 ^{a}	4.75 ± 0.19 ^{a}	4.38 ± 0.02 ^{b}
5	4	-	-	-	4.74 ± 0.07 ^{a}	4.63 ± 0.12 ^{a}
7	4	4.86 ± 0.04 ^{a}	4.44 ± 0.27 ^{ab}	4.30 ± 0.29 ^{b}*	5.66 ± 0.16 ^{a}	5.29 ± 0.13 ^{b}
10	8	-	-	-	6.91 ± 0.20 ^{a}	6.83 ± 0.25 ^{a}
12	8	7.41 ± 0.28 ^{a}	6.36 ± 0.07 ^{b}*	6.48 ± 0.16 ^{b}*	6.93 ± 0.18 ^{a}	6.71 ± 0.17 ^{a}
Day	**Temperature (°C)**	**Molds and yeasts (log CFU/g)**			**Molds and yeasts (log CFU/g)**	
0	4		2.89 ± 0.01		3.45 ± 0.02	
3	4	4.82 ± 0.11 ^{a}	4.27 ± 0.20 ^{b}*	4.16 ± 0.09 ^{b}*	5.62 ± 0.16 ^{a}	5.01 ± 0.12 ^{b}*
5	4	-	-	-	6.07 ± 0.15 ^{a}	5.87 ± 0.16 ^{a}
7	4	6.55 ± 0.22 ^{a}	5.93 ± 0.43 ^{b}*	5.69 ± 0.06 ^{b}*	7.16 ± 0.22 ^{a}	6.83 ± 0.06 ^{a}
10	8	-	-	-	8.33 ± 0.07 ^{a}	8.21 ± 0.15 ^{a}
12	8	9.33 ± 0.05 ^{a}	8.06 ± 0.16 ^{b}*	7.13 ± 0.16 ^{c}*	8.46 ± 0.03 ^{a}	8.19 ± 0.13 ^{b}
Day	**Temperature (°C)**	**Psychrotrophic bacteria (log CFU/g)**			**Psychrotrophic bacteria (log CFU/g)**	
0	4		3.33 ± 0.03		2.73 ± 0.23	
3	4	4.97 ± 0.12 ^{a}	4.43 ± 0.24 ^{b}*	4.43 ± 0.14 ^{b}*	5.72 ± 0.14 ^{a}	5.09 ± 0.10 ^{b}*
5	4	-	-	-	6.29 ± 0.21 ^{a}	6.18 ± 0.21 ^{a}
7	4	6.39 ± 0.13 ^{a}	6.27 ± 0.13 ^{a}	4.75 ± 0.05 ^{b}*	7.14 ± 0.15 ^{a}	6.96 ± 0.10 ^{a}
10	8	-	-	-	8.40 ± 0.03 ^{a}	8.29 ± 0.18 ^{a}
12	8	7.80 ± 0.02	>6.83	>6.83	8.52 ± 0.06 ^{a}	8.28 ± 0.13 ^{b}

Values among same day of analysis within the same row and with a different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis). Statistical analyses were individually performed for each cultivar. DL=1.6 log CFU/g. (-) Test not performed. (*) Significant log reduction (any reduction less than 0.5 log is not considered biologically meaningful, whether statistically significant or not).

The shift of temperature from 4 to 8 °C caused a sharp increase of molds and yeasts numbers packed with control film up to 9.33 log CFU/g and an increase up to 8.06 and 7.13 log CFU/g for PP60/40a_8.5%CHL and PP30/70a_8.5%CHL films, respectively, after 12 days of storage. In this case, significant inhibition, with a reduction of about 1.27 and 2.20 log CFU/g was quantified for PP60/40a_8.5%CHL and PP30/70a_8.5%CHL films, respectively. Again, the highest reduction was observed for the film with highest antimicrobial concentration.

The molds and yeasts enumerated from the Navel cultivar packed with control (P80_2) and active film (PP10/70a_8.5%CHL) significantly increased from 3.45 to above 6 log CFU/g at 7 days of storage at 4 °C, with no significant differences between films. After the change of temperature, the growth of molds and yeasts increased above 8 log CFU/g for control and active film without significant differences in microbial growth.

Psychrotrophic bacteria counts from Lane-late orange packed with control (P100_2) and active films PP60/40a_8.5%CHL were above 6 log CFU/g at day 7 of storage, while PP30/70a_8.5%CHL film kept psychrotrophic bacteria below 4.5 log CFU/g, corresponding to a significant reduction of 1.64 log CFU/g compared to the control film.

Psychrotrophic bacteria from Navel cultivar packed with control (P80_2) and active film (PP10/70a_8.5%CHL) sharply increased from 2.73 to around 7 log CFU/g at 7 days of storage at 4 °C. After the change of temperature, the growth of psychrotrophic bacteria increased above 8 log CFU/g for control and active films, with no significant differences in microbial growth after 10 days of storage.

In general, the maximum microbial load reduction at 4 °C was observed in Lane-Late cultivar for the film with higher antimicrobial concentration although significant reductions were also observed for the film with lower active agent concentration. An increment in temperature, from 4 to 8 °C, accelerated the microbial growth of the three tested group of microorganisms in Lane-Late and Navel cultivars. However, for the Lane-Late cultivar, the effect of active films was enhanced by the increase of temperature, counteracting significantly the microbial growth of total aerobic bacteria and molds and yeasts. This effect was observed to a lesser extent with the Navel cultivar for molds and yeast and psychrotrophic bacteria. In this case, the increase of the vapor pressure and the increase in the release rate of the active agents from the films as a consequence of increasing the

temperature from 4 to 8 °C seemed to counteract the increase of the microbial growth rate in the orange.

Regarding the quality limits of consumption, taking into account the three groups of microorganisms tested, only Lane-Late orange packed with the film with the highest active concentration presented acceptable quality for consumption up to day 7 (commercially estimated best-before date) with microbial numbers below 6 log CFU/g.

Overall, active films containing a mixture of citral, hexanal and linalool showed higher activity in the inhibition of the microbial growth in pineapple than in orange at 4 °C. According to the results observed in the present work, Lopez-Reyes *et al.* (2013) reported that the efficacy of the EOs for controlling the growth of fungal microorganisms during postharvest is dependent on the fruit cultivar, the composition and concentration of the antimicrobials applied, and also the length of storage. This behavior might have also been caused by many other parameters such as the cellular structure of the fruit, fiber content, pH, specific microbial flora, or surface exposed to the active agents. Indeed, the antimicrobial activity of EOs were demonstrated to be enhanced by a decrease in the pH of the food (Skandamis and Nychas, 2000). This effect can be attributed to the fact that the EOs becomes more hydrophobic at low pH and thus can be dissolved better in the lipid phase of the bacterial membrane (Juven *et al.*, 1994).

It is worth noting that pH of pineapple (3.3) is lower than orange (4.0), and it could be possible that the difference in antimicrobial activity observed has to do with the difference in pH between the fruits. This suggestion is supported by Roller and Seedhar (2002) who showed that carvacrol and cinnamaldehyde were very effective at reducing the viable count of the natural flora on kiwifruit when used in dipping solution, but they were less effective on honeydew melon. In this case, the pH of kiwifruit was found to be lower than the pH of the melon.

4.5.1.2. Evolution of the headspace gas composition

The equilibrium concentration of O_2 and CO_2 in the headspace of the packaging depends not only on the respiratory rate of the fruit, but also on the packaging properties and storage conditions. Changes in gases concentration during the storage period are a sensitive indicator of physiological changes in the living cells of the fresh cut fruit.

The gas composition of the packed fruit was monitored during storage as described in section 3.2.8.4. Figure 4.42 to Figure 4.45 show the evolution of the gas composition (O_2 and CO_2) in the headspace of pineapple and orange for control and active films stored at 4 °C for 7 days followed by 5 days at 8 °C. The initial gas composition was air. Results represent the average of five replicates ± standard deviation. Statistical differences on juice leakage within materials were evaluated through ANOVA and Tukey analysis.

In general terms, the concentrations of O_2 and CO_2 for pineapple and orange samples remained quite steady and close to aerobic conditions up to day 7 for both control and active films. The concentration of O_2 was in the range of 18-20% and that of CO_2 in the range of 2-3% for both fruits. Small changes in O_2 composition could be explained not only by the low respiration rate of pineapple and orange at low temperature but also by the high permeability of the films. The micro holes allowed gas exchange with the atmosphere, otherwise anaerobic conditions could appear after one week of storage (Larsen and Liland, 2013).

Changes caused by an increase of the temperature at day 7, from 4 to 8 °C, were also monitored. After an increase of temperature, the equilibrium concentrations of gasses in pineapple (Gold Madura and MD2 cultivars) packed with control films changed, reaching about 14-16 % and 7-10 % of O_2 and CO_2 at day 12, respectively.

Similar trends were observed for orange packed with control films when the temperature of storage changed from 4 to 8 °C. The O_2 concentration of Lane-Late and Navel cultivars decreased to 13-16 % and CO_2 increased up to 7-11 % after 12 d of storage.

A sharp rise in respiration of Gold Madura pineapple packed with active films PP60/40a_8.5%CHL and PP30/70a_8.5%CHL took place after the increase of temperature. The O_2 concentration decreased up to 7-8% and the CO_2 concentrations increased to 29-30% for both active films. However, these changes were more pronounced for the MD2 cultivar packed with PP10/70a_8.5%CHL reaching values around 3% and 50% of O_2 and CO_2, respectively. Film PP10/70a_8.5%CHL was similar to the film PP30/70a_8.5%CHL in active concentration with the only difference being the thickness of the structural layer which would not affect the gas exchange through the package (both films were perforated). Differences in headspace evolution between these two systems could be attributed to differences in the pineapple cultivar or even to differences in maturity stage rather than in differences between the bilayer films.

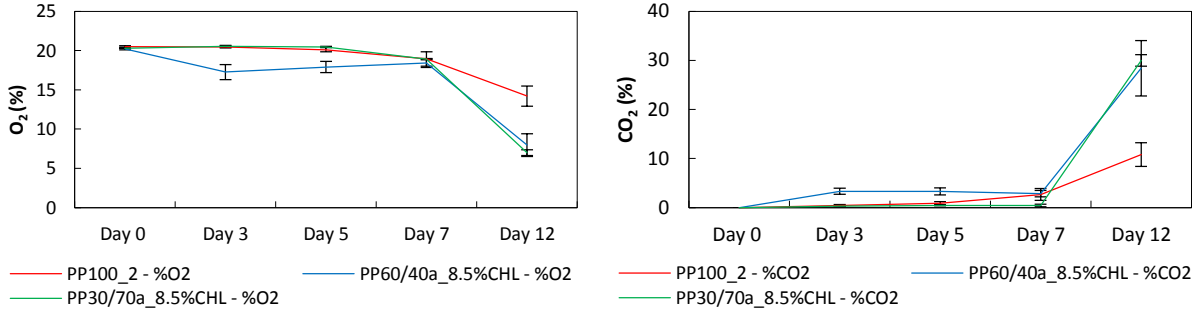


Figure 4.42. Evolution of O_2 and CO_2 concentrations in the headspace of pineapple (Gold Madura cultivar) packed with control and active films stored at 4 °C for 7 days followed by 5 days at 8 °C

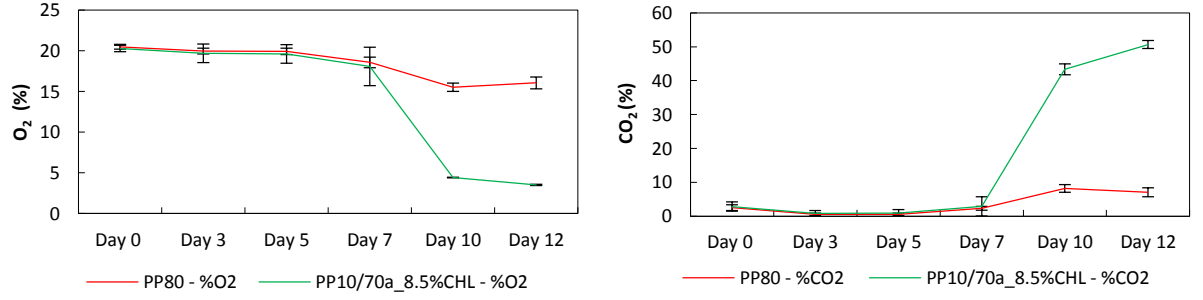


Figure 4.43. Evolution of O_2 and CO_2 concentration in the headspace of pineapple (MD2 cultivar) packed with control and active film stored at 4 °C for 7 days followed by 5 days at 8 °C

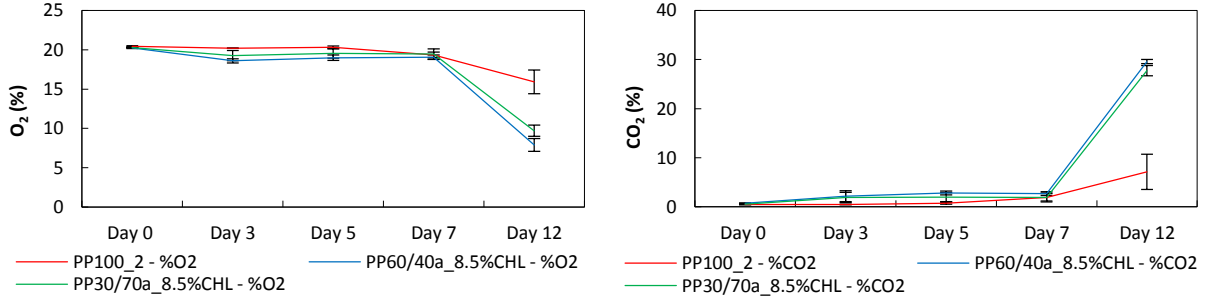


Figure 4.44. Evolution of O_2 and CO_2 concentration in the headspace of orange (Lane-Late cultivar) packed with control and active films stored at 4 °C for 7 days followed by 5 days at 8 °C

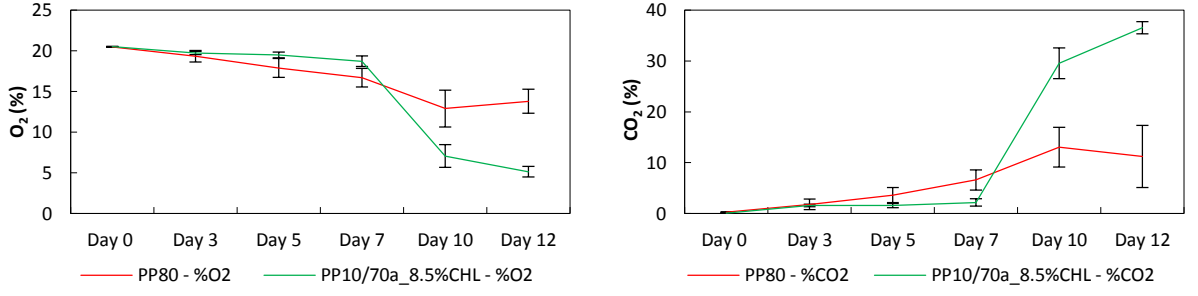


Figure 4.45. Evolution of O_2 and CO_2 concentration in the headspace of orange (Navel cultivar) packed with control and active film stored at 4 °C for 7 days followed by 5 days at 8 °C

An increase in respiration was also observed in Lane-Late and Navel cultivars after the increase of temperature when packed with active films. The O_2 concentrations changed up to 8-10 % and the CO_2 concentrations increased up to 28-30% for PP60/40a_8.5%CHL and PP30/70a_8.5%CHL films. The headspace composition of Navel cultivar packed with PP10/70a_8.5%CHL film only experienced minor differences compared to the headspace composition of PP60/40a_8.5%CHL and PP30/70a_8.5%CHL films.

The increase in respiration observed in the present research (higher CO_2 generation and O_2 consumption) is considered a normal behavior due to the increase in temperature, which is one of the main factors affecting the quality of fresh cut fruit (Marrero and Kader, 2006). An increase of temperature was responsible for the acceleration of the respiration of the fruit and other metabolic processes related to deterioration, as well as for microbial growth. In addition, volatile active compounds accelerated the respiration of the peeled and cut fruit when abuse temperature occurred. However, change in the headspace gas composition was not in accordance with the evolution of the microbial flora during the storage period. In this regard, the microbial loads of pineapple and orange packed with active films were significantly lower than fruit packed with control films during the storage period.

These results are in agreement with Rojas-Graü *et al.* (2007) who reported significant increases in the rates of O_2 depletion and CO_2 production in fresh cut apples containing high concentrations of EOs such as vanillin or oregano. Siroli *et al.* (2015b) also reported an increase of CO_2 production in lamb´s lettuce washed with oregano and thyme or carvacrol and thyme compared to the control. On the contrary, Serrano *et al.* (2008) reported similar gas composition for whole sweet cherries and table grapes packed in control bags and active packages containing thymol, menthol, eugenol, eucalyptol or carvacrol.

Differences observed in the headspace evolution between minimally processed and whole fruit in the presence of EOs or their pure components could be attributed to the higher metabolic activity of cut fruit. Fresh cut fruit physiology significantly differs from that of intact fruit and vegetables. Subcellular compartmentalization is disrupted at the cut surfaces, favoring contact of substrates and enzymes normally separated initiating deteriorative reactions that are usually absent in the whole product (Toivonen and Brummell, 2008). The contact of EOs with damaged tissues could accelerate the enzymatic and physicochemical reactions that take place in cut fruits, accelerating the respiration process, but slowing microbial evolution.

4.5.1.3. Juice leakage

Fruit possesses a high content of water and it can be easily lost when the skin is removed. The process of peeling and cutting destroys surface cells and allows exudate to leak from inner tissues, providing essential growth nutrients to the microbial flora present in these fruit products (Sela and Fallik, 2009).

The effect of the active bilayer film PP10/70a_8.5%CHL on juice leakage from pineapple and orange was evaluated during storage as described in 3.2.8.5. Figure 4.46 and Figure 4.47 show the accumulated juice leakage (g juice/100 g fruit) from fresh cut pineapple and orange, respectively packed with control and active films. Results were the average of three replicates ± standard deviation. Statistical differences of juice leakage within materials were evaluated through ANOVA and Tukey analyses.

The amount of exudate released from pineapple remained quite constant during the storage period and no significant effect was found between control and active films. In addition, no changes were observed from day 7 of storage (abuse temperature) until day 12.

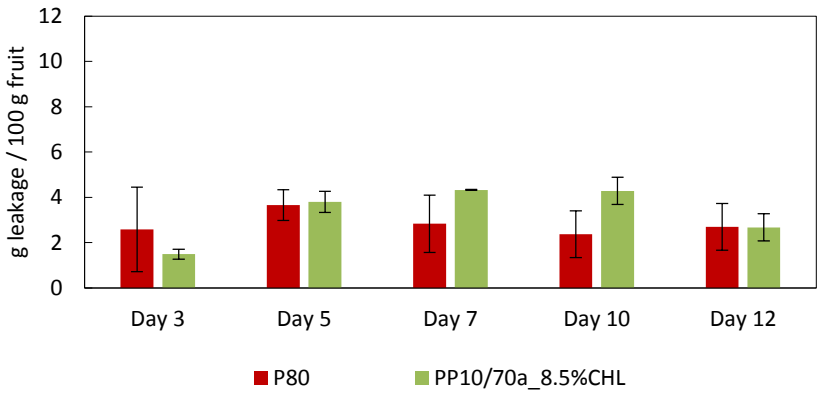


Figure 4.46. Evolution of juice leakage in pineapple (MD2 cultivar) packed with control and active films stored at 4 °C for 7 days followed by 5 days at 8 °C

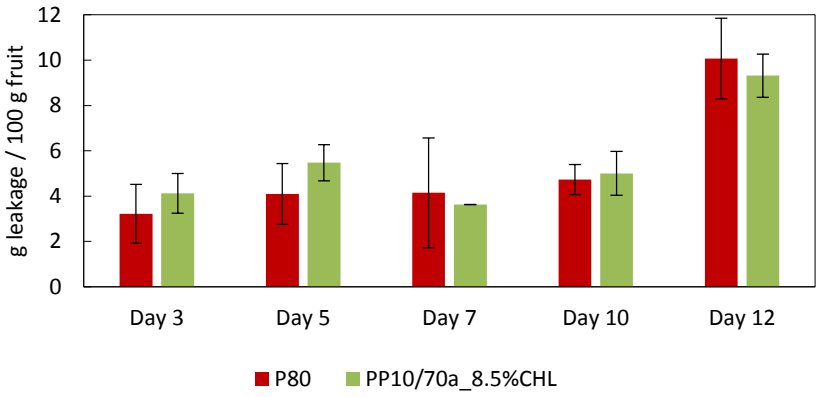


Figure 4.47. Evolution of juice leakage in orange (Navel cultivar) packed with control and active films stored at 4 °C for 7 days followed by 5 days at 8 °C

The amount of juice measured in pineapple was in line with Montero-Calderón *et al.* (2008) and Marrero and Kader (2006), who reported an exudate up to 4-5 mL of juice per 100 g of fruit after 14-15 days of storage at 5 °C in conventional packaging materials. Moreover, Montero-Calderón *et al.* (2008) found no significant differences between three packaging conditions (low O_2 atmosphere, high O_2 atmosphere and air), indicating that headspace gas composition did not affect juice leakage during storage of pineapple. Santos et al. (2005) reported similar results for fresh cut 'Perola' cultivar pineapple, stored under passive and active modified atmospheres.

The amount of exudate released from orange samples remained quite constant during storage from day 3 to day 10 (between 4-5 g/100 g fruit) with no significant differences between control and active films. From day 10 up to day 12, the juice leakage increased from 4-5 g to 9-10 g with no significant differences between films. This increase of exudate could have been related to the increase in temperature and an increase of CO_2 inside the packaging.

The water loss in fresh cut citrus such as lemons were also reported by Artés-Hernández *et al.* (2007) who observed formation of important amounts of exudate after 7 days of storage, but only for the smaller cut size (1/4 slices), concluding that the leakage effect depended overall on how the fruit was cut.

As a main conclusion, active packaging had no influence either on the juice leakage from peeled and cut pineapple or on the juice leakage from orange samples. On the contrary, temperature and high CO_2 concentration could have had some effect on the increase of weight loss in orange samples due to the acceleration of metabolism and cell damage due to peeling and cutting steps.

4.5.1.4. pH

The effect of the active films on fresh cut pineapple and orange pH compared to the control film was evaluated as described in 3.2.8.6. Figure 4.48 and Figure 4.49 show the evolution of the pH of fresh cut pineapple and orange, respectively, during the 7 days of storage at 4 °C followed by 5 days of storage at 8 °C. Results were the average of three replicates ± standard deviation. Statistical differences in the fruit pH within packaging materials were evaluated through ANOVA and Tukey analyses.

As can be observed in Figure 4.48 and Figure 4.49, pH values remained constant during the storage period for both fruits. The pH of pineapple (3.3 ± 0.1) was lower than that of orange (4.0 ± 0.2). No significant changes in pH between control and active packaging were observed, and there were no differences over time. The release of the active agents from the film had no influence on pH of peeled and cut pineapple and orange. Moreover, pH was not altered as consequence of the temperature increase from 4 to 8 °C.

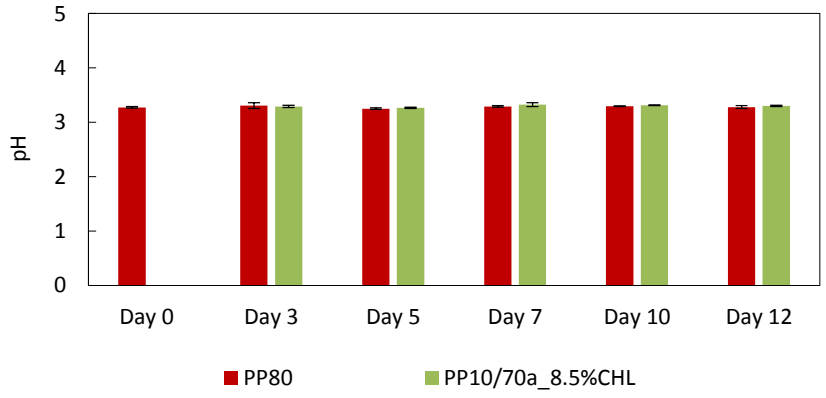


Figure 4.48. Evolution of pH in pineapple (MD2 cultivar) packed with control and active films stored at 4 °C for 7 days followed by 5 days at 8 °C

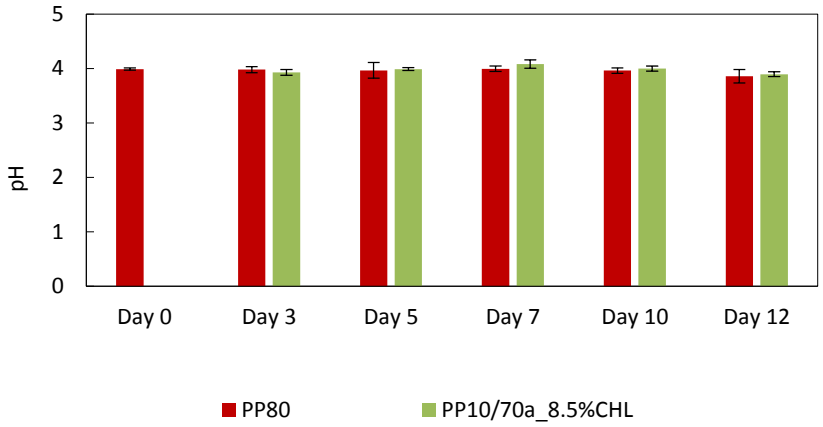


Figure 4.49. Evolution of pH in orange (Navel cultivar) packed with control and active films stored at 4 °C for 7 days followed by 5 days at 8 °C

These results are in agreement with Bitencourt *et al.* (2014) who concluded that the pH of pineapple coated with mint EO was not affected during 6 days of storage at 7 °C, and no difference in pH was observed between coated and uncoated samples. Rocha *et al.* (1995) did not observe significant changes in pH for minimally processed oranges, during 10 days of storage at 4 °C. Values of pH found for both pineapple and orange in the present research were within the pH range for these fruits, 3.3-5.2 for pineapple and 3.1-4.1 for oranges (FDA, 2012).

4.5.1.5. Total soluble solids (TSS)

Total soluble solids (TSS) refers to the total amount of soluble constituents of the fruit. These are mainly sugars, with smaller amounts of organic acids, vitamins, proteins, free amino acids, essential oils and glucosides. Fruit sugar level generally increases as the fruit ripens. However, levels can decrease when fruit is over-ripened, due to the consumption and degradation of these compounds during metabolic processes (Hardy and Sanderson, 2010).

The evolution of TSS during the storage period and the effect of the active film on the peeled and cut pineapple and orange were evaluated as described in 3.2.8.7. Figure 4.50 and Figure 4.51 represent the weight of sugar per 100 g of product expressed as TSS for pineapple and orange packed with control (P80) and active film (PP10/70a_8.5%CHL) during the 12 days of

storage. Results were the average of three replicates ± standard deviation. Statistical differences in the fruit TSS within materials were evaluated through ANOVA and Tukey analyses.

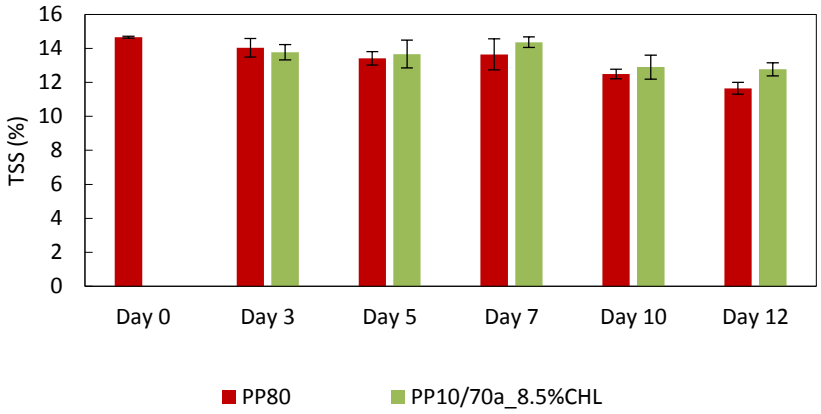


Figure 4.50. Evolution of TSS (%) in pineapple (MD2 cultivar) packed with control and active films stored at 4 °C for 7 days followed by 5 days at 8 °C

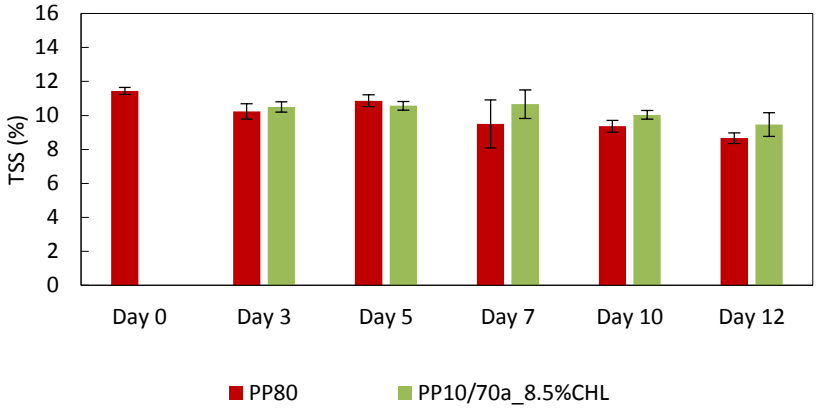


Figure 4.51. Evolution of TSS (%) in orange (Navel cultivar) packed with control and active films stored at 4 °C for 7 days followed by 5 days at 8 °C

In general, a reduction of TSS was observed for orange and pineapple during storage irrespective of the packaging material. No significant differences were observed in TSS for

pineapple or orange packed with control or active films, although the active film showed slightly higher TSS by the end of the storage time.

Specifically, for pineapple packed with control film, no significant changes of percent TSS were reported between day 0 and day 7 of storage (14.7-13.7%). However, a significant decrease of TSS occurred by day 10 of storage (12.5%). This decrease was even greater at day 12 (11.7%). For pineapple packed with active film, significant differences in TSS were observed from the beginning of storage up to day 10 and day 12 when TSS values of 12.9% and 12.8%, respectively, were reached.

For orange packed with control films, TSS values showed the same pattern as pineapple packed with control film, where a significant decrease in TSS occurred from day 0 (11.4%) to day 10 and 12 of storage with values of 9.4 and 8.7 %, respectively. Higher TSS values were also reported at the end of storage for orange packed with active film in comparison with the beginning of the storage period.

Likewise, Gil *et al.* (2006) and Montero-Calderón *et al.* (2008) observed some changes in TSS at the end of storage period of fresh cut pineapple stored at refrigeration temperatures. Authors such as Pretel *et al.* (1998) and Rocha *et al.* (1995) also found changes in TSS content during the storage of fresh cut orange. In general, the reduction of TSS observed during storage might be explained by an increase in respiratory metabolism that could have been enhanced by the increased temperature at day 7, as previously observed in the headspace evolution.

As reported by Garcia *et al.* (2012), non-climacteric fruits such as strawberries, citrus or pineapple normally exhibit a reduction in TSS content during storage. The reason is that at harvest time, these fruits have low or no energy source (starch). Subsequently, sugars are formed and serve as an energy source for respiration, resulting in a reduction TSS content.

The results obtained in the present research showed some effect of the active agents on maintaining the TSS parameter during postharvest ripening of the fruit compared with the control film. The effect of the volatile active agents observed on the TSS of the fruits are in agreement with the results obtained by Mohammadi *et al.* (2014). They observed higher TSS in fruits treated with different concentrations of fennel seed EO, black caraway seed or anise seed EOs in comparison to controls at the end of storage, with fennel EO showing the greatest differences. A dose dependent effect was observed for the three EOs tested. On the

contrary, Serrano *et al.* (2005) did not show significant differences in the TSS of whole sweet cherries with or without eugenol, thymol or menthol treatment and cold storage.

4.5.1.6. Sensory evaluation

A sensory evaluation test was carried out by a panel of 10 consumers who scored the visual quality (color and general appearance) and the odor perception of peeled and cut orange and pineapple packed with control and active films stored at 4 °C for 7 days followed by 5 days at 8 °C as described in 3.2.8.8. Ratings were based on a 5-point hedonic scale.

Figure 4.52 and Figure 4.53 shows the sensory parameters color, odor and general appearance evaluated for pineapple and orange, respectively, during the storage period.

In the case of pineapple, sensory attributes gradually decreased during the storage period when packed with both control and active films. At day 5, pineapple packed with control film was judged to be at the limit of marketability (score 3) in terms of visual quality (general appearance and color) and odor. Brown areas were already present and a hint of musty odor was perceived when the packaging was opened. As reported in section 4.5.1.1, molds and yeasts and psychrotrophic bacteria numbers were above 6 log CFU/g by day 5 in pineapple packed with control film, exceeding the quality limit of 6 log CFU/g. However, microbial growth was not visually evident until day 7, and coincided with a strong musty odor when the packages were opened.

On the other hand, pineapple packed with active film was generally scored higher compared with pineapples packed with control films. In particular, color, odor and general appearance were within the limit of marketability (score ≥ 3) up to day 7 although a slight aroma of volatile active agents was reported from day 3 of storage. Brown areas started to appear by day 7. As reported in section 4.5.1.1, at day 7, molds, yeasts and psychrotrophic bacterial numbers in pineapple were around the limit of 6 log CFU/g, while total aerobic numbers were far below the quality limit. However, it was not until day 10 of storage when visual microbial growth was observed in the fruit. At this stage, total aerobic counts, molds and yeast and psychrotrophic bacteria overcame the 6 log CFU/g quality limit for the active film.

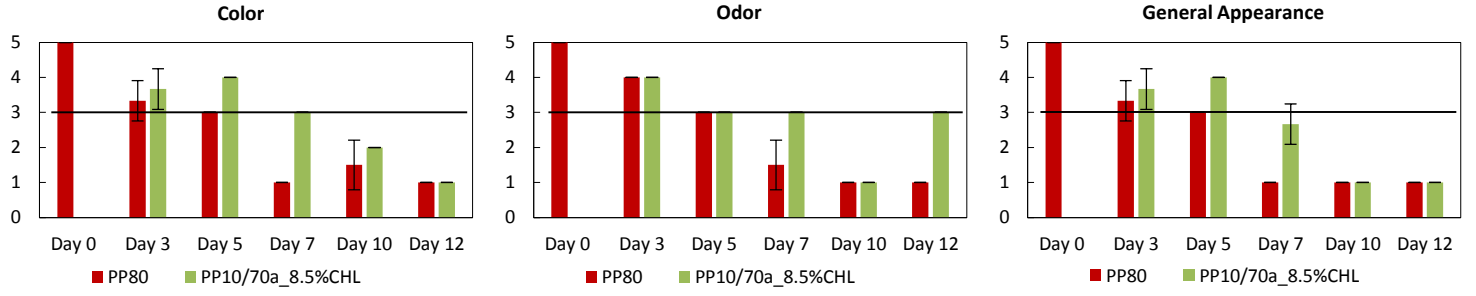


Figure 4.52. Sensory evaluation of pineapple (MD2 cultivar) packed in the control and active films stored at 4 °C for 7 days followed by 5 days at 8 °C
5 = very good; 4 = good; 3= fair (limit of marketability); 2= poor; 1= bad (unusable)

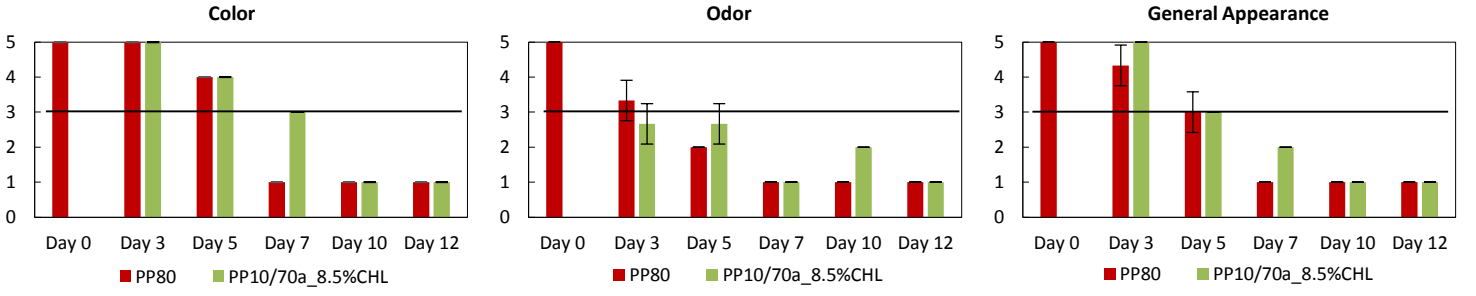


Figure 4.53. Sensory evaluation of orange (Navel cultivar) packed in the control and active films stored at 4 °C for 7 days followed by 5 days at 8 °C
5 = very good; 4 = good; 3= fair (limit of marketability); 2= poor; 1= bad (unusable)

In the case of orange, the sensory attributes also gradually decreased both for control and active films during the storage period. By day 5, orange packed with control film was above the marketability threshold in terms of visual quality but a stale odor was perceived once the packaging was opened (score < 3). Then, at this stage of the storage period, orange packed with control film was placed under the marketability threshold. By day 5, microbial analysis showed how molds and yeast and psychrotrophic bacteria slightly overcame the quality limit (section 4.5.1.1). However, until day 7 microbial growth was not evident for orange packed with control film. Also surface desiccation on the fruit surface was observed at this stage.

Contrary to what was observed for the control film, by day 5, orange packed with active film was still within the marketability limits for the sensory parameters studied, with odor being scored better than for the control film. At day 5, the microbial loads were also below the quality limit except for psychrotrophic bacteria that slightly overcame the limit. Nevertheless, from day 5 the stale odor mixed with the aroma of the active agents and surface desiccation of the fruit tended to gradually increase with storage time and placed the fruit under the threshold of acceptability. By day 7, the microbial loads of molds and yeasts increased over the quality limit while the total aerobic counts remained below the limit at this stage. It was at day 10 when visual growth was observed for orange packed with active film.

From this research it was observed that when spoilage microorganisms exceed the microbiological quality limit it does not always result in occurrence of visual defects, as both microbiological and physiological activity play a role in the spoilage of these products (Ragaert *et al.*, 2007).

Despite the aroma exerted by the volatile active agents released from the active film, their contribution to the odor perception of the fruit did not alter their acceptance, since the fruit packed with active films generally scored better than fruit packed with control films. For attributes such as color and general appearance, the active films also scored better than the controls on some specific days, although at the end of the storage period both control and active films failed to maintain sensory attributes above the marketability threshold.

4.5.1.7. Conclusions on active films containing volatile active agents

The use of active films containing citral, hexanal and linalool delayed the microbial spoilage of peeled and cut pineapple and orange during 12 days of storage at 4/8 °C, although the effectiveness was dependent on the cultivar. Overall, for Gold Madura and MD2 pineapple cultivars, the active materials preserved the microbiological quality from day 3/5 to day 7. For the Lane-Late orange cultivar, the active materials increased the microbiological quality from day 3/5 to day 7. For the Navel orange cultivar, results were not as promising in terms of microbiological quality extension. In this respect, control and active films maintained the microbiological quality of the orange up to days 3/5, although slight lower microbial loads were found for the fruit packed with active films.

These results suggest that these volatile agents have potential for controlling the microbiota of orange and pineapple, supporting previous observations that essential oils and their pure components are a useful tool to increase the shelf life of fruits. The antimicrobial activity of the films was dependent on the active layer thickness, the type of fruit, type of cultivar as well as the temperature of storage.

Regarding physiological quality attributes of the fruit packed with active films, some relevant parameters such as juice leakage, pH and TSS remained within acceptable quality levels longer compared with fruit packed with control films. The biggest changes were observed for the gas composition of the headspace when abuse temperature was used, and were more pronounced for active films.

Finally, from a sensory point of view, active films maintained longer the organoleptic characteristics of the fruit in terms of general appearance, color and odor compared to the control film. However, the detection of aroma associated with the volatile active agents suggests a reduction of the active agent concentrations in further development work.

4.5.2. Validation of the active tray containing solid active agents

The active tray developed at semi-industrial scale containing a mixture of potassium sorbate and sodium benzoate was validated with peeled and cut pineapple and orange. To this end, peeled and cut orange or pineapple were packaged separately with control (PP/PP:10%EVA) and active trays (PP/PP:10%EVA:20%PS_SB) as described in 3.2.8.1.

PP80 control film was used as a lid for this trial. Orange and pineapple were packaged under aerobic conditions. Films were micro-perforated with two holes of 250 µm per tray to allow gas exchange with the environment and avoid anaerobic conditions.

The packed samples were stored at 4 °C for 7 days followed by 3 days at 8 °C in order to simulate possible abuse temperatures. Microbial counts, gas composition of the headspace, and sensory parameters were evaluated in pineapple and orange over storage time. The pineapple and orange cultivars used for the validation of the active tray were MD2 and Navel, respectively.

4.5.2.1. Antimicrobial properties

Peeled and cut pineapple and orange packed with control and active trays were subjected to microbiological analyses during storage at 4 °C for 7 days and 8 °C until day 10. The microflora of the fruit packed with control and active trays was evaluated at day 0, 3, 7 and 10 in terms of total aerobic numbers, molds and yeast and psychrotrophic bacteria. Day 0 was defined as the microbial load of the fruit before the packaging process.

Table 4.44 shows the microbial population of pineapple packaged with control and active trays during storage. Results were the average of three replicates ± standard deviation. In general, the microbial loads of pineapple packed with the active tray were significantly lower than the pineapple packed with the control tray.

Particularly, the total aerobic counts for MD2 pineapple packed with control and active trays remained quite constant during storage, with small but significant reductions caused by the active tray at day 7 and day 10. Total aerobic counts did not exceed the quality limit during the evaluated storage period (10 days).

Molds and yeasts enumerated from the MD2 pineapple cultivar packed in the control tray increased around 1 log CFU/g after 7 days of storage at 4 °C. The shift of temperature from 4 to 8 °C caused an increase of molds and yeasts up to 7.58 log CFU/g. The increase of mold and yeast populations for pineapple packed in the active tray was slowed down up to day 7 compared to the control tray. However, with the increase of temperature, the growth increment from day 7 to day 10 was of the same order of magnitude as for the control tray (around 2 log CFU/g). Significant mold and yeast reductions were observed for pineapple

packed in the active tray at day 7 and day 10 of storage, with 0.87 and 1.18 log CFU/g reductions, respectively, compared with the control tray.

Psychrotrophic bacteria enumerated from MD2 pineapple packed in the control tray increased around 2 log CFU/g after 7 days of storage at 4 °C. The pineapple packed with the active tray experienced slower psychrotrophic bacterial growth with 1 log CFU/g increment after 7 days of storage. As reported for molds and yeast, after the shift from 4 to 8 °C, an increase of around 2 log CFU/g was observed for both control and active trays. However, a significant reduction was still observed for active trays at 7 and 10 days with 1.18 and 1.25 log CFU/g reductions compared to the control tray.

In general, by day 7, neither active tray nor control tray exceeded the quality limit for any microorganism. The maximum microbial reduction was generally observed in pineapple packed in the active tray at day 10 after the shift from 4 to 8 °C. According to these results, pineapple was considered microbiologically acceptable up to day 7 in both trays, but the active tray was able to keep the numbers lower than control tray during storage.

Table 4.44. Microbial population of peeled cut pineapple (MD2 cultivar) packed with control and active trays stored at 4 °C for 7 days followed by 3 days at 8 °C

		PP/PP:10%EVA tray	PP/PP:10%EVA:20%PS_SB tray
Day	**Temperature (°C)**	**Total aerobic numbers (log CFU/g)**	
0	4	4.68 ± 0.10	
3	4	5.14 ± 0.04 [a]	4.78 ± 0.23 [a]
7	4	4.49 ± 0.08 [a]	4.16 ± 0.18 [b]
10	8	5.33 ± 0.09 [a]	4.89 ± 0.25 [b]
Day	**Temperature (°C)**	**Molds and yeasts (log CFU/g)**	
0	4	4.40 ± 0.24	
3	4	4.38 ± 0.15 [a]	4.40 ± 0.10 [a]
7	4	5.38 ± 0.34 [a]	4.51 ± 0.18 [b]*
10	8	7.58 ± 0.08 [a]	6.40 ± 0.16 [b]*
Day	**Temperature (°C)**	**Psychrotrophic bacteria (log CFU/g)**	
0	4	3.49 ± 0.34	
3	4	3.85 ± 0.13 [a]	3.54 ± 0.05 [b]
7	4	5.59 ± 0.35 [a]	4.41 ± 0.20 [b]*
10	8	7.63 ± 0.13 [a]	6.37 ± 0.20 [b]*

Values among same day of analysis within the same row and with a different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis). DL=1.6 log CFU/g. (*) Significant log reduction (any reduction less than 0.5 log is not considered biologically meaningful, whether statistically significant or not).

By day 10, microbial numbers for molds and yeasts and psychrotrophic bacteria in pineapple packed in the control and active trays already exceeded the 6 log CFU/g quality limit. It should be noted that total aerobic numbers remained below 6 log CFU/g over time, irrespective of packaging tray type.

Table 4.45 shows the microbial population of the different microbial groups analyzed for orange packaged in control and active trays during the storage period. Results were expressed as the mean of three replicates ± standard deviation.

Little or no antimicrobial effect was observed for orange packed with active trays compared to control trays. The microbial load for total aerobic numbers remained quite steady during 7 days of storage for control and active trays, with no significant reduction between trays. At day 10 of storage, after the increase of temperature, an increase of total aerobic numbers took place and the same microbial load was observed between trays. Regarding molds and yeast and psychrotrophic bacteria, no significant reductions were observed between trays during the storage period. In this case, active trays did not seem to be effective in terms of microbial reduction.

Table 4.45. Microbial population of peeled cut orange (Navel cultivar) packed with control and active trays stored at 4 °C for 7 days followed by 3 days at 8 °C

		PP/PP:10%EVA tray	PP/PP:10%EVA:20%PS_SB tray
Day	**Temperature (°C)**	**Total aerobic numbers (log CFU/g)**	
0	4	3.59 ± 0.12	
3	4	3.74 ± 0.28 [a]	3.73 ± 0.45 [a]
7	4	3.76 ± 0.14 [a]	3.58 ± 0.19 [a]
10	8	5.78 ± 0.08 [a]	5.78 ± 0.20 [a]
Day	**Temperature (°C)**	**Molds and yeasts (log CFU/g)**	
0	4	2.93 ± 0.09	
3	4	3.29 ± 0.15 [a]	3.35 ± 0.13 [a]
7	4	4.51 ± 0.16 [a]	4.72 ± 0.29 [a]
10	8	6.69 ± 0.06 [a]	6.63 ± 0.10 [a]
Day	**Temperature (°C)**	**Psychrotrophic bacteria (log CFU/g)**	
0	4	3.77 ± 0.10	
3	4	3.79 ± 0.21 [a]	3.77 ± 0.19 [a]
7	4	4.74 ± 0.16 [a]	4.84 ± 0.30 [a]
10	8	6.94 ± 0.11 [a]	6.73 ± 0.16 [a]

Values among same day of analysis within the same row and with a different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis). DL=1.6 log CFU/g. (*) Significant log reduction (any reduction less than 0.5 log is not considered biologically meaningful, whether statistically significant or not).

As reported for pineapple, orange samples kept microbial numbers below the quality limit up to day 7 for both control and active trays. At day 10, orange packed with both trays exceeded the quality limit in terms of molds and yeasts and psychrotrophic bacteria.

Potassium sorbate and sodium benzoate are widely used for inhibiting mold, yeast and some bacterial strains in various foods, including cheese, baked goods, fruits and vegetables, jams and certain meat and fish products (Davidson et al., 2005). The low pH of pineapple and orange studied could support the antimicrobial effect of the potassium sorbate and sodium benzoate (Suhr and Nielsen, 2004). Unexpectedly, evidence on the antimicrobial activity of the trays was only seen in pineapple, although both pineapple and orange had a pH within the range of activity of sorbate (up to 6) and benzoate (up to 4-4.5) (Liewen and Marth, 1985). Unfortunately, the amount of potassium sorbate and sodium benzoate released from the active trays was not measured in this test, but in the active packaging system. Several reasons for the low antimicrobial effectiveness could be a deficient release of the active agents, a deficient absorption or solubility of the salts by the fruit or the media surrounding the fruit, or a low efficacy of the active salts against the microorganisms growing in orange.

4.5.2.2. Evolution of the headspace gas composition

The gas composition of the headspace of fruit packed with control and active trays during storage was monitored as described in 3.2.8.4. Changes caused by an increase from 4 to 8 °C by day 7 and also any possible correlation between the presence of salts and changes in the fruit metabolism in terms of O_2 consumption and CO_2 generation were evaluated. Results were the average of five replicates ± standard deviation. Figure 4.54 and Figure 4.55 show the evolution of the gas atmosphere composition of pineapple and orange, respectively, packed in control and active trays during storage.

As can be observed in these figures, the gas composition remained stable until day 7 both for control and active trays with both fruits. However, when storage temperature shifted from 4 to 8 °C at day 7, the gas composition of fruit packed in control trays changed to about 15% O_2 and 10% CO_2 for pineapple and 16% O_2 and 8% CO_2 for orange (day 10). This is a normal indicator of physiological changes in the living cells of the fresh cut fruit with an increase of temperature and storage time.

On the other hand, the active tray significantly reduced the respiration rate in both pineapple and orange during storage. No changes of O_2 for pineapple and orange were found with the change of temperature and the final O_2 level approached 19%, while CO_2 remained around 3% by the end of storage.

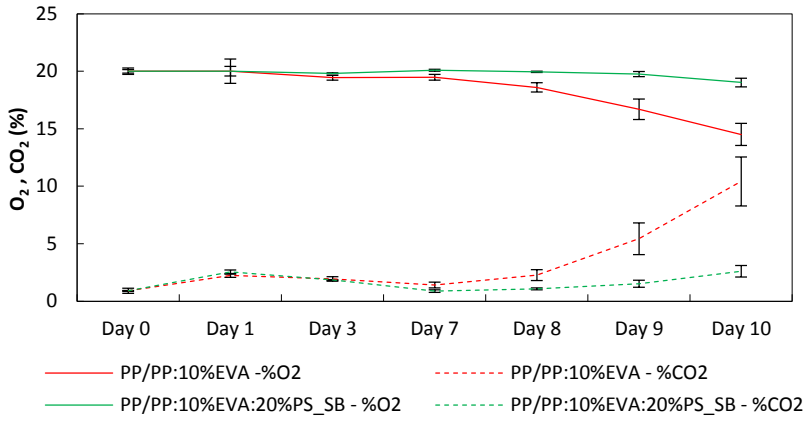


Figure 4.54. Evolution of O_2 and CO_2 concentration in the headspace of pineapple (MD2 cultivar) packed with control and active trays stored at 4 °C for 7 days followed by 3 days at 8 °C

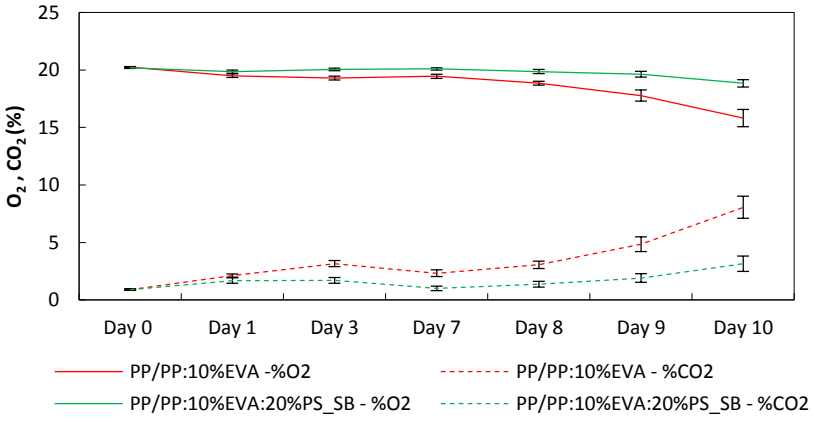


Figure 4.55. Evolution of O_2 and CO_2 concentration in the headspace of orange (Navel cultivar) packed with control and active trays stored at 4 °C for 7 days followed by 3 days at 8 °C

In agreement, Castelló *et al.* (2006) observed that cell respiration in strawberry halves was inhibited in both aerobic and anaerobic pathways in the presence of osmotic dehydration caused by a solution containing potassium sorbate. The action of this antimicrobial on cell membranes probably inhibited enzymatic reactions in the vegetable cells, resulting in the lower respiration rate.

4.5.2.3. Sensory evaluation

A sensory evaluation test of pineapple and orange packed with control and active tray stored at 4 °C for 7 days followed by 3 days at 8 °C was carried out as described in section 3.2.8.8. Ratings were based on a 5-point hedonic scale. Figure 4.56 and Figure 4.57 show the sensory parameters color, odor and general appearance evaluated for pineapple and orange during the storage period, respectively.

As reported in section 4.5.1.6, the sensory attributes of fruit gradually decreased during storage regardless of the packaging material. However, fruit packed in active packaging trays was generally scored slightly higher compared to control packaging.

In detail, pineapple packed in control trays was at the limit of marketability (score 3) in terms of visual quality (general appearance and color) at day 7 of storage. However, the odor attribute was already scored as not marketable since a hint of musty odor was perceived when the packaging was opened. Brown areas were already observed by day 7 for control trays. By day 10, the three attributes were below the marketability limit and microbial growth was visually evident.

As noted, pineapple packed in the active tray was scored higher compared with pineapple packed in the control tray. Color, odor and general appearance were within the limit of marketability (score ≥ 3) up to day 7 and microbial numbers were below the quality limit of 6 log CFU/ml as reported in section 4.5.2.1. Brown areas started to appear by day 7. By day 10 pineapple packed in the active tray was below the marketability limit, but no visual growth was observed even at the end of the storage period. Contrary to pineapple packed in the control tray, an increase of exudate was observed for pineapple packed in active trays. This exudate was increased at the end of storage period.

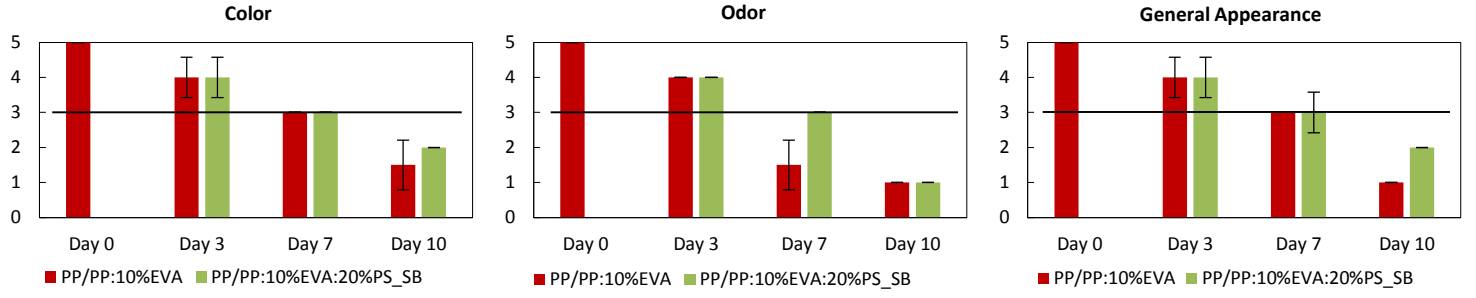


Figure 4.56. Sensory evaluation of pineapple (MD2 cultivar) packed in the control or active tray, stored at 4 °C for 7 days followed by 3 days at 8 °C
5 = very good; 4 = good; 3= fair (limit of marketability); 2= poor; 1= bad (unusable)

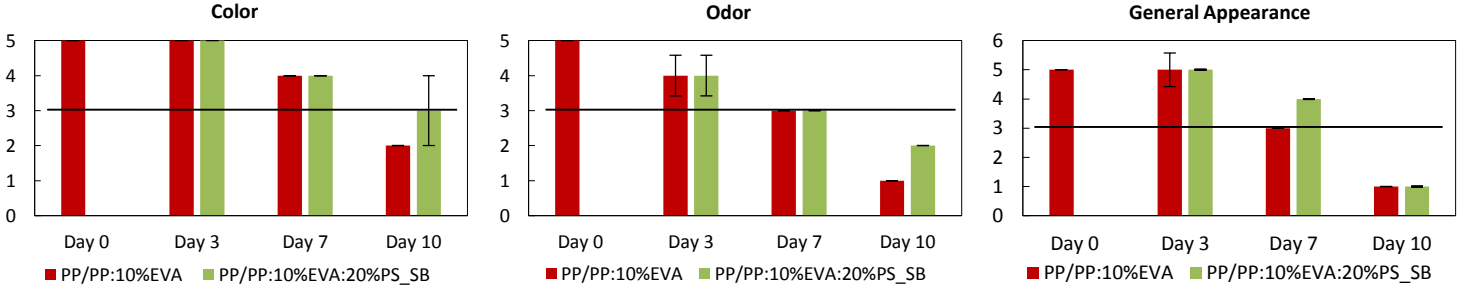


Figure 4.57. Sensory evaluation of orange (Navel cultivar) packed in the control or active tray, stored at 4 °C for 7 days followed by 3 days at 8 °C
5 = very good; 4 = good; 3= fair (limit of marketability); 2= poor; 1= bad (unusable)

In the case of the orange, the fruit packed in the active tray was generally scored higher compared with the one packed in the control tray. In this latter case, orange was scored within the marketability limit in terms of visual quality and odor up to day 7. At day 7, microbial numbers were below the quality limit of 6 log CFU/ml as reported in section 4.5.2.1. Orange packed with active tray followed similar evolution as orange packed with active tray. Decay in the sensory attributes were more pronounce at day 10 while at day 7 orange was still within the marketability threshold. However, they were generally scored higher than for the control. By day 10, microbial growth was observed for both trays.

4.5.2.4. Conclusions on active trays containing solid active agents

The use of the active tray containing potassium sorbate and sodium benzoate in peeled and cut pineapple delayed microbial spoilage of the fruit during 10 days of storage at 4/8 °C, suggesting that these preservatives have potential in controlling the microbiota of pineapple. However, no antimicrobial effect was observed for the Navel orange cultivar packed with the active trays.

The active tray significantly reduced the respiration rate in both pineapple and orange during storage, probably due to inhibition of enzymatic reactions in the cells of the fruit, resulting in lowered respiration.

Finally, from a sensory evaluation point of view, fruit packed in active trays was scored slightly higher in comparison with fruit packed in control trays.

According to these results, the use of active trays based on potassium sorbate and sodium benzoate seems to be a useful tool to maintain microbial quality parameters of fresh cut pineapple longer, improving somehow its sensory attributes and slowing down its metabolism. The use of active trays with minimally processed orange also provided an improvement of sensory parameters and a reduction of fruit metabolism.

4.5.3. Validation of the final active packaging solution

Results presented in this Thesis have shown that the incorporation of citral, hexanal and linalool into PP bilayer films by extrusion has the ability to delay microbial decay and can maintain quality parameters of perishable peeled and cut pineapple and orange. Moreover, some evidence on the delay of microbial growth in pineapple and on the delay of changes in

the sensory attributes of pineapple and orange have also been shown with the use of an active tray containing potassium sorbate and sodium benzoate.

Investigation on the combination of both materials developed, active film containing volatile active agents and active tray containing non-volatiles active agents, has been carried out to evaluate the overall effect of the "active system" and any possible synergy resulting from this combination.

To that, peeled and cut orange and pineapple were packaged with the active antimicrobial system (PP10/70a_8.5%CHL film sealed with PP/PP:10%EVA:20%PS_SB tray) and with a control system (PP80 film sealed with PP/PP:10%EVA tray) as described in 3.2.8.1. Control and active films were perforated with 2 micro holes of 250 µm to allow gas exchange. Packed samples were stored at 4 °C for 7 days followed by 5 days at 8 °C in order to simulate possible abuse temperatures.

Navel and MD2 were the orange and pineapple cultivars supplied for the validation of the active system. Microbial counts, evolution of the gas composition of the headspace, juice leakage, pH, TSS and sensory properties of orange and pineapple were evaluated during storage. The active agents released into the fruit during the storage period were also evaluated.

4.5.3.1. Antimicrobial properties of the active packaging solution

The peeled and cut fruit was subjected to microbiological analyses during the storage period. The microflora of the fruit was evaluated at day 0, 3, 5, 7, 10 and 12 in terms of total aerobic count, molds and yeasts and psychrotrophic bacteria. Day 0 was defined as microbial load of the fruit before the packaging.

Table 4.46 shows the microbial population of pineapple packed with the control and active packaging systems during the storage period. Results were expressed as the mean of three replicates ± standard deviation.

In general, the microbial load of pineapple packed in both packaging systems gradually increased during storage. However, the active system was able to keep the microbiota of pineapple lower than the control system during storage.

Table 4.46. Microbial population of peeled and cut pineapple (MD2 cultivar) packed with the control or active packaging system, stored at 4 °C for 7 days followed by 5 days at 8 °C

		PP80 film + PP/PP:10%EVA tray	PP10/70a_8.5%CHL film + PP/PP:10%EVA:20%PS_SB tray
Day	**Temperature (°C)**	**Total aerobic numbers (log CFU/g)**	
0	4	4.84 ± 0.58	
3	4	5.77 ± 0.71 [a]	4.91 ± 0.52 [a]*
5	4	5.46 ± 0.20 [a]	3.86 ± 0.24 [b]*
7	4	5.99 ± 0.21 [a]	3.99 ± 0.13 [b]*
10	8	6.44 ± 0.14 [a]	4.93 ± 0.45 [b]*
12	8	6.69 ± 0.41 [a]	5.35 ± 0.64 [b]*
Day	**Temperature (°C)**	**Molds and yeasts (log CFU/g)**	
0	4	4.29 ± 0.12	
3	4	6.01 ± 0.23 [a]	4.28 ± 0.34 [b]*
5	4	6.55 ± 0.44 [a]	5.01 ± 0.41 [b]*
7	4	8.06 ± 0.10 [a]	5.21 ± 0.12 [b]*
10	8	8.34 ± 0.05 [a]	6.03 ± 0.43 [b]*
12	8	8.53 ± 0.03 [a]	5.85 ± 0.42 [b]*
Day	**Temperature (°C)**	**Psychrotrophic bacteria (log CFU/g)**	
0	4	2.76 ± 0.08	
3	4	5.97 ± 0.20 [a]	4.84 ± 0.35 [b]*
5	4	6.67 ± 0.53 [a]	5.01 ± 0.47 [b]*
7	4	7.77 ± 0.01 [a]	5.17 ± 0.14 [b]*
10	8	8.23 ± 0.03 [a]	6.00 ± 0.44 [b]*
12	8	8.40 ± 0.16 [a]	5.78 ± 0.54 [b]*

Values among same day of analysis within the same row and with a different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis); DL=1.6 log CFU/g; (*) Significant log reduction (any reduction less than 0.5 log is not considered biologically meaningful, whether statistically significant or not).

For the control system, total aerobic counts enumerated for the MD2 cultivar increased up to 5.99 log CFU/g at day 7 (estimated commercial best-before date). The increase of the storage temperature at day 7 enabled the microbial growth to reach numbers above the quality limit at day 10 of storage. Molds and yeasts numbers rapidly increased up to 8.06 log CFU/g at day 7. In this case, yeasts were much more predominant than molds. This effect was corroborated by Tournas *et al.* (2006) who reported that mold growth was limited in fruit salads possibly due to faster growth of yeasts, which were able to take over before the filamentous fungi. At the end of the commercial shelf life, day 7, psychrotrophic bacterial numbers exceeded the quality limit.

In the case of the active system, after 7 days of storage, total aerobic numbers remained stable or were even lower than the initial load. After 12 days of storage, 5.35 log CFU/g were observed. Molds and yeast numbers slightly increased up to 5.21 log CFU/g after 7 days of storage. After 12 days of storage, molds and yeast numbers did not exceed the quality limit. Psychrotrophic bacteria numbers increased up to 5.17 log CFU/g by day 7 and after 12 days, counts remained below the quality limit.

Microbial population of pineapple packed with the active packaging system remained below the recommended microbiological quality threshold for the entire 12 day study period. In fact, significant reductions of 2.00, 2.85 and 2.60 log CFU/g were observed for total aerobic numbers, molds and yeast and psychrotrophic bacteria by day 7, respectively. After the change of storage temperature from 4 to 8 °C, significant reductions persisted and were 1.34, 2.67 and 2.62 log CFU/g for total aerobic numbers, molds and yeasts and psychrotrophic bacteria at 12 of days storage, respectively.

Table 4.47 shows the microbial population of the peeled and cut orange packed with the control and active system during the storage period. Results were expressed as the mean of three replicates ± standard deviation.

As observed for pineapple, the microbial load of orange packed with control and active packaging gradually increased during the storage period, but the active system was able to keep the microbiota of orange at levels significantly below the control system during the studied period.

In the case of the control system, the total aerobic numbers increased around 1 log CFU/g from day 0 to day 7. The increase in storage temperature at day 7 promoted the microbial growth of total aerobic bacteria above the quality limit at day 10. Molds and yeasts numbers quickly increased up to 7.16 log CFU/g at day 7, reaching numbers above 6 log CFU/g at day 5 of storage. As for pineapple, yeasts were the predominant fungi found in orange. Psychrotrophic bacterial growth also increased very fast from 2.73 log CFU/g at day 0 to 7.14 log CFU/g at day 7, reaching numbers above 6 log CFU/g at day 5 of storage.

In the active system, total aerobic bacterial numbers on orange decreased about 0.5 log CFU/g from day 0 to day 7 of storage. However, the increase in storage temperature at day 7 promoted the growth of total bacteria above 6 log CFU/g at day 10. Molds and yeast remained below the quality limit of 6 log CFU/g at day 7. However, as the temperature increased, microbial growth substantially increased, reaching numbers of around 8 log

CFU/g at the end of storage. The same pattern was observed for psychrotrophic bacteria, where numbers increased up to 5.83 log CFU/g at day 7, reaching 8.05 log CFU/g after 12 days of storage.

In general, a significant microbial reduction was observed for the orange packed with active packaging system compared to the control packaging. A maximum reduction of 1.61, 1.49 and 1.30 log CFU/g was observed for total aerobic numbers, molds and yeasts and psychrotrophic bacteria by day 7 of storage, respectively. Although by day 10 microbial numbers were above the quality limit (6 log CFU/g), microbial reductions were still significant compared to the samples packed with control system, with differences higher than 0.5 log CFU/g for total aerobic bacteria, molds and yeasts and psychrotrophic bacteria.

Table 4.47. Microbial population of peeled and cut orange (Navel cultivar) packed with the control or active packaging system, stored at 4 °C for 7 days followed by 5 days at 8 °C

		PP80 film + PP/PP:10%EVA tray	PP10/70a_8.5%CHL film + PP/PP:10%EVA:20%PS_SB tray
Day	**Temperature (°C)**	**Total aerobic numbers (log CFU/g)**	
0	4	4.49 ± 0.75	
3	4	4.75 ± 0.19 ^{a}	4.28 ± 0.13 ^{b}
5	4	4.74 ± 0.07 ^{a}	3.71 ± 0.13 ^{b*}
7	4	5.66 ± 0.16 ^{a}	4.05 ± 0.34 ^{b*}
10	8	6.91 ± 0.20 ^{a}	6.31 ± 0.19 ^{b*}
12	8	6.93 ± 0.18 ^{a}	6.58 ± 0.04 ^{b}
Day	**Temperature (°C)**	**Molds and yeasts (log CFU/g)**	
0	4	3.45 ± 0.02	
3	4	5.62 ± 0.16 ^{a}	4.73 ± 016 ^{b*}
5	4	6.07 ± 0.15 ^{a}	5.09 ± 0.30 ^{b*}
7	4	7.16 ± 0.22 ^{a}	5.67 ± 0.20 ^{b*}
10	8	8.33 ± 0.07 ^{a}	7.70 ± 0.23 ^{b*}
12	8	8.46 ± 0.03 ^{a}	8.00 ± 0.03 ^{b}
Day	**Temperature (°C)**	**Psychrotrophic bacteria (log CFU/g)**	
0	4	2.73 ± 0.23	
3	4	5.72 ± 0.14 ^{a}	4.52 ± 0.31 ^{b*}
5	4	6.29 ± 0.21 ^{a}	5.92 ± 0.44 ^{b}
7	4	7.14 ± 0.15 ^{a}	5.83 ± 0.18 ^{b*}
10	8	8.40 ± 0.03 ^{a}	7.55 ± 0.12 ^{b*}
12	8	8.52 ± 0.06 ^{a}	8.05 ± 0.06 ^{b}

Values among same day of analysis within the same row and with a different superscript letter are significantly different at $p \leq 0.05$ (Tukey analysis); DL=1.6 log CFU/g; (*) Significant log reduction (any reduction less than 0.5 log is not considered biologically meaningful, whether statistically significant or not).

The antimicrobial effectiveness of the active packaging system was studied with respect to the active film and tray when tested alone. Table 4.48 and Table 4.49 summarise the microbial reduction produced by the active film (PP10/70a_8.5%CHL), the active tray (PP/PP:10%EVA:20%PS_SB tray) and the combination of both active packaging materials (PP10/70a_8.5%CHL film sealed with PP/PP:10%EVA:20%PS_SB tray) in pineapple and orange, respectively. The sum of individual reductions of film and tray and the synergistic effect calculated as the difference between the effect achieved with the active system and the sum of the individual reductions are also shown in the following tables.

Table 4.48. Microbial reduction in peeled and cut pineapple (MD2 cultivar) packed with active film, active tray and active packaging system during storage

					Σ individual reductions	
		Active film[1]	Active tray[2]	Active system[3]	(film + tray)	Synergistic effect
Day	**T (°C)**	**Reduction of total aerobic numbers (log CFU/g)**				
3	4	0.00 ± 0.02	0.36 ± 0.23	0.86 ± 0.88	0.36 ± 0.23	0.5 ± 0.91
5	4	0.98 ± 0.25*	---	1.60 ± 0.31*	---	---
7	4	1.26 ± 0.24*	0.33 ± 0.20*	2.00 ± 0.25*	1.59 ± 0.31	0.41 ± 0.40
10	8	0.37 ± 0.41	0.43 ± 0.26*	1.51 ± 0.47*	0.8 ± 0.48	0.71 ± 0.67
12	8	0.37 ± 0.42	---	1.34 ± 0.76*	---	---
Day	**T (°C)**	**Reduction of molds and yeasts (log CFU/g)**				
3	4	1.01 ± 0.37*	-	1.74 ± 0.41*	1.01 ± 0.37	0.73 ± 0.55
5	4	1.22 ± 0.79*	---	1.54 ± 0.60*	---	---
7	4	2.00 ± 0.12*	0.87 ± 0.38*	2.85 ± 0.16*	2.87 ± 0.40	-
10	8	0.06 ± 0.05	1.18 ± 0.18*	2.30 ± 0.43*	1.24 ± 0.19	1.06 ± 0.47
12	8	0.18 ± 0.03*	---	2.67 ± 0.42*	---	---
Day	**T (°C)**	**Reduction of psychrotrophic bacteria (log CFU/g)**				
3	4	1.10 ± 0.31*	0.31 ± 0.14	1.13 ± 0.40*	1.41 ± 0.34	-
5	4	1.98 ± 0.73*	---	1.66 ± 0.71*	---	---
7	4	1.72 ± 0.04*	1.17 ± 0.40*	2.60 ± 0.14*	2.89 ± 0.40	-
10	8	0.01 ± 0.09	1.25 ± 0.24*	2.24 ± 0.44*	1.26 ± 0.26	0.98 ± 0.51
12	8	0.16 ± 0.16	---	2.62 ± 0.56*	---	---

(-) No reduction. (---) Not tested. (*) Significant differences with respect their control packaging.

[1] Taken from Table 4.42
[2] Taken from Table 4.44
[3] Taken from Table 4.46

Table 4.49. Microbial reduction in peeled and cut orange (Navel cultivar) packed with active film, active tray and active packaging system during storage

		Active film[4]	Active tray[5]	Active system[6]	Σ individual reductions (film + tray)	Synergistic effect
Day	**T (°C)**	**Reduction of total aerobic numbers (log CFU/g)**				
3	4	0.37 ± 0.19*	0.01 ± 0.53	0.47 ± 0.23*	0.38 ± 0.56	0.09 ± 0.60
5	4	0.11 ± 0.14	---	1.03 ± 0.02*	---	---
7	4	0.37 ± 0.21*	0.18 ± 0.24	1.61 ± 0.37*	0.55 ± 0.32	1.06 ± 0.49
10	8	0.08 ± 0.32	-	0.60 ± 0.28*	0.08 ± 0.32	0.52 ± 0.42
12	8	0.22 ± 0.25	---	0.36 ± 0.18*	---	---
Day	**T (°C)**	**Reduction of molds and yeasts (log CFU/g)**				
3	4	0.61 ± 0.20*	-	0.89 ± 0.23*	0.61 ± 0.20	0.28 ± 0.31
5	4	0.20 ± 0.22	---	0.97 ± 0.33*	---	---
7	4	0.33 ± 0.23	-	1.49 ± 0.30*	0.33 ± 0.23	1.16 ± 0.38
10	8	0.12 ± 0.16	0.06 ± 0.12	0.63 ± 0.24*	0.18 ± 0.2	0.45 ± 0.31
12	8	0.27 ± 0.13*	---	0.47 ± 0.04*	---	---
Day	**T (°C)**	**Reduction of psychrotrophic bacteria (log CFU/g)**				
3	4	0.62 ± 0.17*	0.02 ± 0.28	1.19 ± 0.34*	0.64 ± 0.33	0.55 ± 0.47
5	4	0.11 ± 0.30	---	0.37 ± 0.49*	---	---
7	4	0.18 ± 0.18	-	1.30 ± 0.23*	0.18 ± 0.18	1.12 ± 0.29
10	8	0.11 ± 0.18	0.21 ± 0.19	0.84 ± 0.12*	0.32 ± 0.26	0.52 ± 0.29
12	8	0.25 ± 0.14*	---	0.48 ± 0.08*	---	---

(-) No reduction. (---) Not tested. (*) Significant differences with respect their control packaging.

As can be observed in both tables, the active packaging system exerted greater antimicrobial effects on pineapple and orange when compared with the individual film and tray, and the sum of their individual reductions. In this regard, the combination of the active components of the active packaging system increased the antimicrobial protection of the fruit causing a synergistic effect. For pineapple, as Table 4.48 shows, the maximum synergistic effect exerted by the active packaging system was observed at day 10 of storage (at abuse temperature) for total aerobic numbers, molds and yeasts and psychrotrophic bacteria with an extra reduction of 0.71, 1.06 and 0.98 log CFU/g compared with the sum of individual effects, respectively. A synergistic effect was also observed on total aerobic numbers during the storage test. For molds and yeasts, also a synergistic effect was reported at day 3 while

[4] Taken from Table 4.43
[5] Taken from Table 4.45
[6] Taken from Table 4.47

an additive effect was observed by day 7 of storage. For psychrotrophic bacteria, an additive effect was observed for the rest of the storage period.

In orange, a maximum synergistic effect was exerted by the active packaging system at day 7 of storage with 1.06, 1.16 and 1.12 log CFU/g reductions for total aerobic numbers, molds and yeasts and psychrotrophic bacteria, respectively compared with the sum of their individual effects. In this case, the active packaging system showed a synergistic effect against all the microorganisms tested during the storage period.

Other authors have also demonstrated the synergistic activity of essential oils with potassium sorbate or sodium benzoate. The synergistic effects of the combination of volatiles such as cinnamon essential oil at 0.1%, 0.2%, or 0.3% and sodium benzoate or potassium sorbate at 0.1% were demonstrated by Ceylan *et al.* (2004) in apple juice against *Escherichia coli* O157:H7 at 8 °C and 25 °C. Stanojević *et al.* (2010) also reported the antibacterial activity of *Salvia officinalis* L. aqueous extracts and its synergistic action with sodium nitrite, sodium benzoate and potassium sorbate against food spoilage bacteria such as *Agrobacterium tumefaciens*, *Bacillus subtilis* and *Proteus* sp. A synergistic effect of the combination of cinnamaldehyde and other solid preservative compounds such as natamycin was also observed against fungal growth in cheese by Balaguer *et al.* (2014).

In conclusion, higher microbial reductions were observed for pineapple than for orange with the active packaging system. As mentioned before in this chapter, the lower pH of pineapple could have contributed to this behavior as citral, hexanal, linalool, potassium sorbate and sodium benzoate could exert higher antimicrobial effect at lower pH.

4.5.3.2. Evolution of active agent concentrations during fruit storage tests

The release of the active agents, volatiles and non-volatiles, from the active packaging system during the storage of the fruits was evaluated. To this end, the content of active agents (citral, hexanal and linalool in the films and potassium sorbate and sodium benzoate in the trays) was determined over time following the methodology described in 3.2.8.3. Results were the average of three replicates ± standard deviation.

Table 4.50 and Table 4.51 show the amount of citral, hexanal, linalool and the total amount of volatile active agents released per active tray in pineapple and orange, respectively.

Table 4.50. Volatile active agents released from the active lid PP10/70a_8.5%CHL film during storage of pineapple (MD2 cultivar) packed in the active system stored at 4 °C for 7 days followed by 5 days at 8 °C

Day	Temperature (°C)	Citral (mg/tray)	Hexanal (mg/tray)	Linalool (mg/tray)	Total released amount (mg/tray)
3	4	9.41 ± 1.06	7.08 ± 0.34	6.86 ± 0.96	23.35 ± 1.47
7	4	12.27 ± 1.97	7.84 ± 0.45	8.44 ± 1.06	28.55 ± 2.28
10	8	12.24 ± 1.50	8.20 ± 0.18	7.67 ± 1.08	28.11 ± 1.86
12	8	11.39 ± 0.54	8.31 ± 0.27	7.17 ± 0.32	26.87 ± 0.68

Table 4.51. Volatile active agents released from the active lid PP10/70a_8.5%CHL film during storage of orange (Navel cultivar) packed in the active system stored at 4 °C for 7 days followed by 5 days at 8 °C

Day	Temperature (°C)	Citral (mg/tray)	Hexanal (mg/tray)	Linalool (mg/tray)	Total released amount (mg/tray)
3	4	2.23 ± 2.38	4.94 ± 0.55	0.85 ± 1.30	8.02 ± 2.77
7	4	4.76 ± 1.28	7.26 ± 0.48	2.40 ± 0.99	14.42 ± 1.68
10	8	5.19 ± 2.88	8.66 ± 0.18	2.47 ± 0.51	16.32 ± 2.93
12	8	6.54 ± 2.74	9.45 ± 0.97	3.17 ± 2.17	19.15 ± 3.62

The release of hexanal was quite similar for both pineapple and orange during storage of the fruit. Hexanal showed a quick release from the active lid from day 0 to day 3 in the case of pineapple. After day 3, the release of hexanal seemed to reach an equilibrium and it showed a lower release up to day 12. In the case of the orange, hexanal showed a more gradual release from the active film.

The release of citral and linalool was completely different for pineapple as for orange. In the case of the pineapple, both active agents showed a quick release from day 0 to day 3 and a clear slowdown from day 3 to day 12. In the case of the orange, the amount released of citral and linalool from the active film was substantially slower than for pineapple. These results suggested a direct relationship between the higher antimicrobial effect observed for the pineapple in comparison with the orange as a consequence of a larger release of active agents in the pineapple packages.

Contrary to the effect observed in the release tests at 4 °C, the antimicrobial activity of the active films in fruit at refrigeration temperature was originated by the release of the three active agents.

It is difficult to draw conclusions about the reasons behind the different release behavior of the active agents from the films in pineapple and orange. The packaging system was the same in both cases. However, this packaging system is not hermetic since two holes of 250 µm were made and part of the volatile active agents could have been lost through the micro holes. In addition, there may have been diffusion across the 10 µm thickness of the structural layer of the lid to the surrounding atmosphere. Since both fruits were kept at the same temperature, its influence on differences observed were probably minimal. A possible explanation could be related to a different absorption of the volatiles by the fruit due to the effect of the matrix or by differences in solubility in the fruit exudates.

The total concentration of volatile active agents remaining in the active lid after the storage of the fruit at day 12 represented 77% and 83% for pineapple and orange, respectively. In this regard, due to the large amount of volatiles retained in the films, a reduction of active agents concentration during manufacture of these materials would be possible, reducing both the cost of the film and the sensory impact resulting from the release of the volatiles into the fruit.

In the case of the active tray, Table 4.52 shows the amount of salts released into the fruit per tray. The quantification of the two salts in the package was carried out using a muffle furnace as described in section 3.2.5.2.2.

A gradual release of potassium sorbate and sodium benzoate mixture into the fruit was observed both for pineapple and orange during storage. However, the release of the salts was higher for pineapple. Specifically, after 12 days in contact with fruit, the 50.9% of the salts were released into the pineapple, whereas only the 32.2% was released into orange.

The higher release of the potassium sorbate and sodium benzoate mixture in pineapple could be explained due to differences in the amount of potassium present in raw pineapple and orange. In this case, according to the USDA database (USDA, 2015), raw Navel orange has 166 mg of potassium per 100 g of fruit whilst pineapple only has 109 mg. Then, the higher amount of potassium inherently present in orange raw material could have reduced the gradient of salts from the tray to the fruit.

On the other hand, the higher release observed in the case of pineapple was correlated with the higher antimicrobial effect observed in comparison with orange. Nevertheless, it has to borne in mind that the higher antimicrobial effect is not only dependent on the released amount, but also upon the pH of the fruit as mentioned previously. In this way, the lower pH

of pineapple could be supporting the antimicrobial activity of the potassium sorbate and sodium benzoate released from the active tray.

Table 4.52. Potassium sorbate and sodium benzoate mixture released from the active tray during storage of the fruit packed in the active system stored at 4 °C for 7 days followed by 5 days at 8 °C

		Pineapple	Orange
Day	Temperature (°C)	PS_SB released (mg/ tray)	PS_SB released (mg/ tray)
7	4	198.2 ± 83.6	122.7 ± 79.28
12	8	308.9 ± 81.3	195.7 ± 82.58

4.5.3.3. Evolution of the headspace gas composition

The gas composition of the headspace of the fruit packed with control and active packaging system was monitored during the storage period as described in 3.2.8.4. Results were the average of five replicates ± standard deviation.

Figure 4.58 and Figure 4.59 show changes in the gas atmosphere of pineapple and orange pieces, respectively, packed with control and active packaging systems during the storage period.

As observed in previous tests, Figure 4.58 shows that the gas composition of the headspace of pineapple packed with control materials that slightly changed from day 0 to day 7, reaching levels around 18.5% and 2.5% of O_2 and CO_2, respectively. With the increase of temperature, the O_2 decreased and the CO_2 increased, reaching concentrations of 16% and 7% at the end of the storage period (12 days), respectively. On the contrary, the headspace of pineapple packed in the active system remained stable during storage with levels of O_2 and CO_2 around 20% and 0.5%, respectively.

For orange packed in the control system, as Figure 4.59 shows, the headspace started changing at the beginning of the storage period, maybe due to a more advanced ripening stage of the fruit compared with other storage tests. Concentrations of O_2 and CO_2 at the end of storage were 14% and 11%, respectively. Some variation was observed for CO_2 numbers between replicates. As observed for pineapple, the headspace of orange packed in the active packaging system remained within the initial values of O_2 and CO_2 up to day 7. A

slight decrease in O_2 and an increase in CO_2 happened after the increase of temperature with 18.5% and 2% of O_2 and CO_2, respectively, at the end of storage.

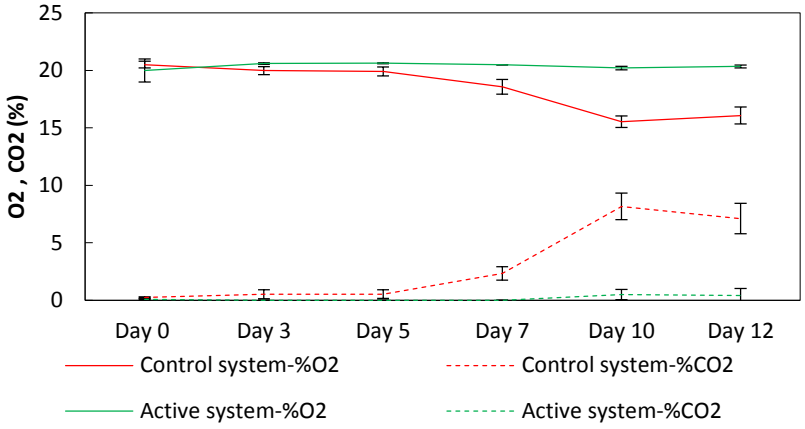


Figure 4.58. Evolution of O_2 and CO_2 concentration in the headspace of pineapple (MD2 cultivar) packed in control and active packaging systems stored at 4 °C for 7 days followed by 5 days at 8 °C

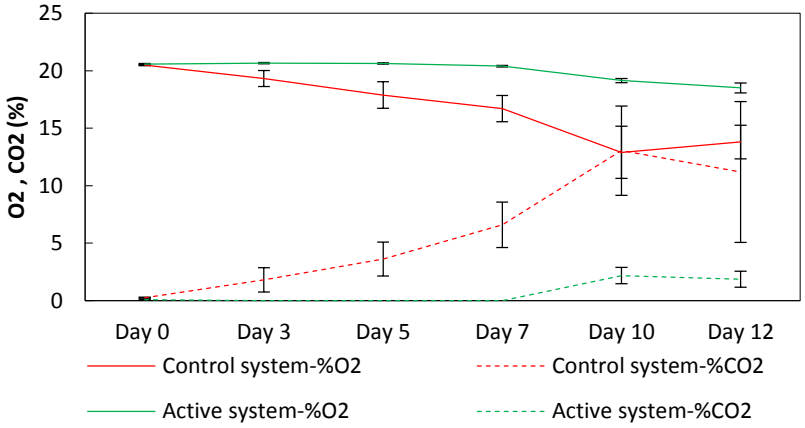


Figure 4.59. Evolution of O_2 and CO_2 concentration in the headspace of orange (Navel cultivar) packed in control and active packaging systems stored at 4 °C for 7 days followed by 5 days at 8 °C

The evolution of the headspace in fruit packed with control packaging materials was within normal levels of O_2 and CO_2 as previously discussed in 4.5.1.2. The effect of the active system on the decrease of the respiration in both fruits was attributable to the presence of

potassium sorbate and sodium benzoate released from the tray that counteracted the increased respiration produced by the volatile active agents. As discussed in section 4.5.2.2, the presence of organic salts could have inhibited enzymatic reactions in the vegetative fruit cell, causing a decrease in the respiration rate (Castelló *et al.*, 2006).

4.5.3.4. Juice leakage

The effect of the active packaging system based on the use of an active film containing volatile agents (citral, hexanal and linalool) and an active tray containing solid acidic salts (potassium sorbate and sodium benzoate) on juice leakage from pineapple and orange pieces was evaluated during storage. Results were the average of three replicates ± standard deviation. Statistical differences in juice leakage within materials were evaluated through ANOVA and Tukey analyses.

Figure 4.60 and Figure 4.61 show the accumulated mass of juice leakage from fresh cut pineapple and orange, respectively, packed with the control and active packaging system.

The amount of exudate released from pineapple packed with the control packaging system was quite constant during the 12 days of storage with an average of exudate below 5%. From day 7 of storage there was a significant increase ($p \leq 0.05$) in the exudate from pineapple packed with the active system compared to the control packaging. This effect might have been produced by the presence of potassium sorbate and sodium benzoate, which could have produced some dehydration of the fruit due to osmotic effects. This effect was corroborated by the gradual release of potassium sorbate and sodium benzoate mixture observed for pineapple during storage, reaching values of 308.9 mg of potassium sorbate and sodium benzoate/tray at day 12 of storage as reported in section 4.5.3.2.

In the case of orange, no significant differences ($p > 0.05$) in the amount of exudate was observed during storage or between control and active systems, except for samples packed in the control at day 12 of storage that showed an increase in juice leakage in both systems. In this case, the combination of organic salts and the volatiles kept exudate levels in orange under the levels generated by the control packaging system.

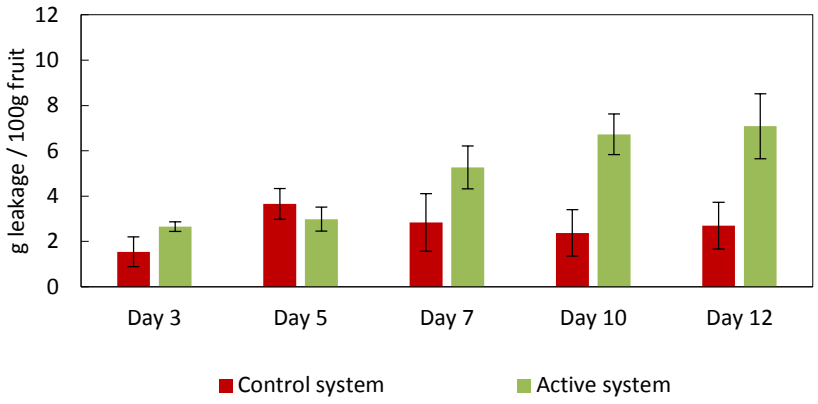


Figure 4.60. Evolution of juice leakage from pineapple (MD2 cultivar) packed in control and active packaging system films stored at 4 °C for 7 days followed by 5 days at 8 °C

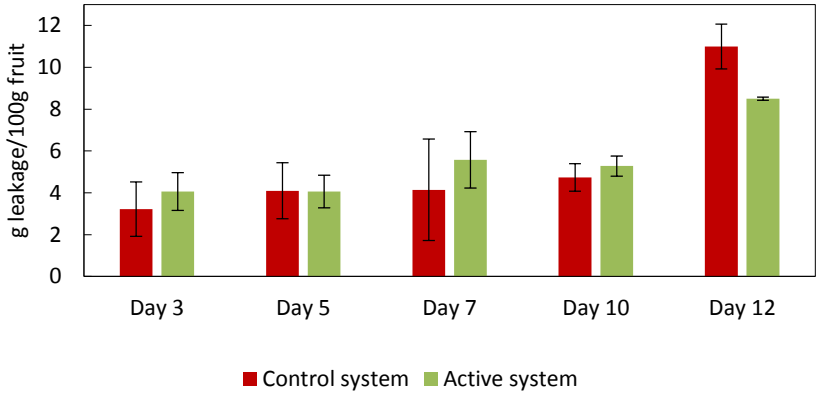


Figure 4.61. Evolution of juice leakage from orange (Navel cultivar) packed in control and active packaging system films stored at 4 °C for 7 days followed by 5 days at 8 °C

4.5.3.5. pH

The effect of the active packaging system on fresh cut pineapple and orange pH was evaluated as described in 3.2.8.6. Results were the average of three replicates ± standard deviation. Statistical differences in the pH of the fruit within materials were evaluated

through ANOVA and Tukey analyses. Figure 4.62 and Figure 4.63 show the pH of fresh cut pineapple and orange, respectively during 12 days of storage packed in control and active packaging systems.

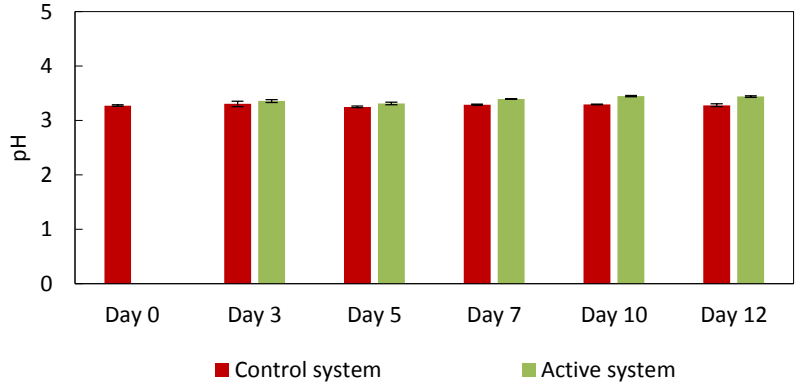


Figure 4.62. Evolution of pH in pineapple (MD2 cultivar) packed in control and active packaging systems stored at 4 °C for 7 days followed by 5 days at 8 °C

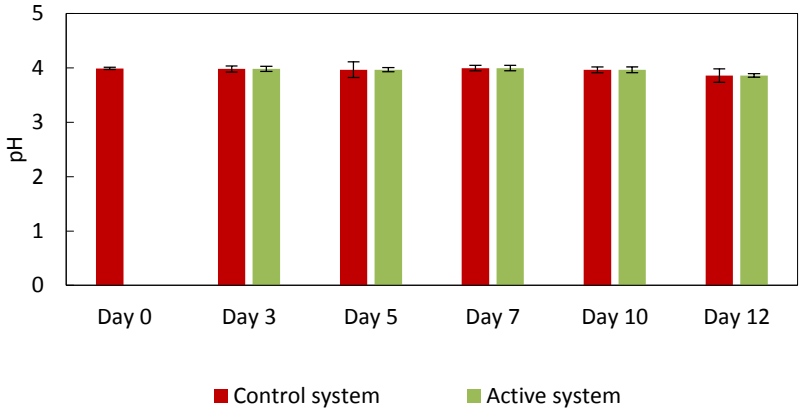


Figure 4.63. Evolution of pH in orange (Navel cultivar) packed in control and active packaging system stored at 4 °C for 7 days followed by 5 days at 8 °C

As can be observed in both figures, pH was quite stable during the whole storage period. No significant differences in pH ($p > 0.05$) were observed for both fruits packed with the control and active packaging system.

As previously observed in this research, the active films with volatile active agents did not affect the pH of the packed fruit. In addition, the release of potassium sorbate and sodium benzoate to the fruit in combination with the volatile active agents did not affect the pH of the fruit. These results are in agreement with those of Garcia *et al.* (2012) who showed how the addition of potassium sorbate to a starch edible coating on strawberries had no significant effect on fruit pH.

4.5.3.6. Total soluble solids (TSS)

The evolution of TSS during storage and the effect of the active packaging system on the peeled and cut pineapple and orange were evaluated as described 3.2.8.7. Results were the average of three replicates ± standard deviation. Statistical differences in the TSS of the fruit within materials were evaluated through ANOVA and Tukey analyses.

Figure 4.64 and Figure 4.65 show the weight of sugar per 100 g of product, expressed as TSS, for pineapple and orange when packed with the control and active packaging systems during 12 days of storage. As can be observed in both figures, TSS decreased significantly during storage of the fruit packed in the control packages from 14.7 to 11.7% for pineapple and from 11.4 to 8.7% for orange samples. On the contrary, the active packaging system maintained the levels of TSS almost stable during storage of both pineapple and orange at values around 14% and 10.3%, respectively by day 12. This effect was slightly different from the effect observed with the fruit packed in the antimicrobial film alone (with no active tray), where TSS gradually decreased during storage from 14.6% to 12.8% and from 11.4% to 9.5% for pineapple and orange, respectively. This effect could have been a consequence of the released potassium sorbate and sodium benzoate that contributed to the final concentration of TSS.

Differences in TSS observed between pineapple and orange packed with the active system regarding their controls at the end of storage period were in agreement with the amount of potassium sorbate and sodium benzoate released in each case. In this perspective, bigger differences in TSS were reported at day 12 for pineapple packed with the active system in comparison with its control.

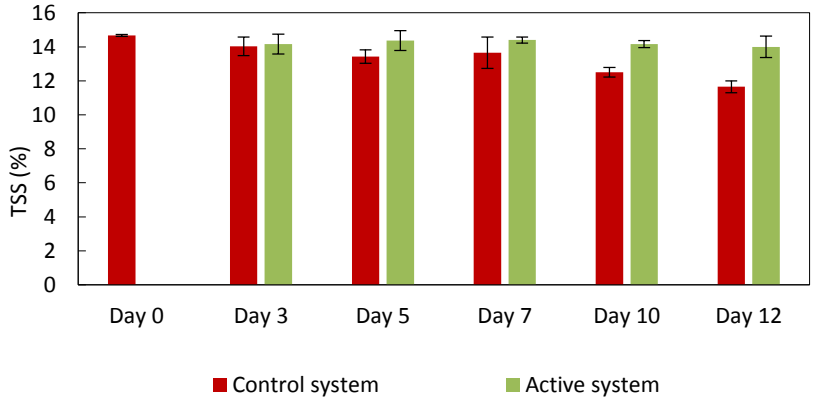


Figure 4.64. Evolution of TSS (%) in pineapple (MD2 cultivar) packed in control and active packaging systems stored at 4 °C for 7 days followed by 5 days at 8 °C

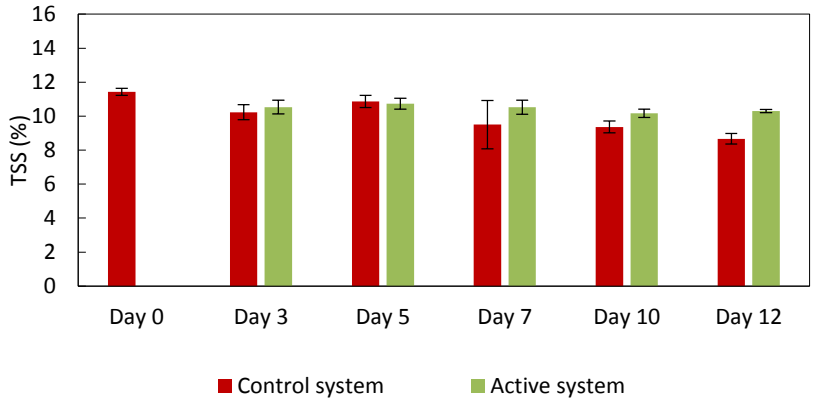


Figure 4.65. Evolution of TSS (%) in orange (Navel cultivar) packed in control and active packaging systems stored at 4 °C for 7 days followed by 5 days at 8 °C

The constant values of TSS for pineapple and orange packed with active packaging systems could be also related to the conclusions drawn by Castelló *et al.* (2010) regarding the reduction in the respiration rate of the fruit caused by the presence of potassium sorbate and sodium benzoate as corroborated in section 4.5.3.3. Therefore, the combination of volatile active agents with the salts was demonstrated to maintain the TSS quality parameter

(related to postharvest ripening of the fruit) at the same levels as the beginning of the storage.

4.5.3.7. Sensory evaluation

A sensory evaluation of peeled and cut pineapple and orange packed in a control (PP80 film sealed with PP/PP:10%EVA tray) and active packaging system (PP10/70a_8.5%CHL film sealed with PP/PP:10%EVA:20%PS_SB tray), stored at 4 °C for 7 days followed by 5 days at 8 °C was carried out. Ratings were based on a 5-point hedonic. Figure 4.66 and Figure 4.67 show the sensory parameters color, odor and general appearance of pineapple and orange, respectively evaluated during storage.

As reported in section 4.5.1.6 and 4.5.2.3, the sensory attributes of fruit gradually decreased during storage for fruit packed with both control and active packaging systems. The pineapple packed with the control packaging system, by day 5 of storage at 4 °C, was judged to be at the limit of marketability in terms of general appearance, color and odor. By day 7, microbial growth was already visually observed and a strong ripe fruit odor was perceived when packaging was opened. In this case, by day 5 the quality limit levels for molds and yeast and psychrotrophic bacteria was already exceeded (Table 4.46).

In contrast, for pineapple packed with the active system, the general appearance and color remained within the limits of marketability during the entire test with no microbial growth visually observed. In addition, total aerobic numbers, molds and yeast and psychrotrophic bacteria did not exceed the quality limit of 6 log CFU/g during the storage period (Table 4.46). The increase of exudate in pineapple during storage as reported in section 4.5.3.4 did not negatively influence its general appearance. Nevertheless, after the shift of temperature, a ripe fruit odor mixed with the active agent aromas was reported by day 10 and enhanced at the end of storage, decreasing the score attributable to odor.

Orange packed with the control system was scored within the marketability limits in terms of general appearance and color up to day 5 of storage. However, a ripe/musty fruit odor was perceived once the packaging was opened at day 5, placing orange under the marketability threshold. Although no visual growth was observed until day 7, the microbial analysis showed that molds and yeast and psychrotrophic bacteria already exceed the quality limit at day 5 (Table 4.47). In addition, surface desiccation of the fruit was observed at day 10 of storage.

Orange packed with active system was within the marketability limits of the sensory parameters studied (general appearance, color and odor) until day 7. Nevertheless, from day 7, when the temperature changed from 4 to 8 °C, the ripe or musty odor of the fruit mixed with the volatile active agents placed orange under the quality threshold of acceptance. Visual growth was present in orange at day 10 of storage, exceeding the microbiological quality limits at this stage of storage.

In general, the sensory attributes evaluated were scored higher for the fruit packed in the active packaging system, where color and general appearance were the attributes scored better. Although the odor attribute usually remained higher for active than for control packaging, the odor generated by the release of volatile active agents mixed with the ripe scent of the fruit generated over time was the most limiting factor for the acceptance of the fruit packed with active packaging. The odor attribute was even more limiting than microbial growth, both for control and active packaging, scoring the fruit below the marketability limits before microbial spoilage occurred.

Evidence for the positive effects of the active packaging system on visual appearance of pineapple and orange pieces during storage are shown in Figure 4.68 and Figure 4.69, respectively. From these analyses, it could be concluded that the active packaging system significantly improved the visual quality of the fruit compared to the fruit packed in the control packaging. Active packaging also improved sensory parameters such as odor, although at some point of the storage test the odor generated by the volatile active agents could lead to rejection by consumers. To mitigate this effect and to improve the acceptance of the fruit packed with active packaging materials, a reduction in the active agent concentrations should be undertaken as the remaining concentration at the end of storage test was still high as described in 4.5.3.2.

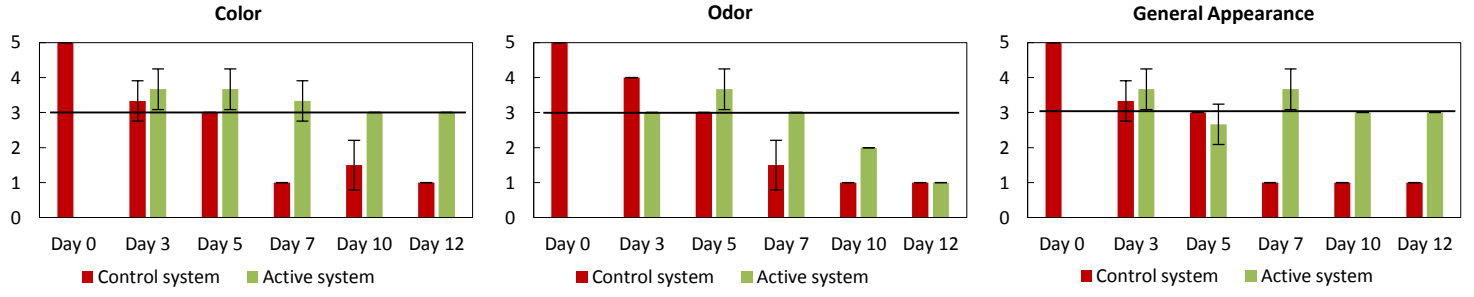


Figure 4.66. Sensory evaluation of pineapple (MD2 cultivar) packed in the control and active system stored at 4 °C for 7 days followed by 5 days at 8 °C
5 = very good; 4 = good; 3= fair (limit of marketability); 2= poor; 1= bad (unusable)

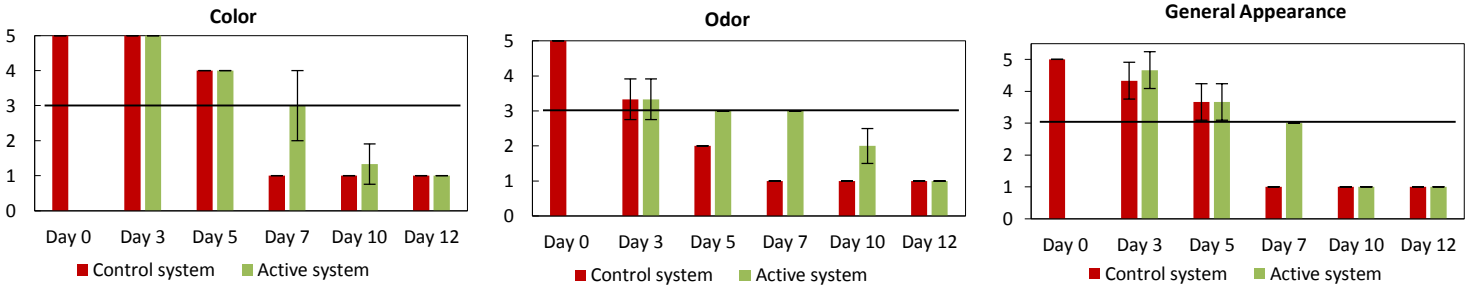


Figure 4.67. Sensory evaluation of orange (Navel cultivar) packed in the control and active system stored at 4 °C for 7 days followed by 5 days at 8 °C

5 = very good; 4 = good; 3= fair (limit of marketability); 2= poor; 1= bad (unusable)

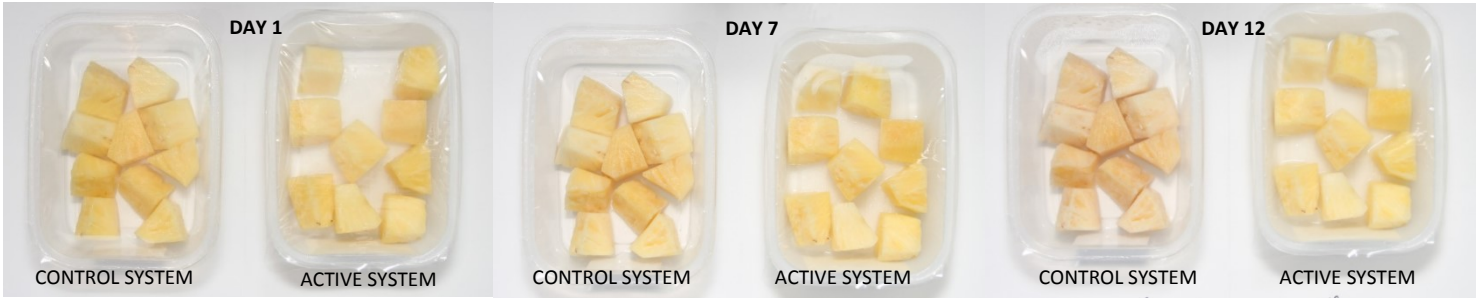


Figure 4.68. Visual aspect of pineapple pieces (MD2 cultivar) packed in the control and active packaging systems and stored at 4 °C for 7 days followed by 5 days at 8 °C

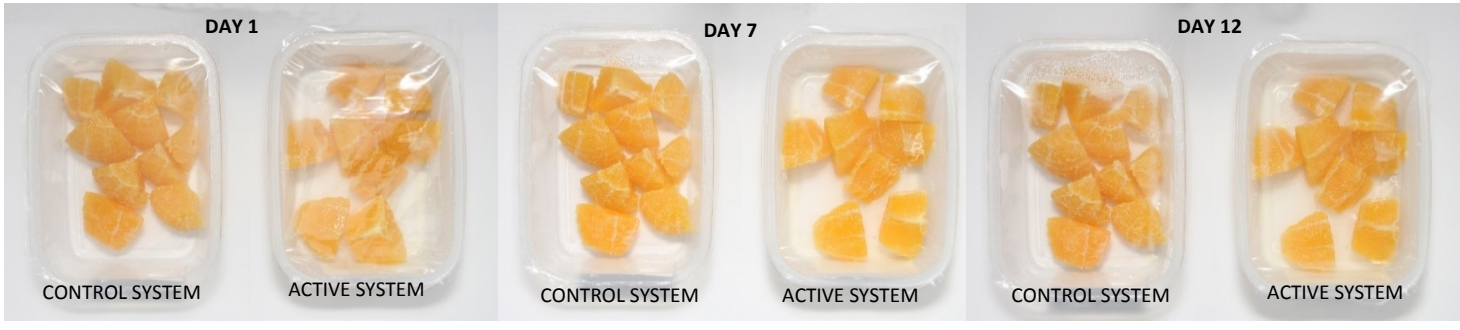


Figure 4.69. Visual aspect of orange pieces (Navel cultivar) packed in the control and active packaging systems, stored at 4 °C for 7 days followed by 5 days at 8 °C

4.5.3.8. Evaluation of the active packaging materials along the storage period of the fruit

4.5.3.8.1. Visual appearance of the film

The effect of the storage time and temperature on active films used to pack pineapple and orange was evaluated. To that end, a visual analysis of the films was conducted during 12 days of storage as shown in Figure 4.68 and Figure 4.69. No differences were observed in the film material during the storage test, either for pineapple or for orange althought condensation of water vapor in the inner face of the film was sometimes observed both for control and active films. Therefore, from this visual inspection it can be concluded the need to add an antifog additive to the inner layer of the film to avoid water condensation.

4.5.3.8.2. Visual appearance of the tray

The effect of the storage time and temperature on active trays used to pack peeled and cut pineapple and orange was also evaluated. A visual analysis of the trays was conducted during 12 days of storage. Figure 4.70 and Figure 4.71 show the visual changes that occurred in the active trays exposed to the pineapple and orange, respectively stored at 4 °C for 7 days followed by 5 days at 8 °C.

From visual monitoring, it was observed that from the beginning of storage of the fruit, the potassium sorbate and sodium benzoate incorporated in the active tray were clearly released into the fruit, altering in some way the appearance of the tray. Due to the visual change of the active trays, the addition of a white food contact colorant (titanium dioxide) could be considered to avoid changes in the visual aspect of the active trays.

4.5.3.9. Conclusions on active packaging system

The combination of an active packaging system consisting of an active film containing citral, hexanal and linalool and an active tray with potassium sorbate and sodium benzoate significantly reduced the growth of spoilage microorganisms of peeled and cut pineapple and orange. In particular, for the MD2 pineapple cultivar, the active materials enhanced the microbiological quality from day 3 to day 12. For Lane-Late orange cultivar, the active materials enhanced the microbiological quality from day 3 to day 7.

The active packaging system also influenced other quality parameters of the fruits. Some positive effects were observed. For instance, respiration was reduced in comparison with control packages. This effect was attributable to the potassium sorbate and sodium benzoate released from the active tray. TSS was also maintained quite stable during storage of pineapple and orange packed with the active packaging system. On the contrary, the active packaging system caused an increase of juice leakage in pineapple probably due to the amount of potassium sorbate and sodium benzoate released. This effect was not observed for orange samples.

In terms of sensory properties, higher sensory scores were observed for the active system. However, a reduction of the content of active volatile agents appears to be needed due to the odor given to the fruit, which was a limiting factor for the consumer acceptability.

Based on the different antimicrobial effectiveness studies carried out during this research, there is some margin to reduce the concentration of the active agents, even more after observing a synergistic effect when the active film is combined with the active tray. A reformulation of both active film and active tray should be performed to balance antimicrobial effectiveness and sensory attributes.

Figure 4.70. Visual aspect of the active trays previously packed with pineapple at 4 °C for 7 days followed by 5 days at 8 °C

Figure 4.71. Visual aspect of the active trays previously packed with orange at 4 °C for 7 days followed by 5 days at 8 °C

4.6. FOOD CONTACT MATERIALS COMPLIANCE

4.6.1. Migration tests of active films

4.6.1.1. Overall migration

As stated by the Regulation (EU) No. 10/2011, the overall migration limit (OML) is the maximum permitted amount of non-volatile released substances from a material or article to a food simulant. In this regard, plastic materials should not transfer their constituents to food simulants in quantities exceeding 10 mg of total constituents released per dm^2 of food contact surface (OML ≤ 10 mg/dm^2).

Food simulant B (3% (w/v) acetic acid) and food simulant C (20% (v/v) ethanol) set for fruit and vegetable juices were selected to verify the compliance of control and active films for fresh cut fruits. Overall migration tests were performed with the selected simulants at 20 °C for 10 days according to Regulation (EU) No. 10/2011. These standardized time and temperature testing conditions represent any food contact under frozen or refrigerated conditions.

The tests were performed in a cell under the European Standard EN 1186-5:2002 where only the active layer was in contact with the food simulant as described in section 3.2.9.1. Table 4.53 shows the results of the overall migration obtained for control and active films. Reported values were the mean of three replicates ± uncertainty. Overall migration was calculated according to Equation 3.12.

Although the overall migration of the active films was higher than overall migration of control film in 3% acetic acid and 20% ethanol, both materials fulfil the OML of 10 mg/dm^2 when they are intended to be in contact with peeled and cut fruit under frozen or refrigerated conditions.

According to the Regulation (EC) No. 450/2009 on active and intelligent materials and articles, there may be a risk of exceeding the OML due to the release of active substances into the food. Then, as the active function is not an inherent feature of the passive material, the amount of released active substances should not be considered in the overall migration value. To this end, Regulation (EC) No. 450/2009 sets that the amount of active agent released could be subtracted from the overall migration obtained. However, in this specific

case, the overall migration results of the active films fulfil the OML limit without subtracting the residue corresponding to the active agents citral, hexanal and linalool.

Table 4.53. Overall migration of control and active films according to the European Standard EN 1186-5:2002 and Regulation (EU) No. 10/2011

Sample	Simulant	Test conditions	Overall migration (mg/dm²) ± uncertainty
PP80	3% (w/v) acetic acid	10 days/20 °C	0.2 ± 0.3
PP10/70a_8.5%CHL		Cell	1.1 ± 0.8
PP80	20% (v/v) ethanol	10 days/20 °C	0.1 ± 0.2
PP10/70a_8.5%CHL		Cell	1.4 ± 0.8

4.6.1.2. Specific migration

The specific migration tests of citral, hexanal and linalool from the active film PP10/70a_8.5%CHL were performed in food simulant C (20% (v/v) ethanol) for 10 d at 20 °C, according to Regulation (EU) No. 10/2011. These standardized test conditions for specific migration represent the usual conditions of contact for refrigerated food between 3 and 30 days and between 5 and 20 °C.

The tests were performed in a cell under the European Standard EN 13130-1:2005 where only the active layer was in contact with the food simulant as described in 3.2.9.2. Simulant C (20% (v/v) ethanol) was considered the worst case simulant in terms of solubility, since these compounds are poorly soluble in water and highly soluble in ethanol and other organic solvents (Pubchem, 2015).

After the migration test, the analysis of citral, hexanal and linalool in the food simulant was carried out by GC-MS as described in section 3.2.5.2.1. Data on the validation of the analytical method was reported in section 4.2.1.1.

Table 4.54 shows the specific migration of citral, hexanal and linalool from the active film. Reported values were the mean of three replicates ± standard deviation. As can be observed in the table, the specific migration of citral, hexanal and linalool were between approximately 20-40 mg/kg of simulant.

Table 4.54. Specific migration of citral, hexanal and linalool according to the European Standard EN 13130-1:2005 and Regulation (EU) No. 10/2011

Sample	Simulant	Test conditions	Antimicrobial agent	Specific migration ± SD (mg/dm²)	Specific migration* ± SD (mg/kg)
PP10/70a_8.5%CHL	Ethanol 20% (v/v)	10 days/ 20°C Cell	Citral	6.29 ± 0.77	37.76 ± 4.62
			Hexanal	6.36 ± 0.23	38.00 ± 1.40
			Linalool	4.05 ± 0.37	24.30 ± 2.20

*6 dm^2 in contact with 1 kg of food.

As no specific restriction has been set up for these substances authorized under Regulation (EC) No. 1334/2008 on flavourings (FL 05.020 citral; FL 05.008 hexanal; FL 02.013 linalool), there is no legal basis for comparing the migration results obtained with the legal limits permitted in food. In fact, according to Regulation (EC) No. 1334/2008, the minimum amount needed to reach the desired technological effect in the food should be added.

In order to adequately consider the overall safety of the active material, the acceptable daily intake (ADI) established for each compound was used as the reference safety limit. In this way, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established in 1979 an ADI of 0.5 mg/kg body weight/day for citral and linalool (Inchem, 2004, 2005a). In addition, an estimated daily intake (EDI) per capita in Europe for citral and linalool was set in 6849 and 4300 µg/person/day, respectively, which corresponds to 0.11 and 0.07 mg/kg bw/day, respectively (considering that 1 kg of food is consumed daily by a person of 60 kg bodyweight). For hexanal, it was concluded in its last evaluation in 1997 that there was no safety concern at current levels of intake when used as a flavouring agent. In this regard, the EDI per capita of hexanal in Europe was estimated in 780 µg/person/day (0.01 mg/kg bw/day) (Inchem, 2001).

According to the EFSA dietary survey data performed to assess food intake in Europe, the average fruit intake is 0.166 kg per day, implying that the average consumption of fruit and vegetables is 0.386 kg per day (EFSA, 2008).

Considering all these data and the specific migration results of citral, hexanal and linalool obtained, exposure through ingestion was calculated according to Equation 3.13, considering that all the fruit is packed with the active film. Table 4.55 summarizes the exposure data of citral, hexanal and linalool from the migration data and their compliance with the set ADI. The levels of exposure in relation with EDI values were also indicated.

Table 4.55. Citral, hexanal and linalool exposure calculated from specific migration test of PP10/70a_8.5%CHL film for 10 days at 20 °C and compliance with ADI and EDI values

Active agent	Exposure specific migration test (mg/kg bw/day) ± SD	Compliance
Citral	0.10 ± 0.01	Compliant with ADI; Below EDI
Hexanal	0.11 ± 0.00	Exceeding EDI
Linalool	0.07 ± 0.01	Compliant with ADI; equal to EDI

Citral: ADI 0.50 mg/kg bw/day; EDI 0.11 mg/kg bw/day
Hexanal: EDI 0.01 mg/kg bw/day
Linalool: ADI 0.50 mg/kg bw/day; EDI 0.07 mg/kg bw/day

The exposure data calculated from the migration test both for citral and linalool were below the ADI and EDI values set for these compounds. However, the exposure of hexanal exceeded the current levels of intake (EDI) (as food flavourings) for which there was no safety concern established.

According to Article 18th of plastic Regulation (EU) No. 10/2011, the results of specific migration testing obtained in foods shall prevail over the results obtained in food simulants. Therefore, the released substances from the active packaging system (active film) into peeled and cut orange and pineapple evaluated in section 4.5.3.2 were also considered for the evaluation of compliance.

Table 4.56 shows the exposure data calculated according to Equation 3.13 considering the amount of citral, hexanal and linalool released from the active film PP10/70a_8.5%CHL during storage (11.39, 8.31 and 7.17 mg/package of citral, hexanal and linalool for pineapple, respectively and 6.54, 9.45 and 3.17 mg/package of citral, hexanal and linalool for orange, respectively). For the calculation, it was considered 0.150 kg of fruit per package and 0.166 kg of fruit as daily intake. Compliance with ADI and EDI values are also indicated in the table. Standard deviation were considered for the compliance evaluation.

As can be observed in Table 4.56, the exposure data calculated in the fruit was generally higher than the exposure calculated from migration tests for the three compounds. The exposure calculation from pineapple and orange storage tests showed that neither citral nor linalool exceeded the set ADI during the study. For hexanal, the EDI value set as reference (0.01 mg/kg bw/day) was exceeded from the beginning of storage for both orange and pineapple.

Table 4.56. Citral, hexanal and linalool exposure calculated from pineapple (MD2 cultivar) and orange (Navel cultivar) storage test at 4 °C for 7 days followed by 5 days at 8 °C

	Pineapple (MD2)		Orange (Navel)	
	Exposure to citral		Exposure to citral	
Day	± SD (mg/kg bw/day)	Compliance	± SD (mg/kg bw/day)	Compliance
3	0.17 ± 0.02	Compliant with ADI Exceeded EDI	0.04 ± 0.04	Compliant with ADI Below EDI
7	0.23 ± 0.04	Compliant with ADI Exceeded EDI	0.09 ± 0.02	Compliant with ADI Below EDI
10	0.23 ± 0.03	Compliant with ADI Exceeded EDI	0.10 ± 0.05	Compliant with ADI Exceeded EDI
12	0.21 ± 0.01	Compliant with ADI Exceeded EDI	0.12 ± 0.05	Compliant with ADI Exceeded EDI
	Exposure to hexanal		Exposure to hexanal	
Day	± SD (mg/kg bw/day)	Compliance	± SD (mg/kg bw/day)	Compliance
3	0.13 ± 0.01	Exceeded EDI	0.09 ± 0.01	Exceeded EDI
7	0.14 ± 0.01	Exceeded EDI	0.13 ± 0.01	Exceeded EDI
10	0.15 ± 0.00	Exceeded EDI	0.16 ± 0.00	Exceeded EDI
12	0.15 ± 0.01	Exceeded EDI	0.17 ± 0.02	Exceeded EDI
	Exposure to linalool		Exposure to linalool	
Day	± SD (mg/kg bw/day)	Compliance	± SD (mg/kg bw/day)	Compliance
3	0.13 ± 0.02	Compliant with ADI Exceeded EDI	0.02 ± 0.02	Compliant with ADI Below EDI
7	0.16 ± 0.02	Compliant with ADI Exceeded EDI	0.04 ± 0.02	Compliant with ADI Below EDI
10	0.14 ± 0.02	Compliant with ADI Exceeded EDI	0.05 ± 0.01	Compliant with ADI Below EDI
12	0.13 ± 0.01	Compliant with ADI Exceeded EDI	0.06 ± 0.04	Compliant with ADI Exceeded EDI

Citral: ADI 0.50 mg/kg bw/day; EDI 0.11 mg/kg bw/day
Hexanal: EDI 0.01 mg/kg bw/day
Linalool: ADI 0.50 mg/kg bw/day; EDI 0.07 mg/kg bw/day

However, this exposure could have been overestimated since it was based on the total amount of active agents released from the active film, which might not represent the actual amount of citral, hexanal and linalool absorbed by the fruit. It should be considered that some losses of volatiles to the atmosphere could take place through both the structural layer and the two micro holes present in the film.

Moreover, the exposure was calculated based on a worst-case scenario. It was assumed that all the fruit that is consumed (0.166 kg per day and person) is commercially packed with the new active packaging system. However, this is an overestimated scenario, since fruit is largely consumed in whole units, and not all the minimally processed fruit would be packed with the active system. Therefore, the actual average exposure coming from the active packaging system would represent values around 10% of the worst-case scenario considering that 25% of fruit intake would be minimally processed and 40% would be packed with an active packaging system. That is 0.013 to 0.015 mg hexanal/kg bw/day for pineapple and 0.009 to 0.017 mg hexanal/kg bw/day for orange.

In conclusion, the approach followed to evaluate the safety of the active material demonstrated that the exposure of hexanal exceeded the EDI values set by JECFA as a flavoring compound both for the migration test with simulants and for the storage test with fruit, considering a worst-case scenario. However, actual exposure would be substantially lower. In this regard, as concluded in section 4.5, a reduction in the concentration of the volatile active agents in the film should be carried out in order to optimize the antimicrobial effectiveness versus the sensory impact. This will further reduce the actual exposure to citral, hexanal and linalool.

In any case, adopting the worst-case situation, where the total amount released from the active film would be absorbed by the fruit, and taking into consideration that each tray contained 150-160 g of fruit, the maximum concentration that will be released into the fruit after 12 days of storage would range between 168-179 mg/kg for pineapple and 120-128 mg/kg for orange.

However, in order to commercialize these active films with peeled and cut fruit, citral, hexanal and linalool would need to be authorized under Regulation (EC) No. 1333/2008 on food additives for their intended use.

4.6.2. Migration tests of the active tray

4.6.2.1. Specific migration

The specific migration of potassium sorbate and sodium benzoate from the active tray was evaluated using food simulant B and food simulant C at 20 °C for 10 days according to European Regulation (EU) No. 10/2011. The tests were performed by total immersion under

European Standard EN 13130-1:2002 where both sides of the tray were in contact with the food simulant as described in 3.2.9.2. According to the standard, if the test is performed by total immersion and the thickness of the material is greater than 0.5 mm, the OM results should be corrected considering both sides of exposure (double area; 2 dm^2). In this case, although the thickness of the tray was greater than 0.5 mm, only one side was considered since the potential source of release was only from the active layer.

After carrying out the migration test, the analysis of the potassium sorbate and sodium benzoate was carried out by HPLC-UV as described in section 3.2.6.2. Table 4.57 shows the results of specific migration of potassium sorbate and sodium benzoate as the mean of three replicates ± standard deviation.

The total amount of potassium sorbate and sodium benzoate mixture migrated into 3% (w/v) acetic acid was 202.98 mg/dm^2 (or 1217.76 expressed in mg/kg of food) and 171.7 mg/dm^2 (or 1030.13 mg/kg) into 20% (v/v) ethanol.

Table 4.57. Specific migration of potassium sorbate and sodium benzoate according to the European Standard EN 13130-1:2005 and Regulation (EU) No. 10/2011

Sample	Simulant	Test conditions	Active agent	Specific migration ± SD (mg/dm^2)	Specific migration* ± SD (mg/kg)
PP/PP:10%EVA :20%PS_SB	3% (w/v) acetic acid	10 days/20 °C Total immersion	Potassium sorbate	91.88 ± 9.76	551.28 ± 58.54
			Sodium benzoate	111.1 ± 34.60	666.48 ± 207.57
	20% (v/v) ethanol	10 days/20 °C Total immersion	Potassium sorbate	86.94 ± 16.15	521.65 ± 96.93
			Sodium benzoate	84.80 ± 17.94	508.48 ± 107.66

*6 dm^2 in contact with 1 kg of food

Sorbic acid (E-200) and its salts such as potassium sorbate (E-202) are food additives authorised under Regulation (EC) No. 1333/2008 on food additives with maximum permitted levels of use ranging from 20 to 6000 mg/kg, according to the type of food. Sodium benzoate (E-211) is also authorised as a food additive under Regulation (EC) No. 1333/2008 with maximum permitted levels ranging from 300 to 6000 mg/kg, also depending on the food. These agents may be added individually or in combination. Potassium sorbate and sodium

benzoate are not approved to be used for minimally processed fruit. Thus, there is no legal basis for the comparison of the specific migration results with legal limits.

Therefore, as carried out for the volatile antimicrobials, the ADI values of potassium sorbate (3 mg/kg bw/day) (EFSA, 2015) and sodium benzoate (5 mg/kg bw/day) (Inchem, 2005b) were taken into consideration for the safety evaluation of the active tray.

In this way, taking into account that 0.166 kg of fruit is consumed daily by a person of 60 kg bodyweight (EFSA, 2008), the exposure of potassium sorbate and sodium benzoate were calculated from the worst-case migration in 3% (w/v) acetic acid simulant (highest migration). To this end, the amount of fruit contained per package (0.150 kg/package) was also considered.

Table 4.58 shows the exposure data of potassium sorbate and sodium benzoate from the migration data and their compliance with established ADI values. Considering these results, it was concluded that the exposure data of both active agents calculated from food simulants comply with the ADI values for potassium sorbate and sodium benzoate.

Table 4.58. Potassium sorbate and sodium benzoate exposure calculated from specific migration tests of the active tray in 3% (w/v) acetic acid for 10 days at 20 °C and compliance with ADI values

Active agent	Exposure ± SD (mg/kg bw/day)	Compliance
Potassium sorbate	1.53 ± 0.16	Compliant with ADI
Sodium benzoate	1.84 ± 0.57	Compliant with ADI

Potassium sorbate: ADI 3 mg/kg bw/day
Sodium benzoate: ADI 5 mg/kg bw/day

On the other hand, the exposure associated with the potassium sorbate and sodium benzoate released from the active trays into peeled and cut orange and pineapple were considered for the evaluation of compliance. Values obtained in section 4.5.3.2 showed maximum levels of 308.9 mg/package of both salts for pineapple and 195.7 mg/package for orange.

Table 4.59 reports the exposure data derived from the amount of potassium sorbate and sodium benzoate mixture released from the active tray to the fruit during storage. Exposure was calculated according to Equation 3.13, considering that a person of 60 kg bodyweight daily consumes 0.166 kg of fruit, and, that 0.150 kg of fruit was present in each pack.

Table 4.59. Potassium sorbate and sodium benzoate exposure calculated from pineapple (MD2 cultivar) and orange (Navel cultivar) storage tests at 4 °C for 7 days followed by 5 days at 8 °C

Pineapple (MD2)				
	Exposure ± SD (mg/kg bw/day)			
Day	PS + SB	PS	SB	Compliance
7	3.66 ± 1.54	1.83 ± 0.77	1.83 ± 0.77	PS and SB compliant with ADI
12	5.70 ± 1.51	2.56 ± 0.68	3.13 ± 0.83	PS not compliant with ADI; SB compliant with ADI
Orange (Navel)				
	Exposure ± SD (mg/kg bw/day)			
Day	PS + SB	PS	SB	Compliance
7	2.26 ± 1.46	1.36 ± 0.73	0.91 ± 0.73	PS and SB compliant with ADI
12	3.61 ± 1.52	2.17 ± 0.68	1.44 ± 0.84	PS and SB compliant with ADI

Potassium sorbate: ADI 3 mg/kg bw/day; Sodium benzoate: ADI 5 mg/kg bw/day
Day 7 PS:SB ratio 50:50
Day 12 PS:SB ratio 45:55

As the release was measured quantifying both salts as a mixture, it was assumed that the percentage of each active salt in the mixture corresponded to the values observed in the release tests carried out in section 4.3.3 (PS:SB ratio of 50:50 at day 7 and 45:55 at day 12). Standard deviation was considered for the compliance evaluation.

As Table 4.59 shows, the exposure calculation from pineapple and orange storage tests showed that potassium sorbate and sodium benzoate complied with the ADI limit during the 12 days of anticipated shelf life of the fruit, except for potassium sorbate at day 12 with pineapple.

As reported for the active films, exposure from the fruit was calculated based on a worst-case scenario assuming that all fruit consumed was packed with the new active packaging system. Therefore, the actual average exposure coming from the active packaging system would represent values around 10% of the worst-case level with exposure values far from the established ADI.

In any case, taking into consideration that each tray contained 150-160 g of fruit, the maximum concentration that would be released into the fruit after 12 days of storage, would range between 1930-2060 mg/kg for pineapple and 1223-1325 mg/kg for orange.

Finally, from a regulatory point of view, these additives are not approved to be used for minimally processed fruit. In this regard, although it has been demonstrated that ADI values were not exceeded, for the commercialized use of the active tray for peeled and cut fruit, potassium sorbate and sodium benzoate will need to be authorized under Regulation (EC) No. 1333/2008 on food additives for their intended used (minimally processed fruit) with a specific restriction on use.

4.6.2.2. Overall migration

The overall migration from the control and active tray was evaluated into food simulant B and food simulant C at 20°C for 10 days according to the European Regulation (EU) No. 10/2011. As reported for the film migration test, the testing conditions, time and temperature, represent any food contact under frozen and refrigerated conditions. The tests were performed by total immersion under the European Standard EN 1186-3:2002 where both sides of the tray were in contact with the food simulant as described in 3.2.9.1.

Table 4.60 shows the overall migration results from control and active trays. Although the thickness of the tray was greater than 0.5 mm, only one side was considered for the calculation as already described in section 4.6.2.1. Overall migration was calculated according to Equation 3.12. Reported values were the mean of three replicates ± uncertainty.

As can be observed in the table, the migration results for the control tray were negligible in both simulants. In contrast, the overall migration for the active tray far exceed the 10 mg/dm^2 set by the legislation.

Table 4.60. Overall migration of control and active trays according to the European Standard EN 1186-3:2002 and Regulation (EU) No. 10/2011

Sample	Simulant	Test conditions	Overall migration (mg/dm²) ± uncertainty
PP/PP:10%EVA	3% (w/v) acetic acid	10 days/20 °C Total immersion	0.0 ± 3.5
PP/PP:10%EVA:20%PS_SB			131.7 ± 39.6
PP/PP:10%EVA	20% (v/v) ethanol	10 days/20 °C Total immersion	0.0 ± 1.2
PP/PP:10%EVA:20%PS_SB			155.5 ± 9.9

Nevertheless, Regulation (EC) No. 450/2009 on active and intelligent materials stipulates that the migration of the active substances should not be included in the calculation of the overall migration limit because they are incorporated in the packaging material to be intentionally released into the food. Because of that, the amount corresponding to potassium sorbate and sodium benzoate quantified in the specific migration test was subtracted from the overall migration results.

The subtraction of the specific migration values obtained for potassium sorbate and sodium benzoate (Table 4.57) from the overall migration results (Table 4.60) gave -16.2 ± 26.1 mg/dm^2 for the case of 20% ethanol and -71.3 ± 53.5 mg/dm^2 for the case of 3% acetic acid as corrected overall migration. In the first case, it can be considered that all the overall migration came as consequence of the migration of the salts. However, in the second case, the corrected overall migration gave an illogical result since the specific migration was higher than the overall migration. This behaviour could be explained by the fact that potassium sorbate and/or sodium benzoate might be undergoing degradation during the evaporation of the simulant in the overall migration test.

In order to understand whether the evaporation conditions of the overall migration test were breaking down the salts, a stability test was performed. For this purpose, 50 mg of potassium sorbate dissolved in 100 mL of 3% (w/v) acetic acid were subjected to evaporation to estimate the dry residue. Results obtained showed that 100% of the amount of potassium sorbate was recovered as residue. The same procedure was applied with sodium benzoate. In this case, results showed that about 30% of the salt was lost during the process of evaporation, and yielded a residue of 70% of the initial amount of salt. Therefore, the overall migration in 3% (w/v) acetic acid should be corrected taking into account that sodium benzoate is not stable during the test.

Considering this result, the overall migration expected according to the specific migration results (91.88 ± 9.76 mg/dm^2 PS and 111.11 ± 34.60 mg/dm^2 SB) would be 169.7 ± 36.0 mg/dm^2 (100% PS + 70% SB) which in comparison with the experimental overall migration value (131.7 ± 39.6 mg/dm^2) provides a corrected overall migration of -38 ± 53.5 mg/dm^2. This value can be considered statistically equal to zero, and, in any case, far below the threshold limit established as 10 mg/dm^2. Therefore, the overall migration from the active tray came as consequence of the migration of the active agents and thus a negligible amount was caused by other additives migrating from the PP.

4.6.3. Conclusions on the food safety evaluation of active materials

The active films showed great inertness for the simulants B and C, complying with the OML of 10 mg/dm^2 set in the Regulation (EU) No. 10/2011. To evaluate the safety compliance of citral, hexanal and linalool, food law was followed as the active agents were deliberately incorporated to the active material to be released into the food. In this regard, and in absence of a limit of use for these substances in minimally processed fruit, the ADI approach was used for compliance assessment. In this case, from the specific migration tests in the food simulant 3% (w/v) acetic acid and from storage tests in fruit, it was concluded that citral and linalool exposure met the ADI limits set for these flavouring agents. However, hexanal exceeded the normal EDI value under a worst-case scenario. The application of more realistic exposure assumptions provided values around the EDI.

The active trays complied with the OML of 10 mg/dm^2 when the amount of migrated potassium sorbate and sodium benzoate was subtracted from the overall migration result. Exposure of potassium sorbate and sodium benzoate calculated from specific migration tests in food simulants 3% (w/v) acetic acid and 20% (v/v) ethanol and from storage tests in fruit were used to evaluate its compliance with food law as set by Regulation (EC) No. 450/2009. In this case, exposure assessment calculated from both scenarios showed that potassium sorbate and sodium benzoate met the ADI limits set for each additive. In any case, an authorisation procedure under Regulation (EC) No. 1333/2008 should be undertaken for citral, hexanal, linalool, potassium sorbate and sodium benzoate in order to use these active materials with peeled and cut orange and pineapple.

5. CONCLUSIONS

1. Citral, hexanal and linalool demonstrated to have potential antifungal activity against spoilage yeasts and molds typically from fruits. Synergistic inhibitory effect for the mixture (1:1:1) was observed.

2. Active polypropylene (PP) films with citral, hexanal and linalool mixture (1:1:1) were successfully processed at lab and semi-industrial scale by extrusion. At the semi-industrial scale there was 95% incorporation of the active volatile mixture.

3. The incorporation of a non-volatile mixture of potassium sorbate (PS) and sodium benzoate (SB) (3:2) in a polypropylene (PP) and ethyl vinyl acetate (EVA) blend was successfully carried out at lab and semi-industrial scale by compounding. This blend was successfully transformed into an active bilayer sheet by coextrusion. The active sheet was successfully converted into an active bilayer tray by thermoforming. There was 100% incorporation of the non-volatile active mixture into the active tray matrix.

4. Functional properties of active films and tray were not negatively affected by the incorporation of the active agents. The incorporation of PS and SB into the tray significantly decreased its transparency.

5. The release kinetics of volatile agents from active films were limited by the refrigeration temperature. An increase of temperature from 4 to 25 °C had a significant effect on the diffusion coefficient (D) of hexanal, citral and linalool. Hexanal showed the highest D value followed by citral and linalool. Active tray showed a clear release of PS and SB into 3% (w/v) acetic acid at 4 °C. The D value obtained for PS and SB was in the same order of magnitude.

6. Active films exhibited excellent antimicrobial properties against *Saccharomyces cerevisiae*, *Penicillium aurantiogriseum* and *Aspergillus niger* during *in vitro* vapor diffusion tests at room and refrigeration temperature with a dose-dependent antimicrobial effect. The antimicrobial effect was enhanced by an increase in temperature.

7. The active tray was strongly inhibitory toward the target microorganisms in broth at room temperature. Under refrigeration conditions, the microbial reduction was much lower, although some significant reduction was still observed.

8. The native microflora of peeled and cut orange and pineapple was significantly reduced by the active packaging materials. In general, the active films and tray were more

effective with pineapple. The combination of active film and tray exerted a synergistic antimicrobial effect in both fruits. The active packaging system also maintained the quality parameters of the fruits longer compared to control packaging.

9. The general appearance and color attributes of fruit packed with the active packaging system was scored higher than the odor attribute. Smell was a limiting factor placing the fruit under the marketability limit before it was microbiologically spoiled. A reduction of active ingredients in the packaging materials should be performed to balance antimicrobial effectiveness and other quality parameters with the sensory attributes of the fruit.

10. Active films and tray were demonstrated to be compliant with the overall migration limit set by the plastic Regulation (EU) No. 10/2011. The level of food additives migrated from the active packaging system to the fruit was not of a safety concern based on the Admissible Daily Intake (ADI) approach. However, the commercialization of these packaging materials requires authorization of these active agents as food additives under the food additive Regulation (EC) No. 1333/2008 for their intended use.

11. The developed packaging materials could have noteworthy value for application in antimicrobial packaging systems for the food industry by reducing spoilage microorganism growth and extending the shelf life of minimally processed fruit, even when stored at refrigeration temperatures.

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7. ANNEXES

ANNEX I

MINISTERIO
DE INDUSTRIA, ENERGIA
Y TURISMO

Oficina Española
de Patentes y Marcas

Acknowledgement of receipt

We hereby acknowledge receipt of your request for grant of a European patent as follows:

Submission number	300171429	
Application number	EP15382475.0	
File No. to be used for priority declarations	EP15382475	
Date of receipt	30 September 2015	
Your reference	P3374EP00	
Applicant	INSTITUTO TECNOLÓGICO DEL EMBALAJE, TRANSPORTE Y LOGÍSTICA (ITENE)	
Country	ES	
Title	Package for minimally processed fruits or vegetables	
Documents submitted	package-data.xml	ep-request.xml
	application-body.xml	ep-request.pdf (5 p.)
	OLF-ARCHIVE.zip\00 memories.zip	SPECTRANONEP.pdf\P3374EPES00_Traducción.pdf (53 p.)
	SPECEPO-1.pdf\P3374EP00_filing.pdf (50 p.)	f1002-1.pdf (2 p.)
Submitted by	CN=Natividad Toledo 25243	
Method of submission	Online	
Date and time receipt generated	30 September 2015, 12:00:50 (CEST)	
Official Digest of Submission	35:CF:17:20:66:03:50:1D:33:8C:0D:34:B4:0E:E1:F6:D8:5C:72:B8	

/Madrid, Oficina Receptora/

Form 1002 - 1: Public inventor(s)

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Application No:

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