Universidade do Minho
Escola de Engenharia

Dalila da Assunção Maia Vieira

Development of Active Food Packaging with Antioxidant and Antimicrobial Properties

janeiro de 2021

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Development of Active Food Packaging with Antioxidant and Antimicrobial Properties

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Ciência e Engenharia de Polímeros e Compósitos

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Professora Doutora Ana Vera Machado

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STATEMENT OF INTEGRITY

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Dalila da Assunção Maia Vieira

RESUMO

Desenvolvimento de uma Embalagem Ativa com Propriedades Antioxidantes e Antimicrobianas

A recente tendência nas mudanças para um estilo de vida mais acelerado e com menos tempo para o consumidor preparar os alimentos constitui um grande desafio para o setor das embalagens alimentares. Nos últimos anos, a indústria alimentar teve de se adaptar às crescentes demandas da segurança alimentar. A procura por produtos pouco processados, como os alimentos naturais, exige que as embalagens possuem certas propriedades e características específicas, afetando a vida útil dos produtos. O principal objetivo deste trabalho consistiu no desenvolvimento de uma embalagem ativa produzida por extrusão de filme tubular. Inicialmente, diferentes extratos naturais aromáticos (alecrim, anis, cidreira, chá verde, curcumina, cravo e canela) foram selecionados para avaliar o potencial no uso como aditivos para embalagens alimentares ativas. Foi demonstrado que os aditivos naturais possuem propriedades antioxidantes e antimicrobianas promissoras e podem ser incorporados em matrizes poliméricas. O desenvolvimento da embalagem ativa consistiu na incorporação do extrato de chá verde numa matriz termoplástica de polietileno de baixa densidade, seguida da produção do filme pela técnica de extrusão de filme tubular. Diferentes filmes foram preparados, variando a sua estrutura (uma camada e multicamada) e a percentagem de extrato incorporada. Os resultados revelaram uma superfície dos filmes homogénea e lisa, tornando-se mais hidrofóbica. Embora as propriedades de tração não tenham sofrido alterações significativas, o coeficiente de atrito, a resistência à penetração e a fissura por flexão foram melhorados. A capacidade antioxidante e as propriedades de barreira (água e oxigénio) dos filmes foram melhoradas com a adição de maiores quantidades de extrato de chá verde. Os filmes mais promissores foram avaliados como uma nova alternativa para preservar e prolongar a vida útil do sumo de laranja natural, sendo que os filmes ativos de LDPE contendo 3% de extrato de chá verde aumentaram a vida útil do sumo. Este processo de produção pode ser facilmente implementado industrialmente, sendo uma alternativa viável às soluções atualmente existentes no mercado.

Desta forma, o aumento da vida útil do produto levará à diminuição do uso de plásticos e, consequentemente, contribuirá para o aumento da sustentabilidade ecológica e planetária.

Palavras-chave: Embalagem Ativa; Extrato de Chá Verde; Antioxidante; Antimicrobiano; Vida útil

ABSTRACT

Development of Active Food Packaging with Antioxidant and Antimicrobial Properties

The recent trend of lifestyle changes with less time for consumers to prepare foods constitutes a great challenge towards the food packaging sector. Over time, the food industry has experienced endless changes to adapt to the rising demands of food safety. The request for slightly processed products, such as natural foods, requires certain properties and specific characteristics of the packaging, mostly affecting the products shelf-life. The main objective of this thesis was the development of an active packaging through the process of extrusion of tubular film. Therefore, different aromatic natural extracts (rosemary, anise, lemon balm, green tea, curcumin, clove, and cinnamon) were selected and evaluated to assess their potential use as additives for active food packaging. It was demonstrated that natural additives have promising antioxidant and antimicrobial properties and can be incorporated into polymeric matrices. The development of the active packaging consisted on the incorporation of green tea extract in low-density polyethylene followed by film production by blown film extrusion. Different films were prepared, varying the structure (one layer and multilayer) and using different amount of extract. The results revealed that the surface of films was homogeneous and smooth and became more hydrophobic. While the tensile properties were not significantly affected, coefficient of friction, penetration resistance and flex-crack were improved. The antioxidant capacity and barrier properties (water and oxygen) of films were enhanced with the addition of higher amounts of green tea extract. The potential film prepared were assessed as a new approach to preserve and extend the shelf-life of orange juice. The results depict that active LDPE films containing 3 wt.% of green tea extract increased the shelf-life of fresh orange juice and can become a promising film to storage this type of product. Moreover, the production process can easily be implemented at an industrial scale.

Since increasing the shelf-life of the product will lead to a decrease in the use of plastics, this will contribute to increase the ecological and planet sustainability.

Keywords: Active Packaging; Green Tea Extract; Antioxidant; Antimicrobial; Shelf-Life

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations

ºBrix - Total soluble solids

AA – Ascorbic acid

AAC – Antioxidant activity coefficient

AAPH - 2,2'-azobis(2-methylpropionamidine)dihydrochloride

AE – Anise extract

AH – Primary antioxidants

AM - Antimicrobial

AO – Antioxidants

ASTM - American Society for Testing and Materials

BHA - Butylated hydroxyanisole

BHT - Butylated hydroxytoluene

C - (+)-Catechin

CA – Contact angle

CCE – Curcumin extract

CE – Cinnamon extract

CG - (-)-Catechin gallate

CLE – Clove extract

COF – Coefficient of friction

DHA - Dehydroascorbic acid

DKG - 2,3-Diketogulonic acid

DMEM – Dulbecco's modified eagle's medium

DMSO – Dimethylsulfoxide

DNA - Deoxyribonucleic acid

DPPH - radical 2,2-diphenyl-1-picrylhydrazyl

DRBC - Dichloran Rose-Bengal Chloramphenicol

EB - Elongation at break

EC - (-)-Epicatechin

EC - European Comission

ECG - (-)-Epicatechin gallate

EDTA - ethylenediaminetetraacetate;

EFSA - European Food Safety Authority

EGC - (-)-Epigallocatechin

EGCG - (-)-Epigallocatechin gallate

EOs – Essential oils

EU – European Union

EVA - Ethylene-vinyl acetate

EVOH – Ethylene-vinyl alcohol

FBS – Fetal bovine serum

FDA – Food and Drug administration

FTIR – Fourier Transform Infrared spectroscopy

GC - (-)-Gallocatechin

GCG - (-)-Gallocatechin gallate

GI_{50} – Sample concentration that inhibited 50% of the net cell growth

GRAS – Generally recognized as safe

GTE – Green tea extract

HBSS – Hank's balanced salt solution

HDPE - High-density polyethylene

IC_{50} – Effective concentration achieving 50% of antioxidant activity or 0.5 absorbance in reducing power assay

INT – Iodonitrotetrazolium chloride

LBE – Lemon balm extract

LDL - Low-density lipoprotein

LDPE – Low-density polyethylene

LLDPE - Linear low-density polyethylene

MBC – Minimum bactericidal concentration

MD - Machine direction

MDA – Malonaldehyde complex

MFC - Minimum fungicidal concentration

MHB – Muller Hinton broth

MIC - Minimum inhibitory concentration

n.a. – Not applicable

O_2P – Oxygen permeability

OPP – Oriented-polypropylene

OxHLIA – Oxidative hemolysis inhibition assay

PA - Polyamide

PBS – Phosphate buffered saline

PCA – Plate count agar

PE - Polyethylene

PET - Polyethylene terephthalate

PG - Propyl gallate

PLA - Polylactic Acid

PLP2 – Primary porcine liver cells

POE - Polyolefin elastomer

PP – Polypropylene

PS - Polystyrene

PVA - Poly(vinyl alcohol)

RE – Rosemary extract

RNA - Ribonucleic acid

ROOH – Lipid hydroperoxides

rpm – Rotation per minute

SEM - Scanning electron microscopy

SM – Secant modulus

SRB – Sulphorhodamine B

TA – Total acidity

TBA – 2-Thiobarbituric acid

TBARS – Thiobarbituric acid reactive substances

TBHQ - Tert-butyl hydroquinone

TCA – Trichloroacetic acid

TD - Transverse direction

T_g - Glass transition temperature

TGA – Thermogravimetric analysis

T_m - Crystalline melting temperature

Tris-HCl - Hydroxymethylaminomethane buffer

Trolox – 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

TS - Tensile strength

TSB – Tryptic soy broth

USDA - United States Department of Agriculture

UV – Ultraviolet

WVTR – Water vapor transmission rate

β-CD - Beta-cyclodextrin

Symbols

% - Percentage

$\mu g.mL^{-1}$ – Micrograms per milliliter

µL – Microliters

$\mu L.mL^{-1}$ – Microliters per milliliter

$CaCl_2$ – Calcium chloride

$CaCO_3$ – Calcium carbonate

CFU/mL – Colony forming unit per milliliter

cm - Centimeters

cm^2 – Centimeters squared

CO_2 – Carbon dioxide

ECE µg/mL – Epicatechin micrograms per milliliter

g – Grams

$g.h^{-1}.m^{-2}$ - Grams per hour square meter

g/L - Grams per liter

g/mL – Grams per milliliter

GAE mg/mL – Gallic acid equivalents milligrams per milliliter

h – Hours

H_2O_2 - Hydrogen peroxide

H_2SO_4 – Sulfuric acid

Kbr – Potassium bromide

mg - Milligrams

$mg.L^{-1}$ - Milligrams per liter

$mg.mL^{-1}$ - Milligrams per milliliter

min – Minutes

mL – Milliliter

mL.mm/MPa/min/cm^2 – Milliliter millimeter per MegaPascal per minute per centimeters squared

mm - Millimeter

mM – Millimolar

mm/min – Millimeter per minute

mm^3 - Cubic millimeters

MPa – MegaPascal

N – Newton

N_2 - Nitrogen

NaCl – Sodium chloride

NaOH – Sodium hydroxide

nm – Nanometer

O_2 - Oxygen

$O_2^{\cdot-}$ - Superoxide anion

°C – Degree Celsius

°C/min - Degree Celsius per minute

U. mL^{-1} – Units per milliliter

v/v - Volume concentration

Wt.% - Weight percent

1 THESIS OVERVIEW

This chapter includes a general introduction of the thesis, the work motivation and the thesis outline.

1.1 Motivation

Food can be subject to microbial contamination, is caused by bacteria, yeasts, and fungi. Microbial growth in food can cause undesirable reactions that result in changes in taste, odor, color, sensory and textural properties of food. Thus, to prevent oxidation and growth of microorganisms in food, various food preservation techniques are used, such as heat treatment. However, the increasing demand of consumers for safe, less processed, fresh food, led to the development of new preservation techniques. One of the techniques that have been studied to answer consumers demand is active packaging, which can provide safe products with a longer shelf-life. This type of packaging, containing additives with antioxidant and/or antimicrobial properties, has been investigated to meet consumer concerns with the environment and health. To reduce the deterioration of food by microorganisms, different antimicrobial agents (mainly synthetic) are commonly incorporated directly into food. However, these synthetic agents have some disadvantages, such as a characteristic flavor that will modify the flavor of the food. To solve this problem and avoid health issues, natural agents started to be incorporated into the packaging as an additional protective barrier.

Thus, the main goal of this thesis is to develop an active food packaging using blown film extrusion. As its main feature, the packaging to be developed must be a flexible film with antioxidant and antimicrobial properties, which will be given by the incorporation of natural compounds. The package that will be developed should increase the shelf-life of the product, which will also contribute to a decrease in the use of plastics and in this way to a decrease in the ecological footprint of the planet.

1.2 Thesis Outline

This thesis is organized in six Chapters:

CHAPTER 1 presents the context and motivation of the present work, as well as its main objectives.

CHAPTER 2 describes a general overview of antioxidants and antimicrobial agents in the food industry, namely the mechanisms of action, the properties of the antioxidant green tea, the importance of packaging and the main characteristics of fruit juices. Besides, a short review of the preparation of multilayer films as well as their main properties, and the recycling processes are also given.

CHAPTER 3 reports the study of thermal and biological properties (antioxidant and antimicrobial) of seven commercial extracts (green tea extract, rosemary extract, cinnamon extract, anise extract, clove extract, lemon balm extract, curcumin extract) to select the most promising ones in terms of potential antioxidant and antimicrobial properties.

CHAPTER 4 describes the development of active films for food packaging based on the incorporation of green tea extract (GTE) in low-density polyethylene (LDPE) and production of films by blown film extrusion. Different GTE concentrations were evaluated, as well as different film structures (monolayer and multilayer). The produced films were characterized through mechanical, physical, optical, microstructure, antioxidant, and antimicrobial properties.

CHAPTER 5 describes the assessment of the active LDPE/GTE packaging developed in chapter 4 as a new approach to preserve and extend the shelf-life of orange juice. For this, the ascorbic acid (AA) content, sugar content, browning index, parameters of color, pH, total acidity (TA), and microbial stability were evaluated after 3, 7, and 14 days of storage of fresh orange juice.

CHAPTER 6 presents the general conclusions regarding the work carried out in this thesis and suggestions for future work.

2 LITERATURE REVIEW

This chapter contains an overview of the thematic of food packaging. This state-of-the-art of food packaging is focused on literature review, more specifically, on the development of active packaging with antioxidant/antimicrobial properties and the incorporation of plant extracts/essential oils. Then, the process to produce packaging films and the main required properties are also discussed.

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2.1 Food Packaging

2.1.1 The role of Food Packaging

Packaging plays a critical role in the food supply chain, its primary function is to serve as a food container, enabling efficient transport within the whole supply chain, preventing any physical damage and protecting against manipulation and theft. Package should also meet the fundamental requirement to maintain food quality from production to final consumption by preventing any unwanted chemical and biological changes. Moreover, it should act as a barrier to protect the food from environmental influences, such as oxygen, moisture, light, dust, pests, volatiles, and both chemical and microbiological contamination [1–4]. The protective role of the packaging is primarily passive, acting as a barrier between the food, atmosphere surrounding the food, and the external environment. However, there are some exceptions, like fresh products, for which highly gas permeable or perforated packaging materials are used to allow gas exchange through the packaging [2, 5]. Such systems, however, are limited in their ability to further extend the shelf-life of the packaged food. There is an increasing trend to natural high-quality foods, which are non-processed or minimally processed, do not contain preservatives, but has to guarantee an acceptable shelf-life [2, 6, 7]. As an answer, the protective function of packaging has been refined and improved leading to the development of new packaging technologies, such as modified atmosphere packaging, active packaging, smart and intelligent packaging [1, 2, 6, 8, 9].

2.1.2 Types of Packaging

Food packaging materials must provide the appropriate mechanical resistance and optimal gas and water vapor permeability [10]. These parameters are difficult to control when using classic packaging materials since direct food contact has to be as inert as possible [11]. The use of innovative materials is envisaged to extend food's shelf-life without compromising its sensory properties [12]. Certainly, active packaging is an attractive technology due to its "hands-on" food overall conditions, in opposition to the simple passive monitoring of the intelligent packaging.

The "active packaging" concept was introduced by the EC 1935/2004 [11] and it was further regulated by Regulation (EC) 450/2009 [13]. The latter covers the definition of active materials as the ones that are designed to deliberately incorporate components that would release or absorb substances into or from the packaged food or the surrounding environment [4, 13–16], and creates a legal framework for

the development of active packaging legislation. Also, this regulation establishes specific requirements for the marketing of active materials and the substances intended to come into contact with food. Considering the diffusion of active packaging in EU market, it should be mentioned that the issues of acceptance by industries, as well as, the more conservative behavior of European consumers regarding innovations in food are key points that still need to be addressed [17].

Active packaging technologies can safely provide products with longer shelf-life. This type of packaging can contain natural additives, such as antioxidants (AO) and/or antimicrobial (AM) that are released along the shelf-life time to assure the food properties.

2.1.2.1 Antioxidant Packaging

2.1.2.1.1 General Concepts

The use of AO in the food industry and their functional mechanisms have been widely studied. During AO selection, the following properties should be taking into account, such as effectiveness at low concentrations (0.001 to 0.01%), absence of undesirable effects on color, odor, taste and other characteristics in the food, food compatibility and easy application. Also, parameters like legislation, cost and consumer preference, stability in processing, storage conditions, compound and non-toxic oxidation products are very important [18].

Several compounds with AO properties present different chemical groups and, therefore, an orderly classification is difficult. Usually, they are classified either by their mechanism of action, which is more comprehensive and theoretically sounder, or by its chemical nature. AO can be subdivided into two main groups according to the mechanism of action accepted: group I (primary AO) and group II (secondary AO) [18, 19].

In group I, primary antioxidants (AH) are phenolic or amine compounds that promote the removal or inactivation of free radicals formed during the initiation or propagation of the reaction. The main mechanism is through the donation of hydrogen atoms to the radical molecules, in order to interrupt the reaction propagation (Figure 2.1), resulting in an inert radical ($A^{\bullet}$) [18, 19]. Hydrogen atoms from the AO are more easily sequestered by free radicals than the ones from unsaturated lipids.

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$$

$$R^{\bullet} + AH \rightarrow RH + A^{\bullet}$$

Figure 2.1 - Mechanism of action for the primary antioxidants.

The most well-known primary AO can be synthetic, namely butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *tert*-butyl hydroquinone (TBHQ) and propyl gallate (PG), or have a natural origin , such as tocopherols and polyphenols from fruits, herbs, and spices [18, 19].

Group II secondary AO are compounds that inhibit lipid oxidation by different mechanisms and convert free radicals to non-radical products. With some exceptions, secondary AO are normally related to the inhibition of the factors initiating oxidation [18, 19]. Secondary AO include chelators of pro-oxidative metals able to alter the metal solubility or its redox potential, and the main two common examples are citric acid and ethylenediaminetetraacetate (EDTA), acting as quenchers of singlet oxygen act by capturing the oxygen present in the medium, making them unavailable for the auto-oxidation process. Another example of this type of AO is ascorbic acid made of molecular oxygen scavengers acting as strong reducing agents, such as ascorbate and sulfur dioxide, and are particularly useful in foods with headspace or dissolved oxygen. Ultraviolet (UV) light absorbers, such as carbon black, are substances capable of absorbing the harmful UV light. Inhibitors of pro-oxidative enzymes reduce the metal ion in their active site to its inactive reduced form (e.g. Fe^{3+} to Fe^{2+}), and in the case of phenolics it may inactivate pro-oxidative enzymes such as lipoxygenases. This type of enzymatic AO includes enzymes like glutathione peroxidase and catalysts able to deactivate oxidation intermediates such as the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) or lipid hydroperoxides (ROOH).Also, hydroperoxide decomposers induce decomposition of these hydroperoxides through non-radical routes or “bind” the generated secondary products will favorably affect the ultimate AO effect [18, 19]. The decomposition of lipid hydroperoxides can produce new radicals or unwanted secondary oxidation products, representing a key feature in lipid oxidation [19].

Nowadays, AO from natural resources have been investigated to replace the synthetic ones, and some examples are further discussed.

2.1.2.1.2 Tea (*Camellia sinensis* (*L.*))

Tea is the second most consumed beverage around the world, following water. It is native from China and has a long history that spans across numerous countries over thousands of years [20–24]. Since from ancient times, tea has always been regarded as the traditional Chinese medicine capable of ameliorating or preventing all sorts of disorders. Nowadays, more than 30 countries around the world produces different varieties of tea, not only as a relaxation drink but also due to its documented health benefits supported by a myriad of scientific studies [20, 24]. Tea drinking is associated with a reduction of serum cholesterol, prevention of low-density lipoprotein (LDL) oxidation and a decreased risk of

cardiovascular disease and cancer. Due to these facts, the popularity of this beverage has grown all over the world [20].

Tea derived from leaves of the plant *Camellia sinensis* can be categorized into three main types depending on the level of oxidation: green (unfermented), oolong (partially fermented) and black (fermented) tea [20–22, 24]. The chemical composition of green tea varies with genetic strain, climatic conditions, soil properties, plucking season, the position of the leaf, processing, and storage. Some factors are more important than others, for example, the highest quality green teas are plucked during the first flush in late April and early May and quality declines in later harvests. Usually, the buds and the first two to three leaves are plucked by hand or a mechanical tea plucked for processing, and this process is generally repeated every two weeks. These basic types of tea have different quality characteristics, including appearance, flavor, taste, and color [21, 22].

2.1.2.1.3 Green Tea

In the case of green teas, the initial stages of manufacturing involve steaming or roasting, which will inactivate the activity of polyphenol oxidase hence preventing any oxidation from occurring during subsequent processing steps. As such, green tea preserves the native structure of its polyphenolic compounds as well as its overall compositions [24]. In green tea, polyphenols have a general designation for catechins, flavones, anthocyanins, and phenolic acids. Some minor polyphenols that also exist are epigallocatechin gallate, a flavonol glycoside, and tannins. In green tea, the polyphenol compounds are the main constituents accounting 24-36% in dry weight, followed by its protein content (15%), lignin (7%), amino acids (3-4%), caffeine (2-4%), organic acid (2%), and chlorophyll (0.5%) [24].

The relationship between the quality and chemical components in green tea have been studied and have shown that free amino acids, caffeine, and polyphenols are qualitatively important components. Catechins, the main component of polyphenols, are well known for their AO properties, which have led to their evaluation in many diseases associated with free radicals, including cancer, cardiovascular and neurodegenerative diseases. Generally, the major catechins of tea leaves are (+)-catechin (C), (-)-epicatechin (EC), (+)-gallocatechin (GC), (-)-epigallocatechin (EGC), (–)-catechin-3-gallate (CG), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin-3-gallate (EGCG), and (+)-gallocatechin gallate (GCG) [22, 24, 25] (for structures, see Figure 2.2). The AO properties of catechins are mainly related to the number and position of the hydroxyl group in the molecules and, consequently, it has the ability to bind and neutralize the free radicals [22, 24]. Previous studies have shown that tea catechins are excellent electron donors and effective scavengers of physiologically relevant reactive oxygen species *in vitro*, including superoxide anions, peroxyl radicals, and singlet oxygen [22, 24]. Most studies on the AO effects of green

tea are directly related to the total phenolic extracts, not considering the contributions of individual molecules, although several catechins such as EGCG, ECG, and EGC, have been linked to its strong AO activity [22]. Given the contribution of oxidative stress to the onset and progression of chronic pathological conditions, the antioxidative activity found in green tea was documented to prevent a variety of diseases. Moreover, EGCG was also described as a second signal messenger, which is a stimulator of plasma membrane proteins and a modulator of metabolic enzymes. EGCG was also reported to be involved in cell signaling and transcription pathways [24] and this way has been claimed to have chemopreventive actions on a variety of health-related endpoints in humans [21].

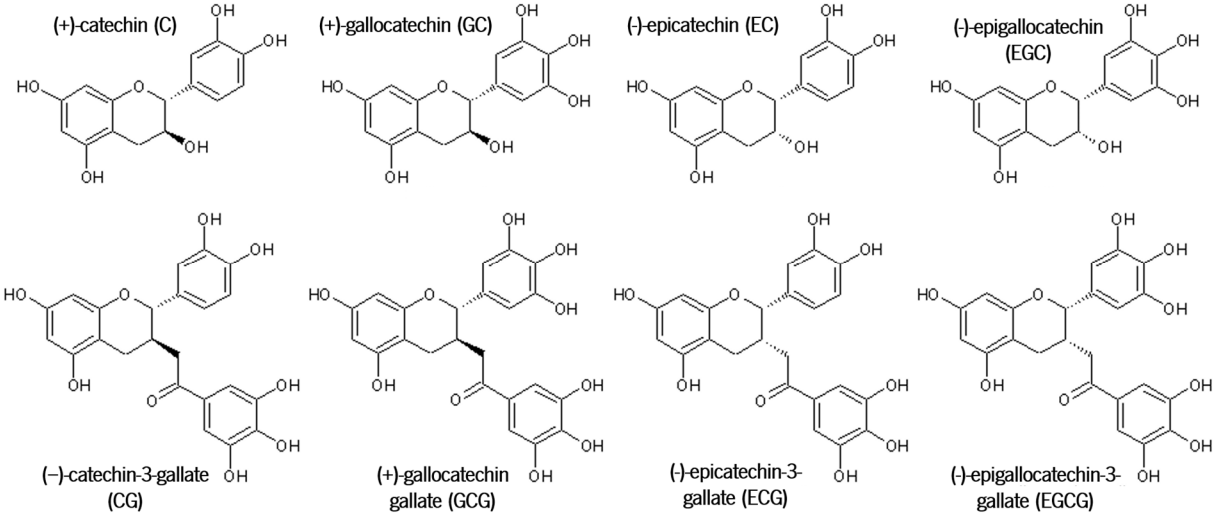

Figure 2.2 - Structures of catechins.

According to the United States Department of Agriculture (USDA) Flavonoid Database, brewed green tea contains an average of 126.6 mg total catechins and 77.8 mg EGCG per 1 g leaf/100 mL infusion. Consequently, each 240 mL serving of brewed green tea may provide an estimated 304 mg total catechins, with 187 mg EGCG. Therefore, the estimated daily intake of catechins and EGCG through green tea consumption can reach approximately 912 and 560 mg/day, respectively, for individuals consuming an average of three 8 oz. cups of green tea daily [23]. The European Food Safety Authority (EFSA) reported that in average European consumers have an estimated daily intake of green tea and EGCG of 95.2 and 147.7 mg, and could reach up to 288.5 and 447.6 mg, respectively[21]. Numerous *in vitro* and *in vivo* studies suggested the beneficial health properties of green tea and tea polyphenols including AO, antitumor, hypertension and hyperlipidemia reduction, and antimicrobial (AM) activity [20, 25]. But there are other compounds in green tea with interest for human health like fluoride, caffeine, minerals and trace elements like chromium and manganese. The regular consumption of tea can contribute to the daily dietary requirements of some of these elements [20]. Due to the beneficial effects

of green tea and catechins, green tea extracts have been widely applied to different fields, particularly in the food and beverage industries [25].

2.1.2.1.4 Applications

A wide variety of organic molecules are susceptible to be chemically attack by oxygen and among them more attention has been paid to lipids due to their tendency to oxidate [26, 27]. Lipid oxidation is the most important cause of food quality deterioration, the destruction of valuable nutrients and the production of toxic compounds. During the last decades, the lipid oxidation has been considered as an important challenge for package manufacturers and researchers to prevent food degradation [26].

Synthetic AO are frequently used to stabilize fats, oils, and lipids containing foods [26]. However, their use in food has been questioned by the scientific community because of the potential toxicity over the foodstuff damage [18].

Nowadays, synthetic AO, such as BHA and BHT are regulated by Codex Alimentarius, by the European Comission (EC) and by the Food and Drug Administration (FDA) Food Additive status list. The alternative approach is the use of natural AO, such as tocopherol, plant extracts, and essential oils (EOs) from herbs and spices. These natural compounds have been used to produce packaging with AO properties. According to Nerín *et al.* [28] the active films containing natural AO enhance the stability of both myoglobin and fresh meat against oxidation processes. These evidences accelerated the search for natural AO, which led to the identification of natural resources to isolate the active AO molecules [26].

Pokorny [29] established some advantages and disadvantages of natural AO compared to synthetic ones. Thus, the possible advantages are the following: numerous AO available, accepted by legislation, can be replaced by other food components and be used as flavorings with good sensory properties and good preservation agents. The possible disadvantages are variable composition and activity, limited purity, need for higher amounts, negative sensory properties, and complicated availability. For these reasons, the concomitant use of natural of AO in active food packaging could be an interesting future strategy, since it has been demonstrated that these substances possess AO activity when added to film matrices [30].

A suitable selection of AO compound to be incorporated in packaging material is crucial. The AO compound should be compatible with the polymer matrix to achieve a homogeneous distribution, and the partition coefficients of the AO in the different phases should favor its release to the food or headspace. Once released, the solubility characteristics of the AO can determine its effectiveness, thus the type of AO should be selected as a function of the type of food. Nonpolar AO seem to be more suitable for foods with a high lipid content and *vice versa* [31].

There are basically two methodologies for producing AO packaging systems. The first one, denominated independent devices, which can be a sachet, pad or label containing the agent separately from the food product. The food product is then added to a conventional 'passive' package. The AO packaging materials methodology consists in the manufacture of the package, in which the active agent is incorporated in the matrix, exerting its action by absorbing undesirable compounds from the headspace or by releasing AO compounds to the food or the headspace surrounding it [31].

AO packaging materials are being developed by incorporating the active compounds in the polymer matrix or on the polymeric film surface. The manufacturing process should be selected taking into consideration the type of polymer, and the characteristics of the AO agents, like thermal stability, mechanism of action and type of food. If the AO activity of the material is based on a migration process into the food, the substances released should be also suitable as food additives and fulfil the present regulations in terms of maximum concentration [31].

From a technological point of view, the agent (or the reactive substances which produce the agent) is intimately mixed with the polymer, either by dissolving both into an appropriate solvent followed by application of the solution to a substrate by coating technologies, or by melting mixing, where the incorporation of the agent in the polymer occurs in the melt using extrusion technologies [31].

The inclusion of plant extracts represents an interesting ingredient for food packaging materials, mainly due to its natural origin and potent source of AO. Such type of package could be used to inhibit or reduce oxidative degradation of inside food. AO packaging is a major category of active packaging and a very promising technique for enhancing the shelf-life of food products. Furthermore, enriching films with AO allows nutritional and aesthetic quality aspects to be extended without affecting the integrity of the packaged product [32].

Concerning films with added EOs, their AO activity could vary depending on the EOs used or on the film composition. Several authors have described a lower AO activity of films with added EOs compared to the oils alone. This fact could be due to interactions between the components of films and EOs, such as polyphenols and proteins, reducing the availability of the AO compounds, and could also be due to the loss of volatile compounds during film preparation. Moreover, depending on the simulant used for the assays, the AO activity could also change. Thus, López-de-Dicastillo *et al.* [33] found that film activity was simulant-dependent, depending on the hydrophilic or lipophilic character of the EOs, a simulant or other (water or ethanol) must be chosen for the evaluation of the AO activity of the incorporated films. Finally, there are also studies where the film activity was assessed after simulated digestion, resulting in a decrease of the activity compared with the activity of the original film. These authors attributed this finding

to the transformation of the components from the extract during the digestion process or to an interaction between compounds and film [30].

Incorporating plant extracts into starch or protein-based edible films could create packaging with AO properties. Siripatrawan and Harte [34] reported that the total phenolic content and free radical scavenging activity of chitosan films improved up to 15% when green tea extract (GTE) concentration increased from 0 to 20%.

Nerín *et al.* [28] have shown the effectiveness of rosemary EOs and its components included in active food packaging to increase the food shelf-life. Also, Sanches-Silva *et al.*[35] studied the AO activity of natural substances in active packaging and concluded that it is necessary to study the safety and the effectiveness of these materials in to order to evaluate which food products are suitable to be packed by each material [36].

Zhang *et al.* [37] introduced blueberry extract into soybean isolate films and noticed that the AO capacity of soybean-protein-isolate film incorporated with the blueberry extract was greater than with vitamin E and similar to BHA.

Active packaging using green tea has been reported to delay lipidic oxidation in different foods models, due to catechins being actively involved in the scavenging process of free radicals, as well as to the stimulation of transcription factors and mitogen-activated protein kinases dependent cell cycle regulation. Films incorporated with oregano and thyme EOs have shown different AO activity depending on the content in carvacrol and thymol, although it has also been demonstrated that there is a synergism between both components. As GTE, the activity of oregano and thyme is related with the retardation of lipid peroxidation through their potent radical scavenging activity derived from their composition.

Peng *et al.* [38] studied the effect of GTE and black tea extracts on AO properties of chitosan films. The study showed that the addition of tea extracts significantly increased the AO ability of films. The radical scavenging capacity of GTE films was stronger than that of black tea. This investigation revealed an active chitosan film incorporated with tea extracts able to provide a new option to develop an AO active packaging system [32].

GTE has been added to films based on chitosan and agar-gelatin, the strong radical scavenging activity might be due to an increase in intermolecular interactions, such as hydrogen bonding between phenolic compounds and chitosan [34, 39]. Films containing tea and rosemary extract showed greater AO effects on the storage stability of lipidic foods, such as peanuts, beef, and fish, as an alternative to synthetic α-tocopherol [40, 41]. The use of thyme extract alongside chitosan and starch films provides remarkable

AO activity [42]. Norajit *et al.* [43] reported that the ginseng extract could be successfully incorporated into alginate films and retain excellent AO properties, since it increased free-radical scavenging activities. The total phenolic content of films containing mint extract was higher than in the films with pomegranate peel extract. Furthermore, the amount of phenolics compounds released from the developed films varied significantly according to the testing temperature. At higher temperature (37 °C), the phenolics released from the film was maximum and the release of phenolics were low for films kept at 15 °C. The total phenolic content of films is directly correlated with the AO activity measured by radical scavenging activity. Films containing mint extract had significantly higher AO activity compared to those containing pomegranate peel extract [32].

Li *et al.* [44] evaluated the radical scavenging activity of gelatin-based films after adding natural plant extracts. The results suggested that the addition of 1.0 $mg.mL^{-1}$ gingko leaf extracts made film with strong scavenging activity against DPPH radical. At the same time, adding the GTE and grape seed extract exhibited a similar AO property to film incorporated with gingko leaf extract. These extracts showed higher AO activity in the film than vitamin C at 1.0 $mg.mL^{-1}$. The higher AO activities in the film with GTE and grape seed extract were due to high amounts of caffeic acid and epicatechin in GTEs and epicatechin and catechin in grape seed extracts. The flavonoids contained in gingko leaf extract enriched film can be responsible for its high AO activity. As to gelatin-based film mixed with ginger extract, the radical scavenging capacity could be negligible compared to that of films with other extracts.

The highest value of reducing power was obtained from film incorporated with GTE. The gelatin film containing different extracts exhibited good potential in food packaging for self-life extension due to a rich source of AO [32].

2.1.2.2 Antimicrobial Packaging

Food products can be subjected to microbial contamination mainly caused by bacteria, yeasts, and fungi. Many of these microorganisms can cause undesirable reactions that deteriorate the flavor, odor, color, sensory, and textural properties of the food. Moreover, microbial growth is a major concern because microorganisms can potentially cause foodborne illness [45, 46]. To prevent the growth of spoilage and pathogenic microorganisms on food, various traditional preservation techniques have been used in food industry, such as heat treatment, salting, acidification, and drying [47]. In recent years, an increase in consumer demand for safe, fresh, and minimally processed foods has led to the development of new preservation techniques [45]. The active packaging technologies designed primarily to protect food products from deterioration and microorganism growth can involve the use of synthetic or natural AM agents. To decrease food spoilage by microorganisms, different AM agents (primarily synthetic) are

commonly incorporated directly into the food. This method has many disadvantages: (i) can have side-effects, thus consumers prefer foods with no or minimal synthetic additives; (ii) since food spoilage occurs primarily on the surface, incorporation of relatively large quantities of the gents in the bulk of the food is not justified; (iii) some synthetic agents have a distinct flavor that may be rendered to the food flavor, and (iv) synthetic additives have to be declared on the package. Therefore, active packaging is preferred as preservation method. The active agent is incorporated into a polymer matrix, which is placed close to the food surface, enabling the control or prevention of spoilage and pathogenic microorganisms. Thus, a packaging film impregnated or coated with natural AM agents, could potentially extend the shelf-life and improve the microbial safety of food products.

The use of active packaging offers several advantages compared to the direct addition of preservatives to the food products since the only low levels of this additives come into contact with the food. Typically, AM packaging systems can be classified as migrating or nonmigrating with the distinction depending on the specific AM agents used and how it interacts with the packaging and the food matrix. In preservative releasing or migrating approaches, preservatives are introduced into the bulk, or applied to their surfaces, which subsequently migrate into the food or the head-space surrounding the food. These systems are most useful when direct contact between the packaging film and food product is not required for efficient AM activity. The nonmigrating systems involve packaging materials in which the AM agents is immobilized within the material [25] and these systems can be applied where direct contact between the food and the material can be achieved or is required for effective AM activity [22, 26].

The additives used to prepare AM packaging materials include inorganic, organic and biological active substances. Especially the AM tagged as natural, efficient and non-toxic agent are preferred due to health and ecological concerns. EOs is a kind of natural substance with powerful AM activity against a variety of foodborne pathogens and are categorized as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration (FDA, cite:21CFR582.20), which indicates that they can be used in food industry without further approval. However, the use of EOs in food preservation is limited in some extent due to their insolubility in water and special flavor, which would change the original sensory property of food [45, 48].

Han [49] suggested that the mass transfer rate of an AM agents should not be higher than the growth rate of the target microorganism, otherwise the AM agents might be diluted on the surface of the packaged food product, thus limiting the AM activity [45].

The EOs extracted from plants possess antibacterial, antifungal and antiviral properties that have been studied as alternatives to synthetic compounds. The mode of action of AM agents and/or AM activity of

plant EOs is related to their chemical structure, namely, the presence of hydrophilic functional groups, such as the hydroxyl groups of phenolic components and/or lipophilicity of the components in the EOs, which depends on their concentration. EOs and their principal constituents inhibit microorganisms by a range of mechanisms, such as disruption of the cytoplasmic membrane, leakage of intracellular constituents, coagulation of cell content, inhibition of protein synthesis, enzymes associated with cell wall synthesis, DNA/RNA synthesis, general metabolite pathways; and/or the destruction of the osmotic integrity of the cell membrane, this mechanism can be observed in Figure 2.3.

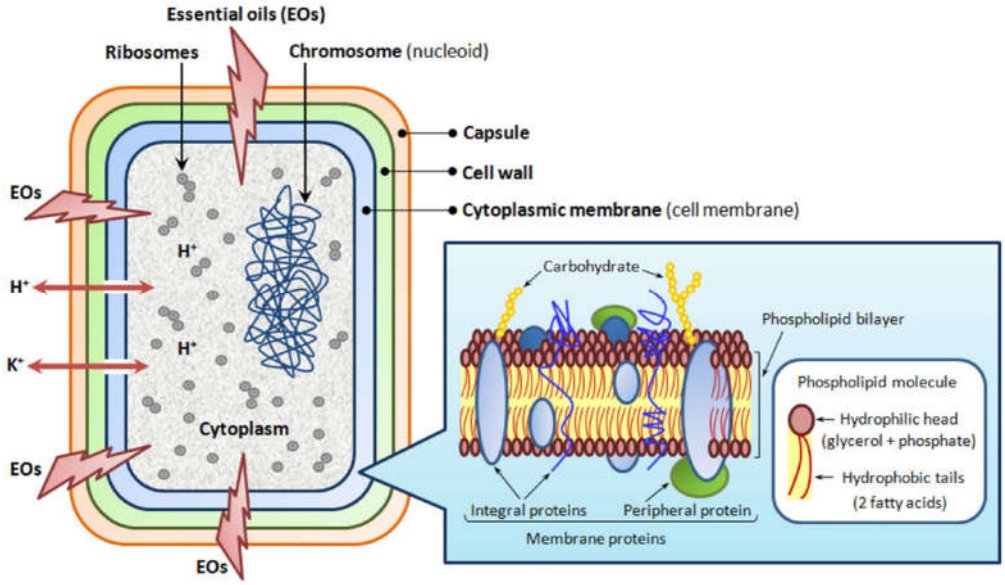

Figure 2.3 - The effect of EOs on bacteria cell [50].

There has been an extensive pursuit for potential natural food additive candidates that retain a broad spectrum of AO and AM activities able to improve the quality and shelf-life of perishable foods. The emergence of antibiotic-resistant bacterial and the negative consumer attitudes toward food preservatives has led to an increased interest in the use of plant components that contain EOs and essences as alternative agents for the control of food spoilage and harmful pathogens [51].

According to Davidson and Zivanovic [52], natural AM agents are classified by their sources: AM agents derived from plant EOs (for example, basil, thyme, oregano, cinnamon, clove, and rosemary) contain a range of natural compounds, such as thymol, linalool, and carvacrol that have a broad AM spectrum against different pathogenic and spoilage microorganisms including Gram-negative species, such as *E. coli*, *Yersinia enterocolitica*, *P. aeruginosa*, and *Sal. choleraesuis*; Gram-positive bacteria such as *L. monocytogenes*, *S. aureus*, *B. cereus*; yeasts such as *S. cerevisiae*, *Candida albicans*, *Debaryomyces hansenii*; and molds such as *Alternaria alternate*, *A. niger*, *Botrytis cinerea*, *A. flavus*, *Penicillium roqueforti*; animal sources (for example, lysozyme, lactoferrin); microbial sources (nisin, natamycin) and naturally occurring polymers (chitosan).

EOs are very difficult to compare given the variation of EO compositions among the plant species, differences in the geographic origin of the plants, harvesting season, extraction methods, and the part of plant that is used [45]. They have also been traditionally used as additives to extend the shelf-life of perishable products due to their AM or AO properties. Burt [53] concluded that EOs exhibit antibacterial activity against food-borne pathogens *in vitro* and in foods, being more sensitive for Gram-positive microorganisms.

According to Davison and Zivanovic [52], the concentration of AM agents required to demonstrate AM activity against various microorganisms on food products might be higher than the concentration applied for flavoring purposes. As a result, this might cause food tainting and/or adverse sensorial effects. The adverse sensorial effects of AM agents can be overcome by masking the odor of AM agents with an approved aroma compound as suggested by Gutierrez *et al.* [54]. The understanding of the relationship between minimum inhibitory concentration and acceptable organoleptic properties of AM agents and/or their constituents is crucial. In some cases, the replacement of EOs by one or some of their principal constituents may provide equal AM effectiveness but with milder flavoring attributes [45, 55].

Suppakul *et al.* [56] prepared low-density polyethylene (LDPE) blown films containing basil EO and noticed a decrease in the agent concentration due to volatilization during extrusion, which resulted in partial loss of AM activity, confirmed by the tests with cheese samples. Ethylene vinyl alcohol (EVOH) copolymers have been studied as a hosting medium for the incorporation of AM agents. This polymer is commonly used in multilayered structures due to its exceptional barrier properties to gas (oxygen in particular). Due to the hydrophilicity of EVOH, its oxygen permeability can increase depending on the relative humidity. This intrinsic sensitivity has been exploited to modulate the release of AM compounds from EVOH layers in contact with foods. Muriel-Galet *et al.* [57] reported the incorporation of oregano essential oil and GTE in EVOH, and observed that the presence of strong binding forces between water and EVOH provides the film with AM properties when in contact with food. In this context, oregano EO and GTE impregnated into EVOH copolymers have the potential to be used as an AM packaging film as it was demonstrated by the inhibition of microbial growth in vapor phases and liquid media [58].

The Table 2.1 summarized some examples of active food packaging and its effects on packaging.

Table 2.1 - Spice extracts or EOs and their application in active food packaging.

Essential oil (EO)/Spice extract	Polymer	Effect on food packaging	References
Rosemary extract	PVA	Antioxidant	[59]
	Whey protein	Antioxidant and Antimicrobial	[60]
Rosemary EO	Whey protein	Antioxidant	[61]
Cinnamon EO	PVA/β-CD	Antimicrobial	[62]
	Whey protein	Antioxidant	[61]
Oregano EO	EVOH	Antioxidant and Antimicrobial	[57]
	Cellulose acetate	Antimicrobial	[63]
	Multilayer PET/PE/EVOH/PE	Antioxidant and Antimicrobial	[64]
	Polyethylene	Antioxidant	[65]
Green tea extract	EVOH	Antioxidant and Antimicrobial	[57]
	PLA	Antioxidant	[66]
Thyme extract	Whey protein	Antioxidant and Antimicrobial	[60]
Olive leaf extract	Not specified	Antioxidant and Antimicrobial	[67]
Clove EO	Chicken feather	Antioxidant and Antimicrobial	[68]

2.1.3 Packaging in Fruit Juices Industry

2.1.3.1 Fruit Juices

Fruits are parts of flowering plant derived from the fertilization of specific tissues, such as one or more ovaries. Fruits are highly perishable, non-staple foods, which make-up about 39% of the food intake (fresh state or processed form). Fruits have been shown to contain a high amount of minerals, moisture, low ash, and crude fiber and are sources of sugar, vitamin A, C and B groups, low protein and lipid. Their juice is recognized for its nutritive value, mineral and vitamin contents, important source of bioactive compounds like phenolics (flavanone glycosides, hydroxycinnamic acids), vitamin C and carotenoid, which is an excellent source of bioavailable AO phytochemicals and improves blood lipid profiles especially for people affected with hypercholesterolemia [69]. Fruit juices are liquid, non-alcoholic products with the certain degree of clarity and viscosity obtained through pressing or breaking up of fruits with or without sugar or carbon dioxide addition. Fruits and its juices constitute one of the most important foods for

human. Their regular consumption maintains health and makes up for the losses in the human diet. Costescu *et al.* [70] recommended the consumption of juices with pulp from nutritional and medicinal points of view.

Sensory quality attributes and nutritive value of fruit play an important role in consumer satisfaction and influence for further consumption. Sensory ratings of fruit juice by-products and physical measurements of fruit juice properties are useful methods in the evaluation of fruit juice quality. Sensory quality is a difficult concept to define; it should be comprehended as the interaction between the product and the consumer. It is necessary to establish a relationship between the physical and chemical composition of the product and its sensory attributes, such as color, texture, aroma (volatile compounds) and taste (sweet, sour, salty and bitter sensations) as well as between the sensory perceptions and the acceptability for the consumer [69].

Several factors have to be considered when assessing the fruit juice quality, such as composition that depends on the variety, origin, and growing conditions of the fruit, its quality, processing and stored procedures. Apart from the its nutritive value, it should have acceptable organoleptic and physicochemical characteristics as well as free from microbial and chemical contaminants.

The physicochemical characteristics of juices considered in the quality assessment are pH, titratable acidity (TA), total soluble solids (ºBrix), dry matter content, ash content, crude protein, ascorbic acid (AA), total sugar, reducing sugar and ºBrix (sugar)/acid ratio. The predominant constituent of juice is water, and also carbohydrate, sucrose, fructose, glucose, sorbitol and a small amount of protein. Fruit juices have a low pH (2-5) since they are rich in a mixture of organic acids, whose composition varies depending on fruit nature and maturity. The total soluble solids (TTS) content is significantly influenced by the combined effect of stages of maturity and ripening conditions [69].

2.1.3.2 Microorganisms and Juices

Fruit juices contain a microflora which is normally presented in the surface of fruits during the harvest and postharvest processing including transport, storage, and processing [71, 72]. Many microorganisms, such as acid tolerant bacteria and fungi (molds, yeasts) use them as a substrate for their growth. The major genera include *Candida*, *Dekkera*, *Hanseniaspora*, *Pichia*, *Saccharomyces*, and *Zygosaccharomyces*. *Penicillium*, *Byssochlamys*, *Aspergillus*, *Paecilomyces*, *Mucor*, *Cladosporium*, *Fusarium*, *Botrytis*, *Talaromyces*, and *Neosartorya* are filamentous fungi most frequently isolated from fresh fruits and juices. Among bacteria, lactic acid bacteria and acetic acid bacteria have been isolated from fruit juices [72, 73].

The critical factors affecting the spoilage of juices include juice pH, oxidation-reduction potential, water activity, availability of nutrients, the presence of AM compounds, and competing for microflora. The spoilage caused by microorganisms in juices includes cloud loss, development of off-flavors, CO_2 production, and changes in color, texture, and appearance resulting in degradation of the product [72, 74, 75]. The most commonly reported bacterial genera include *Acetobacter*, *Alicyclobacillus*, *Bacillus*, *Gluconobacter*, *Lactobacillus*, *Leuconostoc*, *Zymomonas*, and *Zymobacter*. Among yeasts, *Pichia*, *Candida*, *Saccharomyces*, and *Rhodotorula* have commonly encountered genera responsible for spoilage of juices [76]. Certain common molds, such as *Penicillium* sp., *Aspergillus* sp., *Eurotium*, *Alternaria*, *Cladosporium*, *Paecilomyces*, and *Botrytis* have been reported in spoilage of fruit juices [72–74].

Microbial contamination decreases the juice shelf-life and increase the risk of food borne illness. Hence, there is a critical requirement in food packaging industry for effective methods to inactivate or eliminate spoilage and food borne pathogens.

2.1.3.3 Packaging Fruit Juices

In earlier days, fruit juices were packed in glass containers, as it has many attractive features with excellent protection quality. The traditional glass bottles used for fruit juices and fruit juices beverages provide many advantages, such as inertness, easy cleaning, durability, rigidity and very good barrier properties [77]. Due to heavyweight and brittleness, only small volumes of juices are still packed in glass bottles.

Nowadays, tinplate containers, tin-free steel or chromium-coated steel plate and aluminum cans have been used for packaging of fruit juices. Although tin is considered to be a nonpoisonous metal, its presence in large concentrations causes serious digestive disturbances. Some years ago, polyethylene (PE)/paper/PE/aluminum foil/PE laminate used to form in-line boxes for aseptic packaging is the path-breaking development in the packaging of beverages. It provided a combination of protection, lightweight, alongside economic solutions. Several plastics have been used in packaging, such as high and low-density polyethylene (HDPE, LDPE), polystyrene (PS) and polyethylene terephthalate (PET), the last two to produce bottles and cups. PET can also be laminated with HDPE or EVOH to produce films with very good barrier properties [77].

The use of plastics in beverage packaging is increasing due to the inherent low cost and functional advantages, such as thermo-sealability, microwavability, weight, optical properties, and unlimited sizes and shapes, compared to traditional materials, such as glass and tinplate. Plastic materials can be manufactured either as a mono-film or a combination of multiple polymers by co-extrusion or lamination [77].

2.1.3.4 Shelf-life of Juices

The progress on beverage markets for fruit juices and soft drinks also encompasses the development of suitable packaging able to provide physical support an acceptable shelf-life for the contents. Traditionally, long-life fruit juices and carbonated products were packaged in the glass. Recent solutions use a form of plastic container, plastic laminated paperboard or a flexible packaging. Metallic cans still provide an important alternative to the other types of packaging.

It is self-evident that the primary function of any beverage packaging, which must include the closure, is to provide the physical retention of the contents. Container leakage may also result in damage to other properties; thus, the primary evaluation of beverage packaging is concerned with the retention of liquid content. This performance characteristic is determined not only by the container itself but also by the effectiveness of the seal between container and closure. However, it is not usual to produce containers with a defect in the body that permits leakage. For packages that are produced on line, such as in "form-fill-seal" operations, there is a significantly increased risk of the failure of seals and, therefore, an appropriate regular quality check is required [78].

Assuming that the contents of a beverage container are retained satisfactorily, there are other quality parameters, such as, protection against external contamination, light effect, and limitation or prevention to oxygen permeability. These factors determine the suitability of the beverage/packaging system [78].

The performance of any package is measured by its ability to keep the contents in a condition as close to the taste, appearance, and nutritional qualities or other additional required characteristic, within the period between the manufacture and expiration dates [78].

Juice can last from weeks to months beyond the date printed on the label since the shelf-life of fruit juice depends on a variety of factors, such as the type of juice, the best by date, how the juice was stored, the packaging and the actual content of the juice package. The term "juice fruit" is a broad term that can mean anything from 100% fruit content to less than 1% fruit content with a lot of added sugar that greatly increases the shelf-life. Juices that are mainly fruit will spoil sooner than with added sugar and preservatives, as the natural sugars in the fruit will begin to ferment over time.

Food and drink can last for a shorter period of time if they are not stored properly. Juice should be stored in a cold dark place (like inside the pantry) and once opened it should be stored in a tightly closed container to keep out moisture and contaminants. Some benefits of proper food storage include eating healthier, cutting food cost and helping the environment by avoiding waste [79]. Long life juices usually keep their properties for 6-12 months on a sealed package, and do not require chilling if a pasteurization

process is applied alongside a proper packing method. Short life juices have a shelf-life up to 30 days and must be kept chilled [80].

The unopened juice package shelf-time depends on the storage conditions, which is maximized in cold and dry places. At room temperature unopened juices packaging, properly stored, will generally stay at best quality for about 12-18 months, although it is safe to consume after that. The manufacturer storage time is an indication of the period where the food can maintain its original properties, like texture, color or flavor[81].

2.2 Preparation of Multilayer Films Packaging

One of the most common processes to produce films is by film blowing extrusion, this is widely used to manufacture thin biaxially-oriented thermoplastic films [82]. The semi-crystalline polymers have two transition temperatures, glass transition (T_g) and melting point (T_m), and that the maximum crystallization rate occurs in the interval between the two, although closer to T_m.

In a typical tubular film manufacturing line, the film (with annular cross-section) is extruded vertically upwardly, through a tower containing a tent that systematizes it, and a pair of pull rollers which determines its linear speed and thereafter down again until it reaches a winding unit. As between the die exit and the pull rollers the interior of the film forms a closed volume, the inflation of air by the axis of the extrusion direction causes the formation of the bubble. Once the desired bubble diameter has been reached, the air to be subsequently blown is only to compensate for any pressure losses therein. After a few initial centimeters, the diameter progressively increases, and the bubble cylindrical shape is kept due to the film solidification upon being cooled by an annular jet of cold air from an outer ring positioned above the lips of the die. It is readily understood that the construction of the cooling ring is complex, since it is the task of the aircraft to uniform the air velocity around the perimeter, although it is supplied to it through a relatively small number of individual inlets [83]. The melt leaving the die must be cooled as quickly as possible to solidify and, in most cases, maintain high brightness and transparency.

The process is very flexible in terms of produced film dimensions, while the diameter of the bubble is determined by the inflation pressure, the thickness of the film that can be adjusted by screw rotation speed of the and/or the pull rollers (Figure 2.4). The operating strategy should consider the induced molecular orientation (or rather, the balance between longitudinal and transverse molecular orientation) [83].

The height of the line is determined by the cooling capacity of the bubble (if it is planned at too high temperature, the tightening pressure of the pull rolls welds the inner faces of the film). In practice, to produce significant flows, the towers of the tubular film extrusion lines can reach heights equivalent to those of buildings with 3 to 4 floors for bubble with one to three meters in diameter, with thicknesses of a few tens of microns, that is, containing significant volumes of air under pressure. This is a process that is particularly sensitive to the environmental conditions, both temperature and convective currents that can destabilize the bubble [83].

The film essentially cools from the outside to the inside, that is, as the film layers near the inner surface cool more slowly, there are conditions for increased spherulite growth - in case the material is partially crystalline, the which affects the brightness and transparency of the film [83].

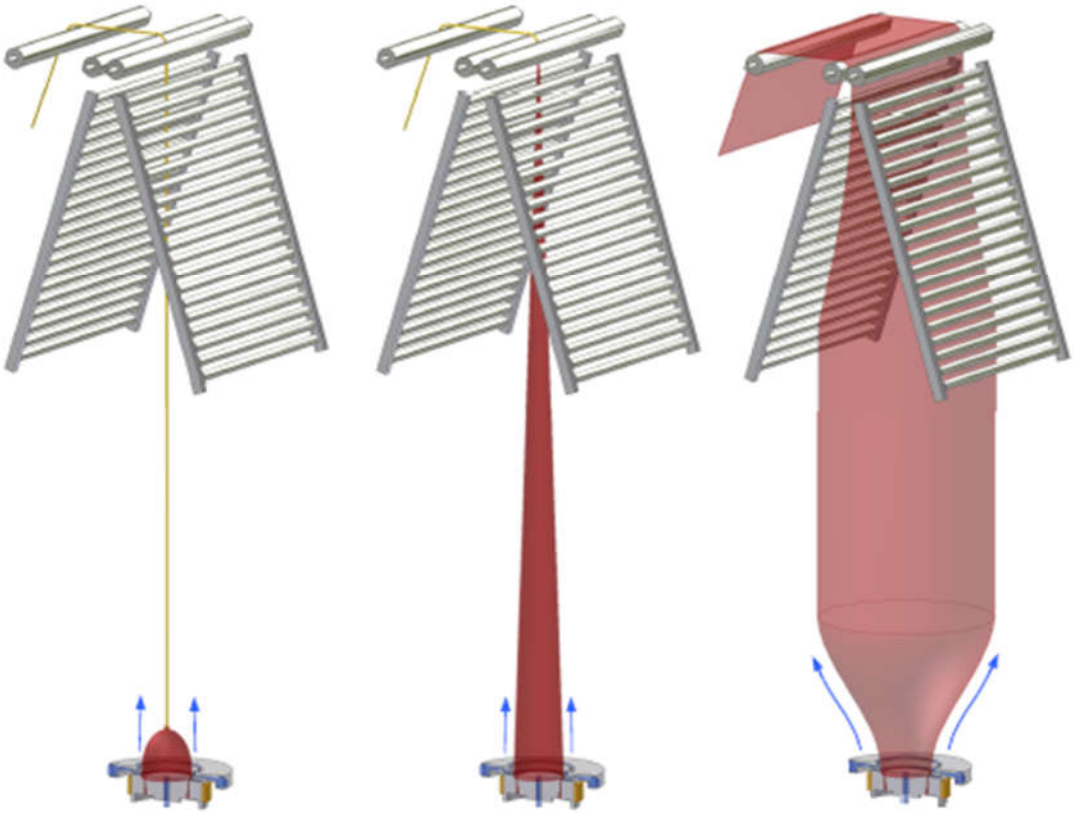

Figure 2.4 - Procedure used to start the film blowing process [82].

The blow-up ratio and draw-down ratio are two of the most important parameters for the blown film extrusion process.

The blow-up ratio represents the size of the melt stretching in the transverse direction and is expressed as the ratio of the final bubble diameter at the freeze line height to the bubble diameter at the die exit. Die diameter is fixed and it is identified by the producer. The most frequent blow-up ratio is in the range of 2 to 10 [82].

The draw-down ratio is characterized by the film stretching in the machine direction and expresses the total stretching degree because the thickness reduction occurs simultaneously in the transverse and machine direction. The draw-down ratio describes the thickness reduction from the die gap thickness to the final film thickness [82].

The blow-up ratio together with the draw-down ratio describes two directions of the molten bubble extension. This is one way to change the film thickness. The second extension direction is circumferential and is generated by the air pressure inside the bubble. So, the axial and circumferential extensions produce the final shape and thickness of the bubble [82, 84].

Film thickness is determined by the nip roll speed and also by the internal air pressure. After the dimensions control the final film can be one-side or two-side split to produce a thin film. Finally, the film is spooled on the cylinder of a wind-up device where it is cut on a required length by a radial cutting mechanism [82, 85, 86].

Plastic packaging generally consists of a combination of different materials to meet all demanding requirements. Since each layer has its functionality, the combination of layers allows to produce a film structure with enhanced properties.

The mechanical and barrier properties can be improved by increasing the adhesion on the interphase between the immiscible components. Additives can also be used to enlarge physical properties like viscosity modifiers, plasticizers, etc. Likewise, mineral fillers (e.g. $CaCO_3$, talc) are often used to optimize mechanical properties or to reduce cost [87].

The most widely used polymers to produce mono and multilayer films packaging are: PET, LDPE, ethylene-vinyl acetate (EVA), linear low-density polyethylene (LLDPE), EVOH, nylon, high-density polyethylene (HDPE), polyamide (PA) and PE.

PA for example, is frequently used in multilayered structures because of its excellent barrier properties to gases, its tear resistance, and strength. But the hygroscopic behavior of PA makes the polymer permeable for humidity. To overcome this, PA is often combined with polyolefins, which have a good moisture barrier, low cost, great salability, outstanding toughness and flexibility [87].

2.3 Properties of Multilayer Films Packaging

2.3.1 Mechanical Properties

Mechanical properties of packaging films and flexible packaging are important to maintain their integrity and endure external stress during processing, transport, storage and handling of packaged materials [61, 88, 89]. Sufficient mechanical strength and extensibility are generally needed for use in food packaging applications [89].

The mechanical properties of the packages will depend on the characteristics of each individual material, the adhesion characteristics, as well as on the process conditions employed, that compromise the integrity of the packaging, barrier properties, and mechanical strength. Moreover, changes in the heat-seal strength may occur, which is strongly influenced by the properties of the inner layer of the packaging material and is fundamental to keep the integrity of the package to preserve the food quality [96].

One of most important mechanical property is the coefficient of friction.

Coefficient of friction, commonly referred as COF, is a ratio of the force required to move one surface over the other to the force applied normally to the surface, i.e., COF measures how slick or slippery the surface is [90]. Therefore, COF provides a relative indication of frictional characteristics, allowing manufactures to optimize performance and prevents problems in forming, transporting, and storing of packages [91].

During the production of flexible packaging materials, frictions can occur between different surfaces (Inner/Inner, Inner/Outer, Outer/Outer) in different processes (unwrapping, bag making, product filling or heat sealing). The effect of COF of different surfaces also varies to the final packaging materials. For example, if COF I/I is too low, the packaging materials may slip and cause incorrect sealing edges. Conversely if it is too high, it may be difficult to open the packaging while filling. The COF I/O may influence the unwrapping of packaging materials [92].

COF comprises two stages, static and Kinetic, as it can be seen in Figure 2.5. While static COF is the ratio of force required to move one surface over the other from the instant that motion starts, kinetic COF is the resistance of one surface being dragged across another in motion [90].

COF values range from 0.00-1.00, values of COF<0.25 indicates lower resistance to sliding (high slip), while COF>0.45 indicates high resistance (low/non- slip) [91].

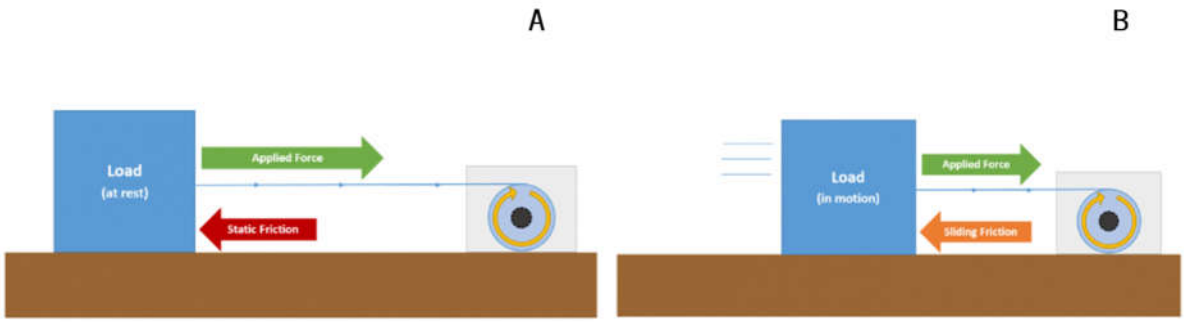

Figure 2.5 - Coefficient of friction (COF). A – Static COF and B – Kinetic COF, adapted from [90].

Customized COF's are achieved by adding a "slip agent" to the polymer during production. This additive is incompatible with the polymer and will migrate to the surface of the film over time, thus reducing COF. Non-migratory slip agents offer benefits in thermal stability and consistency but can affect film clarity [91]. COF can be affected by several factors including anti-block additives, corona treatment, anti-stats, inks, varnishes and adhesives [91, 92]. Since laboratory tests cannot simulate every element of any packaging process, COF should not be considered as an indicator of the system – specific performance. It is, instead, a way of reproducing frictional properties shown to be successful under a given set of conditions [91]. COF number is a great tool to evaluate and to specify flexible packaging and label constructions. It is important due to keeping the correlation between the material real performance and the packaging equipment machine and/or the stacking. Therefore, static COF is used for stacking, and kinetic COF to evaluate output on the packaging equipment [93]. COF on distinct surfaces of same material may be different, thus a suitable surface should be selected to measure the COF of flexible packaging materials. The COF evaluation reproducibility depends on the respect to the applied methodology and also the "environmental conditions" to make the analysis. Some factors that could disturb COF reproducibility:

- Surface smoothness or surface roughness;
- Compatibility between surfaces;
- Plastic material composition (slip agents, anti-blocking, pigments);
- Electrostatic energy;
- Surface treatment (corona, flame, plasma, chemical);
- Warehouse conditions (temperature and relative humidity);
- Film manufacturer process.

An example of the importance of COF in packaging is the case of Catty corporation, has solved a variety of wrapping problems through modification of COF. When the wrap was not wrapping tight enough around a customer's chocolate pieces, they lowered the slip on the wrap to allow the ends folds to slide more

easily over one another leaving a tighter overall seal. When they observed errors in another customer's lines while their chocolate piece was transferring from one grabbing mechanism to another, lowered the COF to allow a cleaner release from the grab [90].

Currently the COF test of flexible packaging film is mainly following standard ASTM D1894: Standard Test Method for Static and Kinetic Coefficients of Plastic Film and Sheeting [92].

2.3.2 Barrier Properties

An important requirement to select food-packaging systems is the barrier properties of the packaging material. It includes permeability to gases (O_2, CO_2, N_2, and ethylene), water vapor, aroma compounds and light, which are crucial factors to preserve the food quality. For example, a good barrier to moisture and oxygen preserves the product crispy and freshness and reduces oxidation.

The factors that affect the barrier properties of the polymers are:

- Affinity with the permeant;
- Crystallinity, and consequent orientation - the more crystalline the polymer, the more oriented it will be, then the better physical properties, and thus the better barrier properties;
- String rigidity;
- Sensitivity to humidity;
- High glass transition temperature (T_g) [94].

Plastics allows the transportation of compounds from package to the environment (and the opposite) due to permeation, migration, and absorption, Figure 2.6.

The quality and shelf-life of plastic-packaged food depends mostly on the physical and chemical properties of the polymeric film and the interactions between food components and package during storage. Several studies showed that substantial amounts of aroma compounds can be absorbed by plastic packaging materials, resulting in loss of aroma intensity or an unstable flavor profile. Absorption might also indirectly affect the food quality by producing delamination of the multilayer packaging or by changing the barrier and mechanical properties of plastic packaging materials.

In contact with the packaged product, oxygen can oxidize fats, denature proteins, decompose vitamins and affect taste, odor, and color [94, 95]. The barrier requirements must be defined for each product according to their composition, presentation form, distribution and useful lifetime. For some products, it is necessary to use a packaging with barrier properties to the transfer of gases, this type of barrier is used in foods that may lose their specific aroma (e.g. coffee) or when it comes to foods (e.g. fresh meat

or fish) in which the dehydration packaging, where the gas mixture introduced for help in product conservation, do not get lost [95].

Not only is the O_2 barrier fundamental, but also the CO_2 barrier is important, especially in carbonated beverages, because CO_2 must remain and should not be release through the packaging, to preserve the quality of the drink [94].

In 1990, according to Sadler and Braddock (1990), it was possible to verify through its study that attachment of volatile molecules at the polymer surface (adsorption) might hinder oxygen permeation, which would lower the oxygen permeation, or leave it unaffected. The increased oxygen permeability of LDPE indicated that the absorption of volatiles must be responsible for structural changes in the polymer. Flavor absorption can have a major impact on the oxygen permeability of plastic packaging materials, and therefore on the shelf-life of a food product, making it necessary to investigate this important aspect more carefully [96].

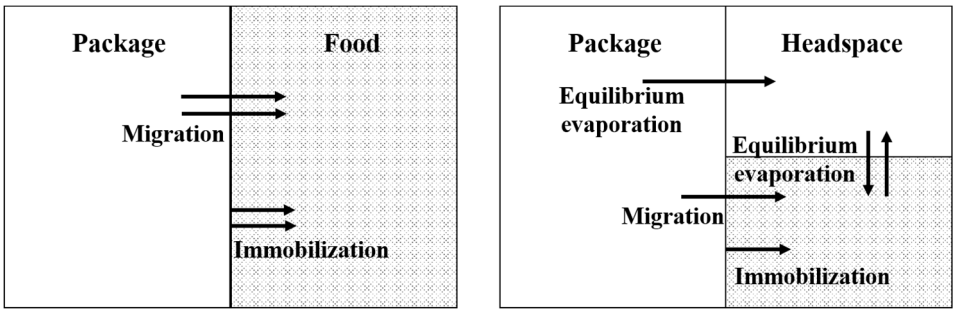

Figure 2.6 - Food packaging systems and migration phenomena, adapted from [97].

2.4 Sustainability/Recycling of the Package after Life-Time

Polymer-based multilayer packaging materials are commonly used in order to combine the respective performance of different polymers. By this approach, the tailored functionality of packaging concepts is created to sufficiently protect sensitive food products to extend shelf-life. However, due to incompatibility, most multilayers cannot be recycled and usually are incinerated or disposed in landfill, counteracting the efforts towards a circular economy and crude oil independence [98].

A proper end-of-life treatment of post-consumer packaging waste comprises three steps: collection, sorting and reprocessing. An appropriate collection is a prerequisite for effective sorting and collection. The sorting process of postconsumer waste is essential to generate a decent amount and quality of a recycling material [98].

Overall, there are two ways to recycle multilayered packaging: the first option is to separate the different components and make them available for recycling in separated recycling streams, the second option is to process the used components together in one compatibilization step.

For the separation of the different components, two methods are applied: a separation can be performed either by delamination of the system or by selective dissolution-precipitation of the different components. While the delamination methods can be based on the chemical decomposition of an inter- or adhesive layer, methods based on selective dissolution and methods based on the combined processing of the different constituents can be described by the thermodynamics of polymers solutions [98].

Mechanical recycling will lead to the formation of polymer blends, but the immiscibility between different polymers has a negative effect on the mechanical properties and processability, which turns an efficient mechanical recycling quite challenging [87].

Tandt *et al.* [87], examined the effect of different additives on a possibility for open-loop recycling of a post-industrial multilayered waste stream. Therefore, a three-layered film consisting of PA and PE, initially used for the sheet molding compound process, were investigated and subjected to an intensive mechanical and rheological characterization. Additionally, the waste was melt-blended with different additives to improve both mechanical and rheological properties as well as the processability. The different upcycled blends were then used to produce injection molded samples to determine a difference in the properties and processability. Improvements in flow properties were observed by adding both polyolefin elastomer (POE) and/or talc, which significantly improved the processability into injection molded test samples. The presence of talc also increased the stiffness of the material, but significantly decreased the elongation at break and impact strength. The same trends, although at a lower extent, were observed for when POE was added [87].

In 2003, a method was introduced by Mäurer *et al.* [99], for the treatment of polymer-containing waste, which was also specified for packaging recycling. In the case of packaging recycling, the method aimed to recover PE, since it is the polymer component with the highest value-adding potential, due to its higher mass fraction in the film. PE can be recovered in a quality close to that of the virgin material, while the undissolved components remain as a residue of little value. Solvents applied by Mäurer *et al.* [99] are not classified as dangerous materials and, therefore, do not require special labeling. In 2017, it was announced that this method was going to be performed in a pilot scale in Indonesia to recycle plastic sachet waste from landfills.

Lindner *et al.* [100], described a method to obtain LDPE from presorted plastic films, consisting of four steps for the removal of inks, removal low-molecular-weight components, the solution of the film

components, precipitation and removal of undesirable polymers, and finally recovering of LDPE from the solution. A patent specification from 2000, in WO 2000077082 A1 [101], the polyolefin components were dissolved from commingled postconsumer plastic packaging and separated from the undissolved residue by common mechanical separation technologies. Subsequently, the different polyolefins, HDPE, PP, and LDPE were successively precipitated from the solution by crystallization, by applying shear forces at different temperatures, while oligomers, inks, and additives were intended to remain in the solution. WO2005118691A2 [102], a patent specification from 2005, described the recycling of PA-6 and PA-6,6 commingled polyolefin polymer waste. By blending the waste with an ester solvent and heating the mixture to a temperature above the melting temperature of the contained polymer, an ester solvent composition with dissolved polyamide and a separate immiscible liquid polyolefin phase was formed. The separation of the discrete molten polyolefin phase from the ester solvent composition could be performed by skimming, decantation, filtration, centrifugation, or combinations thereof [102].

One advantage of the dissolution-precipitation method is that the input does not have to run through a complex sorting scheme. Furthermore, the precipitated polymer can be expected to be of very high-quality, even competing with the virgin polymer. The biggest drawbacks of this method are the energy-intensive drying of the polymer and the fact that all polymer components that do not dissolve remain as a residue of little value [98].

The mechanism of multilayer delamination can be induced physically by the dissolution and mechanically by the decomposition of an interlayer or by reactions at the interface [98]. In 2015, Lovis *et al.* [103], introduced a method that is based on a microemulsion including swelling agents, carboxylic acids, water, and surfactants. These microemulsions had low enough interfacial tensions to penetrate the interphase and neutralize adhesion [103].

A method introduced by Patel *et al.* [104], addressed a multilayer packaging consisting of PET and PE, sulphury acid with concentrations ranging from 68 to 98% was used to degrade the PET component. The PE film was not affected and could be reused after several washing steps.

The blending of polymers is an approach to recycling polymer-based multilayers without components separation. A compatibilization process can improve the blend performance by making blend components more miscible through the addition or *in situ* generation of copolymers that act at the interface, which decrease de interfacial tension and decrease the particle size. Therefore, the material can be considered macroscopically homogeneous [98].

The blending of polyolefins with PA produces thermodynamically immiscible two-phase systems. In literature, several techniques for the compatibilization of those blends can be found, a method commonly

used to induce the compatibility between PA and polyolefins is by addition of the polyolefin component grafted with maleic anhydride (PO-*g*-MA). This concept was employed by Choudhury *et al.* [105], in 2006 to recycle a postconsumer oil pouch material consisting of a coextruded film made of LDPE, LLDPE, and PA-6. During the melt extrusion of the pouch components together with the compatibilizer, intermolecular reaction between the primary amine end group of PA-6 and MA groups take place, Figure 2.7.

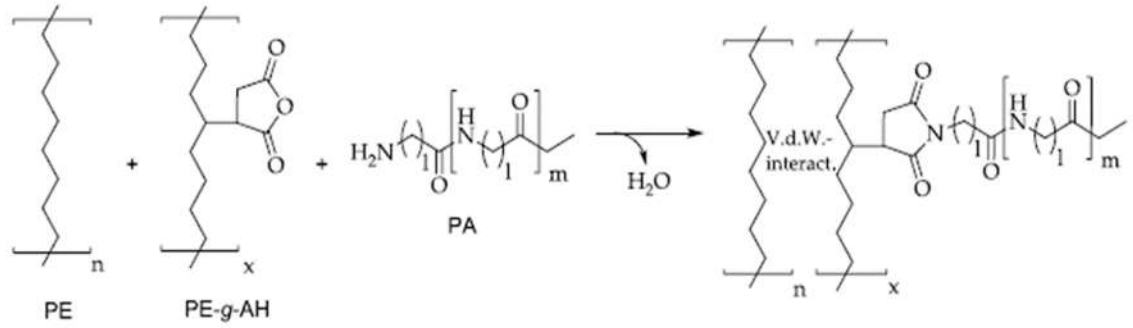

Figure 2.7 - Compatibilization mechanism of PE and PA using PE-*g*-MA [98].

With this method, tensile strength, hardness, and percent elongation at break of the compatibilized blend could clearly be improved by the increased interfacial adhesion [105].

In general, to recycle multilayered packaging items describes two ways: the first option is to separate the different multilayer components and make them available for recycling in separated recycling streams, the second option is to process the used components together in one compatibilization step, as can be seen in Figure 2.8.

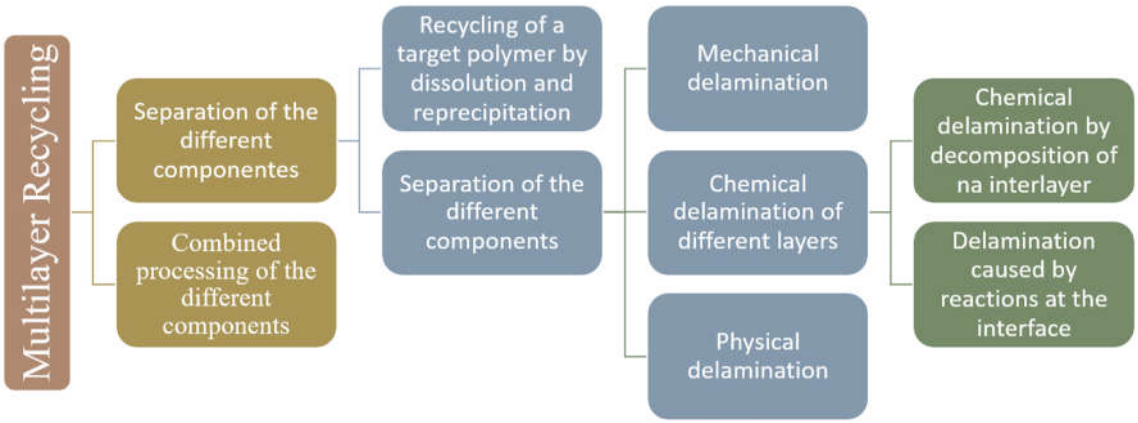

Figure 2.8 - Schematic overview of the introduced recycling methods of multilayer packaging, adapted from [98].

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3 EVALUATION OF PLANT EXTRACTS AS POTENTIAL ADDITIVES FOR ACTIVE FOOD PACKAGING

This chapter reports on thermal and biological properties (antioxidant and antimicrobial) of natural extracts investigating their potential to be incorporated in the polymeric matrix for food packaging.

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Abstract

Natural aromatic extracts are used in several traditional medicines and also for culinary purposes around the world, to add taste or enhance the diet quality. The biological properties, such as antioxidant and antimicrobial activities of extracts of aromatic plants are due to the several active compounds that they have in the composition. Herein, different aromatic natural extracts (rosemary, anise, lemon balm, green tea, curcumin, clove, and cinnamon) were evaluated to assess their potential use as additives for active food packaging. While rosemary extract presents the higher thermal stability with a degradation starting only at 327 ºC, lemon balm extract was the less stable with an onset degradation temperature of 180 ºC. For the thiobarbituric acid reactive substances assay, all extracts presented antioxidant activity, except clove extract, while for the oxidative hemolysis inhibition assay, the extracts that did not present activity were anise extract, cinnamon extract, and clove extract. Cinnamon, lemon balm, and curcumin extracts exhibited slight cytotoxicity against non-tumor cells. Natural extracts from aromatic plants are promising antioxidant and antimicrobial natural additives that can be incorporated into polymeric matrices to produce active food packaging that will increase the products shelf-life.

Keywords: Aromatic extracts; Antioxidant; Antimicrobial; Cytotoxicity; Active food packaging

3.1 Introduction

Food safety is one of the main issues in food industry and apart from spoilage of foodstuff, there are always concerns about the outbreak of foodborne illnesses among food manufactures, regulatory agencies, researchers, and consumers. Therefore, food industry priority is to produce safe food [106]. Foodborne illness resulting from the consumption of foods contaminated with pathogenic bacteria and yeasts have been of great concern of public health. Nowadays, consumers are looking for natural and healthier products, decreasing the consumption of synthetic [107]. A new trend in food packaging is the incorporation of bioactive natural compounds in package materials to preserve food quality and extend its shelf-life [59].

Aromatic plants are strongly linked to the human civilization and its evolution. Since ancient time, plants have been used in several traditional medicines, such as Indian and Chinese, and also in all cuisines around the world as condiments or to enhance diet. Pharmaceutical industry uses more than 25% of the products worldwide derived from plants. Several active compounds responsible for their biological

properties, such as antioxidant and antimicrobial are part of extracts of aromatic plants. Their constitution depends on several factors, such as the extraction method, solvent used, plant edaphoclimatic conditions, chosen plant part, among others. Phenolic compounds are responsible for plants biological properties and are present in the majority of edible plants. These compounds are part of the secondary metabolites of plants, contributing for their protection against ultraviolet radiation and against pathogens, parasites, and predators. They are responsible for the color and organoleptic properties contributing, for example, to the better taste of fruits. Their distribution through the plant is not quantitatively the same, varying according to the plant part [60]. Above one hundred extracts from aromatic plants have been approved by FDA (Food and Drug Administration) as GRAS (Generally Recognized as Safe). The extracts are prepared in order to have compounds with relevant biological properties and be in its most purified form. They have been used as food additives to stop or inhibit lipid oxidation and the growth of pathogen microorganisms.

Therefore, in this study, aromatic extracts from different plant parts (leaves, flowers, and seeds) were selected to evaluate their antioxidant and/or antimicrobial properties. Table 3.1 presents the selected plants and the part from which the extract was obtained, as well as its main properties described in literature.

Rosmarinus officinalis (rosemary) (RE), originated from the Mediterranean region, is categorized as a woody and aromatic plant. Their leaf extracts have been used in traditional medicine, to treat several human diseases and in food preservation. The several RE bioactivities include antioxidant, anti-inflammatory, antimicrobial, hepatoprotective, and antidiabetic properties. Caffeic acid derivatives, like rosmanaric acid that has the main content in phenolic compounds, is responsible for the medicinal properties [108]. The European Commission has approved the use of RE as food additive E 392 by the Directives 2010/67/EU and 2010/69/EU [60].

Green tea extract (GTE) is taken from the leaves of *Camellia sinensis L.* and is recognized by its antioxidant, antimicrobial, anticarcinogenic, and anti-inflammatory properties. GTE is a rich source of polyphenol antioxidants, particularly catechins, being the major catechins (+)-catechin (C), (-)-epicatechin (EC), (-)-catechin gallate (CG), (-)-epicatechin gallate (ECG), (-)-gallocatechin (GC), (-)-epigallocatechin (EGC), (-)-gallocatechin gallate (GCG), and (-)-epigallocatechin gallate (EGCG) [109]. These compounds are responsible for its antioxidant capacity together with gallic acid, GTE also contains other flavonoids and phenolic acids, but in lower proportion.

Lemon balm (*Melissa officinalis*) (LBE) is a plant, belonging to Lamiaceae family, used in traditional medicine. Even though, native from the Mediterranean region, this plant contains high levels of phenolic

acids, mainly hydroxycinnamic acid derivatives, like rosmarinic acid and can cultivated all over the world. It is used as aromatic, antimicrobial, antioxidant, and antiseptic additive for food and drugs applications [109, 110].

The most largely extracts natural preservative used in food are Cinnamon (CE) and clove (CLE). CE is mainly composed of cinnamaldehyde and has good inhibitory effect on many food spoilage microorganisms [111]. The antioxidant, anti-inflammatory, and antitumor are other properties reported in several studies [111–113]. CE cam have several applications, like traditional kitchen, incense, perfumes, and pharmaceuticals products [113]. Eugenol is the major active component of CLE and has strong antibacterial, antioxidant, and insecticidal effects [111, 112, 114]. Both CE and CLE are natural preservatives and flavor substances, which are safe to consume and have been approved by the FDA and the European Commission as natural food additives [61, 111, 112].

Anise (*Pimpinella anisum*) (AE) is a plant from the Umbelliferae family and is a popular aromatic herb and spice from ancient times, its seeds are used in folk medicine and as food ingredient for cooking. Chemical studies demonstrated that the AE contains anethole, estragole, eugenol, pseudoisoeugenol, methylchavicol, anisaldehyde, coumarins, scopoletin, umbelliferone, estrols, terpene hydrocarbons, polenes, and polyacetylenes. Anetholes are the main active compounds responsible for the antimicrobial activity [115, 116].

Curcumin extract (CCE) is a natural hydrophobic yellow-orange compound derived from the root of the herb *Curcuma Longa L.*, which is widely used for medicinal and food purposes. CCE exhibits potent antioxidant, antitumor, antibacterial, and anticancer properties [117–120]. Clinical trials have shown that CCE is safe, even when consumed at a daily dosage of 12 g for 3 months [119].

Table 3.1 - The main characteristics of selected extracts.

Extract	Latin Name	Plant Part	Activity	References
LBE	*Melissa officinalis L.*	Leaves	Antimicrobial, Antioxidant and Antiseptic	[109, 110]
GTE	*Camellia sinensis L.*	Leaves	Antioxidant, Antimicrobial, Anticarcinogenic and Anti-inflammatory	[66]
RE	*Rosmarinus officinalis L.*	Leaves	Antioxidant, Antimicrobial, Anti-inflammatory, Hepatoprotective and Antidiabetic	[60, 108]
CLE	*Eugenia carvophyllata*	Flowers	Antibacterial, Antioxidant, Anti-inflammatory, Analgesic, Anti-stress, Antiseptic and Insecticidal	[111, 112]
AE	*Pimpinella anisum*	Seeds	Antioxidant and Antimicrobial	[115, 116]
CE	*Cinnamomium zeylanicum*	Bark	Antimicrobial, Antioxidant, Anti-inflammatory and Antitumoral	[111, 113]
CCE	*Curcuma longa*	Rhizome	Antioxidant, Antitumoral, Antibacterial and Anticarcinogenic	[117–120]

Thus, the aim of this study is to screen in terms of biological properties (antioxidant and antimicrobial) a set of commercial extracts be able to select the most promising ones in terms of potential additive to produce polymeric film for active food package. Therefore, properties assessment was made both in the extract and the extract incorporated in a polymeric matrix.

3.2 Materials and Methods

3.2.1 Samples

Low-density polyethylene (LDPE) was kindly provided by Vizelpas. The green tea extract (GTE), rosemary extract (RE), cinnamon extract (CE), anise extract (AE), clove extract (CLE) and lemon balm extract (LBE) were acquired from ESSÊNCIAD'UMSEGREDO, LDA. Curcumin extract (CCE) (from Curcuma longa (Tumeric) powder, ≥65%) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2.2 Reagents and standards

Potassium Bromide (KBr) (Acros Organics, spectroscopic standard) was used to produce the pellets for Fourier Transform Infrared Spectroscopy. Trichloroacetic acid (TCA), ascorbic acid, iron sulfate, sodium chloride, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azobis(2-

methylpropionamidine)dihydrochloride (AAPH), phosphate buffered saline (PBS), hydroxymethylaminomethane buffer (Tris-HCl), dimethyl sulfoxide (DMSO), and 2-thiobarbituric acid (TBA) were purchased from Sigma Aldrich (St. Louis, Missouri, EUA) and used in OxHLIA and TBARS assays. For cytotoxic assay, ellipticine, sulforhodamine B (SRB), Hank's balanced salt solution (HBSS), trypan blue, nonessential amino acid solution (2 mM), tris-(hydroxymethyl)aminomethane (TRIS), RPMI-1640, fetal bovine serum (FBS), and penicillin/streptomycin solution (100 $U.mL^{-1}$ and 100 $mg\ mL^{-1}$, respectively) were acquired from Gibco Invitrogen Life Technologies (Paisley, UK). The culture media Muller Hinton broth (MHB) and Tryptic Soy Broth (TSB) were purchased from Biomerieux (Marcy l'Etoile, France) was used antimicrobial activity. Blood agar with 7% sheep blood and Mac Conkey agar plates were purchased from bioMérieux (Marcy l'Etoile, France). Antifungal agents, ketoconazole and bifonazole, were purchased by Srbolek, Belgrade, Serbia and Zorkapharma, Sabac, Serbia, respectively.

3.2.3 Films and extracts preparation

The extracts were dried at 60 °C overnight under vacuum and then incorporated in an LDPE matrix. LDPE with 2 wt.% of each extract was prepared in the Xplore MC15 micro single screw extruder at 145 °C, 90 rpm and a residence time of 2 min. From the prepared materials, thin films were produced by compression molding in a hot press at 140 °C under a pressure of 10 tons.

For bioactive properties evaluation, and due to compounds insolubility in water, the dried extracts were dissolved in a PBS/DMSO mixture (95:5, v/v), and the recovery of the extracts from the films obtained by grinding followed by shaking during 2 days in 20 mL PBS with 5% DMSO, getting a 10 $mg.mL^{-1}$ concentration. Successive dilutions were prepared from the stock solutions.

3.2.4 Structural and Thermal characterization

Infrared Fourier Transform Spectroscopy (FTIR) analysis of the extracts and films was performed in a 4100 Jasco (Japan) spectrometer in transmittance mode at 32 $scans.min^{-1}$, 4 cm^{-1} resolution in a wavelength range of 4500-400 cm^{-1}. Each extract (10 wt.%) was mixed with KBr > 99%, to obtain a translucent sample.

Thermogravimetric analysis (TGA) of the extracts was accomplished using a TGA Q500 (TA Instruments, New Castle, EUA) under nitrogen atmosphere at 10 ºC/min in a temperature range from 40-900 ºC.

3.2.5 Antioxidant Activity

3.2.5.1 Thiobarbituric acid reactive substances (TBARS) formation inhibition assay

The capacity of the extracts and films incorporated with the extracts to inhibit the formation of thiobarbituric acid reactive substances (TBARS), such as malondialdehyde (MDA) generated from the ex vivo decomposition of lipid peroxidation products, was evaluated using porcine brain cell homogenates, according to the protocol previously described by the authors [121]. The color intensity of the malonaldehyde-thiobarbituric acid complex, (MDA)-TBA, was read at 532 nm. Trolox was used as positive control and the results were expressed as IC_{50} values ($\mu g.mL^{-1}$), i.e., compound concentration providing 50% of antioxidant activity.

3.2.5.2 Oxidative hemolysis inhibition assay (OxHLIA)

The antihemolytic activity was assessed using the method described by Takebayashi *et al.* [122], with some modifications. Sheep blood samples were gathered from healthy animals and the assay was done as defined in literature [123]. The percentage of erythrocyte population that remained intact over the experimental time and the delayed time of hemolysis (Δt), comparing to the control sample, were calculated. Results were expressed as IC_{50} values ($\mu L.mL^{-1}$) at Δt 60 min, i.e., the compound concentration required to keep 50% of the erythrocyte population intact for 60 min.

3.2.6 Cytotoxic assay

To assess the cytotoxicity of the extracts and films, the sulforhodamine (SRB) assay was performed. The cytotoxicity was evaluated by SRB assay in a primary culture of porcine liver cells (PLP2), as previously described by the authors [124]. Phase contrast microscope was used for direct monitoring of cell cultivation during 48 h. The results were expressed as GI_{50} values, i.e., compound concentration providing 50% of net cell growth inhibition, using ellipticine as positive control.

3.2.7 Antimicrobial activity

The extracts were dissolved in 30% ethanol solution and added to Tryptic soy broth (TSB) medium. The antibacterial activity was evaluated against three Gram positive (*Staphylococcus aureus* (ATCC 11632), *Bacillus cereus* (food isolate), and *Listeria monocytogenes* (NCTC 7973)) and three Gram negative (*Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 35030), and *Salmonella typhimurium* (ATCC 13311)) bacteria. The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations

were determined by the microdilution method. Briefly, overnight bacterial cultures were adjusted using the spectrophotometer to a concentration of $1x10^5$ CFU/mL using a spectrophotometer at 625 nm (OD 625). Extracts testing was carried out in different dilutions over the wells containing 100 mL of Tryptic Soy Broth (TSB) and further, 10 µL of the bacterial suspension was added to all the wells. The microplates were incubated for 24 h at 37 °C. The MIC concentrations were reached by adding 40 mL of iodonitrotetrazolium chloride (INT) (0.2 $mg.mL^{-1}$) and incubation at 37 °C for 30 min. The lowest concentration providing a significant inhibition (around 50%) of the bacterial growth in comparison to the positive control was considered as the MIC. Minimal bactericidal concentrations (MBC) were defined by sub-cultivation of 10 mL into microplates comprising 100 mL of TSB. The lowest concentration that shows no bacterial growth is considered as the MBC. Standard antibiotics, namely streptomycin and ampicillin, were used as positive controls and 5% DMSO was used as the negative control.

The antifungal activity was estimated against six micromycetes: *Aspergillus fumigatus* (ATCC 9197), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Penicillium funiculosum* (ATCC 36839), *Penicillium verrucosum* var. *cyclopium* (food isolate), and Trichoderma viride (IAM 5061). The microorganisms are placed at Mycological laboratory, Department of Plant Physiology, Institute for biological research "Sinisa Stanković", University of Belgrade, Serbia [125]. The MIC and MFC (minimum fungicidal concentrations) were achieved by using the microdilution method. Briefly, the rice extracts dissolved in a DMSO solution were add to broth malt medium containing fungal inoculum. The microplates were incubated for 72 h at 28 °C. The lowest concentrations without visible growth were defined as MIC. The minimum fungicidal concentrations (MFCs) were determined by serial subcultivation of 2 mL in microtiter plates containing 100 mL of malt broth per well and further incubation for 72 h at 28 °C. The lowest concentration with no visible growth was defined as the MFC, indicating 99.5% killing of the original inoculum. DMSO 5% was used as a negative control, while bifonazole and ketoconazole were used as positive controls.

3.3 Results and discussion

3.3.1 Fourier Transform Infrared Spectroscopy

The RE contain several organic molecules that presents aromatic and phenolic groups. FTIR spectrum shows (Figure 3.1 - A) a strong band corresponding to O-H stretching of phenol group (3000-3600 cm^{-1}), C-H stretching (2932 cm^{-1}), C=C ring stretching (1688 cm-1), (1458 cm^{-1}) and C-OH stretching of phenolic

groups (1275 cm^{-1} and 1029 cm^{-1}). The spectrum of CCE exhibited the characteristic absorption bands at 3513 cm^{-1} (OH stretching vibration), 1622 cm^{-1} (carbonyl group (C=O)), 1512 cm^{-1} (C=C bonds), 1426 cm^{-1} (C-H bending vibration), 1278 cm^{-1} (aromatic C-O stretching) and 1032 cm^{-1} (C-O-C stretching vibration). CE presents its characteristic band at 1613 cm^{-1} that indicates the presence of the unsaturated vibration of benzene ring. The band at 1151 cm^{-1} corresponded to the C-O-H stretching of other trace phenolic compounds. The characteristic peaks of LBE were seen at 1448 cm^{-1} (aromatic nitro compounds), 1603 cm^{-1} (amide, carboxylate or aromatic ring stretch), 2926 cm^{-1} (methylene C-H stretch) and 3372 cm-1 (O-H stretching of phenol group). It was also possible to identify the presence of GTE through its characteristic vibrational bands, such as the O-H stretching centered at 3349 cm^{-1}, C=C stretching at 1611 cm^{-1}, O-H bending at 1341 cm^{-1} and the C-O stretching at 1234 cm^{-1} and 1033 cm-1. The FTIR of CLE characterized by eugenol peaks at 3385 cm^{-1} (O-H stretching), 1239 cm^{-1} (C-O bending) and at 1601 cm^{-1} and 1416 cm^{-1} (C-C stretching vibrations in the phenyl ring) and still at 1024 cm^{-1} (C-O-C stretching vibration). In case of AE the FTIR spectrum exhibited characteristic peaks at 3381 cm^{-1} (O-H stretching of phenolic compounds), 2924 cm^{-1} (aromatic C-H stretching), 1631 cm^{-1}(C=C or COO stretching) and 1034 cm^{-1} (C=O).

Figure 3.1 - B clearly shows the successful incorporation of the CLE, through the evidence of its characteristic bands at 1024 cm^{-1} (C-O-C stretching vibration), perceived among the characteristic LDPE bands.

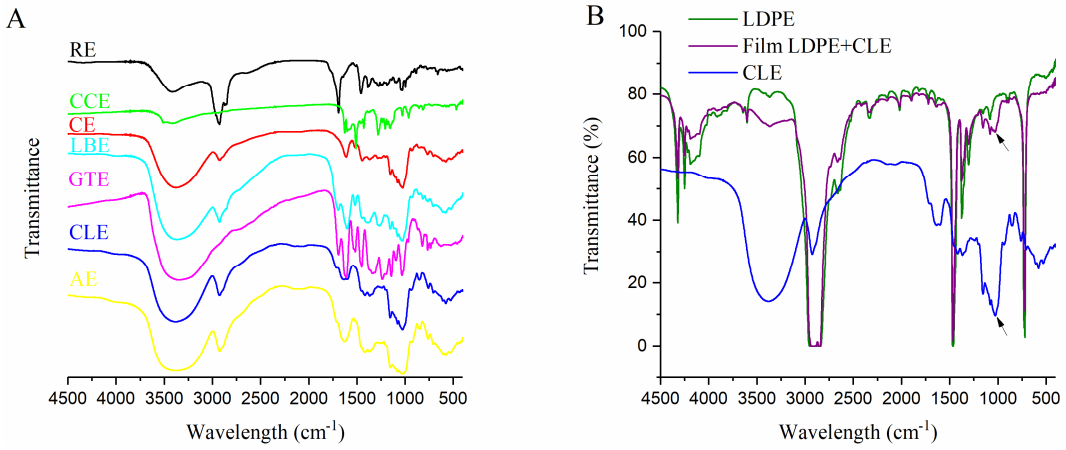

Figure 3.1 - FTIR spectra of various extracts (A) and comparison of extract and extract incorporate in LDPE matrix (B). AE: anise extract, CCE: curcumin extract, CE: cinnamon extract, CLE: clove extract, GTE: green tea extract, LBE: lemon balm extract, RE: rosemary extract.

3.3.2 Thermal Analysis

The thermograms of all extracts are depicted in Figure 3.2, with the exception of CCE and RE since they exhibited a weight loss at low temperature (between 70 and 150 °C), which can be associated to the presence of moisture and/or traces of ethanol, the solvent used to obtain some the extracts.

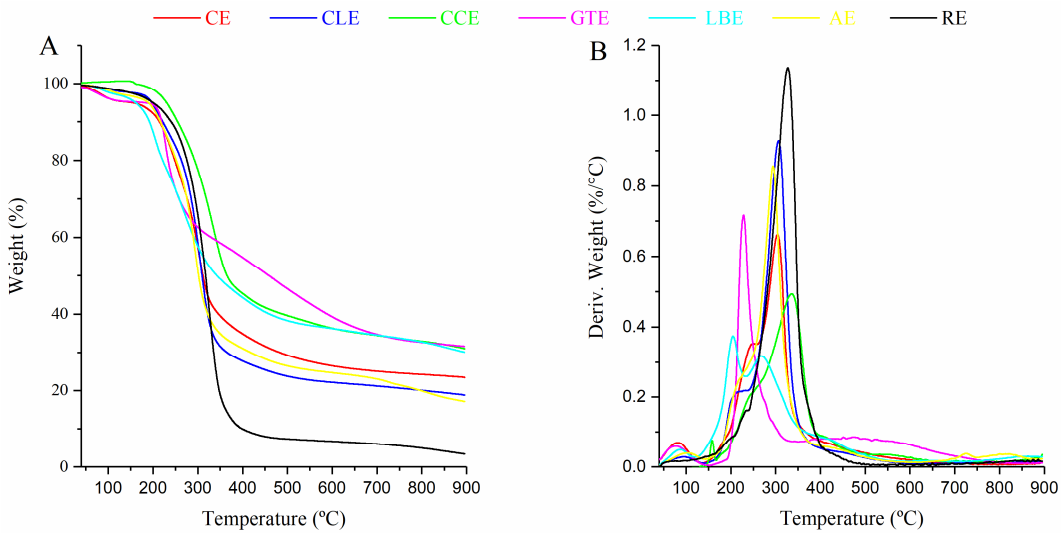

Figure 3.2 - Thermogravimetric (TG) (A) and derivative TG (DTG) (B) curves of different extracts.

Onset of degradation temperature and the residual mass at 900 °C are presented in Table 3.2. While LBE presents the lower onset temperature (temperature of initial degradation), 180°C, CCE, CLE and RE are the ones with higher thermal stability, with onset temperatures above 250°C. GTE, CE and AE exhibit onset values between 210 and 242°C. The differences noticed in the degradation temperatures (temperature of maximum rate decomposition) are due to the volatilization and/or degradation of the compounds contained in each extract. It is possible to observe that LBE presents the lowest thermal stability, showing two degradation peaks, which can be, probably, attributed to the decomposition of bioactive compounds, such as rosmarinic acid. CE, AE, CLE, and RE exhibit a very similar thermal behavior, as can be seen in Figure 3.2 - B, with very close onset and degradation temperatures. The greatest loss of mass verified in the thermogram of CE may be due to the decomposition of one of the main constituents, the cinnamaldehyde, both procyanidins, and catechins. The CLE and AE show mass loss with values of approximately 60% that can be associated with the volatilization/decomposition of bioactive constituents, in case of AE probably from anethole or eugenol and for CLE to eugenol. For RE, between 190 to 398 °C, it has a mass loss of 86% with onset around at 327 °C. This may also attribute to the volatilization/decomposition of bioactive constituents, possibly carnosic acid, and carnosol, as well as from the rosmarinic acid and/or ursolic acid. GTE presented a more complex degradation profile, which is related to the glycosylation of catechins and other components, resulting in a weight loss of 41%.

CCE has greater thermal stability, with the loss of mass associated with terpenoids presents in your constitution, as curcuminoids (diphenylheptanoids), demethoxycurcumin, and bisdemethoxycurcumin. The high values of residual mass at the end of the analysis can be explained by the origin of the extracts, all of them were commercial and were analyzed without further purification.

Table 3.2 - Onset and degradation temperatures and residual mass of the extracts given by thermal analysis.

Extracts	Onset temperature (≈ °C)	Degradation temperature (°C)	Residue mass (%)
AE	242	294	17
CE	231	304	24
GTE	210	228	32
LBE	180	268	30
RE	270	327	3
CCE	255	336	31
CLE	255	306	19

Therefore, through the thermal analysis it is possible to determine that the GTE, AE, CE, RE, and CLE are the extracts that exhibited higher thermal stability. Consequently, they can be incorporated in the polymeric matrix of LDPE since the processing temperature is around 180 °C, which is lower than the onset decomposition temperature of the extracts.

3.3.3 Biological activity

3.3.3.1 Antioxidant activity

The results of the TBARS and OxHLIA assays, presented in Table 3.3, allow to find out the samples concentration providing 50% of the antioxidant activity. The lower IC_{50} values correspond to a higher antioxidant capacity. The OxHLIA assay is a suitable method to study free radical-induced oxidative damage of biological membranes and the antioxidant effects of extracts and correspondent films. Sheep erythrocytes were subjected to the oxidizing action of the hydrophilic free radical initiator AAPH. Initially, the (peroxyl) radicals formed from the thermal decomposition of this oxidizing agent attack the erythrocytes membrane, eventually causing its lysis. Consequently, lipophilic (alkyl) radicals are generated through a lipid peroxidation phenomenon, which also attack the membranes. In fact, the erythrocytes membrane is rich in polyunsaturated fatty acids and, thus, very susceptible to free radical-mediated peroxidation. As shown in Table 3.3, RE has the highest antioxidant activity, with an IC_{50} value of 0.58±0.04 $\mu g.mL^{-1}$; this activity was even significantly higher than the one found for the positive control, trolox (85±2 $\mu g.mL^{-1}$). With the exception of AE, CE, and CLE extracts that did not present antioxidant

activity in this assay, all the extracts revealed a higher antihemolytic capacity than the positive control (1.04±0.07 to 40±3 μg.mL^{-1}). On the other hand, most of the studied film extracts presented the capacity to delay for 60 min the oxidative hemolysis, although in higher concentrations (309±10 to 1603±91 μg.mL^{-1}). As expected, LDPE did not present activity, but similar observations were also made for LBE and CLE. Indeed, none of the CLE (compound or film) was able to promote the hemolysis delay. Regarding LBE, the fact that the IC_{50} value of the compound extract was the highest one, could possibly explain that the film extract did not present activity at the tested concentrations. On the contrary, AE and CE revealed to be more active in the film form.

The TBARS assay provided information on the compounds capacity to inhibit the formation of thiobarbituric acid reactive substances, such as malondialdehyde generated by the ex vivo decomposition of the lipid peroxidation products. Porcine brain cells are used for this purpose, as they are biological substrates rich in polyunsaturated fatty acids. The results (Table 3.3) demonstrate that all the extracts showing activity, except for AE (447.3±0.2 μg.mL^{-1}), were more active than the positive control (139±5 μg.mL^{-1}), with GTE presenting the highest antioxidant capacity (0.65±0.03 μg.mL^{-1}). CLE was the only extract that was not able to prevent the oxidation mechanisms, but was the most active when incorporated in the LPDE matrix (816±31 μg.mL^{-1}), contrarily to LBE and RE that did not present the capacity to prevent lipid peroxidation in the film form.

The results obtained for the extracts and their active compounds are very reliable, since the phenolic compounds are primarily responsible for the antioxidant activity of the extracts, as is the case of GTE, RE, and CCE that are extracts rich in phenolic compounds, such as catechins and carnosic acid, and even in the case of curcumin, which is a diarylheptanoid belonging to the group of curcuminoids that are natural phenols. Thus, as expected, the presence of these active compounds influences the antioxidant capacity of the studied extracts.

The differences found between the antioxidant activity in the form of extract or when incorporated into the polymeric LDPE matrix are possibly associated to the main antioxidant compounds degradation at the processing temperature, or to the inefficiency of the method of extraction of the active compounds from the film.

Table 3.3 - Bioactivity (TBARS and OxHLIA) of different extracts, films incorporated with extracts, and positive control (trolox).

Extracts	Form	TBARS (IC_{50}; $\mu g.mL^{-1}$)	OxHLIA (IC_{50}; $\mu g.mL^{-1}$)*
AE	extract	447.3±0.2	n.a.
	film	1080±26	432±19.
CE	extract	12.4±0.3	n.a.
	film	1086±15	309±10
GTE	extract	0.65±0.03	2.4±0.2
	film	4074±74	704±19
LBE	extract	8.7±0,4	40±3
	film	n.a.	n.a.
RE	extract	5.65±0.07	0.58±0.04
	film	n.a.	1603±91
CCE	extract	7.9±0.01	1.04±0.07
	film	2706±135	352±11
CLE	extract	n.a.	n.a.
	film	816±31	n.a.
LDPE	film	n.a.	n.a.
Trolox		139±5	85±2

n.a.: no activity.

Results are expressed as mean ± standard deviation.

*extract concentration required to keep 50% of the erythrocyte population intact for 60 min (Lockowandt et al., 2019).

3.3.3.2 Cytotoxic activity

The results of the cytotoxicity of the extracts and films incorporated with extracts are shown in Table 3.4. The only extracts revealing toxicity for the primary culture of porcine liver cells were CE, LBE, and CCE, in concentrations of 263.87±20.37, 366.36±25.30, and 141.62±5.01 $\mu g.mL^{-1}$, respectively. Notwithstanding, the GI_{50} values were significantly higher than the concentrations in which these extracts revealed antioxidant properties. Thus, the application of these extracts in lower concentrations can be considered. Regarding the films, none of the studied samples presented cytotoxic properties at the tested concentrations.

The cytotoxicity presented by CE, LBE, and CCE is possibly related to their higher concentration in total phenolic acids and flavonoids. Similar observations were previously made with other extracts, as Portuguese propolis and *Alnus rugosa L.*, and reported by Calhelha *et al.* [126], Rashed *et al.* [127], and Jabeur *et al.* [128].

Table 3.4 - Cytotoxic (GI50 values μg.mL^{-1}) activity of the extracts, films incorporated with extracts, and positive control (ellipticine).

Extracts	Form	Cytotoxic activity (GI_{50}; μg.mL^{-1})
AE	extract	>400
	film	>400
CE	extract	263.87±20.37
	film	>400
GTE	extract	>400
	film	>400
LBE	extract	366.36±25.30
	film	>400
RE	extract	>400
	film	>400
CCE	extract	141.62±5.01
	film	>400
CLE	extract	>400
	film	>400
LDPE	film	>400
Ellipticine		2.31±0.09

Results are expressed as mean ± standard deviation.

3.3.3.3 Antimicrobial activity

The antibacterial activity (Table 3.5) of the different extracts was evaluated against Gram positive (*Staphylococcus aureus, Bacillus cereus*, and *Listeria monocytogenes*) and Gram negative (*Escherichia coli, Enterobacter cloacae,* and *Salmonella typhimurium*) bacteria. Generally, LBE was the most active extract, being able to inhibit all the bacterial strains in a concentration of 1.09 mg.mL^{-1}. All the extracts that inhibited the bacterial growth, also revealed bactericidal capacity at twice the concentration. CE was the only extract that did not show inhibitory nor bactericidal capacity, at the maximum studied concentration (8.50 mg.mL^{-1}).

Regarding antifungal activity (Table 3.6), RE revealed the lowest MIC and MFC against *A. versicolor* (0.55 and 1.09 mg.mL^{-1}), *P. funiculosum* (0.27 and 0.55 mg.mL^{-1}), *P. verrucosum* (0.55 and 1.09 mg.mL^{-1}), and *A. niger* (0.55 and 1.09 mg.mL^{-1}). This latest was also sensitive to the same concentration of LBE. In the case of *P. funiculosum*, RE revealed MIC and MFC values close to the ones found for the positive control, the antifungal drug ketoconazole (0.20 and 0.50 mg.mL^{-1}). All the extracts show inhibitory and fungicidal capacity.

In a study previously performed by Stefanovic and Čomić [129], RE showed antibacterial activity with MIC values in the range of 5-20 mg.mL-1 for *Bacillus subtilis*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Escherichia coli* (Stefanovic and

Čomić, 2012). Nabavi *et al.* [130] demonstrated that CE showed good antibacterial activity against Gram negative bacteria (*E. coli* O157:H7, *Yersinia enterocolitica* O9, *Proteus* spp. and *Klebsiella pneumonia*) with very low MIC values (12.5 μL.mL^{-1}, 6.25 μL.mL^{-1}, 1.5 μL.mL^{-1} and 3.125 μL.mL^{-1}, respectively) [130]. Also, in results reported by Zhang *et al.* [131], CE presented MIC and MBC values of 1.0 and 4.0 mg.mL^{-1} for *E. coli* and 1.0 and 2.0 mg.mL^{-1} for *S. aureus* [131]. Comparing with the obtained results, a higher concentration of extract was required for antimicrobial activity manifestation. CLE was previously characterized as having antimicrobial activity, by Xu *et al.* [132], who verified that CLE exhibited strong antibacterial activity against *Staphylococcus aureus* ATCC 25923 with a MIC of 0.625 mg.mL^{-1} [132]. The aqueous extracts of *Curcuma longa* rhizome also demonstrate antibacterial activity (MIC=4-16 mg.mL^{-1}; MBC=16-32 mg.mL^{-1}), mainly against strains such as *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Escherichia coli* [133]. In the present study, the results obtained for antibacterial activity were better than the ones mentioned in the literature. For *Staphylococcus aureus* and *Escherichia coli* bacteria, according to Thielmann *et al.* [134], RE and AE did not present antibacterial activity, which is not in agreement with the results obtained in this study, while LBE revealed a MIC of 100 μg.mL^{-1} for *S. aureus* and 800 μg.mL^{-1} for *E. coli* [134]. Nieto *et al.* [135] reported that RE did not inhibit *E. coli* ATCC 25922, with the oil showing a MIC >6.4 mg.L^{-1}. Tariq and Patole [136] also tested the antimicrobial activity of GTE and reported MIC values of 11-12 mg.mL^{-1} and MBC values of 12-13 mg.mL-1, fairly constant for all seven pathogens tested (3 *Escherichia coli*, 2 *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Citrobacter amalonaticus*) [136].

Table 3.5 - Antibacterial activity (mg.mL^{-1}) of extracts.

Extracts	*S. aureus* (ATCC 11632)		*B. cereus* (food isolate)		*L. monocytogenes* (NCTC 7973)		*S. typhimurium* (ATCC 13311)		*E. cloacae* (ATCC 35030)		*E. coli* (ATCC 25922)	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
AE	2.19	4.38	2.19	4.38	2.19	4.38	4.38	8.75	4.38	8.75	1.09	2.19
CE	>8.50	>8.50	>8.50	>8.50	>8.50	>8.50	>8.50	>8.50	>8.50	>8.50	>8.50	>8.50
GTE	1.09	2.19	2.19	4.38	1.09	2.19	2.19	4.38	1.09	2.19	2.19	4.38
LBE	1.09	2.19	1.09	2.19	1.09	2.19	1.09	2.19	1.09	2.19	1.09	2.19
RE	1.09	2.19	2.19	4.38	1.09	2.18	1.09	2.18	1.09	2.18	1.09	2.18
CCE	1.09	2.19	2.19	4.38	1.09	2.18	1.09	2.18	1.09	2.18	1.09	2.18
CLE	2.19	4.38	2.19	4.38	2.19	4.38	2.19	4.38	2.19	4.38	1.09	2.19
Streptomycin	0.006	0.012	0.10	0.20	0.20	0.30	0.20	0.30	0.003	0.006	0.20	0.30
Ampicilin	0.012	0.025	0.25	0.40	0.40	0.50	0.75	1.20	0.006	0.012	0.40	0.50

Table 3.6 - Antifungal activity (mg.mL^{-1}) of extracts.

Extracts	*Aspergillus fumigatus* (ATCC 9197)		*Aspergillus versicolor* (ATCC 11730)		*Aspergillus niger* (ATCC 6275)		*Penicillium funiculosum* (ATCC 36839)		*Penicillium verrucosum* var. *cyclopium* (food isolate)		*Trichoderma viride* (IAM 5061)	
	MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
AE	1.09	2.18	1.09	2.18	1.09	2.18	0.55	1.09	1.09	2.18	0.27	0.55
CE	1.09	2.18	1.09	2.18	1.09	2.18	0.55	1.09	2.18	4.38	0.27	0.55
GTE	0.55	1.09	1.09	2.18	1.09	2.18	0.55	1.09	1.09	2.18	0.27	0.55
LBE	0.55	1.09	1.09	2.18	0.55	1.09	0.55	1.09	1.09	2.18	0.55	1.09
RE	1.09	2.18	0.55	1.09	0.55	1.09	0.27	0.55	0.55	1.09	0.55	1.09
CCE	2.18	4.38	2.18	4.38	1.09	2.18	0.55	1.09	2.18	4.38	1.09	2.18
CLE	2.18	4.38	1.09	2.18	1.09	2.18	0.55	1.09	2.18	4.38	0.55	1.09
Ketoconazole	0.20	0.50	0.20	0.47	0.20	0.50	0.20	0.50	0.20	0.30	0.20	0.30

3.4 Conclusion

The application of extracts and essential oils of aromatic plants is a current topic of research and a promising approach in terms of its use in active food packaging, due to antioxidant and/or antimicrobial properties. Several aromatic natural extracts from different plant parts: leaves (LBE, GTE, and RE), flower (CLE), seed (AE), bark (CE), and rhizome (CCE) were assessed in terms of thermal stability, antioxidant and antimicrobial activities.

While RE presented higher thermal stability, CCE and LBE exhibited lower stability in comparison with the remaining extracts. According to the OxHLIA assay, RE and CE were the most active extract and film, respectively, with IC_{50} values of 0.58±0.04 μg.mL^{-1} and 309±10 μg.mL^{-1}. The TBARS assay demonstrated that GTE (extract) and CLE (film) were the ones with highest activity, with IC_{50} values of 0.65±0.03 μg.mL^{-1} and 816±31 μg.mL^{-1}, respectively. Concerning cytotoxicity, only the extracts CE, LBE, and CCE presented toxicity. Although all the extracts, except for CE, presented antimicrobial activity, LBE was the most active, with MIC and MBC values of 1.09 and 2.19 mg.mL^{-1}, respectively. RE was the extract showing the strongest antifungal activity, with MIC and MFC values ranging from 0.27 to 0.55 and 0.55 to 1.09 mg.mL^{-1}, respectively. Generally, the extracts revealed stronger antifungal than antibacterial activity.

Based on the thermal characterization, antioxidant capacity, cytotoxic activity, and antimicrobial activity, it was possible to conclude that the extracts that gather the conditions as candidates to be incorporated into the polymeric matrix of LDPE are RE, GTE, and CCE, offering potential to be used as additives in active food packaging. From the point of view of different parts of the plants (leaves, flower, seeds, etc), the results of the aromatic extracts demonstrated that the parts of the plants that presented the best performance were the leaves (RE and GTE) and the rhizome (CCE). Thus, natural extracts from aromatic plants are promising antioxidant and antimicrobial natural additives that can be incorporated into polymeric matrices to produce active food packaging that will increase the food shelf-life.

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4 PREPARATION AND CHARACTERIZATION OF ACTIVE FILMS FOR JUICE PACKAGING

This chapter describes the development of active films for food packaging based on the incorporation of green tea extract (GTE) in low-density polyethylene (LDPE) and production by blown film extrusion, as well as its mechanical, physical, optical, microstructure, antioxidant, and antimicrobial characterization.

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Abstract

A new active packaging film of one and two layers structures based in low-density polyethylene (LDPE) with incorporation of 1.5 and 3 wt.% green tea extract (GTE) in different amounts has been successfully produce by blown film extrusion. Microstructure, physical, optical, mechanical, antioxidant and barrier properties of the films were characterized using several techniques. The results revealed a homogeneous, smooth more hydrophobic surface. While the tensile properties were not significantly affected, coefficient of friction (COF), penetration resistance and flex-crack were improved. The antioxidant capacity and barrier properties (water and oxygen) of the films were enhanced with the addition of 3 wt.% of GTE. This study demonstrates the benefits of GTE incorporation of into LDPE matrix and its potential to be used in food packaging at an industrial scale.

Keywords: Active packaging; Blown extrusion; Green tea extract; Antioxidant capacity; Barrier properties

4.1 Introduction

The packaging allows the preservation, safety and quality of food during storage, transportation, and above all, extended shelf-life, avoiding unfavorable factors or conditions, such as deteriorating microorganisms, chemical contaminants, oxygen, moisture, light, external forces, among others [1–4]. The request for packaged food has increased due to population growth and the globalization phenomenon, encouraging the food industry to develop new solutions to protect the food against internal and external factors for longer periods of storage time [5].

In the last years, with technological advances allows to develop active packaging solutions, which incorporates active agents in the polymer matrix to interacts with the product aiming to extend the shelf-life [5–7]. Active packaging is a promising alternative compared to the conventional preservation methods and is one of the most innovative food packaging concepts being introduced due to the continuous changes in current consumer demand and market trends. The release of active agents, usually antioxidants, antimicrobial and/or nutrients, in a controlled way to the surface or the inner part of the product can stop or delay microbial, oxidative, and enzymatic spoilage, minimize weight loss and contaminations, as well as to ensure the texture and color of the products during storage [8–10].

Consequently, due to consumer health concerns and environmental problems, the current research has been focused on the use of natural components as active additives [8, 11].

Several approaches have been proposed, such as mixing or coating in single and multilayer systems with several compounds, such as green tea (GTE), curcumin, and cinnamon extract [12–17]. Incorporation of active agents in an efficient and feasibly way, transferable to industry, would help to successfully apply the innovative packaging materials. GTE is a rich source of polyphenols, mainly catechins, that provide high antioxidant control. GTE was used in previous research to produce active packaging in combination with several polymers, such as ethylene-vinyl alcohol (EVOH) [18], poly (L-lactic acid) [1], polypropylene (PP) [19], oriented-polypropylene (OPP) multilayer films [11], chitosan [20, 21], gelatin [22], agar [23], and soy protein [24].

GTE is considered to be a healthy product with its consumption linked to lower incidences of various pathological conditions due to its reported anti-inflammatory, anti-tumor, antioxidative and antimicrobial properties. Also, it has polyphenols or flavonoids in its constitution, among which the catechins, amino acids, alkaloids like caffeine, volatiles, and minerals. Environmental factors, such as the climate and growth seasons influence the activity of the active components. It is known that the mixture of catechins is responsible for the high antioxidant effect of GTE, and other reports have shown that different types of catechins are responsible for its overall antimicrobial activity [23–25]. Due to the bioactive properties and its classification as a food additive by the European Union (EU), green tea extracts (GTEs) have been incorporated into different food packages in order to extend product shelf-life [1, 18, 19, 25].

Multilayer packaging film systems have been used to increase the shelf-life and safety of food products, as these systems offer multiple benefits, for example, higher barrier for both water and gases and an increase in mechanical strength. Moreover, the multilayer structure allows to incorporate the essential oils (EOs)/extracts in the layer that is more appropriate to preserve the food. Therefore, the incorporation of active compounds into multilayer packaging polymers will be an effective approach to enhance product shelf-life.

Low-density polyethylene (LDPE) is a polyolefin with low water vapor transmission rate (WVTR), medium gas permeability, good resistance to greases and chemicals, good abrasion resistance, high-temperature stability and gloss and high transparency. All of these features make LDPE one of the most used polymers in industry to produce films for food packaging [10].

Therefore, the aim of the current study was to develop an active film for food packaging based on incorporated GTE into LDPE by blown film extrusion. Different structured films (monolayer and multilayer)

were prepared using 1.5 and 3.0 wt.% of GTE. The produced films were characterized through mechanical, physical, optical, microstructure and antioxidant properties.

4.2 Materials and methods

4.2.1 Materials

Low-density polyethylene (LDPE) was kindly provided by Vizelpas. The green tea extract (GTE), was supplied from ESSÊNCIAD'UMSEGREDO, LDA. Potassium Bromide (KBr) (Acros Organics, spectroscopic standard) was used to produce the pellets for Fourier Transform Infrared Spectroscopy (FTIR). Acetic acid glacial (from Fisher Scientific UK) was used for the contact angle (CA) test. Extra pure calcium chloride ($Cacl_2$) (Riel de Haën) was used for the water vapor transmission rate (WVTR) test.

For the antioxidant activity, absolute ethanol, chloroform, methanol, sodium carbonate anhydrous, sodium nitrite, and sodium hydroxide were acquired from Merck (Darmstadt, Germany). The gallic acid, radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), epicatechin, β-carotene, linoleic acid, Tween®40, Folin–Ciocalteu reagent were acquired from Sigma-Aldrich (Madrid, Spain). Also, a rotary evaporator Büchi model R-210 (Labortechnik, Switzerland), a Thermo Scientific Evolution 300 LC spectrophotometer and a Grant Instruments™ QB Series Dry Block Heating System (Cambridge, England) were used.

4.2.2 Preparation of a LDPE/GTE masterbatch

Previous to blown film extrusion, a LDPE/GTE masterbatch was produced to ensure better GTE dispersion in the LDPE matrix. Initially, LDPE and GTE were dried at 80 ºC, overnight, in a vacuum oven. Thereafter, a masterbatch with 10 wt.% of GTE was produced in a Leistritz AG LSM 34 6L co-rotating twin-screw extruder at an average melt temperature of 170 ºC, at 125 rpm and an output of 4 $Kg.h^{-1}$. To minimize the degradation of GTE, a secondary feed zone near the dye was used. The extruded filaments were air cooled and granulated.

4.2.3 Blown film extrusion

The pristine LDPE and LDPE/GTE masterbatch were dried in a vacuum oven at 60 ºC. Different quantities of masterbatch were added to LDPE to produce films containing 1.5 and 3 wt.% of GTE. A Periplast

extruder was used to produce the films through blown film extrusion process, using one extruder for monolayer films of the LDPE with GTE, and an additional extruder to produce the coextruded films LDPE/LDPE+GTE (Figure 4.1). An extrusion line encompassing a single screw at a speed of 50 rpm, a blow-up ratio of 1.71, a draw-down ratio of 12.39, and a temperature profile of 170 ºC on the first heating zone, 175 ºC on the second zone and 180 ºC on the remaining zones.

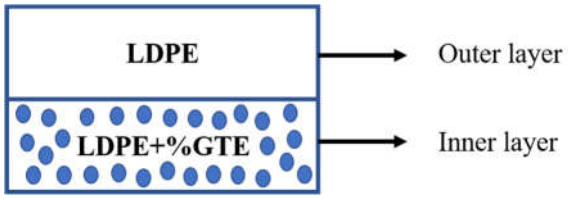

Figure 4.1 - Structure of coextruded film.

The description of various films developed for food packaging based on LDPE containing GTE is described in Table 4.1.

Table 4.1 - Description of films based on LDPE/GTE.

Film	Description
LDPE	Film without GTE
LDPE_1.5GTE	Film extruded (monolayer) with 1.5 GTE
LDPE_3GTE	Film extruded (monolayer) with 3 GTE
CO_LDPE_1.5GTE	Film coextruded (two layers) with 1.5 GTE
CO_LDPE_3GTE	Film coextruded (two layers) with 3 GTE

4.2.4 Scanning electron microscopy (SEM)

Morphological analysis was performed in an Ultra-high resolution Field Emission Gun Scanning Electron Microscopy (FEG-SEM), NOVA 200 Nano SEM (FEI Company). Samples were previously fractured in liquid nitrogen and covered with a thin film (2 nm) of Au-Pd (80-20 weight %); in a high-resolution sputter coater (208HR Cressington Company), coupled to an MTM-20 Cressington High Resolution Thickness Controller.

4.2.5 Fourier Transform Infrared (FTIR) analysis

FTIR spectra were obtained in a 4100 Jasco spectrometer apparatus in transmittance mode using the following parameters: 32 scans.min^{-1}, 4 cm^{-1} resolution and a scanning range of 4500-400 cm^{-1}. Translucent thin films, prepared by compression molding in a hot press at 140 ºC under a pressure of 10 tons, of masterbatch mixture, extruded and coextruded films were used in the analysis. A translucent sample disc of GTE (10 wt.% in KBr, > 99%, Acros Organics) was used to acquire the FTIR spectrum.

4.2.6 Film thickness

The film thickness of LDPE/GTE films was determined using a hand-held digital micrometer screw gauge (Mitutoyo Absolute, N°.547-301, Japan). The reported values are the average of, at least 5 random readings on each film sample.

4.2.7 Opacity and light transmission

The opacity of the films was calculated using Equation (1):

$$\text{Opacity} = \frac{Abs_{600}}{X} \quad (1)$$

Where Abs600 is the value of absorbance at 600 nm using UV-vis spectrophotometer (Shimadzu UV-2401 PC, Japan) and X is the thickness of the film (mm). At least 5 samples of each film type were tested.

The ultraviolet (UV) and visible light barrier properties of the films were measured on dried films at 200 to 800 nm, using a UV-vis spectrophotometer (Shimadzu UV-2401 PC, Japan) based on the procedure described by Han and Floros [26]. At least 5 samples of each film type were tested.

4.2.8 Contact angle (CA) measurement

The CA was used to estimate the surface hydrophobicity of the films and was measured using a goniometer (Contact Angle System OCA 20 Dataphysiscs, Germany). CA measurement using water and acetic acid 3% solution (simulant of acidic foods, i.e. aqueous foods having a pH≤4.5) on a horizontal film surface was carried out using image software. Distilled water/acetic acid 3% solution (3 µL) was dropped on the film surfaces with a precision syringe using the sessile drop method. In the case of coextruded films, the measurement was performed on both sides of the film, the outer layer (LDPE) and the inner layer (LDPE+%GTE). The image of the drop, initial (taken at 0 seconds) was recorded with a video camera. At least 5 measurements per film were carried out and the mean value was taken.

4.2.9 Mechanical properties

Tensile strength at break (TS), elongation at break (EB) and secant modulus (SM) of LDPE/GTE films were determined according to the ASTM standard test method D882-02, using the INSTRON 3345 machine with a 500 N load cell and speed of 500 $mm.min^{-1}$, under controlled temperature (23±2 °C) and relative humidity (50±5%). The films were cut into rectangular strips (10 mm width), and their thickness was measured at 5 points. The films were held parallel with an initial grip separation of 50 mm. This measurement was repeated at least 5 times (n>5) for each film, and the mean values were reported. The calculated parameters (TS, EB and SM) were performed in the extrusion direction (MD) and transverse direction (TD) of the tubular film.

The penetration resistance of films is based on ASTM F1306, which defines the test parameters, such as speed, metal probe shape, and sample area. To perform the test, 10 circular specimens with a diameter of 5 cm were used for each sample with the aid of a mold through LLOYD Instruments LF plus equipment. The test piece was placed on the equipment with the test layer facing upwards, i.e. the surface where the food will be in contact with - the inner layer. During the test, each specimen was loaded with a metal tip until the film was ruptured. The force applied by the metal probe has a biaxial direction. The analysis was performed at a speed of 25 mm/min, and under controlled temperature (23±2 °C) and relative humidity (50±5%).

The coefficient of friction (COF) was determined according ASTM D1894 using an RDM CF-800XS equipment. For this test, a sample with 130x320 mm was cut to cover the horizontal plane test site. Another smaller sample (110x170 mm) was cut to cover the block used the test under the horizontal plane. The speed test was 150 ± 30 mm/min. Three specimens were cut to each side of the film, outer and inner for coextruded films, the specimens were cut in the machine direction. The analysis was performed at temperature (23±2 °C) and relative humidity (50±5%).

Flex-crack resistance indicates how much the packaging can be flexed or bend before it cracks. A sample (210x297 mm) is attached to the flex-tester mandrels using a Brugger KFT-C equipment. The flexing action consists of a twisting motion combined with horizontal motion (compression), thus repeatedly twisting and crushing the sample. For polymeric film, the flexing cycle number used was set to 500, before the sample is examined. The formation of pinholes is determined using a pink dye solution (Rhodamine B) that stains through the pinholes onto an absorbent white backing. The number of pinholes is a measure of the flex-crack resistance of the material and it is reported as, for example, 3 pinholes/500 flexes. Flex-crack resistance was determined according to the ASTM F392 standard test method for flex

durability of flexible barrier materials. The analysis was performed at 23±2 °C and relative humidity (50±5%). Three replicas were performed for each sample.

4.2.10 Antioxidant activity release tests

The extruded films (6 cm2) were placed in contact with 10 mL of ethanol 95% (v/v), the alternative food simulant for fatty foods according to the Commission Regulation No. 10/2011 [27], for 10 days at 40 °C. Then the antioxidant activity was determinate by the DPPH Radical Scavenging Assay and the β-carotene bleaching method. Also, the total phenolic compounds and the total flavonoids were quantified.

4.2.10.1 Determination of total phenolics content

The total phenolic content was determined by the method described by Erkan *et al.* [28]. Briefly, 1 mL of each sample was mixed with 7.5 mL of Folin-Ciocalteu reagent (10%, v/v). After 5 minutes, 7.5 mL of an aqueous solution of sodium carbonate 60 mg/mL (w/v) were added. The solutions were homogenized, and the samples were kept for 120 minutes, in the dark. After this time, the absorbance was measured in an Evolution™ 300 UV-Vis Spectrophotometer at 725 nm.

4.2.10.2 Determination of flavonoids content

For the determination of the total flavonoids content, the method described by Yoo *et al.* [29] was applied. In short, 4 mL of ultrapure water were added to 1 mL of sample. Then, 300 μL of an aqueous solution of sodium nitrite (5%, w/v) and the mixture was homogenized. After 5 min, 600 μL of an aqueous solution of aluminum chloride (10%, w/v) were added and the solution was homogenized again. After 6 min, 2 mL of an aqueous solution of sodium hydroxide (1 M, w/v) and 2.1 mL of ultrapure water were added. The samples were homogenized, and the absorbance were measured at 510 nm.

4.2.10.3 DPPH radical scavenging method

Regarding the DPPH radical assay, this is an easy and fast method to evaluate the in vitro antioxidant activity of a given sample. Briefly, 2 mL of a methanolic DPPH solution (14.2 μg/mL) were added to 50 μL of sample. The solutions were homogenized and protected from the light for 30 min. The absorbance was measured at 515 nm. The Inhibition Percentage was measured through the Equation (2).

$$IP\ (\%) = \frac{AC\text{-}AS}{AC} \times 100 \quad (2)$$

In which, AC stands for the control' absorbance and AS stands for the sample' absorbance. The applied method was in accordance with the method described by Andrade *et al.* [30].

4.2.10.4 β-carotene bleaching method

First, an emulsion of β-carotene and linoleic acid was made using 20 mg of linoleic acid, 200 mg of Tween®40 and 1 mL of a chloroform solution of β-carotene (2 mg/mL). The chloroform was evaporated using a rotary evaporator at 40 °C. Then, 50 mL of ultrapure water was added, and the solution was vigorously shaken. Once the emulsion was prepared, 5 mL were immediately added to 200 μL of sample. The samples were kept at 50 °C for 120 minutes, in a Grant Instruments™ QB Series Dry Block Heating System (Cambridge, England). The absorbances of the hot samples were measured at 470 nm and the Antioxidant Activity Coefficient (AAC) was calculated through the Equation (3). The applied method was described by Miller [31] and adapt by Andrade *et al.* [30].

$$AAC = \frac{AS - AC2}{AC0 - AC2} \times 1000 \quad (3)$$

In which, AS stands for the absorbance of the samples, AC0 stands for the absorbance of the control before heating and AC2 stands for the absorbance of the control after the heating.

4.2.11 Water vapor permeability

The water vapor transmission rate (WVTR) of films was determined by the desiccant method based on the ASTM method E96/E96 M-05. The films were placed in a circular metal test cups, with a surface diameter of 69.5 mm, and filled with approximately 25 g of $CaCl_2$, previously dried at 150 ºC in a vacuum oven. The films were sealed to the cups with paraffin wax to ensure that humidity migration occurred exclusively through the film. Then, the test cups were placed in a desiccator and maintained at 20.78±1.37 ºC, 94.26±4.33% RH and weighed daily for one month. The measured WVTR of the films was calculated using Equation (4):

$$WVTR(g\ water/(m^2.\ hour)) = \frac{G}{t \times A} \quad (4)$$

Where G/t (g water/hour) is the slope (weight versus time plot) and "A" is effective film area (m^2). WVTR was calculated using three replications and expressed in $g.h^{-1}m^{-2}$.

4.2.12 Oxygen permeability(O_2P)

A gas permeability tester (DP-100A, Porous Materials, Inc.) was used to measure oxygen transmission rates through the films at 21 ºC and 55% RH according to standard method D1434-82. The film samples were placed in the testing area with 4 cm of diameter. Tests were performed in triplicate and mean values were expressed as mL.mm/MPa/min/cm^2.

4.2.13 Statistical analysis

Microsoft Windows Excel 365 and OriginPro software (Version 17) were used to analyze the resulting data. Results were expressed as mean ± standard deviations of at least three replicates.

4.3 Results and discussion

4.3.1 Microstructure Properties

The visual aspect of the films produced is depicted in Figure 4.2, films containing GTE presented a brownish color, which is dependent on the amount of GTE added.

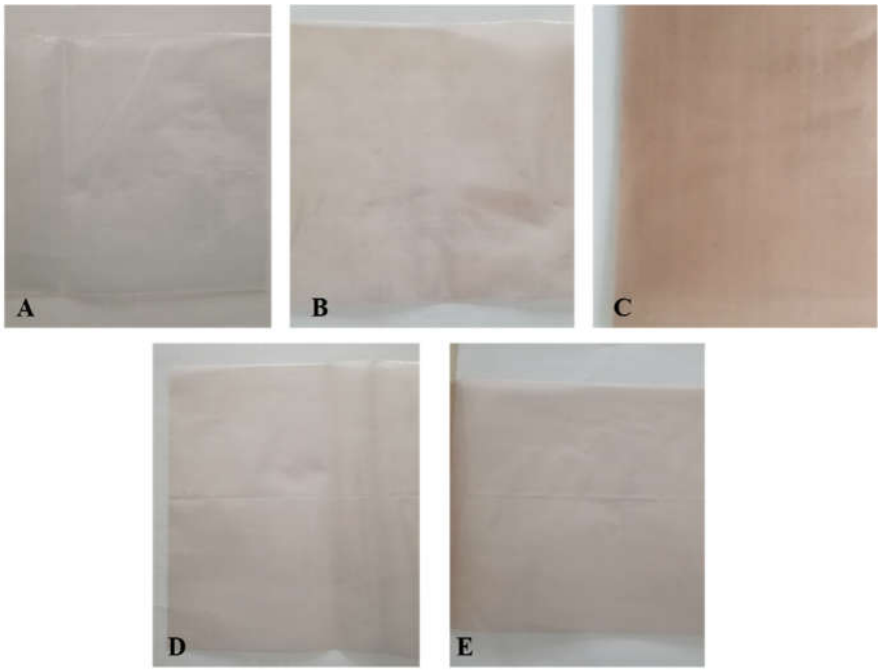

Figure 4.2 - Visual appearance of the films LDPE (neat) (A); LDPE_1.5GTE monolayer film (B); LDPE_3GTE monolayer film (C); LDPE_1.5GTE coextruded film (D) and LDPE_3GTE coextruded film (E).

The GTE dispersion into the LDPE matrix is demonstrated by SEM micrographs of the surface and cross-section of the films in Figure 4.3. The surface and cross-section of pure LDPE film exhibited smooth and homogeneous surfaces, which is in agreement with the results reported by Dong *et al.* [10]. The surface roughness of the films increased slightly with GTE. However, GTE aggregates at the surface were not detected, even for higher amounts of GTE. The cross-section images present a smooth and homogeneous surface with low porosity. Micrographs with high magnification confirm a very good dispersion of the GTE in all samples.

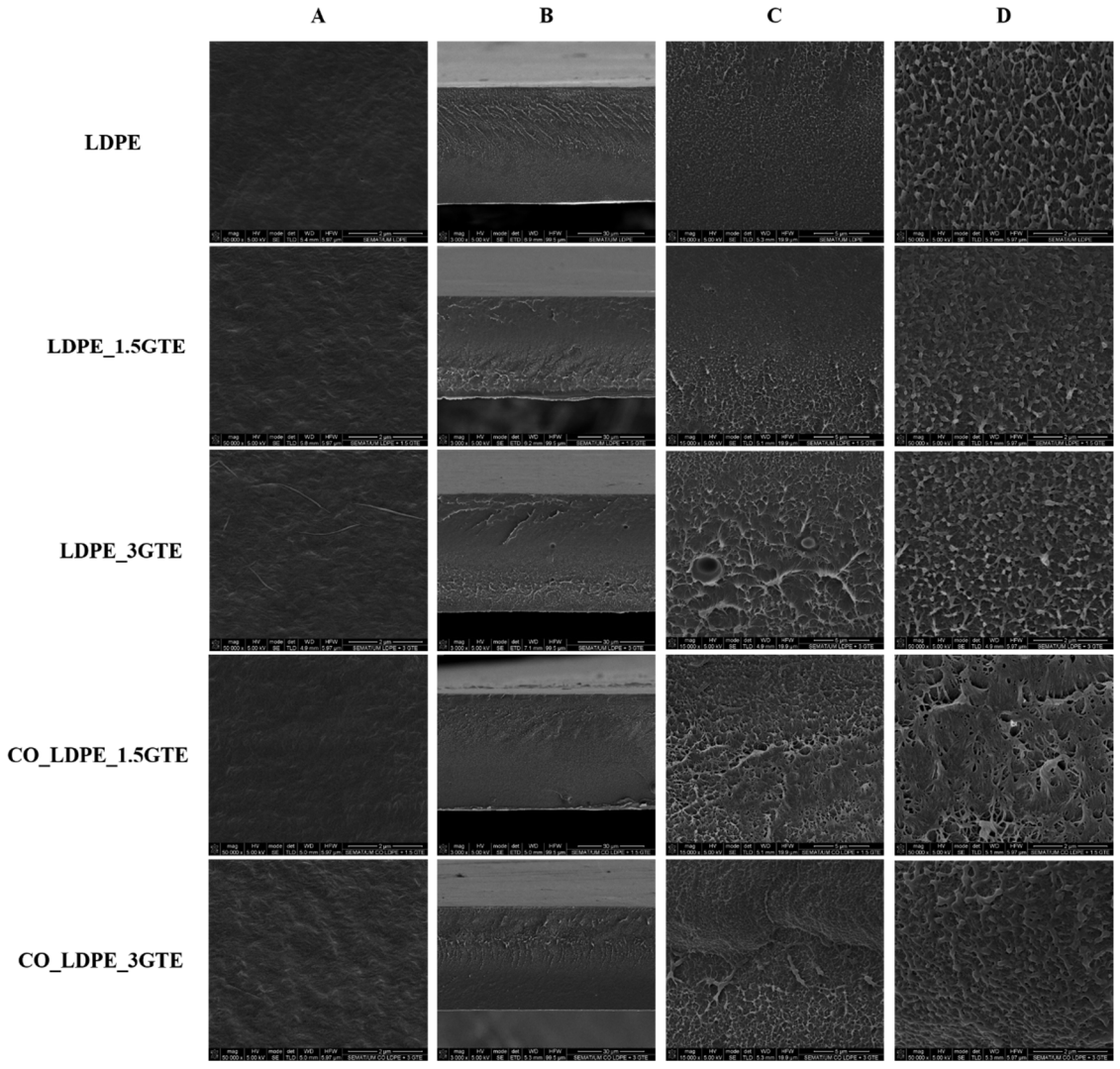

Figure 4.3 - SEM micrographs of surfaces, magnification 50 000× (A), cross-sections magnification 3000× (B), magnification 15000× (C) and magnification 50000× (D) of LDPE containing GTE.

FTIR spectroscopy was used as a tool to investigate the interaction between LDPE film and GTE polyphenols. FTIR spectrum of an LDPE/GTE masterbatch (Figure 4.4 - A), presents LDPE characteristic vibrational bands, (CH_2 asymmetric and symmetric stretching at 2918 and 2851 cm^{-1}, respectively, , bending deformation at 1465 cm^{-1}, CH_3 symmetric deformation at 1376 cm^{-1}, twisting at 1303 cm^{-1} and

rocking deformation at 722 cm^{-1}). The presence of GTE can be confirmed through its characteristic vibrational bands, such the O-H stretching at 3349 cm^{-1}, the C=C stretching at 1614 cm^{-1}, a band shift of C-O bending for 1374 cm^{-1} and the C-O stretching at 1237 cm^{-1} and 1030 cm^{-1} typical of aromatic ether groups.

FTIR analysis was also performed to confirm the presence of GTE in LDPE films (Figure 4.4 - B and C). The FTIR spectra of extruded and coextruded films are similar to the one obtained for the masterbatch (Figure 4.4 - A). The characteristic peaks of GTE related to O-H stretching at 3349 cm^{-1} and C=C stretching at 1611 cm^{-1}, associated to the presence of polyphenols, are noticeable on the FTIR spectra of the films. Spectra exhibited in Figure 4.4 do not show any difference between the two amounts of GTE used for both extruded and coextruded films, as no perceptible difference in intensity of GTE characteristic bands was detected. This can be explained by the small difference between the two percentages.

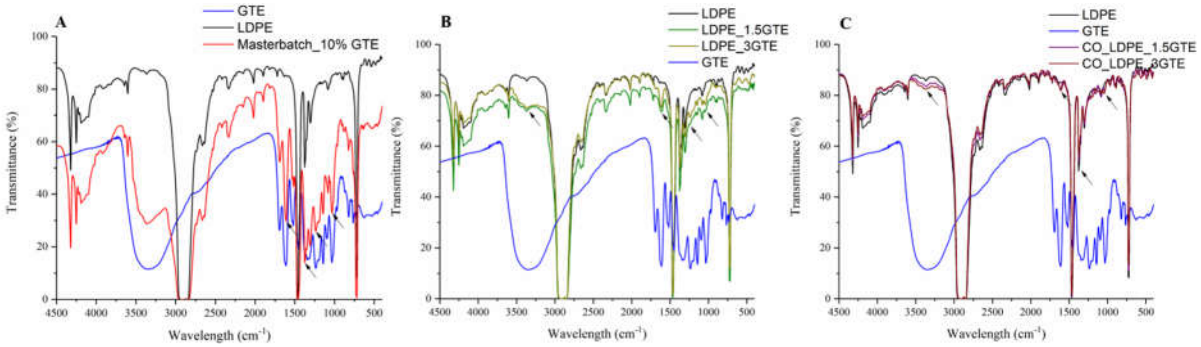

Figure 4.4 - FTIR spectrum of LDPE/GTE masterbatch (A), extruded films (B) and coextruded films (C).

4.3.2 Physical Properties

LDPE films were used as control specimens to evaluate the influence of GTE addition. The film thickness is presented in Table 4.2, and ranges between 49.3 and 54.0 µm for all LDPE/GTE films and 48.7 µm (± 0.012) for the control film. Under equivalent processing conditions, increasing the GTE concentration resulted in slightly thicken films.

The CA between liquid-solid interfaces is a good indicator of adsorption because it increases the capacity of the liquid to spread, thereby CA decreases [32]. Consequently, films with a lower CA will have a greater capacity to spread liquids and penetrate into the surface [32].

The measured CA of each sample using two solutions (distilled water and acetic acid 3%) is represented in Table 4.2. Regarding water, the control film has a CA of 86.36º, i.e., due to the hydrophobic nature of PE, however, it has the lowest value compared to the other films. Extruded and coextruded films, the CA values increase as GTE increases, meaning that the surface of the film became more hydrophobic. Since the GTE has phenolic compounds, the opposite would be excepted, and as a possible explanation is that

the GTE particles are covered by very thin layer of polymer and can also be associated with differences in films surface roughness, as observed in SEM analysis. No significant variation was observed for the different layers of the coextruded film. It can also be confirmed that the results are consistent since the outer side of the film (LDPE only) which presents the same CA value for the two concentrations of GTE studied. For acetic acid solution, there is no increase in CA with increasing GTE concentration showing approximately the same value ($\theta \approx 89^{o}$) for mono and coextruded films. In general, taking into account the error associated with the measurement, comparing the two fluids used, the CA results were very similar between them.

Table 4.2 - Physical properties of LDPE films containing GTE.

Film	Thickness (μm)	Contact angle (θ)	
		Distilled water	Acetic acid 3%
LDPE	48.7±0.012	86.36±3.04	84.46±4.95
LDPE_1.5GTE	50.0±0.010	90.21±3.94	89.76±2.21
LDPE_3GTE	54.0±0.006	93.39±2.88	89.13±4.52
CO_LDPE_1.5GTE (Outer)	49.3±0.006	88.18±3.41	86.92±3.67
CO_LDPE_1.5GTE (Inner)		89.63±3.67	88.34±2.96
CO_LDPE_3GTE (Outer)	50.7±0.006	88.91±1.93	88.65±2.31
CO_LDPE_3GTE (Inner)		90.19±3.58	93.56±2.79

4.3.3 Optical Properties

Optical properties are of extreme importance in the film appearance because they directly influence the consumer's acceptability, contributing to the consumer's willingness to buy a particular food product. To compare the differences among prepared films, Table 4.3 reports the parameters of light transmission (%) and opacity measured.

The UV transmittance of the control films ranged from 9.13 to 73.32% in a wavelength range of 200-400 nm. However, the UV transmittance of the films containing GTE decreased as the GTE content increased. While, at 400 nm, LDPE_1.5GTE films exhibited 66.89% light transmission, LDPE_3GTE films presented 52.98%, indicating an increase in opacity. Nevertheless, the trade-off of this outcome was a decrease in the transparency of the films. In the visible light range (300-800 nm), the light transmission decreased with the increasing GTE concentration. Films extruded with 3 wt.% of GTE exhibited the highest block properties in the visible light, in fact it is known that EOs/extracts influenced the light transmission. The results obtained for light transmission are similar to those reported by other authors as Lee *et al.* [33], Mulla *et al.* [34], Hosseini *et al.* [35], and Ribeiro-Santos *et al.* [5].

Opacity values demonstrated that GTE content affected significantly the transparency of the film (Table 4.3). The films containing GTE were darker than the control film, and as GTE concentration increases the tendency toward redness and yellowness of the films is noticed. The increase in the brown coloration can be explained by the brown color of the GTE, which is in agreement with published results [1, 18, 36, 37]. Similar results were described for chitosan films containing GTE [20, 38, 39] and in PVA films with GTE [36]. For industrial application the amount of GTE incorporated into the LDPE matrix has to be taken into account due to its effects on the color and opacity of the films, especially for packaging materials with high transparency requirements.

Table 4.3 - Light transmission (%) and opacity of LDPE films containing GTE.

Film	Light transmission (%) at different wavelengths (nm)									Opacity
	200	250	300	350	400	500	600	700	800	
LDPE	9.13 ±2.42	57.43 ±1.86	64.13 ±1.76	69.95 ±1.44	73.32 ±0.98	77.77 ±0.72	80.75 ±0.57	83.01 ±0.53	84.79 ±0.57	1.77±0.47
LDPE_1.5GTE	9.73 ±4.08	52.23 ±4.03	56.26 ±4.85	62.65 ±4.21	66.89 ±3.15	71.57 ±2.60	74.89 ±2.15	77.90 ±1.68	80.29 ±1.41	2.68±0.93
LDPE_3GTE	6.68 ±0.94	42.14 ±1.40	45.71 ±1.47	50.61 ±1.41	52.98 ±1.27	56.46 ±1.20	59.50 ±1.19	63.23 ±1.14	66.84 ±1.05	4.17±0.85
CO_LDPE_1.5GTE	9.73 ±1.65	54.41 ±1.29	60.21 ±1.36	65.98 ±1.16	69.18 ±1.34	73.63 ±1.39	76.87 ±1.26	79.64 ±1.08	81.88 ±0.95	2.14±0.27
CO_LDPE_3 GTE	9.49 ±2.02	50.91 ±2.01	55.56 ±2.09	60.86 ±2.01	64.18 ±2.06	68.61 ±2.07	72.18 ±1.92	75.59 ±1.68	78.44 ±1.38	2.76±0.39

4.3.4 Mechanical Properties

Mechanical properties (TS, EB, and SM) were measured in the machine direction (MD) and the transversal direction (TD) of the tubular film extruded, Figure 4.5. Upon incorporation of 1.5 and 3 wt.% GTE in LDPE (monolayer film), and 3 wt.% GTE coextruded film, the TS reduced from 23.35 MPa to 18.46, 15.05 and 18.42 MPa, respectively, with the exception for 1.5 wt.% GTE coextruded film where the TS increase (26.09 MPa). The decrease in TS might be ascribed to the presence of active compounds and its interactions with the polymer matrix. In fact, similar results were already reported by Sung *et al.* [40] who tested the effect of Allium sativum on the TS of LDPE-based film and by Dong *et al.* [10] which studied the TS performance in new active bilayer structure based on LDPE films containing rosemary and cinnamon essential oils. Contrarily, the presence of GTE seems not significantly affect the EB. As expected, SM follows the same trend as TS.

The TD results, represented in Figure 4.5- B, show a decrease of 8.6 to 11.4% compared to the control

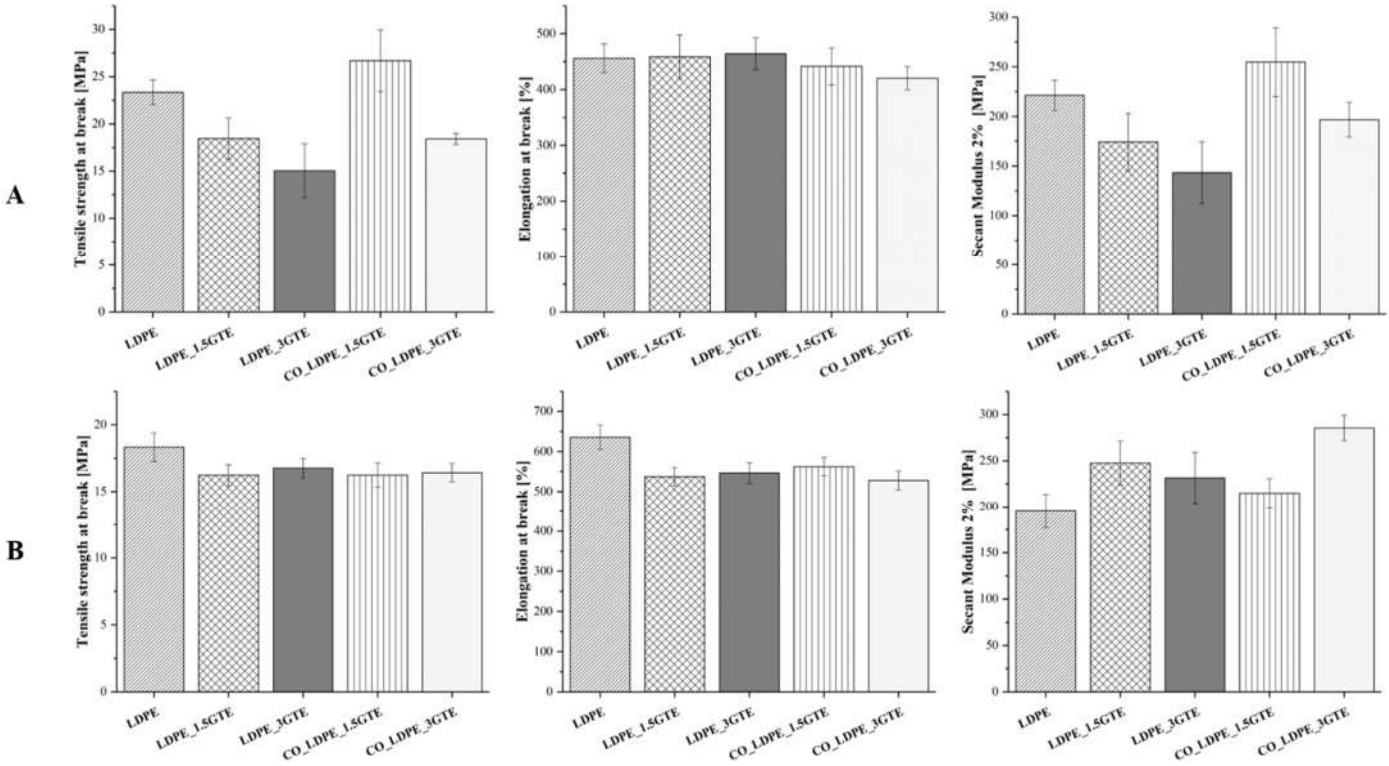

Figure 4.5 - Mechanical properties of LDPE/GTE films: tensile strength at break (TS), elongation at break (EB) and secant modulus 2%, (A) in the machine direction (MD) and (B) transverse direction (TD).

film, the effect of GTE was minor for TD rather than the MD direction. The values obtained for EB decrease in comparison with the LDPE value.

MD analysis show that SM value increases with 1.5 wt.% GTE and then decreases slightly for higher GTE concentration. This can be associated to the poorest dispersion of the GTE particles in the LDPE matrix.

For multilayer films SM increases with the addition of GTE. Even though the highest value is reached for 3 wt.% GTE coextruded film, a real correlation with the addition of GTE cannot be obtained.

The penetration resistance test is very important once it intend to simulate forces which the package may be subjected, caused by objects with sharp corners, for example. The obtained results obtained are depicted in Figure 4.6, and it can be seen an increase of the penetration resistance force with the increase in GTE concentration. The LDPE_3GTE film has the highest penetration resistance, with an increase of 23.5% over the LDPE control film (5.65 ± 1.11 N). This is probably due to the plasticizing effect of GTE on the polymer matrix, thus reinforcing it with increasing extract concentration.

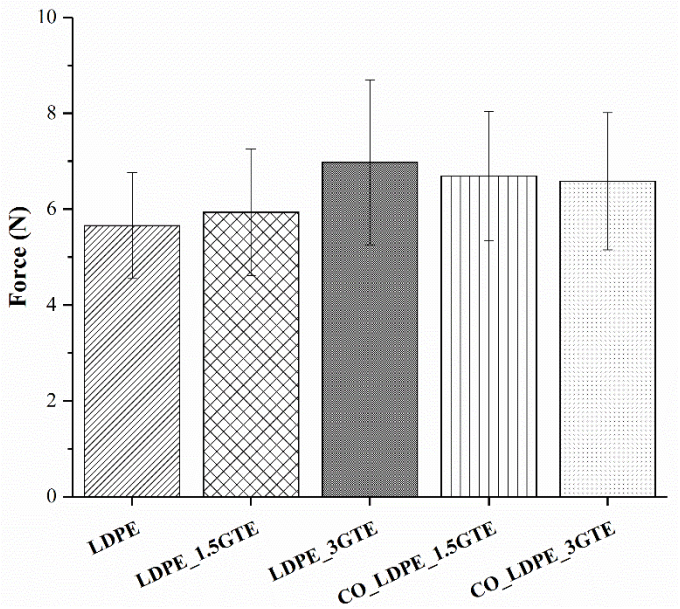

Figure 4.6 - Results obtained in the penetration resistance tests for LDPE/GTE films.

The COF is directly related to the machinability of the film, and the results are shown in Figure 4.7. Through the, it can be noticed that most of the films present a low COF (COF <0.25), indicating smaller resistance to friction. The addition of GTE decreases COF by more than half. This behavior occurs for both kinetic and static COF. Thus, using GTE in the LDPE matrix will aid in the film's machinability, making the process more reproducible since GTE acts as a “slipping agent”.

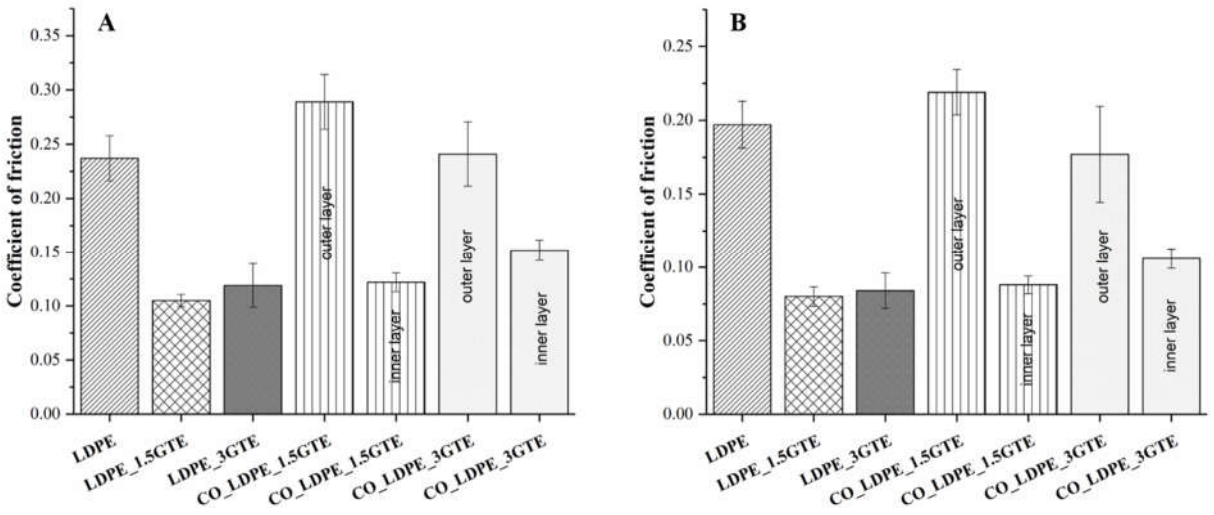

Figure 4.7 - Coefficient of friction results, (A) static and (B) kinetic for LDPE/GTE films.

Through the analysis of the results presented in Figure 4.8, it can be noticed that the incorporation of GTE decreases the values of the flex-crack fatigue test, this one evaluates the resistance of brittlely on the flexible packaging. Nevertheless, increasing the concentration of GTE in the polymer matrix improves flex-crack. The results also indicate that the coextruded films performed worse, presenting an average of holes of approximately 9, but the holes presented for this type of film are quite small, and are called "micro holes". Based on what was previously mentioned, the number of permissible holes based on the number of cycles performed should equal or less than 3, considering this, only the LDPE_3GTE film would pass the test.

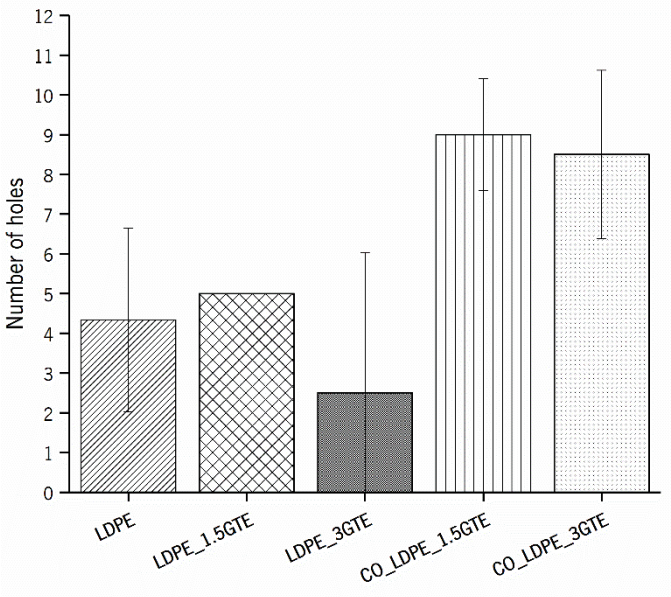

Figure 4.8 - Results of Flex-crack test for LDPE/GTE films.

4.3.5 Determination of antioxidant activity

One of the main groups of compounds that act as primary antioxidants or free radical scavengers in the extracts are polyphenols. These are extremely effective as oxygen scavengers, reducing agents and hydrogens donors. The content of phenolics was calculated using a regression equation of the gallic acid calibration curve, "y=0.0065x-0.1473", with a coefficient of determination of a 0.998, expressed in GAE as milligrams per milliliter.

As far as total flavonoids are concerned, these are some of the main chemical antioxidant components, being potential metal chelators and inhibitors of lipoperoxidation. Flavonoids, including flavones, flavonols and condensed tannins, are plant secondary metabolites, and their antioxidant activity depends on the presence of free OH groups, especially 3-OH. Flavonoids have antioxidant activity *in vitro* and also act as antioxidants *in vivo*. Epicatechin was used to construct the standard curve using 9 calibration points in a range of 10-200 $\mu g.mL^{-1}$, the results are expressed as µg ECE equivalents per milliliter. The assays mentioned were carried out for all the prepared films (Table 4.4).

Analyzing the results, it was confirmed that CO_LDPE_3GTE showed a higher content of total phenolic compounds (25.31 GAE mg/mL), whereas the CO_LDPE_1.5GTE presented a higher content of total flavonoids (32.27 ECE µg/mL). It can also be noticed that all active films have a similar content of total phenolic compounds, with a range of 24.63 to 25.31 GAE mg/mL and similar results obtained for the total flavonoids compounds.

Even though several studies in literature [1, 20, 38, 41] determined the content of total phenolic compounds and total flavonoids in GTE, when GTE is incorporated in for polymeric films, these parameters are not calculated. Since the values of total phenolic compounds and total flavonoids, change according to the different edaphoclimatic conditions of the tea plant, the extraction solvent and the methodology used, it is difficult to compare de values of different studies.

Table 4.4 - Values of the total phenolic compounds and total flavonoids results for the active films.

Film	Total content of phenolic compounds (GAE mg/mL)	Total content of flavonoids compounds (ECE µg/mL)
LDPE	18.01±6.04	30.3±0.15
LDPE_1.5GTE	24.63±0.03	32.23±0.72
LDPE_3GTE	25.17±0.09	31.25±0.77
CO_LDPE_1.5GTE	25.04±0.004	32.27±0.20
CO_LDPE_3GTE	25.31±0.25	30.5±0.20

The determination of the radical-scavenging activity of packaging films is very important due to the harmful effects of free radicals in foods and biological systems. DPPH· assays depended on the reducing ability

of the antioxidant (electron transfer) of nitrogen radical, with a marginal radical reaction via hydrogen transfer mechanism [12]. This assay was based on the ability of DPPH, a stable free radical, to be quenched and thereby decolorize in the presence of antioxidants resulting in a reduction in absorbance values [42]. The percentage inhibition values of the DPPH, radical-scavenging ability, of the active film samples are shown in Table 4.5. As expected, all film presented antioxidant activity, with the exception of the film without GTE that produced a null result. The percentage inhibition values of DPPH radical-scavenging ability increased as the GTE concentration in the film increased. The antioxidant ability of the active films coextruded containing 3 wt.% GTE was higher than other film. This can be associated to the higher concentration of GTE that resulted in an increase of antioxidant activity that is largely associated with the phenolic content, specifically the presence of catechin compounds, reported to be mainly responsible for the antioxidant activity of green tea in numerous reports. The increase in the amount of extract added into films is generally proportional to the total phenolic content and radical-scavenging activity [43].

Table 4.5 also presented the results of the β-carotene bleaching test. The active film with 3 wt.% of GTE, present similar antioxidant capacity among those evaluated. It is possible to noticed that in DPPH inhibition, the active film containing 1.5 wt.% GTE (LDPE_1.5GTE) showed the lowest antioxidant capacity (1.77%), the same is confirmed in the β-carotene bleaching test (99.6 $\mu g.mL^{-1}$).

Table 4.5 - Values of the DPPH radical inhibition results, expressed in % inhibition and values of the inhibition of β-carotene bleaching, expressed by the coefficient of antioxidant activity (AAC) for the active films.

Film	DPPH· method (% inhibition)	The β-carotene bleaching assay (AAC)
LDPE	0	94.5±22.4
LDPE_1.5GTE	1.77±0.74	99.6±8.42
LDPE_3GTE	3.30±0.31	130±6.18
CO_LDPE_1.5GTE	2.39±0.15	125±14.2
CO_LDPE_3GTE	5.03±0.77	128±12.6

4.3.6 Active films permeability

Barrier properties to oxygen of LDPE/GTE active films were studied by the determination of the O_2P, Figure 4.9 - A. The decline in the O_2P values was observed from $1.886x10^{-4}$ $mL.mm/MPa/min/cm^2$ of control film to $1.365x10^{-4}$, $1.601x10^{-4}$ and $1.630x10^{-4}$ $mL.mm/MPa/min/cm^2$, where LDPE_3GTE active film presents the smaller value. However, there was an exception for the LDPE_1.5GTE film, where the O_2P value increases for $2.304x10^{-4}$ $mL.mm/MPa/min/cm^2$. Thus, there is a tendency to lower the values of O_2P with the incorporation of GTE on the active films, indicating that the addition of the GTE to LDPE

films improved the oxygen barrier property. This is closely related to the effective distribution of the extract into the LDPE matrix, which follows previous findings [10, 44].

The O_2P of LDPE/GTE active films decreased for higher concentration of GTE for the monolayer films (LDPE_3GTE), which indicated that the addition of the GTE to LDPE films improved the oxygen barrier property. In the case of coextruded films this effect was not so notorious. The active film with better O_2P is the LDPE_3GTE, which decreased 27.6% when compared with the control film. This fact may be explained mainly by the improvement of LDPE film hydrophobicity with the incorporation of the GTE. The diffusion of oxygen in the active films was reduced with the addition of GTE. The interaction through LDPE/GTE hydrogen bonding also contributed to this change. McHugh and Krochta [45] indicated that the films containing EOs exhibit relatively poor oxygen barrier properties. This can be explained by the grater oxygen solubility in the non-polar phase that contributes to an increase in the transfer rate of oxygen molecules into the plasticized polymer matrix. Similar results were found by Atarés, *et al.* [46] in hydroxy-propyl-methylcellulose, Fabra *et al.* [47] in sodium caseinate films, Jouki *et al.* [42] in quince seed mucilage films and Pola *et al.* [48] in cellulose acetate.

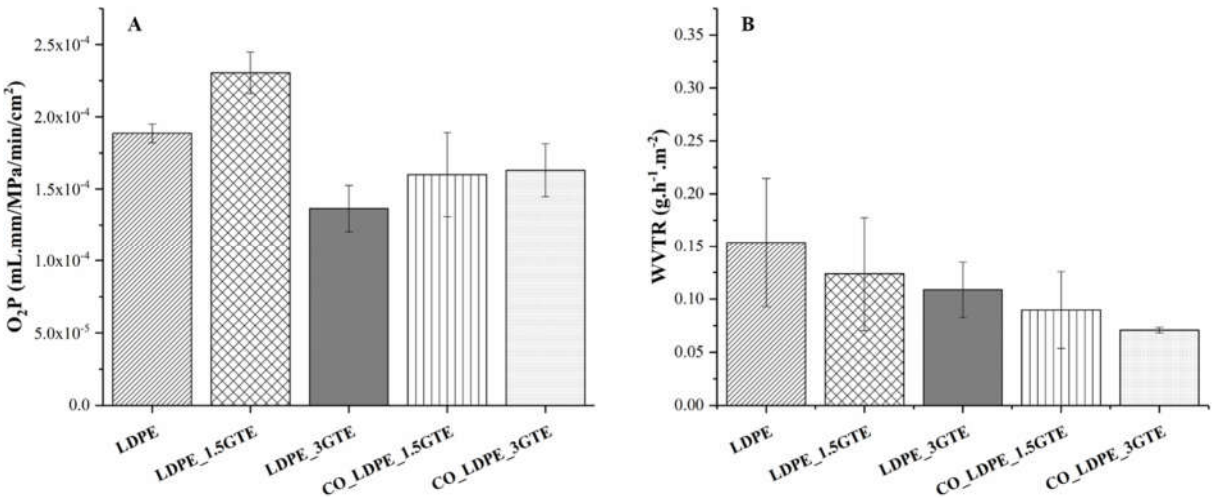

Figure 4.9 - Oxygen permeability (O2P) (A) and WVTR (B) of LDPE/GTE active films.

The WVTR characterizes the ability of moisture to penetrate and pass through the active film. Good water vapor barrier property is crucial for packaging, which not only prevents excessive water loss from food products but also avoid moisture transfer from the atmosphere. This aspect is very important, since water can promote the growth of the microorganisms and decrease food shelf-life [10]. The hydrophobic nature of EOs/plant extracts could have an influence on the properties of polymer-based films developed with EOs/plant extracts due to the decrease in water sorption capacity of the films [5].

The WVTR of LDPE-based films was evaluated to understand the effect of GTE on LDPE water barrier properties, Figure 4.9 - B. The results show that the addition of GTE in the LDPE matrix decreased the

WVTR of the active films from 0.15± 0.061 $g.h^{-1}m^{-2}$ (control film) to 0.12±0.054; 0.11±0.026; 0.090±0.036, and 0.071±0.003 $g.h^{-1}m^{-2}$ corresponding to the LDPE_1.5GTE, LDPE_3GTE, CO_LDPE_1.5GTE, and CO_LDPE_3GTE film, respectively. The addition of higher amounts of GTE improves the LDPE-based film's water vapor barrier properties. These results are in accordance with studies in the literature [49].

Water vapor barrier property of LDPE-based active films can be enhanced by varying the addition of GTE. The polyphenolic compounds, presented in GTE, contain many hydrophobic groups, like benzene ring group, that consequently improves the hydrophobic property of LDPE-based films with the incorporation of GTE. The interaction between LDPE and GTE through hydrogen bonds is another possible reason for the hydrophobic character of the film, which led to a more compact structure, these aspects were also reported by Chen *et al.* [36].

4.4 Conclusion

LDPE-based in natural extract packaging material for food has been successful developed, consisting in a LDPE active film with different concentrations (1.5 and 3 wt.%) of GTE and with different structures, monolayer and coextruded films (two layers). While the tensile properties were similar when compared to LDPE film, the incorporation of GTE improves resistance to penetration, COF and flex-crack.

Films LDPE containing GTE exhibited a small increase in hydrophobicity and a small decrease in transparency. The characteristic brownish color of GTE-based films tends to increase as the concentrations of GTE increases. The antioxidant capacity was significantly improved and increases with concentration.

GTE incorporation improves LDPE barrier properties giving to this active film a great potential to be used in the food packaging industry. Nevertheless, further studies are necessary to evaluate the migration of GTE from the polymer matrix to the atmosphere or into food simulants, to establish the subsequent GTE activity and safety effects.

The results of this work revealed that GTE in low concentrations (1.5-3 wt.%) have a great potential as a natural antioxidant to produce active films to preserve food and promotes the consumer health by the reduction or replacement of synthetic additives. This study also demonstrate that LDPE/GTE can be produced in industry using commercial blown-film extrusion lines.

4.5 References

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5 EVALUATION OF ACTIVE LDPE FILMS FOR PACKAGING FRESH ORANGE JUICE

In this chapter is analyzed the effectiveness of the active LDPE/GTE packaging developed as a new approach to preserve and extend the shelf-life of orange juice

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Abstract

Microbial development, enzymatic action, and chemical reactions influence the quality of fresh orange juice without any type of treatment, which may compromise the sensory characteristics and cause nutritional losses. Active low-density polyethylene (LDPE) films containing green tea extract (GTE) were prepared by blown extrusion process. Packages prepared from the films were then filled with fresh orange juice and stored at 4 °C. Ascorbic acid (AA) content, sugar content, browning index, parameters of color, pH, total acidity (TA) and microbial stability were evaluated after 3, 7, and 14 days of storage.

Packages of film containing GTE, kept the microbial load of fresh juice below the limit of microbial shelf-life (6 log CFU/mL) for the bacterial growth, towards the yeasts and molds only the CO_LDPE_3GTE package kept the microbial load of fresh juice below the limit, up to 14 days. The least degradation of AA (32.60 mg/100 mL of juice), development of brown pigments (browning index=0.139), pH with the value of 3.87, and sugar content (11.4 g/100 mL of juice) were observed for the packages containing 3 wt.% of GTE, after the same time. Active LDPE films containing 3 wt.% of GTE increased the shelf-life of fresh juice and can became a promising film to storage this food product.

Keywords: Active packaging; Orange juice; Physicochemical characteristics; Microbial stability; Shelf-life

5.1 Introduction

Fruit juices are considered a source of vitamins, soluble/insoluble fibers and minerals and their characteristic flavor makes it a product of high consumption [1]. Processed products, especially juice, have become more popular because they are easier to be consumed [2]. Even in refrigerated conditions, natural juices have a short shelf-life. Processing and storage conditions, packaging and raw material are very important factors for the stability of citrus juice. These factors cause microbiological, enzymatic, chemical, and physical changes that will spoil the sensory and nutritional characteristics [1].

The high content of vitamin C in orange juice and the pleasant taste makes it the most appreciated and consumed citrus juice [1–3]. Vitamin C is an essential nutrient for humans and due to its high antioxidant properties, it protects the presence of free radicals helping to prevent many diseases [2]. However, due its nature, vitamin C can oxidize and be lost during the juice storage period. The rate of vitamin C degradation highly depends on storage conditions. Among the factors affecting vitamin C loss in packed

orange juice are, temperature, dissolved oxygen and oxygen permeability through the packaging material [2].

Microflora present in fruit juices is generated during harvest and postharvest, including storage, transport and processing. Lactic and acetic acid bacteria have been isolated from fruit juices, many microorganisms are acid-tolerant bacteria and fungi. [4, 5]. The most commonly reported bacteria generation includes *Acetobacter, Alicyclobacillus, Bacillus, Gluconobacter, Lactobacillus, Leuconostoc, Zymomonas, and Zymobacter* [6].

Availability of nutrients, the presence of antimicrobial compounds, oxidation-reduction potential, water activity, and pH are the critical factors that affect the spoilage of juices, being the last two factors of key importance. The spoilage in juices results from degradation of the product, which induces changes in appearance, color, texture, CO_2 production, cloud loss and development of off-flavors [4, 7, 8].

Fruit juices have a pH in acidic range (<4.5) serving as an important barrier to microbial growth. However, foodborne pathogens such as, E. coli and Salmonella survive in an acidic environment of fruit juices due to acid stress response. Therefore, in the last two decades, several foodborne outbreaks associated with unpasteurized fruit juices have been documented in many countries [4, 9, 10].

Shelf-life is generally known as the period of time during which a food product maintains acceptable characteristics under specified storage conditions. On a food label, shelf-life can be indicated by either a “best before” date that correlates to the quality of the food or “use by” date that is linked to food safety. Sensory characteristics of food, such as, color, aroma, or taste determine its shelf-life. The accurate shelf-life prediction in the package is important not only for food industries but also for consumers [11].

Active compounds can provide several functions when incorporated into the packaging materials, which can be an alternative to conventional packaging systems [12, 13]. There are several natural antioxidants, sweeteners, coloring and antimicrobial agents that have their origin in animals, plants, or even microorganisms, although has not been defined as a specific category for natural additives [14]. Several natural substances can have an active function in the package, such as essential oils (EOs)/extracts of plants, which are generally recognized as safe (GRAS). They may be used as food additives to extend the shelf-life and maintain the food’s quality for a prolonged period of time [15–19]. The EOs/extracts have the potential function of inhibit microorganisms and reduce the lipid oxidation because they are rich in phenolic compounds and volatile terpenoids [18–20]. These compounds have several biological properties, such as antioxidant and antimicrobial activities [18, 19, 29–31, 21–28]. Through disturbance of the cytoplasmic membrane, the active components of plants EOs/extracts inhibit microorganisms. These components disrupt the electron flow, active transport, proton motive force, and inhibition of

protein synthesis [19, 20]. The use of EOs/extracts in food could decrease or substitute the application of synthetic antioxidants and antimicrobial compounds, meeting the consumer's demand for more natural products [18].

Since consumers are increasingly looking for natural products, a lot of research is going on, aiming to replace synthetic compounds by natural ones. Particularly plant extracts, and EOs, which are much safer and offer multiple health benefits to humans. The GTE is extracted from green tea and rich in flavonols and a gallic acid derivatives, namely (+)-catechin, (-)-epicatechin, (+)-gallocatechin, (-)-epicatechin gallate, (-)- epigallocatechin, and (-)- epigallocatechin gallate. The green tea extract (GTE) can be mentioned as a rich source of polyphenol antioxidants, particularly catechins and has the status of food additive [13, 21, 28, 32, 33]. Good antioxidant activity and nontoxicity in various food model systems of GTE have been demonstrated, which led to its incorporation into polymers matrix to developed active packaging films to inhibit food oxidation [32–35].

This active packaging could help to solve a problem of the food packaging industry to eliminate or reduce spoilage and foodborne pathogens on the surface of the products, i.e., increasing product's shelf-life. Thus, active packaging, namely antioxidant and antimicrobial, is one the most promising methods to extend shelf-life while sustaining the nutritional and sensory quality of food [36]. Having this in mind, the main objective of this study is to assess the potentially of GTE in active LDPE packaging as a new approach to preserve and extend the shelf-life of fresh orange juice.

5.2 Materials and methods

5.2.1 Materials

Low-density polyethylene (LDPE) was kindly provided by Vizelpas. The green tea extract (GTE) was supplied from ESSÊNCIAD'UMSEGREDO, LDA. Distilled water, phenolphthalein, and sodium hydroxide (NaOH) were used in total acidity (TA). Ethanol was used in the browning index assay. Sulfuric acid (H2SO4), starch, iodine solution, and sodium thiosulfate were used for determination of ascorbic acid (AA) content. Peptone water, plate count agar (PCA), and Dichloran Rose-Bengal Chloramphenicol agar (DRBC) were used in microbiological tests. All the reagents used are analytical grade.

5.2.2 Preparation of LDPE active films

LDPE active films were prepared according to the methodology described in section 4.3.3 - Blown film extrusion in the previous chapter (Preparation and Characterization of Active LDPE Films for Food Packaging).

The description of different active films produced for juice packaging based on LDPE containing GTE is designated in Table 5.1.

Table 5.1 - Packing films based on LDPE/GTE.

Film	Description
LDPE	Film without GTE
LDPE_1.5GTE	Film extruded (monolayer) with 1.5 GTE
LDPE_3GTE	Film extruded (monolayer) with 3 GTE
CO_LDPE_1.5GTE	Film coextruded (two layers) with 1.5 GTE
CO_LDPE_3GTE	Film coextruded (two layers) with 3 GTE

5.2.3 Packaging orange juice

For the evaluation of the packaging potential, fresh orange juice was selected, it was produced under ideal hygienic-sanitary conditions at the time of application, without the addition of preservatives and preservation processes. The packages, of 14 x 13 cm were prepared sealing at the bottom, filled with 300 mL of juice and closed aseptically.

5.2.4 Storage

Packages containing orange juice were stored in dark and cool conditions (4 °C). The samples were evaluated in a single package for each treatment, in a total of 5 trials for their physicochemical properties of color, pH, sugar content, TA, browning index, AA content and microbiological growth, tested immediately after packaging and after 3, 7, and 14 storage days.

5.2.5 Measurement of color, pH and sugar content

The juices were evaluated for color variations using the colorimeter (Minolta Chroma Meter, CR-400) through triplicate measurements. The equipment uses the CIELab measurement system that measures the L* parameter (lightness index scale) ranges from 0 (black) to 100 (white), the a* parameter indicates

the degree of red (+a) or green (−a*) color and the b* parameter measures the degree of yellow (+b) or blue (−b*) color.

The pH measurement of the juices was performed using a pH meter (Even, PHS -3E, USA) at room temperature. The sugar concentration of the juices was measured using a refractometer (Hanna, HI 96801, Romania) which provides values referring to the amount in mg of sugar in 100 mL of juice.

5.2.6 Total acidity (TA)

To determine the TA, 5 mL of previously filtered juice were used. The juice was homogenized with 25 mL of distilled water and 2 drops of 1% phenolphthalein. The mixture was titrated with 0.1 M NaOH until a pink color appeared. TA was calculated using the Equation (1) [37], expressed in %.

$$TA = \frac{(V \times M \times 100)}{p} \quad (1)$$

Where, V (mL), the volume of NaOH spent in the titration of the juice; M, the molarity of standardized NaOH solution and p (mL), amount of juice used.

5.2.7 Browning index measurement

The juices were collected from the bags (10 mL) and centrifuged at 2000 rpm for 20 min. The supernatant was homogenized in a 1:1 ratio with ethanol and filtered with a 0.45-mm filter paper to obtain a clarified extract. The absorbance of the extracts was read on a spectrophotometer at a wavelength of 420 nm [38].

5.2.8 Determination of ascorbic acid (AA)

The AA content was calculated according to Zambiazi [37], the juice (20 mL) was transferred volumetrically and homogenized with 3 mL of H2SO4 (12 M) and 3 mL of starch (0.5% (m/v)). The mixture was titrated with a standardized 0.01 M iodine solution until the dark color appeared. Afterward, the solution was titrated again using 0.01 M sodium thiosulfate until the dark color disappears, finally, the solution is titrated with 0.01 M iodine until the dark color appears again. The amount of AA present was calculated using the Equation (2).

$$AA = [(Vi \times Fi) - (Vt \times Ft)] \times 0.88 \quad (2)$$

Where, AA, the content of ascorbic acid present in the juice expressed in mg of ascorbic acid/mL of juice; Vi (mL), total volume of iodine used in the titrations; Fi, correction factor obtained in the standardization of the iodine solution; Vt (mL), volume of sodium thiosulfate used in the titration and Ft, correction factor for the standardized sodium thiosulfate solution.

5.2.9 Microbiological growth tests

The juices were evaluated for the microbiological growth of bacterial, molds and yeasts. The juice samples (1 mL) were collected aseptically from each different treatment and the packages were closed again and stored. To count of microorganisms, dilutions from 10^0 to 10^6 were made using 0.1% (m/v) peptone water and juices. To count the bacterial growth, 1 mL of juice in depth was inoculated and PCA was added over it. The inoculated plates were placed in an oven at 37 °C for 48 hours and after colonies were counted according to the dilution method. For the counting of molds and yeasts, the same dilutions were used, however, the inoculation was done on the surface using 0.1 mL of each dilution on DRBC. The plates were incubated in an oven at 25 °C for five days, after the colonies appearance they were counted as described above. All microbiological tests were performed in triplicate [39].

5.2.10 Statistical analysis

Microsoft Windows Excel 365 and OriginPro software (Version 17) were used to analyze the resulting data. Results were expressed as mean ± standard deviations of at least three replicates. For color analysis, analysis of variance (ANOVA) and Tukey's test were applied to determine significant differences with a 95% significance interval. The software used was Statistics 5.0.

5.3 Results and discussion

5.3.1 Ascorbic acid and browning index

The evolution of AA content of orange juice packed in active LDPE films containing GTE and LDPE film without GTE, stored at 4 °C during 14 days, is shown in Figure 5.1. The film that presented the best retention of AA was LDPE_3GTE followed by CO_LDPE_3GTE. The LDPE without GTE presented poor retention of AA, as expected. Since oxygen is one of the main factors that contribute to AA degradation and taking into account that headspace was the same for all packages, the only factor that can explain

these differences in AA retention is oxygen permeability [2]. Results indicate that LDPE_3GTE was the film that presents the lowest permeability followed by CO_LDPE_3GTE, LDPE_1.5GTE, and CO_LDPE_1.5GTE, respectively. Considering the limit of 20 mg/100 mL of AA for shelf-life estimation [40], after stored for 14 days all active LDPE films presented values higher than 20 mg/100 mL of AA. The lowest value of 25.83 mg/100 mL in the juice was obtained for LDPE_1.5GTE and CO_LDPE_1.5GTE film.

At early stage of storage, the results indicate that was a rapid degradation of vitamin C, followed by a gradual loss. This is in agreement with results obtained by other researchers [41, 42], this behavior was attributed mostly to the oxygen that was dissolved in the juice and in the headspace in the early stages of storage [38].

The rate of oxidation of AA is highly dependent on the dissolved oxygen concentration. Solomon *et al.* [43] and Wilson *et al.* [44] reported results exhibiting that the rate of oxidation of AA is significantly associated with the level of dissolved oxygen and with the time length of storage. Oxygen permeation through the packaging during storage is another factor contributing to the extension of the aerobic mechanism of AA oxidation in the active LDPE films [38]. Parameters such as light, heat, oxygen, enzymes, and peroxides stimulated the oxidative process of AA [3].

The final AA content after 14 days of storage varied from 18.36 to 32.60 mg/100 mL in the juice. When these data were compared with the minimum values recommended for processed orange juice it was noticed that they were lower than 40 mg/100 mL referred to as minimum for industrialized juice [45].

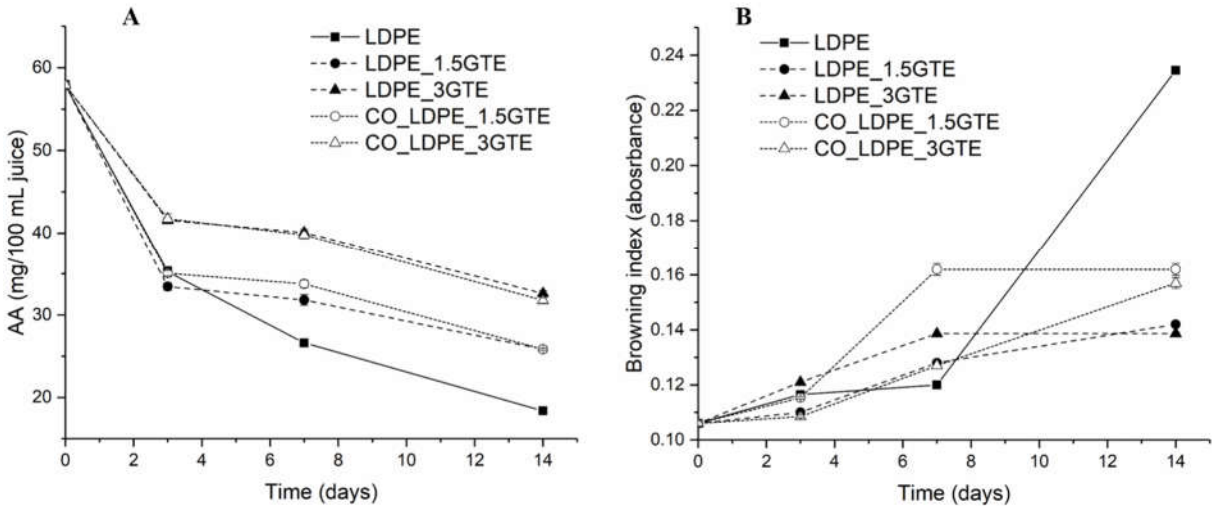

Figure 5.1 - The content of AA (A), and browning index (B) in orange juice packed with active LDPE films containing GTE stored at 4 °C for 14 days.

The values of the browning index in fresh juice measured immediately after packaging were 0.106. Leizerson and Shimoni [46] reported values of the browning index up to 0.367, which leads to the conclusion that is still undetected. Increasing the temperature has a major effect on the increased the rate of browning reactions in fruit juice. According to Figure 5.1 – A and B, a significant decrease is observed in the AA content of all the experimental packages during the storage at 4 °C, while browning index increased slightly in all the test packages.

It can also be seen in Figure 5.1 – B that the LDPE packaging without GTE is the one with the highest browning index, as expected. From the day 7 onwards, there is a greater increase in the browning index in the juice. The package that exhibited a lower browning index is LDPE_3GTE, which demonstrate the influence of GTE as an antioxidant agent.

Roig *et al.* [41] found a relationship between the browning index and the oxidative loss of L-ascorbic acid in citrus juices. The rate of browning is also temperature-dependent, the average browning index of freshly squeezed orange juice.

The results obtained for the browning index are similar to the study of Zerdin *et al.* [38] that determined the extent of AA loss due to oxygen and temperature for orange juice packed in oxygen scavenging film and oxygen barrier film. For the browning index, Cortés *et al.* [47], obtained values lower (0.093) than those obtained in the present study, but the temperature used in the study was lower (2 °C). According to Emamifar *et al.* [3], the browning index increased significantly for all packaging tested in the study, storage at 4 °C, agreeing with the values reached in this study.

Dehydroascorbic acid (DHA) is the major degradation product of AA and is converted to 2,3-diketogulonic acid (DKG), forming xylosone through the aerobic pathway, which is degraded to form reductones or ethylglyoxal, then react with amino acids and contribute to the browning of the orange juice. DHA degrades to form 3-deoxypentosone and the latter degrades to furfural, which also reacts with amino acids [38].

5.3.2 Color

Orange juice color is mainly due to the presence of carotenoid pigments and it is influenced by product ripening, processing treatments, storage conditions and browning reactions [3]. Table 5.2 depicts the evolution of color parameters for orange juice packed in active LDPE films with and without GTE, stored at 4 °C. It can be notice that color parameters did not present significant variations until 3 days of storage. After this time, values of L* start to increase, which indicates an increase in the brightness and light, a* and b* parameters did not present significant variations until 14 days of storage. Thus, there was no

significant change in the color of the juice. The L* parameter changed in LDPE_1.5GTE after 7 days but at a lower rate than CO_LDPE_1.5GTE. It possible to observe that LDPE_3GTE and CO_LDPE_1.5GTE had a better brightness after 14 days. The average of a*, that indicates the variation between red and green color, the increase a* value is verified with a higher amount of GTE (LDPE_3GTE =-0.13±0.03) when compared with LDPE without GTE (-1.26±0.05) after packed 14 days. Parameter b*, that indicates the variation between yellow and blue color, presents an increase of this parameter for all packaging after 14 days of storage, with the largest increase for packaging with 3 wt.% GTE. Thus, a color shift toward positive b* and negative a* directions indicate greater value of yellow and green color in the orange juice. The changes show the progressive deterioration of the juice due to changes in the color spectrum.

These changes have a good correlation with the reduction of AA and the production of brown pigments during storage. Bleaching effect in orange juice might be due to the oxidative degradation of carotenoids, thus, the free radical formed in orange juice packaging might be responsible for the changes in the juice color [3]. Bull *et al.* [48] reported an increase in the total color differences with time in fresh orange juice during storage, regardless of treatment. Esteve *et al.* [45] obtained slight decreases in L* at 4 °C for different commercial orange juices. Lee and Coates [49] studied pasteurized orange juice and reported a small increase in CIE L* value from 40.22 to 41.22. Rivas *et al.* [50] describe a decrease of the parameter L* on pasteurized orange-carrot juice during refrigerated storage. Cortés *et al.* [47] observed that L* values increase in a significant way after one week of refrigerated storage, which is in agreement with the findings of this study.

Table 5.2 - The color parameters obtained for orange juice packaged active LDPE films containing GTE during 14 days of storage.

Film	Day 0			Day 3			Day 7			Day 14		
	L*	a*	b*	L*	a*	b*	L*	a*	b*	L*	a*	b*
LDPE	41.47^{a} ±0.51	-1.67^{d} ±0.09	20.1^{a} ±0.66	40.67^{c} ±0.18	$-0.50^{b,c}$ ±0.06	20.17^{c} ±0.31	43.05^{b} ±0.33	-0.64^{c} ±0.06	$23.36^{b,c}$ ±0.13	46.65^{a} ±0.38	-1.26^{c} ±0.05	28.03^{a} ±0.43
LDPE_1.5GTE	40.95^{a} ±0.61	-1.10^{c} ±0.04	20.04^{a} ±0.29	$41.59^{b,c}$ ±0.56	-0.37^{b} ±0.16	22.77^{b} ±0.56	42.56^{b} ±0.47	-0.91^{d} ±0.09	22.46^{c} ±0.38	43.33^{c} ±0.20	-0.59^{b} ±0.12	$24.27^{c,d}$ ±0.41
LDPE_3GTE	39.04^{b} ±0.38	0.30^{a} ±0.06	18.32^{b} ±0.15	42.98^{a} ±0.51	0.87^{a} ±0.10	25.96^{a} ±0.62	46.15^{a} ±0.65	0.79^{a} ±0.08	29.07^{a} ±0.47	$45.65^{a,b}$ ±0.59	-0.13^{a} ±0.03	26.40^{b} ±0.13
CO_LDPE_1.5GTE	40.96^{a} ±0.13	-1.25^{c} ±0.05	18.80^{b} ±0.30	42.94^{a} ±0.32	-1.25^{d} ±0.02	22.89^{b} ±0.15	47.10^{a} ±0.92	-0.09^{b} ±0.01	28.79^{a} ±0.76	45.51^{b} ±0.02	-1.10^{c} ±0.06	$25.40^{b,c}$ ±0.57
CO_LDPE_3 GTE	$40.29^{a,b}$ ±0.08	-0.87^{b} ±0.04	18.78^{b} ±0.29	$42.57^{a,b}$ ±0.27	-0.77^{c} ±0.06	23.42^{b} ±0.18	43.67^{b} ±0.49	-0.94^{d} ±0.05	24.26^{b} ±0.66	43.41^{c} ±0.07	-0.96^{c} ±0.15	23.68^{d} ±0.25

Values are given as mean ± standard deviation. Different superscript letters in the same column indicate a statistically significant difference ($p<0.05$).

5.3.3 pH and total acidity (TA)

The pH values of the juice studied in five different packages are within the normal range (3-4), after storage for 14 days at 4 °C and the differences among them are significant, as presented in Figure 5.2 - A. As it would be expected, higher acidity corresponds to lower pH value. Owing to the presence of a natural buffer medium in orange juice (mainly potassium citrates and malates), variations in pH with storage are more pronounced than the variations in acidity. After 7 days of storage at 4 °C, the values of pH of the juice in different packages under study decrease, going from 4.73 (initial) to close 3.85 (day 7). Juices with a greater decrease in pH values (Figure 5.2 - A) are the ones in films of CO_LDPE_3GTE and LDPE. The results obtained are in agreement with the study of Touati *et al. [*51], that verified that pH values were significantly lower, independently of the temperature.

Bull *et al.* [50] studied pasteurized nor high pressurized orange juices stored for 12 weeks and noticed a significant modification in pH. Contrarily, Esteve *et al.* [45] do not detected significant modification in pH values of various pasteurized orange and carrot-orange juices, during their storage in refrigeration at 4 °C and 10 °C. In the study made by Cortés *et al.* [47] there was a statically increase in pH values for all the juices analyzed. This increase can be related to a microbiological deterioration of juice, as described by Del Caro *et al.* [52], who studied the changes of pH in citrus segments and juices during storage at 4 °C.

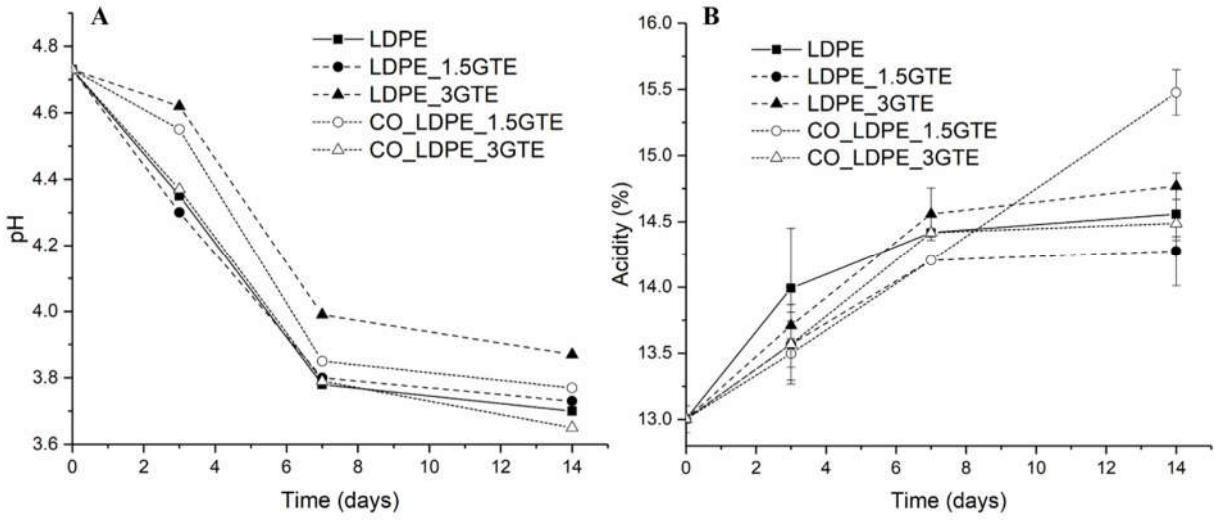

Figure 5.2 - pH indexes (A), and TA (B) of orange juice packed from active LDPE films containing GTE stored at 4 °C for 14 days.

When fermentation of orange juice occurs, the organic acids are responsible for particular flavor and palatability, that is the result of the biochemical process, through the development of certain spoilage microorganisms. To a large extent, acidity protects against the development of pathogens. In orange juice,

citric acid is the most abundant, followed by malic acid, both being present mostly as free acids, although in limited quantities they are also combined as citrates or malates, which gives orange juice its buffer effect. Non-volatile free acids, such as oxalic, galacturonic, quinic, and many others are found in smaller quantities [45]. The TA in the five packages studied have similar behavior (Figure 5.2 - B), except packaged using CO_LDPE_1.5GTE film. Along storage, an increase is visible in acidity for all juices until day 7 and after a plateau is reached. CO_LDPE_1.5GTE film has a different behavior, it exhibits a linear increase in acidity with time. This increase in acidity indicates the start of spoilage or fermentation of the sample. These results are in agreement with those verified by Esteve *et al.* [45], where a significant increase in acidity with storage time was verified. Similar results were reported by Supraditareporn and Pinthong [53] at 4 °C, the acidity began to increase in 3-6 days. The low pH values of orange juices significantly limit the number and types of bacteria that can survive or grow. The typical pH of orange juices is usually between 3 and 4. In this pH range, the lactic acid bacteria to be spoilage microorganisms causing the development of slime, gas, off-flavor, turbidity, and changes in acidity [53].

5.3.4 Microbiological analysis and sugar content

Average initial population immediately after packaging was determined to be $1.79x10^3$ CFU/mL for yeast and molds and $4.57x10^2$ CFU/mL for bacterial growth in orange juice. The variations in the population of yeast and molds and total aerobic bacteria are shown in Figure 5.3.

The average population of yeast and molds increased in juices packaged as well as bacterial growth over of storage. It is possible to verify that the yeast and molds are better adapted to orange juice under refrigeration than bacteria, which is in agreement with the findings of Sadler *et al.* [54] and Emamifar *et al. [3*]. Significant decreases were perceived over 7 days of storage in total count of bacteria and yeast and molds population of the juice in LDPE_3GTE film when compared with the juice in CO_LDPE_3GTE that containing the same concentration of GTE. Figure 5.3 shows that the level of population of yeast and molds, and bacterial growth increased to $1.55x10^6$ CFU/mL and $6.15x10^4$ CFU/mL, respectively, after 14 days of storage in LDPE_3GTE package. The shelf-life of fresh orange juice is defined as the time to reach a microbial population of 6 log CFU/mL [55]. The average population of bacterial growth remained below 6 log CFU/mL until 7 days, in all the packages, and in case of yeast and molds only CO_LDPE_3GTE remained below 6 log CFU/mL. As the GTE concentration increase to 3 wt.%, the antimicrobial activity of coextruded films decreased (Figure 5.3). In contrast, as the GTE concentration increases in monolayer films , the antimicrobial activity increases (Figure 5.4). LDPE_3GTE exhibited a higher antibacterial activity when compared to CO_LDPE_3GTE after 7 days of storage. It is possible to

verify that the increase of the concentration of GTE in the packages has a more prominent effect on antibacterial activity than antifungal, after a week of storage. Therefore, for orange juice at 4 °C, it is possible to verify that LDPE_3GTE keeps the same behavior along the time, it has always higher antimicrobial activity than the other active packages. Previous studies have shown a shelf-life of 14 days for natural, cold orange juice (4 °C) [48, 56, 57]. Yeast, molds, and bacteria exhibit different levels of susceptibility to GTE that was incorporated in active LDPE films. Published studies demonstrate that the yeast growth during storage is the principal parameter that affects the shelf-life of fresh orange juice [1, 57].

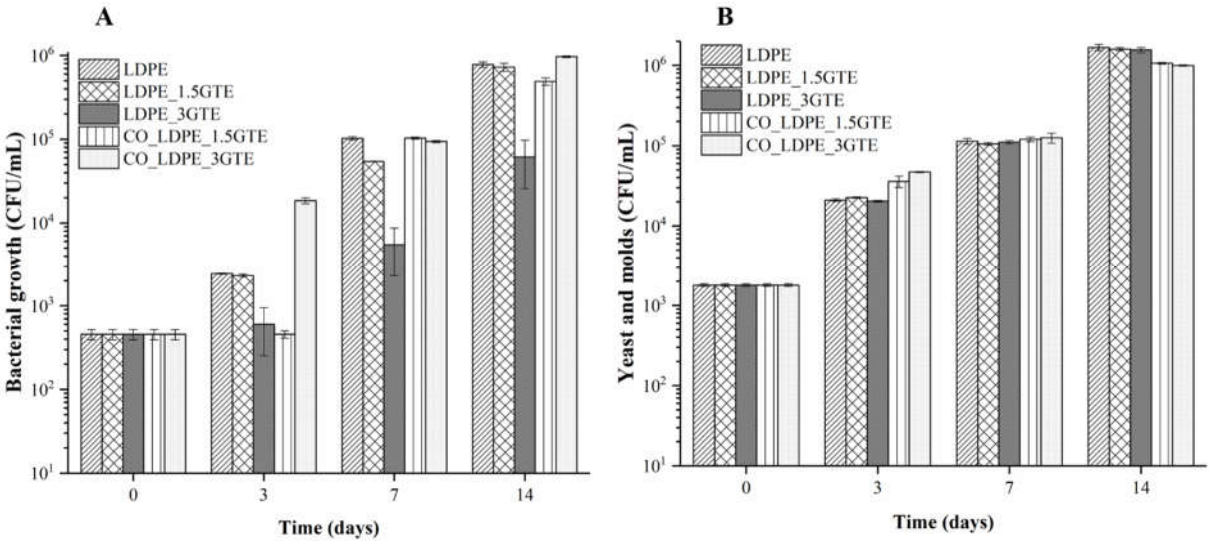

Figure 5.3 - Count of bacterial growth (A) and Yeast and molds (B) in orange juice packed from active LDPE films containing GTE stored at 4 °C for 14 days.

Muriel-Galet *et al.* [60], characterized the antimicrobial efficiency of polypropylene (PP)/ ethylene-vinyl alcohol (EVOH) films with oregano essential oil and citral, and verified that antimicrobial activity reduced spoilage flora on salad, being more effective against Gram-negative bacteria. Another study [21], assessed the antimicrobial effect of GTE and oregano essential oil incorporated in an EVOH film, which showed strong antimicrobial activity against the tested microorganisms, the films containing GTE also inhibited the growth of L. monocytogenes and E. coli in liquid media, but the synergistic antimicrobial effect was not detected. The study of Dong *et al.* [19] based in active packaging film of bilayer structure based on LDPE with incorporation of rosemary and cinnamon essential oils revealed that the active LDPE-based films effectively retarded the growth of total viable count for the Pacific white shrimps, verifying that the cinnamon essential oil exhibited stronger antimicrobial effects than rosemary essential oil.

The orange juice used in this study is a natural juice without the any additional preservatives, usually with a short shelf-life, between 3-4 days. Microbiological activity analysis demonstrates that at least 7 days are

necessary for the growth of bacteria, yeasts and molds. The fact that the juice still good for consumption after a week is probably due to the phenolic compounds present in the GTE, namely catechins, such as epigallocatechin gallate since they are possibly the main responsible for the reduction of microbiological growth.

The initial value of sugar concentration in orange juice for the different packages was 12.7 g/100 mL of juice. After 3 days of storage, orange juice showed a decrease in sugar concentration of about 9.45% in all packages, juice in LDPE_1.5GTE packaging film present a greatest reduction (≈11%). From the day 3 until the end of storage (14 days), the sugar concentration remained practically constant, as can be seen in Figure 5.4.

The reduction in the concentration of sugars is correlated with the increase in the production of microorganisms in juice, as can be seen in Figure 5.3 and 5.4. As yeasts and molds increase, there is a consumption of sugars that are transformed in carbon dioxide, through the fermentation process, which contributes for a decrease in the value of sugar concentrations.

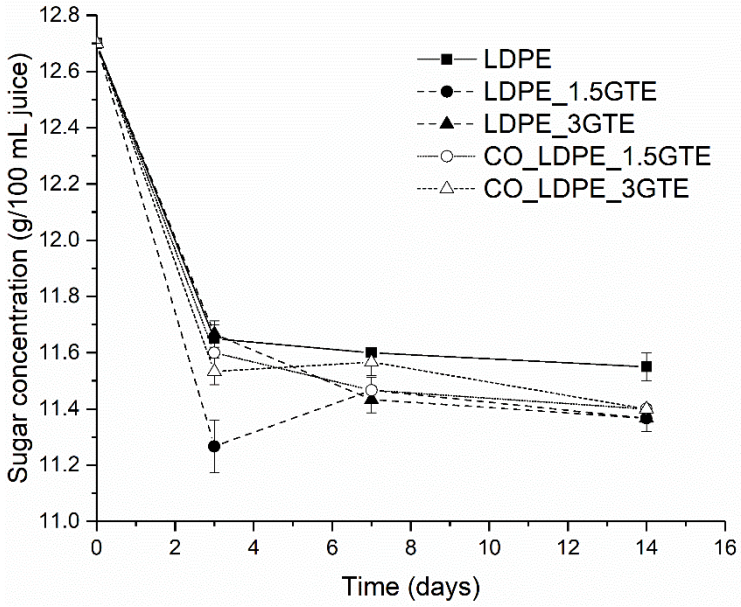

Figure 5.4 - The sugar content in orange juice packed in active LDPE films containing GTE stored at 4 °C for 14 days.

5.4 Conclusion

In general, the packages with a greater concentration of GTE showed better results for juice storage. The greatest effect was verified on the monolayer package with 3 wt.% of GTE.

The results show that final content of AA varied from 18.36 to 32.60 mg/100 mL, which is lower than the reference for industrialized juice (40 mg/100 mL). Other parameters, such as pH and TA, show

different behavior, pH decreases by approximately 21% as a result of an increase of TA. The decrease in the amount of AA correlates with an increase in browning, thus the packaging with the greatest decrease in the AA content is also the one with the highest browning index, which corresponds to the control packaging, LDPE without GTE. The juice that presents higher AA content and lowest browning index are packed in film that contain 3 wt.% GTE (both monolayer and coextruded). The most prominent result of increasing the juice shelf-life is verified through microbiological analysis. The analysis of microbiological activity showed that the increase of concentration of GTE in the LDPE films had a more prominent effect over the bacteria than the fungi, after a week of storage. It can be concluded that GTE is more effective in inhibit bacterial growth in orange juice.

All the results of this investigation allow to conclude that the LDPE_3GTE packaging was the most appropriated to storage orange juice during 14 days at 4 °C. Thus, active LDPE films containing GTE can be a new approach to preserve and extend the shelf-life of fresh orange juice at 4 °C.

5.5 References

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6 CONCLUSIONS AND FUTURE WORK

This chapter presents the general conclusions, the suggestions for future works, and the further contributions of this thesis.

6.1 Conclusions

The research presented in this thesis aimed to develop an active food package using a multilayer blown film extrusion and an active agent in the inner layer. Since natural extracts from aromatic plants are promising antioxidant and antimicrobial that can be incorporated in polymers to produce active food packaging that increases the shelf-life of food, several species were selected. Therefore, this research began (Chapter 3) with the evaluation of their potential biological properties (antioxidant and/or antimicrobial). Several aromatic natural extracts: aqueous (AE and CLE), hydroethanolic (GTE, CE, RE, and LBE) and ethanolic (CCE) were assessed in terms of thermal stability, antioxidant and antimicrobial activities. Based on the thermal characterization, antioxidant capacity, cytotoxic activity, and antimicrobial activity, it was possible to confirm that the extracts that meet the conditions as candidates to be incorporated into the polymeric matrix of LDPE were RE, GTE, and CCE.

In Chapter 4, various active films by blown extrusion process were produced. Different concentrations of GTE were assessed, as well as several film structures (monolayer and multilayer). After the development of active films, they were evaluated for microstructure property, where it was found that analysis through SEM evidenced that the films had a homogenous and smooth surface with the incorporation of GTE into the LDPE matrix. The addition of GTE affected the film transparency and contact angle demonstrated an increase in surface hydrophobicity. It was found that for TS, with an increase of GTE amount the mechanical property slightly decreased. However, for COF and penetration resistance, the opposite was seen, incorporation of GTE improves these properties. Still, concerning COF, the film that presented the best results was the LDPE_1.5GTE monolayer. The flex-crack test revealed that the coextruded films have worse results, presented “micro-holes”. The films exhibited antioxidant activity and the best antioxidant capacity were for those that contained 3 wt.% GTE, as well as mono and multilayer structure. The barrier properties (water and O_2) were also improved for films with highest concentration, multilayer films have the most effective response to water vapor permeability.

Chapter 5 assessed the feasibility of active LDPE films in food packaging applications using orange juice as a model food system. Packages containing the GTE kept the microbial load of fresh juice below the limit of microbial shelf-life (6 log CFU/mL) for the bacterial growth, towards the yeasts and molds only the CO_LDPE_3GTE package kept the microbial load of fresh juice below the limit, up to 14 days. It was also found that the least degradation of AA (32.60 mg/100 mL of juice), development of brown pigments (browning index=0.139), pH with the value of 3.87, and sugar content (11.4 g/100 mL of juice) were observed in film containing 3 wt.% of GTE. The evaluation of color for different packages did not vary

significantly and the changes undergone were correlated with the reduction of AA and the production of brown pigments during storage. Sugar concentration did not show significant variation after 14 days of storage. In general, the LDPE_3GTE packaging was the packaging that showed the best results for all parameters analyzed in orange juice after 14 days stored at 4 °C.

Through this study, it is possible to conclude that active LDPE films packaging containing GTE were successfully produce by blown extrusion, which are able to be used to preserve and extend the shelf-life of fresh orange juice at 4 °C. Moreover, this production process can easily be implemented at industrial scale.

6.2 Future Work

The results obtained with this research, particularly the ones presented in the last two chapters, confirm that GTE demonstrated to be an active additive in the food packaging, presenting both antioxidant and antimicrobial activity, as well as good barrier properties. However, as a package that will be used in the food industry, additional studies must be carried, like migration and absorption.

Given the results and conclusions of the present research, some future work can be recommended:

- To use an intermediate value of the concentration of GTE, to check from which concentration the GTE is effective;
- To investigate the migration of active agents (GTE) from films to the atmosphere and into food simulants to postulate the subsequent AO and/or AM activity and safety effects;
- Use the microencapsulation technique, to make the extract more stable and minimize losses during processing;
- To investigate the potential use of other extracts as active additives for food packaging alone and in combination with GTE, to verify if synergistic effects exist.