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Mariana Alvoco Andrade

ACTIVE FOOD PACKAGING INCORPORATING EXTRACTS FROM SEAWEED, PLANTS AND FRUITS BY-PRODUCTS: DEVELOPMENT, CHARACTERIZATION AND EFFECTIVENESS

Tese no âmbito do Doutoramento em Ciências Farmacêuticas, ramo de Bromatologia e Hidrologia orientada pelo Professor Doutor Fernando Jorge Ramos e pela Doutora Ana Teresa Sanches Silva apresentada à Faculdade de Farmácia da Universidade de Coimbra

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Framework

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Contents

Abbreviations

2-AAF	2-Acetil Aminofluorene
AA	Arachidonic acid
AAC	Antioxidant Activity Coefficient
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AIDS	Acquired immunodeficiency syndrome
AV	*p*-anisidine value
BC	Bacterial cellulose
BHA	Butylated hydroxyanisole
BHI	Brain Hearth Infusion Broth
BHT	Butylated hydroxytoluene
CMC	Carboxymethyl cellulose
D	Dried
DAD	Diode array detector
DEN	Diethylnitrosamine
DHA	Docosahexaenoic acid
DM	Dry mass
DMA	Dynamic mechanical analysis
DPPH	2,2-diphenyl-1-picrylhydrazyl
DS	Dry sample
DW	Dry weight
EAB	Elongation at break
EC	European Commission
ECE	Epicatechin equivalents
EDTA	Ethylenediamine tetraacetic acid
EFSA	European Food Safety Authority
EM	Elastic modulus
EOs	Essential oils
EPA	Eicosapentaenoic acid
ESI	Electrospray ionization
EU	European Union
EUROSTAT	European Statistical Office
F1	Formulation 1, apple and ginger (50:50, w/w)
F2	Formulation 2, apple, carrot, beet and ginger (50:29:20:1, w/w)
FAO	Food and Agriculture Organization of the United Nations
FD	Freeze-dried
FD&C	Federal Food, Drug, and Cosmetic Act
FDA	Food and Drug Administration
FSIS	Food Safety and Inspection Service

FTIR	Fourier-transform infrared spectroscopy
GAE	Gallic acid equivalents
GRAS	Generally Recognized As Safe
GTE	Green tea extract
HACCP	Hazard Analysis and Critical Control Point
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
IC50	Half maximal inhibitory concentration
IP	Inhibition Percentage
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LAB	Lactic acid bacteria
LD50	Median lethal dose
LDPE	Low-density polyethylene
LDPE/4LE	LDPE with 4% (w/w) of Lemon extract
LE	Lemon extract
LOD	Limit of Detection
LOQ	Limit of Quantification
LSWE	Lychee seed water extract
MAE	Microwave Assisted Extraction
MAP	Modified Atmosphere Packaging
MDA	Malonaldehyde
MS/MS	Tandem Mass Spectrometry
MSG	Monosodium glutamate
OP	Oxygen permeability
OS	Oxygen scavengers
PCA	Plate count agar
PCL	Polycaprolactone
PDCAAS	Protein digestibility-corrected amino acid score
PET	Polyethylene terephthalate
PG	Propyl gallate
PGE	Phloroglucinol equivalents
PHA	Polyhydroxyalkanoate
PHB	Poly-hydroxybutyrate
PLA	Polylactic acid
PLA/2GTE	PLA with 2% of Green tea extract
PLA/2GTE/2RE	PLA with 2% of Green tea extract and 2% of rosemary extract
PLA/2RE	PLA with 2% of Rosemary extract
PLA/3PP	PLA with 3% of Pomegranate peels
PLA/3PP	PLA with 3% of pomegranate peels
PLA/3PPE	PLA with 3% of Pomegranate by-products extract

PLA/3PPE	PLA with 3% of freeze-dried pomegranate peels
PLA/4GTE	PLA with 4% of Green tea extract
PLA/4RE	PLA with 4% of Rosemary extract
PLA/6LE	PLA with 6% (w/w) of Lemon extract
PLA/PEG/4LE	PLA with 15% (w/w) of poly(ethylene glycol) and 4% (w/w) of Lemon extract
PPE-FD	Freeze-dried pomegranate peels extract
PPE-N	Natural pomegranate peels extract
PTFE	Polytetrafluoroethylene
PUFA	Polyunsaturated fatty acids
PV	Peroxide value
PVA	Polyvinyl alcohol
RE	Rosemary extract
RH	Relative humidity
SCDLP	Soybean Casein Lecithin Polysorbate
SD	Standard deviation
SEM	Scanning electron microscope
SFE	Supercritical fluid extraction
TBA	Tiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBHQ	*tert*-Butylhydroquinone
TCA	Tricloroacetic acid
TE	Trolox equivalents
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
TS	Tensile strength
TSA	Tryptone Soya agar
UHPLC	Ultra-High Performance Liquid Chromatography
USA	United States of America
USDA	United States Department of Agriculture
UV	Ultraviolet
WHO	World Health Organization
WPV	Water Vapor Permeability

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Resumo

O conceito de embalagens alimentares ativas é relativamente recente, tendo como objetivo aumentar o tempo de vida útil dos alimentos e manter ou melhorar as características organoléticas do alimento, através da interação deliberada entre a embalagem e o alimento embalado. Esta interação pode ser feita através da migração de compostos ativos da embalagem para a superfície do alimento embalado atuando, neste caso, como um sistema de libertação.

Algas, plantas aromáticas e subprodutos de fruta são conhecidos pelas suas poderosas atividades biológicas, nomeadamente as atividades antioxidantes e antimicrobianas. Através destas matrizes, extratos e óleos essenciais com alto teor em compostos bioativos podem ser obtidos e aplicados em matrizes poliméricas de forma a obter uma embalagem ativa.

O principal objetivo desta tese de doutoramento consistiu em desenvolver, caracterizar e avaliar a eficácia de novos materiais alimentares ativos, incorporando extratos de algas, plantas aromáticas ou subprodutos de fruta. Estes novos materiais ativos terão uma ação antioxidante e/ou antimicrobiana e a sua eficácia será avaliada em alimentos modelo. Extratos da macroalga *Fucus vesiculosus* L., folhas de chá verde (*Camelia sinensis* L.) e de alecrim (*Rosmarinus officinalis* L.), subprodutos de várias formulações de sumos de fruta e subprodutos de limão (*Citrus limon*), laranja (*Citrus* × *sinensis*), romã (*Punica granatum*) e uva (*Vitis vinifera*) foram obtidos através de um método de extração com solvente utilizando etanol, um solvente de grau alimentar. A maior capacidade antioxidante foi observada nos extratos de alecrim e subprodutos de romã.

O extrato de *F. vesiculosus* L. foi incorporado numa embalagem ativa à base de proteína de soro de leite pelo método de *casting*. A incorporação do extrato melhorou a espessura, a resistência à tração e a elasticidade do filme à base de proteína de soro de leite. Ao mesmo tempo, diminuiu a permeabilidade ao vapor de água do filme. O filme ativo foi eficaz na redução da oxidação lipídica na carne de frango crua por um tempo máximo de armazenamento de 25 dias a 4 °C.

Dos estudos comparativos entre os subprodutos de sumos e frutas cítricas, o extrato de subprodutos de limão revelou a maior capacidade antioxidante. O extrato foi então incorporado em embalagens à base de ácido poliláctico (PLA) e polietileno de baixa densidade (LDPE), que foram posteriormente testadas em amêndoas e carne de vaca crua. A embalagem ativa à base de LDPE apresentou a maior eficácia em retardar o aparecimento de produtos resultantes da oxidação lipídica das amêndoas. Os ensaios

de migração realizados mostraram também que a embalagem de LDPE retém durante mais tempo os compostos fenólicos do que a embalagem ativa de PLA. No que diz respeito aos ensaios com a carne de vaca, a embalagem ativa à base de ácido poliláctico foi eficaz em inibir o crescimento de microrganismos ao longo de 6 dias de armazenamento.

O extrato e os subprodutos de romã foram incorporados em embalagens de PLA e as suas propriedades químicas, mecânicas, óticas e de barreira foram avaliadas. Com a incorporação dos subprodutos e do extrato da romã, foram produzidas duas embalagens: com 3% de subprodutos de romã (PLA/3PP) e 3% de extrato de romã (PLA/3PPE). Os compostos fenólicos, punicalagina (A+B) e ácido elágico, foram ambos identificados e quantificados no extrato e nas cascas de romã. As embalagens ativas foram utilizadas para embalar amêndoas e carne de vaca crua para avaliar o seu potencial antioxidante e antimicrobiano. Apesar do efeito das embalagens ativas não ser claro na amêndoa, ambas as embalagens foram eficazes na inibição da formação de malonaldeído durante 11 dias de armazenamento como na inibição do crescimento microbiano durante o mesmo período.

Embalagens ativas de PLA com extrato de chá verde (GTE), extrato de alecrim (RE) e uma mistura de ambos foram concebidas e utilizadas para embalar amêndoa e carne de vaca crua. Os principais compostos fenólicos identificados no GTE foram a catequina, epigalocatequina galato, epicatequina, rutina e ácido gálico, enquanto no RE os principais compostos fenólicos foram o ácido carnósico, o carnosol e o ácido rosmarínico. No entanto, o RE apresentou um maior conteúdo em compostos fenólicos totais do que o extrato de chá verde. Ao todo, foram concebidos cinco filmes de PLA: PLA com 2 e 4 % de GTE (PLA/2GTE e PLA/4GTE), PLA com 2 e 4 % de RE (PLA/2RE e PLA/4RE) e PLA com 2% de GTE e 2 % de RE (PLA/2GTE/2RE). O PLA/2RE e PLA/4RE foram os mais eficazes em retardar a oxidação lipídica da amêndoa.

Ao todo, foram produzidas 11 embalagens alimentares ativas com extratos naturais, das quais 10 com base em produtos biodegradáveis (proteína de soro de leite e PLA). Relativamente às embalagens de PLA, nenhum dos extratos alterou significativamente a estrutura do mesmo. Todas as embalagens se mostraram eficazes na redução da oxidação lipídica, especialmente quando aplicadas à carne crua, um alimento modelo ideal para a avaliação *in vivo* das propriedades antioxidantes e antimicrobianas das embalagens ativas com extratos naturais.

Abstract

Food active packaging is a relatively recent food packaging concept whose main goal is to increase foods' shelf-life and to maintain or improve organoleptic characteristics, by deliberated interaction with the packaged food. This interaction may be due to the transfer of active compounds from the package to the food surface and in this case active food packaging works as a releasing system.

Seaweeds, aromatic plants, and fruits by-products are well-known for their powerful biological activities, namely antimicrobial and antioxidant activity. From these matrixes, extracts, and essential oils with high content in active compounds can be obtained and applied in polymeric matrices in order to obtain active packaging.

The main goal of this PhD thesis was to develop, characterize and evaluate the effectiveness of new active food packaging materials incorporating extracts from seaweeds, aromatic plants, or fruits by-products. The new active packaging will be antioxidant and/or antimicrobial and their effectiveness will be evaluated using model foods.

Extracts from the brown seaweed *Fucus vesiculosus* L., green tea (*Camellia sinensis* L.) leaves, rosemary (*Rosmarinus officinalis* L.) leaves, by-products of several juices' formulations and by-products of lemon (*Citrus limon*), orange (*Citrus* × *sinensis*), pomegranate (*Punica granatum*) and grape (*Vitis vinifera*) were obtained through solvent extraction with ethanol, a food-grade solvent. The highest antioxidant capacity was found in the rosemary and pomegranate by-products extracts.

The *F. vesiculosus* L. extract was incorporated into a whey-protein based packaging by casting method. The addition of the extract to the whey-protein based film successfully improved the thickness, tensile strength, and the film' elastic modulus, but diminished the film' water vapor permeability. Also, the active film was able to inhibit the fresh poultry meat' lipid oxidation for a maximum storage time of 25 days at 4 °C.

From the comparative studies among the juices and *Citrus* fruits' by-product extracts, lemon by-products revealed the highest antioxidant activity. The extract was incorporated into polylactic acid (PLA) and low-density polyethylene-based (LDPE) packages and used to pack almonds and raw beef meat, as model foods. The LDPE showed higher efficiency in delaying the almonds lipid oxidation, and the migration assays showed that the LDPE retain more phenolic compounds than the PLA active packaging. Furthermore, the PLA with the lemon by-products extract successfully inhibit the meat microbiological count until 6th day of storage.

Pomegranate peels and pomegranate peels extract were incorporated into PLA active films, and their chemical, mechanical, optical and barrier properties were evaluated. Two active PLA-based packages were produced: with 3% of pomegranate peel (PLA/3PP) and with 3 % of pomegranate peel extract (PLA/3PPE). Phenolic compounds, punicalagin (A+B) and ellagic acid, were both successfully quantified both in the pomegranate by-products and extract. The films were also applied to meat and grounded almond to evaluate their antioxidant and antimicrobial potential. Despite the films' effect on the almond were not clear, both PLA/3PE and PLA/3PP showed antimicrobial and antioxidant activity when applied to meat, having inhibited the malonaldehyde formation for 11 storage days and microbiological growth in the same period.

PLA packages containing green tea extract (GTE), rosemary extract (RE) and a mixture of both, were produced and used to pack ground almonds and beef meat. The main identified phenolic compounds in the GTE were catechin, epigallocatechin gallate, epicatechin, rutin and gallic acid, while in the RE the main phenolic compounds were carnosic acid, carnosol and rosmarinic acid. However, the RE presented a higher content in total phenolic compounds than the GTE. Five active PLA-based films were manufactured: PLA with 2 and 4 % of GTE (PLA/2GTE and PLA/4GTE), PLA with 2 and 4 % of RE (PLA/2RE and PLA/4RE); and PLA with 2% of each extract (PLA/2GTE/2RE). The PLA/2RE and PLA/4RE were the most effective in delaying the almonds lipid oxidation.

Altogether, 11 active food packages were produced with natural extracts, of which 10 were based on biodegradable polymers (whey protein or PLA). Regarding the PLA packaging, none of the extracts significantly altered the PLA structure. All the active packaging were effective in reducing lipid oxidation, especially when applied to raw beef, an ideal model food for *in* vivo evaluation of antioxidant and antimicrobial properties of active food packaging with natural extracts.

Thesis Outline

The concept of food packaging greatly evolved in the beginnings of the XIX century to respond to the needs of the French army during the Napoleonic Wars. Nicolas Appert, the father of canning, developed a method to preserve foods in hermetically closed glass jars which boosted the invention of the tinplate by Peter Durand. With the I and II World Wars, the first petroleum-based materials emerged, which culminated in the modern industry of food packaging based in plastic materials [1,2]. Having emerged only in the 20th century, the annual production of plastic increased from 50 million tons in 1976 to 367 million tons in 2020 [3,4]. This 634 % increase in the worldwide plastics' production exceeds the production of other man-made materials, with the exception of construction materials, such as cement and steel [5]. The vast majority of the produced plastic is used for packaging, which, over the years, changed from reusable to single-use containers [5,6]. However, plastics are not the most sustainable and eco-friendly materials.

Plastic can be defined as synthetic or semi-synthetic organic polymers, which are constituted by long-chain molecules, made by repeating chemical structural units. Derived from fossil hydrocarbons, ethylene and propylene are the most common monomers used in the plastics' manufacture and are not biodegradable. The only way to permanently eliminate plastic is through the incineration process which, besides being an expensive process, can emit large quantities of carbon dioxide and chemicals, contributing to the global warming [5–7]. These factors lead to the increase of plastic waste and its accumulation in the environment. From shorelines and beaches to deep seas and artic sea ice, plastic waste has been recommended as a geological indicator of the proposed Anthropocene era [5,8]. Globalization allows a global market with a perfect mobility of capital, labor and goods which allows almost every consumer a relatively easy access to foods from all over the world [9]. This phenomenon created a new challenge for the food industry to develop more resistible and efficient packaging, allowing foods to reach to its final destination intact and safe for consumption. Plastics are almost the perfect materials to reach this end, being lightweight, low-cost, resilient, durable, versatile, flexible and resistant to corrosion [7].

Packaging is the main responsible for plastic waste. In 2015, 141 million tons of plastic waste was generated by packaging [5]. Being such a versatile and reliable material, the use of plastic and its production tends to increase in the next few years, worsening the accumulation of plastic in the natural environment, endangering ecosystems and both human and animal lives. It is increasingly urgent to find sustainable and biodegradable

alternatives. The search for eco-friendly and biodegradable polymers, such as polylactic acid (PLA), whey protein-based materials, pectin, and starch, has been increasing [10]. These polymers can be applied in active food packaging, whose main function is to interact with the packaged food, by emitting or absorbing compounds from the packaged food.

The purpose of packaging is so much more than a way to preserve and transport food stuffs. It is a way to communicate and inform the consumers, though the nutritional label, ingredients list, possible allergens, and beneficial factors for health, and a key marketing tool. Through the passage of time and with new developments in the food industry, active and intelligent packaging have emerged. These new types of packaging are designed to interact with the packaged food to prolong its' shelf-life and to monitor a condition of the packaged food, respectively. According to the European Regulation No. 1935/2004 [11] the "*active food contact materials are designed to deliberately incorporate 'active' components intended to be released into the food or to absorb substances from the food*.". The same Regulation also states that these materials can change the organoleptic or the composition properties of the packaged food, since the active components are authorized by the European Regulations No. 1333/2008 [12] and No. 1129/2011[13].

Food additives, from synthetic or natural origin, are used to add certain characteristics to foods or only to increase foods' shelf-life and assure its safety. Often used together, synthetic and natural food additives have comparable performances [14]. In the literature, some studies reported that synthetic food additives have harmful health effects in liver, kidneys and microbiota [14], intestinal mucus layer [15], cytotoxicity [16], among others. Nevertheless, the harmful effects for health are closely related to the ingested dose, which is the responsibility of the manufacturer and the consumer. Active food packaging can help diminish the ingestion of these additives, since they are incorporated into the food packaging and can gradually migrate to the food' surface during the storage time.

One of the major problems of the modern world is food waste. According to the Food Waste Index Report 2021 of the Food and Agriculture Organization of the United Nations (FAO) [17], in 2019, 17 % of the worldwide total food production is wasted, which corresponds to, approximately, 931 million tons of food. 61 % of this waste is caused by households, 26 % from food service and 13 % from retail. The amount of food waste produced by households appears to be more than twice estimated in the 2011' report [18]. This suggests that the consumers need/look for more extended shelf-life. In addition to this major concern, and according to the Guide to World Food Safety

Day 2022 from the World Health Organization, one in ten people fall ill from food borne diseases. The public health burden from foodborne diseases is comparable to the burden caused by malaria or human immunodeficiency virus/ acquired immunodeficiency syndrome (HIV/AIDS) [19].

On the other hand, it is well established that plants and fruits are endowed with powerful antioxidant and antimicrobial activities. Some of their extracts and essential oils have Generally Recognized as Safe (GRAS) status by the Food and Drug Administration (FDA) and are authorized by the European Union [13,20]. Phenolic compounds are the main responsible compounds for these biological properties, present in all plants and fruits. These compounds contribute for the plants' defense against pathogenic agents, radiation, predators and parasites, as well as, are responsible for the plants' colors and their organoleptic characteristics [21].

The present PhD thesis aims to incorporate natural extracts from seaweeds, aromatic plants and fruits by-products into polymeric matrices in order to develop active food packaging, with the goal of delaying the lipidic oxidation of high fatty foods and/or inhibit the growth of pathogenic microorganisms. The new active films are characterized and their effectiveness in contact with model foods is evaluated.

This PhD thesis is divided into ten chapters: three reviews, six research studies and one general conclusions and future perspectives.

Chapter I reviews the inclusion of meat in the human diet, as well as, its composition, shelf-life and legislation applied to meat and meat products, food additives and food packaging. It also reviews the direct and indirect, though active packaging, application of additives in meat and meat products. Plant natural extracts, such as rosemary, oregano and thyme direct application to meat products showed to have tremendous antioxidant potential, by reducing lipid oxidation and enhancing some sensory attributes, such as appearance, color and taste. Also, essential oils from plants present antimicrobial activity against foodborne pathogens like *Listeria* spp., *Salmonella* spp., *Escherichia coli* and *Campylobacter* spp. Regarding the active packaging solutions, oxygen and carbon dioxide scavengers, which aims to remove any residual oxygen, carbon dioxide, moisture present in the package, are examples of this technology that have been extensively studied and applied. Antimicrobial and antioxidant active packaging containing natural agents such as essential oils, have been intensively studied. Several examples are presented in this first chapter.

Chapter II & III review the principal compounds and principal biological activities of pomegranate, grape and *Citrus* spp. by-products, accessing their potential applications.

Peels constitute 50 % of pomegranate, which means that the processing of this fruit generates a high quantity of by-products. These by-products and their extracts have reported antioxidant and antimicrobial activities. Grape is one of the most produced crops in the world, being mainly used in wine production, which originates a great quantity of by-products composed by seeds, peels and stems, generally designated as grape pomace. Being a major source of anthocyanins, grape pomace is a great candidate to be applied as antioxidant and/or antimicrobial in foodstuffs. *Citrus* spp. are the most produced crops in the world, being a large amount of this production used to make juice, originating by-products. In general, fruits by-products have powerful antioxidant and antimicrobial activities which makes them ideal for their use in the food industry.

The Chapters IV to IX, are research studies, that describe the incorporation of natural extracts into whey protein-based, PLA and Low-Density Polyethylene (LDPE) packages and their application to model foods, namely almond, chicken and beef meat.

In Chapter IV the *in vitro* antioxidant capacity of *Fucus vesiculosus* L. of five different ethanolic extracts was evaluated through the DPPH Radical Scavenging Assay, the β-carotene Bleaching Assay, and their total content on phenolic compounds content was also determined. The extract obtained through ethanol 75 % (v/v) was then incorporated into a whey protein-based film. The extract increased thickness, tensile strength and elastic modulus of the film and also delayed the formation of malonaldehyde in chicken meat for 25 days of storage.

Chapters V and VI analyze the antioxidant activity and the chemical composition of extracts from fruit juices formulations, lemon, and oranges by-products. The extracts were evaluated through the DPPH Radical Scavenging Assay, the β-carotene Bleaching Assay, and their total content on phenolic compounds and total flavonoids content were also determined. Also, the major phenolic compounds were identified and quantified. Between the fruit juices by-products extracts and the by-products from the individual fruits, lemon extract presented the highest antioxidant activity and the highest content in phenolic compounds, being the major ones eriocitrin, hesperidin, chlorogenic acid, isoquercetin, quercetrin, rutin and naringenin.

Chapter VII describes the incorporation of an ethanolic extract obtained from lemon by-products in LDPE and PLA in different percentages. The incorporation of the lemon extract managed to improve the water barrier properties in the LDPE and the PLA films but had no significant effect in the oxygen permeability of the LDPE based films and reduced it in the PLA based films. In the migration assays, the PLA active films showed higher migration of phenolic compounds than the LDPE active films. In the lipid

oxidation analysis, the LDPE showed more effectiveness in preventing almonds' lipid oxidation for 30 days. Regarding meat, the active PLA successfully inhibit the microbial growth until the 8th storage day.

In Chapter VIII the incorporation of pomegranate by-products extracts and pomegranate peels into PLA based active packaging is described. Antioxidant and antimicrobial activity of the pomegranate extract is addressed as well as the active PLA antioxidant and antimicrobial activity. Although the extract presented antimicrobial activity against *Stapylococcus aureus*, *Escherichia coli* and *Enterococcus faecalis*, once it was incorporated into the PLA, the film only showed antimicrobial activity against *S. aureus*. The content of punicalagin (A+B) and ellagic acid were also quantified in the pomegranate by-products, extract and active PLA based films. Even though the antioxidant activity of the PLA based active films were not clear in the almond assay, regarding the meat, it proved significant inhibition of products from lipid oxidation for 11 storage days and inhibit the microbial growth in meat for 11 days.

Regarding the Chapter IX, green tea and rosemary ethanolic extracts were produced and incorporated into active PLA packaging. The rosemary extract presented a slightly higher *in vitro* antioxidant activity and presented high content in carnosol, carnosic acid and rosmarinic acid. On the other hand, the green tea extract presented high content in catechin, epigallocatechin gallate, epicatechin, rutin and gallic acid. PLA incorporated with different percentages of the rosemary extract showed more effectiveness in reducing the production of lipid oxidation products in almond.

Finally, in the last chapter, the general conclusions of this work are drawn. Also, future perspectives, highlighting the need to promote change in the area of food packaging towards a new paradigm based on the principles of sustainability and circular economy.

Objectives

The major objective of this PhD thesis is to develop active and sustainable food packaging, incorporated with extracts of seaweeds, aromatic plants and/or fruits by-products, that will be able to stop or inhibit the lipid oxidation, and/or inhibit the growth of pathogenic microorganisms of model foods.

The objectives of this PhD are in line with the following Sustainable Development Goals of the United Nations, namely:

- Goal 2 "End hunger, achieve food security and improved nutrition and promote sustainable agriculture", since active food packaging makes it possible to increase the shelf-life of foods, assuring food safety and improving nutrition using natural additives or by reducing the use of direct food additives.
- Goal 3 "Ensure healthy lives and promote well-being for all at all ages", Active packaging makes it possible to reduce the consumption of food additives, maintaining or even improving the characteristics of the food.
- Goal 12 "Ensure sustainable consumption and production patterns", since this PhD thesis explores a possible use for the by-products originated by companies, promoting a circular economy.

To achieve this, the following specific objectives were defined:

- Obtain ethanolic extracts from the seaweed *Fucus vesiculosus* L., by-products of juices formulations, and rosemary and green tea; and evaluate their antioxidant capacity.
- Incorporate the extracts with higher antioxidant capacity into whey protein-based PLA and LDPE films.
- Evaluate the mechanical properties, water vapor and oxygen permeability or the new active films.
- Evaluate the effectiveness of the active packaging in delaying lipid oxidation of model foods (almond, chicken and beef meat) and inhibiting microbial growth during different storage periods.

Chapter I

Application of Releasing Systems in Active Packaging of Meat Products

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1. Introduction

The concept of "meat" is applied to the flesh of consumed animals and often widened to the animal's musculature, organs and other edible tissues. Meat is originated from several mammalian species which can differ depending on the culture, country or continent [22]. Regarding the evolution of human diet, about 5% of the diet of primates was composed by insects, eggs and small animals [23,24]. According to paleo dental evidence, meat consumption by humankind can be traced back to 2.5 million years ago. The size of molar teeth and the robustness of the mandibular and cranium reduced, the shape of incisor teeth altered, jaws and front teeth became stronger. These alterations suggest an increase of more soft foods which requires more tearing and less grinding from the dental apparatus, which suggest the inclusion of meat in the human diet [24,25].

With the introduction of meat consumption in the humankind diet, the intake of animal origin protein and fat increased. This increase is directly related to the calorie intake rise which, in turn, is directly related to the stature increase from the australopithecines to the *Homo habilis* and *H. erectus* [24]. The current brain chemistry also supports the human consumption of meat. Brain's communication network is essentially composed by lipids (60%) which, in turn, are rich in long chain fatty acids, namely docosahexaenoic (DHA) (22:6n:3) and arachidonic (AA) (20:4*n*:6) fatty acids [25]. DHA and AA, as well as eicosapentaenoic acid (EPA, 20:5*n*-3) and other polyunsaturated fat acids (PUFA), perform important roles in human health, such as gene expression modulation and the regulation of the physical properties of membranes [26]. PUFA and the respective derivatives can be mainly obtained from animal sources, namely meat and fish [25–27]. Besides meat, there are also several derived meat products consumed by humans, such as chorizo, salami, sausages, patties, among others. In general, these products are produced using specific parts of meat carcasses, blood, fat, gut lining, and aromatic plants, salt, spices and wine.

Unlike meat, that has a short shelf-life, meat products usually undergo through several processes to increase their shelf-life and/or several additives are added to prevent spoilage and to prevent changes in color, flavor, taste and appearance of the final product. The added additives to these products are, mainly, antioxidant and antimicrobial agents from synthetic origin. The consumption of these additives has been questioned due to their still unknown long-term effects in human health and their association to carcinogenic promotion, allergies and development of neurodegenerative diseases [28,29]. Also, the consumer's concern for the presence of these compounds in food products are increasing, which leads for a growing demand for more natural

products [30] . The application of active packaging can be a viable way to mitigate this problem. In a product packaged with an active releasing packaging, the additive can be incorporated in the package polymer being gradually released to the surface of the packaged food preventing food' spoilage and decreasing the additive' concentration in the food itself.

This chapter will focus on several releasing active packaging systems that were developed to be applied to meat and meat products. The legislation related with these systems will also be addressed.

2. Meat

The presence of meat in human diet has been increasing. The consumption of meat is related to wealth and socioeconomic status of the consumers [31]. According to Ritchie and Roser [32], in 2017 the world's meat production was 333.59 million tones, being Asia the continent with the highest production (141.93 million tons) followed by America (Central, South and North America, 103.12 million tons), Europe, Africa and Oceania. Regarding the meat consumption, USA and Australia present the highest values per person, 124.1 kg and 121.6 kg, respectively. On the contrary, India and Bangladesh present the lowest values of meat consumption per person, 3.78 kg and 4.04 kg, respectively. Regarding the choice of the meat type, poultry is the most consumed and produced meat followed by pork, beef and buffalo [32].

2.1. Composition and Lipidic Content

Meat and meat by-products are a known source of protein, fat, iron, zinc and vitamin B12. Table 1.1 compiles the composition of some common cuts of meat and meat products, according to the United States Department of Agriculture (USDA) Food Data Base [33]. According to this database, besides clams, liver from beef, lamb and veal present the highest content of vitamin B12 (96.00, 90.05 and 84.60 μg/100 g, respectively). Regarding the iron content, dried thyme, basil and spearmint lead the chart with 123.60, 89.80 and 87.47 mg/100 g. According to USDA database, the meat product with highest iron content is beef spleen with 44.55 mg of Fe/100 g, followed by lamb spleen (41.89 mg/100 g), duck liver (30.53 mg/100 g) and goose liver (30.53 mg/100 g). Looking for the zinc highly rich foods, mollusks lead the chart followed by ready-to-eat cereals (64.33 to 12.50 mg/100 g), peanut butter (15.10 to 14.40 mg/100 g), baby food (wheat vanilla biscuits) (14.15 to 12.68 mg/100 g), cottonseed meal seeds (12.32 mg/100 g) and crude wheat germ (12.29 mg/100 g). The first raw meat form is veal liver with

12.02 mg/100 g of zinc. As expected, the food with the highest EPA content is menhaden fish oil (13.17 g/100 g) followed by other fish oils (salmon, sardine, cod). The meat with the highest EPA content, with 0.13 g/100 g, is lamb liver followed by beef liver with 0.11 g/100 g and lamb kidney with 0.09 g/100 g. Regarding the content of DHA, fish oil from salmon, cod and sardine lead the chart with 18.23, 10.97 and 10.66 g/100 g, respectively. The first raw meat entry is pork brain, with 0.45 g DHA/100 g, followed by raw lamb brain with 0.36 g DHA/100 g and lamb testes with 0.18 g DHA/100 g.

Lipids are very important for human health, presenting important roles in human body. In terms of energy, lipids (9 kcal/g) provide more than twice the energy provided by proteins and carbohydrates (4 kcal/g). They also provide a feeling of satiety and are very important in the palatability of foods. Nutritionally speaking, they provide the human body with essential fatty acids, such as linoleic and arachidonic acids, and fat-soluble vitamins (A, D, E, and K). Biologically speaking, lipids act as structural elements of cell walls, form a protective barrier to vital organs and are involved in the body's heat regulation [34,35]. Generally, meat and meat products are considered to have a high lipidic content and associated with health problems such as type 2 diabetes, obesity and coronary heart disease. In contrast, meat and meat products' lipidic content is highly variable from specie to specie and from product to product, being the unsaturated fats the predominant lipids (Table 1.1) [35]. Meat' fat content is directly linked to the breeding measures, the meat cut, environmental conditions to which the animal is exposed, composition and nutritional value of the feed and the age of the animal at the moment of its death [35]. In meat and meat products, lipids are responsible for some desirable characteristics such as flavor and aroma profile, and contribute for the tenderness and juiciness of meat [36].

As can be observed in Table 1.1, the protein content in meat and meat products, although high, can vary. The meat product with the lowest protein value is pork ham with 5.05 g/100 g and the meat with the lowest protein value is pork belly with 9.34 g/100 g. These values contrast with the protein values from pork feet, with 23.16 g/100 g and cured dried beef with 31.1 g/100 g. When comparing food protein contents, the Protein Digestibility-Corrected Amino Acid Scores (PDCAAS) must be considered. The PDCAAS is described and recommended by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), and is based on the combination of age-related scoring patterns, calculated through the age-related amino acid requirement levels, and the digestibility measurements, to calculate the level considered safe of a diet intake or the effective intake when compared with a reference protein [37,38].

Table 1.1. Composition of different meats and meat products in 100 g, according to the USDA Food Database [33].

Meat form	Protein (g)	Total fat (g)	Iron (mg)	Zinc (mg)	Vitamin B12 (µg)	Saturated fat (g)	Monounsaturated fat (g)	Polyunsaturated fat (g)	EPA (g)	DHA (g)
Chicken breast, skinless and boneless, raw	22.5	2.62	0.37	0.68	0.21	0.563	0.689	0.424	0.003	0.004
Chicken breast, meat and skin, raw	20.85	0.25	0.74	0.8	0.34	2.66	3.82	1.96	0.01	0.02
Chicken leg, skinless and boneless, raw	19.16	4.22	0.78	1.76	0.57	1.05	1.44	0.962	0.004	0.011
Chicken leg, meat and skin, raw	16.37	15.95	0.69	1.47	0.56	4.366	6.619	3.352	0.004	0.01
Chicken wing, skinless and boneless, raw	21.97	3.54	0.88	1.63	0.38	0.94	0.85	0.8	0.01	0.05
Chicken wing, meat and skin, raw	17.52	12.85	0.46	1.21	0.25	3.535	5.422	2.498	0.003	0.006
Chicken giblets, raw	17.89	9.21	5.93	3.01	10.83	2.63	2.75	2.1	0	0
Chicken heart, raw	15.55	9.33	5.96	6.59	7.29	2.66	2.37	2.71	0	0
Chicken liver, raw	16.92	4.83	8.99	2.67	16.58	1.563	1.249	1.306	0	0
Chicken gizzard, raw	17.66	2.06	2.49	2.72	1.21	0.529	0.512	0.357	0	0
Ground beef, raw	17.44	19.07	1.97	4.23	2.15	7.291	8.48	0.505	0	0
Ground beef, grass-fed, raw	19.42	12.73	1.99	4.55	1.97	5.335	4.8	0.532	0.001	0
Beef tripe, raw	12.07	3.69	0.59	1.42	1.39	1.291	1.533	0.18	0	0
Beef tongue, raw	14.9	16.09	2.95	2.87	3.79	7	7.24	0.9	0	0
Beef pastrami, cured	21.8	5.82	2.22	4.98	1.87	2.681	2.118	0.145	0	0
Dried beef, cured	31.1	1.94	2.42	4.93	1.59	0.95	0.84	0.07	0	0
Beef sausage	15.5	37.57	1.53	2.92	2.03	15.098	16.387	1.025	0	0
Pork ground, raw	16.88	21.19	0.88	2.2	0.7	7.87	9.44	1.91	-	-
Pork carcass, raw	13.91	35.07	0.69	1.59	0.61	12.44	15.93	3.8	-	-
Pork spareribs, raw	15.47	23.4	0.91	2.5	0.38	7.529	8.542	3.953	0	0

Meat form	Protein (g)	Total fat (g)	Iron (mg)	Zinc (mg)	Vitamin B12 (µg)	Saturated fat (g)	Monounsaturated fat (g)	Polyunsaturated fat (g)	EPA (g)	DHA (g)
Pork loin, raw	20.65	3.53	0.97	1.87	0.52	1.181	1.355	0.562	0	0
Pork belly, raw	9.34	53.01	0.52	1.02	0.84	19.33	24.7	5.65	0	0
Pork back ribs, raw	19.07	16.33	0.76	2.54	0.56	5.783	6.861	2.676	0	0.002
Pork feet, raw	23.16	12.59	0.58	0.76	0.52	3.57	6.289	1.092	0	0
Pork kidney, raw	16.46	3.25	4.89	2.75	8.49	1.04	1.07	0.26	0	0
Pork liver, raw	21.39	3.65	23.3	5.76	26	1.17	0.52	0.87	0	0.02
Pork tongue, raw	16.3	17.2	3.35	3.01	2.84	5.96	8.13	1.78	0	0
Pork, chorizo, raw	13.63	25.1	1.41	1.68	2	8.595	10.603	4.296	0.002	0.003
Italian pork salami	21.7	37	1.52	4.2	2.8	13.1	18.2	3.6	-	-
Pork ham, cured	5.05	80.5	0.44	0.9	0.29	29.38	37.94	9.4	0	0
Pork bacon, cured	13.66	37.13	0.38	1.14	0.5	12.615	15.922	5.757	0.004	0.005
Pork sausage	18.53	27.25	1.2	2.45	0.98	8.826	11.541	5.115	0.002	0.003
Italian pork sausage, mild, raw	13.9	24.26	1.77	1.91	1	8.615	11.024	4.386	0.002	0.003
Ground turkey, raw	19.66	7.66	1.09	2.35	1	2.024	2.635	2.205	0.006	0.008
Whole turkey, meat and skin, raw	21.64	5.64	0.86	1.78	1.22	1.461	1.826	1.466	0.002	0.003
Whole turkey, meat only, raw	22.64	1.93	0.86	1.84	1.24	0.459	0.477	0.411	0	0.002
Turkey giblets, raw	18.18	5.09	5.92	3.23	13.06	1.452	1.016	1.425	0.005	0.025
Turkey heart, raw	16.7	7.44	3.7	3.21	13.3	1.923	2.045	2.153	0.002	0.006
Turkey liver, raw	18.26	5.5	8.94	3.37	19.73	1.664	0.817	1.684	0.009	0.045
Turkey gizzard, raw	18.8	3.37	2.78	3.03	3.61	0.929	0.795	0.708	0	0.007
Turkey sausage, raw	18.79	8.08	1.17	3.06	1.30	1.96	2.62	2.39	0.00	0.00

2.2. Meat Products

There are several types of meat products commercialized. Looking only at Portugal, a relatively small country, several of these products can be easily found in local markets such as farinheira, alheira, morcela, morcela de arroz, morcela de *assar*, *salpicão de vinhais*, *salsichão*, *chouriço*, *chourição*, *chouriço de ossos*, *paio*, *salpicão*, *salsichão de fígado*, *linguiça à moda de Valpaços*, *tripa enfarinhada*, *presunto* (smoked ham), *fiambre* (ham) and bacon. Meat by-products are also part of the gastronomic world of Portugal, such as cow's hand and tongue and, pork ear and feet [39]. These products are rich in PUFA, being highly subjected to lipid oxidation.

2.3 Meat Degradation and Shelf-Life

Lipid oxidation is still one of the main reasons for food spoilage. This natural occurring phenomenon leads to the development of off flavors, nutritional loss, texture and color changes, and reduced shelf-life, resulting in the rejection of these foods by the consumers [34,40]. It can also lead to the formation of toxic compounds, which their consumption is associated with liver hypertrophy, coronary artery disease, atherosclerosis, angiotoxicity and tumor frequency [41–43]. Lipid oxidation can occur by autoxidation and photochemical reactions.

$$RH + ROS \rightarrow R\cdot \qquad \text{Initiation (1)}$$

$$R\cdot + O_2 \rightarrow ROO\cdot \qquad \text{Propagation (2)}$$

$$ROO\cdot + RH \rightarrow ROOH + R\cdot$$

$$R\cdot + R\cdot \rightarrow Nonradical\ products \qquad \text{Termination (3)}$$

$$R\cdot + ROO\cdot \rightarrow Nonradical\ products$$

$$ROO\cdot + ROO\cdot \rightarrow Nonradical\ products$$

Figure 1.1. Lipid oxidation phases adapted from Nawar [34] and Frankel [44].

Autoxidation occurs by free radical mechanisms, and it comprises three phases: initiation, propagation and termination (Figure 1.1). Briefly, the first phase starts when reactive oxygen species reacts with lipids resulting in a fatty acid radical. In propagation, the fatty acid radical, a non-stable molecule, reacts with molecular oxygen creating a peroxyl-fatty acid radical which, in turn being also a non-stable molecule, reacts with other fatty acid resulting in fatty acid radical and a lipid peroxide. The second phase continues until there is no more radical species, resulting in the third phase, termination [34,44]. The first originated lipid peroxyl radicals are colorless, odorless and tasteless

but, being a non-stable molecule, they will originate lipid peroxides which are a complex mixture of lower molecular weight volatile and non-volatile compounds, characterized for having off-flavors and off-odors, normally associated with food rancidity [40,45].

Regarding meat and meat products, lipid oxidation depends on the meats' (or meat product) chemical composition, oxygen and light access, storage temperature and some technological procedures which meat products and meat are subjected during processing. The meat and meat products high content in lipids, especially unsaturated fatty acids, makes these products highly vulnerable to these reactions, which can significantly affect their shelf-life. Besides, the presence of metal ions as copper and iron can triggered lipid oxidation because they can donate electrons leading to increased rate of production of free radicals. In addition, muscles with higher content of myoglobin protein and presence of lipoxygenase enzyme are more susceptible to lipid oxidation [36].

In order to overcome this issue, food industry resorts to the addition of antioxidant compounds to foods, usually synthetic ones. The main goal of these compounds is to 'capture' the free radicals before they interact with fatty acids. These antioxidants can also be from natural origin, extracted from plants, seaweeds and fruits, in the form of extracts and essential oils.

In meat and meat products, spoilage can occur by the contamination with microorganisms, besides lipid oxidation. According to the WHO, foodborne diseases affects, every year, 600 million people and are responsible for the death of 420,000 people [46]. *Listeria* sp., *Escherichia coli*, *Salmonella* and *Staphylococcus aureus* are some of the most common bacteria present in meat and meat products. Several synthetic and natural antimicrobial agents are directly added to meat and meat products in order to assure the safety of these products. However, the safety levels of synthetic additives is constantly being updated to ensure consumer safety. A good alternative to reduce the consumption of synthetic additives is the use of natural antimicrobial agents and/or to incorporate the antimicrobial compounds in the packaging matrix. This way, the surface of the food is not contaminated with microorganisms and the concentration of the additive in the food can be reduced.

3. Legislation

3.1. Meat Legislation

To ensure food safety and quality, countries adopt several measures and laws, which may be specific or general for all food products. The WHO in cooperation with the

FAO created the Codex Alimentarius, which purpose is to promote and guide the elaboration of foods definitions and requirements to assist in their harmonization and, consequently, to facilitate international trade [47].

The European Union (EU) established several measures and legislation in this context. The EU food laws aim to achieve a high level of human and animal life protection and health and the protection of consumers' interests. These objectives should be accomplished through fair practices in food trade and the protection of the environment, animal welfare and health, and plant health. Also, the European Commission (EC) has established an Animal Health policy that aims, besides others, to raise the health status and improve the conditions of the food-producing animals in the EU [48–50].

Meat and meat products are under several EU rules regarding food hygiene that cover all stages of the production, processing, distribution, and market placement. According to the Annex I of Regulation No. 853/2004 [49], meat is defined by the edible parts, including blood, of poultry, domestic ungulates, lagomorphs, wild game, small wild game, farmed game, and large wild game. Still, and according to the Regulation No. 853/2004 [49], fresh meat refers to the "*meat that has not undergone any preserving process other than chilling, freezing, or quick-freezing, including meat that is vacuum-wrapped or wrapped in a controlled atmosphere*". Offal is the fresh meat besides the carcass, including viscera and blood. While viscera are "*the organs of the thoracic, abdominal, and pelvic cavities, as well as the trachea and esophagus and, in birds, the crop*". Minced or ground meat refers to the "*boned meat that has been minced into fragments and contains less than 1% salt".* Meat preparations are known as fresh meat, including meat fragments, which has had foodstuffs, additives, or seasonings added to it or which has undergone insufficient processes to modify the internal fiber muscle structure of the meat and thus eliminating the characteristics of fresh meat. Finally, meat based products means "*processed products resulting from the processing of meat or the further processing of such processed products, so that the cut surface shows that the product no longer has the characteristics of fresh meat*" [49]. These products are under the Regulation No. 178/2002 [48] that establishes the general principles and requirements of food law.

Also, the Regulation No. 852/2004 [51], on the hygiene of foodstuffs, where the EU determined several rules whereas food business operators are responsible for all stages of the production, processing, and distribution of foodstuffs under their control, ensuring the compliance of the hygiene requirements, through the implementation of procedures, such as the hazard analysis and critical control point (HACCP) principles. Besides, all food businesses must comply with the Regulation No. 853/2004 [49], which

sets hygiene rules for animal origin foods and, Regulation No. 854/2004 [52], which specific rules for the official control organizations on products of animal origin intended for human consumption.

To ensure meat quality and safety, the EC enforce measures during animal production, ensuring animal welfare traits. The Council Directive 2002/99/EC [53] presents the animal health rules governing the production, processing, distribution, and introduction of animal origin products for human consumption. Also, the EU establish microbiologic criteria, through Commission Regulation No. 2073/2005 [54], to provide objectives and reference points to assist competent authorities and businesses in their activities to monitor and manage the safety of foodstuffs.

In the USA, the food processing sector is not only regulated by federal agencies, but also by local and state agencies. The meat and poultry sector is regulated by the USDA specifically the Food Safety and Inspection Service (FSIS). Together they are responsible for ensuring the safety, wholesomeness, and accurate labeling of meat [33,55]. FSIS is responsible to ensure that the meat and poultry are within the food safety requirements and that the facilities are under HACCP principles. The US has also its codex program, based on the Codex Alimentarius which motivates stakeholders in advancing science-based international food standards to protect the consumers' health and ensure fair practices in the food trade [33,55].

3.2. Food Additives Legislation

According to the WHO, food additives are substances that are added to food to maintain or improve its safety, taste, freshness, appearance and/or texture. WHO, in cooperation with the FAO, created an international and independent scientific expert group – the Joint FAO/WHO Expert Committee on Food Additives (JECFA). JECFA is responsible for assessing the risks of food additives to human health. After the JECFA proves that the additive poses no risk to human health, national authorities can authorize the use of food additives at specified levels for specific foods, either based on the JECFA assessment or a national assessment [56].

The EU regulates food additives through the Regulation No. 1333/2008 [12], and respective amendments. The Regulation EC 1333/2008 defines food additive as a substance not normally consumed as a food itself and not normally used as a food' characteristic ingredient. Whether or not it has nutritive value, the intentional addition of additives to foods for a technological purpose in the stage of preparation, manufacture, treatment, processing, packaging, transport, or storage results, or can be

reasonably expected to result, in it or its derivatives become, directly or indirectly, a component of such foods. The food additives can be used for several technological objectives such as colors, preservatives, and antioxidants. In the EU, all food additives are identified by an E number which is mandatory to include in the ingredient's list of the food in which they are used [12].

The safety assessment of food additives in the EU is assured by the Scientific Committee on Food and/or the European Food Safety Authority (EFSA) [12,57]. The annex II of the Regulation No. 1333/2008 list all food additives that are authorized in foodstuffs and their conditions of use. In the case of fresh meat, since it is an unprocessed food, no additive can be added, and no food color additive can be added in meat, poultry, and game as well as their preparations. The Regulation No. 1333/2008 divided meat and its products in eight groups, and specified the additives that are allowed, including the authorized maximum level, and the restrictions and/or exceptions that may occur [12].

In the USA, food additives are under the FDA regulation. According to Section 201(s) of the Federal Food, Drug, and Cosmetic (FD&C) Act, a food additive is "*any substance the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of any food*". These substances can be added in the production, treatment, processing, packaging, transportation, or storage of food. This regulation does not include the substances that are generally recognized as safe (GRAS), such as sugar and salt, nor sanctioned before 1958 [58,59]. According to the USA regulation, the FDA is the authority responsible for the approval of a food additive. The process is started through a petition that requires evidences that the new substance is safe under the conditions for which it will be applied. In the case of food additives used in meat and poultry products, the FDA consults with the USDA during the process of approval [58].

3.3. Food Packaging Legislation

The EU regulates food contact materials through Commission Regulation No. 1935/2004 [11]. This Regulation states that materials should not release or transmit their constituents into foods at levels harmful to human health and should not change food composition, taste and odor in an unacceptable way [11]. The regulation is applied for active and intelligent materials and articles, adhesives, ceramics, cork, rubbers, glass, ion-exchange resins, metals and alloys, paper and board, plastics, printing inks, regenerated cellulose, silicones, textiles, varnishes and coatings, waxes, and wood. The Commission Regulation No. 1935/2004 defines general and specific measures for the

groups of materials and articles above mentioned, general requirements for the authorization of substances, labeling and traceability [11]. Active and intelligent materials are also regulated by the Commission Regulation No. 450/2009 [60].

Active materials and articles are "*materials and articles that are intended to extend the shelf-life or to maintain or improve the condition of packaged food; they are designed to deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food*". On the other hand, intelligent materials and articles are "*materials and articles which monitor the condition of packaged food or the environment surrounding the food*" [60]. The EC states that only substances that are included in the Community list of authorized substances may be used in components of active and intelligent materials and articles. It also refers to the conditions for the inclusion of substances in the Community list and established additional rules on labeling [11,60].

The Commission Regulation No. 10/2011 is a specific measure within the meaning of Article 5 of Regulation No. 1935/2004, that refers to the specific measures for groups of materials and articles, and establishes specific requirements for the manufacture and marketing of plastic materials and articles [11,61]. Plastic materials and articles are "*materials and articles and parts thereof consisting exclusively of plastics; plastic multi-layer materials and articles held together by adhesives or by other means; or those printed and/or covered by a coating; plastic layers or plastic coatings, forming gaskets in caps and closures, that together with those caps and closures compose a set of two or more layers of different types of materials; and plastic layers in multi-material multi-layer materials and articles*" [61]. The Commission Regulation No. 10/2011 and its amendments regulate the specifications of plastic materials and articles should fulfill to be included in the union list of authorized substances and the overall and specific migration limits [61].

The US regulation for food contact substances is under the section 409 of the FD&C Act which defines it as "*any substance that is intended for use as a component of materials used in manufacturing, packing, packaging, transporting, or holding food if such use of the substance is not intended to have any technical effect in such food*" [62,63]. The FDA distinguishes food contact substances from food contact materials and food contact articles. Food contact material is made with the food contact substance and (usually) other substances and it is often (but not necessarily) a mixture, such as an antioxidant in a polymer. Food contact article is the finished film, bottle, dough hook, tray, or whatever that is formed out of the food contact material [64]. According to the FDA, it is the responsibility of the manufacturer of a food contact substance to ensure that food

contact materials comply with the specifications and limitations in all applicable authorizations [62,63].

4. Additives Applied Directly and Indirectly to Meats

Following the current legislation, as specified in section 3.2, the use of food additives must be safe and highly justified [12]. They can occur in foods as the result of direct or indirect addition. Direct additives are substances added to foods to provide a desired effect, i.e. for a specific purpose. Whereas indirect additives are substances that migrate from the package into foods, becoming part of it due to its packaging, storage or other handling. Examples of indirect additives include compounds from packaging materials such as butylated hydroxytoluene (BHT). When added in the packaging, as coating or packaging additives, it is possible to reduce or eliminate the addition of large amounts of food additives that are usually integrated in foods [65].

Food additives can be natural, (e.g. from plants, animals, or minerals), or synthetic. In response to recent claims that synthetic additives have the potential to cause adverse health effects and consumers' increased interest in purchasing natural products, food, pharmaceutical and cosmetic industries together with the scientific community, are seeking sources of natural additives. Thus, several studies have directed their research in the search for safe natural food additives for consumption [66,67].

4.1. Antioxidant Additives

Antioxidants are used to minimize the oxidative changes in food, prevent lipid rancidity, off-flavors, and to stabilize color. Regarding the particular case of meat, oxidative changes may have negative effects in the meat' quality, causing modification in their sensory properties (color, texture and flavor), and reduction of nutritional value [67]. The lipid oxidation in meat is the most common chemical deterioration since lipids are one of the most chemically unstable food components that participate in oxidative reactions [67].

Antioxidant additives perform their function either as free-radical scavengers or chelators of pro-oxidant metal ions. These compounds and substances are capable of donating hydrogen radicals to the available free-radicals preventing oxidative damages, delaying or inhibiting lipid oxidation and rancidity emergence, without affecting sensory and nutritional properties [68]. Synthetics antioxidant additives as butylated hydroxyanisole (BHA), BHT, ethylenediamine tetraacetic (EDTA), propyl gallate (PG) and tert-butylhydroquinone (TBHQ), have been widely used in meat. They are aromatic

rings that can donate hydrogen radicals to a free radical and, consequently, stop the oxidative process by forming a more stable compound [69].

In recent years, the processing of foods with the partial and/or total substitution of synthetic by natural additives has been increasing, with the intention of obtaining a healthier product with less risks for Human Health. Phenolic compounds are one of the major groups of natural antioxidants found in nature, among which are α-tocopherols (vitamin E), flavonoids, and phenolic acids, which can be found in plant parts (like leaves, roots, stems, fruits, seeds and bark) and extracted through different techniques [67,70,71].

For instance, Rodrigues et al. [72] obtained an extract from male flowers (part of banana inflorescences) with a high content of phenolics, namely flavonoids, and, consequently, with a high antioxidant capacity. When applied in sausage formulation (concentration of 2%), stored under refrigeration for 28 days, they showed major potential to be used as a natural antioxidant [72]. In another study, rosemary extract was used in the production of a cooked meat storage for 0, 30 and 60 days, with partial replacement of animal fat by alkylglycerols rich oil. In this case, the antioxidant capacity of the extract was measured by determining the carnosic acid (major bioactive compound in the rosemary extract) content at the end of each storage time [73].

Other plant extracts have been used as natural antioxidants in meat products like extracts from thyme, green tea, oregano, lychee, marjoram and lemon balm [71,74]. Lychee seed water extract (LSWE) antioxidative properties in raw meat paste, during a storage period of 0–15 days, were evaluated, as well as, the sensory properties of the cooked meat paste supplemented with different amounts of LSWE [75]. LSWE was effective in retarding lipid oxidation of the meat paste and it significantly improved the sensory properties of the meat paste during the late stage of the storage period [75].

Other natural antioxidants extracted from plants are essential oils. In the study carried out by Fratianni et al. [76], fresh slices of chicken breasts were dipped, for 15 min, in sterile agar solution, with or without 0.5% of thyme and balm essential oils. The slices were then kept at 4 °C, for a 3-week period storage. The treatment with the 2 essential oils effectively limited lipid oxidation, reducing the deterioration of chicken meat and extend the shelf-life of the fresh product [76].

Natural antioxidants as tocopherols and polysaccharides also show potential antioxidant capacity in meat. Gadekar et al. [77] evaluated the effect of sodium ascorbate and α-tocopherol acetate on the quality of a restructured goat meat product. Natural antioxidants significantly reduced lipid oxidation and the value of free fatty acids. Besides,

sensory attributes such as appearance, color and taste were enhanced due to the use of the antioxidants. The results of the study revealed that the restructured goat meat product can be safely stored for 20 days at 4 °C with the addition of these antioxidants [77]. In the study performed by Hamed et al. [78] polysaccharides from pistachio (*Pistacia vera* L.) external hull were isolated. The results of thin layer chromatography showed that the carbohydrate fraction was mainly composed of rhamnose, glucose, galactose, mannose, xylose, arabinose, and galacturonic acid. Findings showed that these crude polysaccharides might have the ability to exhibit strong antioxidant capacities. Thus, the application of these polysaccharides on minced meat, reduced the lipid oxidation during chilled storage for 9 days and, also, showed significant improvement of meat color stability [78].

Aiming of reducing the use of synthetic antioxidants, a study was carried out by Doolaege et al. [70] who investigated the oxidative stability during the preparation of liver pate with different doses of rosemary extract and sodium nitrite (E249) before and after the cooking process, before and after exposure to light and air for 48 h at 4 °C. The addition of rosemary extract had a clear positive effect in delaying lipid oxidation and in maintaining higher levels of other antioxidants (ascorbic acid, α-tocopherol and carnosic acid). In addition, the dose of sodium nitrite added to the liver pâté was reduced when rosemary extract was added, without negative effects on lipid oxidation and color stability [70]. In another study, several plants extracts were used to replace the synthetic antioxidant, sodium erythorbate (E316). Among the 13 plant extracts evaluated by Fernandes et al. [71], the most promising were the extracts obtained from oregano, marjoram, lemon balm and rosemary. Therefore, the authors used these extracts in the production of lamb burgers as substitutes of the sodium erythorbate without compromising the sensory acceptance of this meat product.

Comparing synthetic and natural antioxidants, Waters et al. [79] revealed that sorghum bran varieties delayed lipid oxidation with equal efficacy to BHA/BHT, and significantly more effective than rosemary extract in cooked pork bran and chicken patties, without negatively affecting sensory attributes. In another study performed by Yu et al. [80], the antioxidative effect of apple phenolics extract (chlorogenic acid, phlorizin and phloretin) on lipid oxidation in Chinese-style sausage was compared with BHT and the natural antioxidant ursolic acid. At the optimum addition level, apple phenolics extract (0.5 g/kg in total fat) was more effective at inhibiting meat's lipid oxidation than BHT (0.15 g/kg in total fat) and ursolic acid (0.5 g/kg in total fat) for 120 days storage. Amin & Edris [81], showed that the addition of grape seed extract

significantly retarded the oxidative rancidity of minced beef, during refrigerated storage (4 °C, for 10 days) than BHT. This study introduces grape seeds extract, a natural agro-waste, as an effective alternative in extending meat's shelf-life and delaying lipid oxidation of meat without affecting sensory attributes [81]. Júnior et al. [82], compared the effect of the direct addition of a synthetic antioxidant and curcumin microcrystals to mortadella. The addition of microcrystals of curcumin in mortadella produced a more yellowish color that negatively affected the food. However, the addition of this natural antioxidant (curcumin) can prevent lipid oxidation in meat during storage more efficiently than with synthetic antioxidant (composition: sugar; sodium isoarcorbate and citric acid) [82].

4.2. Antimicrobials

Antimicrobial agents are added during meat processing to prolong their shelf-life and to control foodborne pathogens such as *Salmonella* spp., *E. coli*, *Campylobacter* spp., and *Listeria* spp. A variety of natural and synthetic antimicrobial agents are used in meat products to prevent microbial contamination against bacteria, yeast and molds, ensuring the quality and safety of meat. Synthetic antimicrobials are mostly considered safe, but several have negative and side effects that can cause serious health hazards [83], among theses, includes: organic acids and their derivatives, nitrites, nitrate, bacteriocins, and sulfites [45]. On the other hand, studies with natural preservatives such as nisin, chitosan, plant extracts and essential oils, lysozymes and others, have increased [45].

Nitrates and nitrites are widely used preservatives, commonly used in the cured meat production, usually in the form of sodium nitrite ($NaNO_2$) and potassium nitrate (KNO_2). Between the two, nitrate is the most stable ion, although can be easily transformed in nitrite through microbial action, being usually used as a nitrite "reservation" [84,85]. These compounds are used as food additives due to their antimicrobial action, namely against *Clostridium botulinum*, and to provide cured meats, such as bacon or ham, with the pink-reddish color that the consumers are familiar with and love [84,86]. Nitrates and nitrites have distinct behaviors inside the human body. Nitrates are immediately absorbed by the upper gastrointestinal tract (mouth, pharynx, esophagus and stomach) and 5 to 10% of the ingested nitrates can be reduced to nitrites by the bacteria present in the tongue, stomach and intestine [84,87,88]. On the other hand, nitrites, when they reach the stomach, can originate N-nitroso compounds which are carcinogenic [85,87–89]. Due to health hazard, surveys from various countries indicate a decline in the nitrites content in meat products over the last years [86]. In this

context, it must be ensured that additive' residual contents do not exceed the established legislation limits. This provides microbiological safety to the final product and preserves the consumers' health [90].

Accordingly, lysozyme has been used as an effective nitrite replacer in an Italian-type chicken sausage as reported by Abeyrathne [91]. This study focused on the evaluation of the antimicrobial effects of lysozyme extracted from egg white as a replacer of nitrite in cured meat products. It was concluded that the mixture of 50% nitrite +50% lysozyme was as effective as the control (100% nitrite) in suppressing the growth of *E. coli* and *Salmonella*. In addition, no negative effects were observed in color changes, and sensory characteristics were improved in the Italian type chicken sausage. Ozaki et al. [92] tried to replace the use of nitrite, with radish powder (0.5%) and chitosan (0.25%, and 0.5%) in fermented cooked sausages. The meat was prepared and evaluated against aerobic mesophyll bacteria during the ripening process and storage time. Pure chitosan exhibited *in vitro* antimicrobial activity against *Enterobacter aerogenes*, *Listeria innocua* and *Lactobacillus rhamnosus*. Chitosan (0.5%) with radish powder (0.5%) showed promising results indicating that the products (despite the absence of nitrite) were safe for consumption. With the exception of the aroma, sensory attributes were affected by the addition of chitosan [92].

Bacteriocins, such as nisin, are antimicrobial peptides produced by lactic acid bacteria (LAB) such as Lactococcus, *Enterococcus*, *Pediococcus*, and *Lactobacillus* that inhibit the growing of pathogenic and/or deteriorating bacteria [93]. Organic acids as citric, acetic, lactic and tartaric acids, individually or in combination can result in effective shelf-life extension of meat and meat products. Dipping solutions containing organic acids (lactic and acetic acids), bacteriocins produced by *Lactobacillus curvatus* and *Lactobacillus sakei*, and nisin were tested alone and in combination against *L. monocytogenes* on vacuum-packaged frankfurters stored at 10 °C for 36 days. The organic acids mix reduced the pathogenic population during storage. Solutions containing bacteriocins prevented microbial growth, while nisin was not able to avoid its regrowth after 20 days. The combined addition of the solutions containing bacteriocins + organic acids mix was the most effective approach for pathogen reduction during refrigerated and vacuum-packaged storage [94].

On the other hand, extracts of carvacrol and green tea showed to be weaker antimicrobial agents than sulfite, as revealed by the study of Bellés et al. [95]. The authors evaluated the use of different concentrations of carvacrol and green tea extracts and their combination in preserving lamb burger meat packaged aerobically and displayed for

8 days at 4 °C. The study tested the antimicrobial properties of extracts against total Aerobic viable counts, *Pseudomonas* spp., *Enterobacteriaceae*, lactic acid bacteria and *Brochotrix thermosphacta*, as well as compared them with sulfite (400 ppm). It was concluded that sulfite provided a higher color stability and lower microbial counts than both natural compounds. However, carvacrol seems to be a capable alternative to replace sulfite in lamb burger meat, whereas green tea should be combined with an antimicrobial agent [95].

Studies with cinnamon, rosemary, oregano, thyme and others have shown positive results for their capacity to act as antimicrobial in meat [96,97]. Cegiełka et al. [98] studied the effect of sage (*Salvia officinalis* L.) preparations on the storage stability of vacuum-packed low-pressure mechanically separated meat from chickens stored at −18 °C for 9 months. An aqueous extract, ethanolic extracts, and an essential oil were prepared and added to the meat. The growth of mesophilic aerobic bacteria and psychrotrophic bacteria, coliforms, enterococci and enterobacteriaceae was significantly restricted by all tested sage preparations, especially sage oil and ethanol extracts [98].

Andrés et al. [99], evaluated the effect of the addition of the aqueous extracts obtained from tomato, red grape, olive and pomegranate into lamb meat patties. In general, microbial counts were reduced by the addition of extracts, mainly grape and olive pomaces [99]. In another study, the combination of fresh herbs (*Coriandrum sativum* L. and *Rosmarinus officinalis* L.) with supercritical carbon dioxide treatment, did not increase the inactivation of either *E. coli* or natural flora (total mesophilic bacteria, yeasts, and molds) of raw chicken meat. However, the use of 0.5% v/w pure coriander essential oil, instead of the fresh herbs, showed increased inhibition. The same was not observed for the rosemary essential oil [100]. Thymol, cinnamaldehyde, allyl isothiocyanate, citric acid, ascorbic acid, rosemary extract, and grapefruit seed extract were tested against the spoilage bacteria: *Lactobacillus algidus, Leuconostoc mesenteroides*, *Leuconostoc carnosum*, *Carnobacterium maltaromaticum*, *Carnobacterium divergens*, *Brochothrix thermosphacta*, and *Serratia proteamaculans*. Single and combined antimicrobials were added to vacuum-packed pork meat to evaluate preserving effects. Although antimicrobial concentrations have showed in vitro activity, the same result was not observed when applied in meat at the same concentrations [101]. Badawy et al. [102], applied chitosan nanoparticles loaded with four monoterpenes (limonene, linalool, menthol and thymol) for preservation of minced meat. *In vitro*, *E. coli* was more susceptible than *S. typhimurium* to these products. Among chitosan nanoparticles loaded

monoterpenes, the chitosan nanoparticles loaded with limonene exhibited the highest in vivo antimicrobial activity for the minced meat samples during refrigerated storage.

Sharma et al. [103] studied blends of essential oils as bio-preservatives to increase the shelf-life of emulsion based ready-to-eat chicken sausages, aerobically packaged and stored under refrigerated (4 °C) conditions. The microbiological analysis (total plate count, psychrophilic, yeast and mold and coliform count) was performed in the ready-to eat chicken sausages elaborated with four different treatments which were tested, separately: B-1 (clove oil: 40%, holy basil oil: 20%, cassia oil: 40%); B-2 (clove oil: 40%, holy basil oil: 20%, thyme oil: 40%); B-3 (clove oil: 30%, holy basil oil: 20%, cassia oil: 25%; thyme oil: 25%) and B-4 (clove oil: 25%, holy basil oil: 20%, cassia oil: 20%, thyme oil: 15%, ajowan oil: 10%, betel oil: 10%). The authors found that B-1, 2 and 3 (each at 0.25%) and B-4 (0.125%) enhanced the shelf-life of chicken sausages by 13–14 days, 16–17 days, 10–11 days and 6–7 days, respectively [103].

In the study carried out by Amariei et al. [96], the antimicrobial activity of three essential oils (thyme, rosemary and oregano), was assessed. The essential oils (0.5, 1.0 and 1.5%) were added to a mixture composed by raw minced pork and beef meat. The antimicrobial activity was monitored throughout 4 days. The results indicated that essential oils have a significant influence on the microbiological stability of meat, when compared to control samples (without oil). It was observed that the microorganism's quantity depends on the type and levels of essential oil. Besides, the pleasant taste and smell support their use as additives to prevent bacterial contamination [96]. Amentoflavone is a known bioflavonoid occurring in many natural plants. This polyphenolic compound isolated from *Nandina domestica* Thunb. significantly reduced the cell counts of Staphylococcus aureus and Escherichia coli, in minced chicken [104].

4.3. Coloring Agents

A color additive can be defined as a pigment or substance which, when added or applied to a food, is capable of add or restore color. They can be used in food for many purposes such as, to offset color loss that has been affected by processing (exposure to light, air, temperature extremes, moisture), storage conditions, packaging and distribution; to correct natural variations in color, making foods more visually appealing; or to provide color to colorless [12]. Food coloring agents may act as emulsifying agents, stabilizers, sweeteners, antioxidants and preservatives [105]. Coloring agents can represent a risk to human health; therefore, in consequence, there are many studies and researches for

natural food coloring agents such as carotenoids, chlorophyll, and anthocyanins aiming to replace or reduce artificial coloring [105].

Nitrite, alone or in combination with sodium nitrate, act, among other functions, as a color fixative in cured meat and poultry products (bologna, hot dogs, bacon); however, as stated previously, nitrites have a health risk associated. In this line of though, Bolognesi and Garcia [106] applied annatto carotenoid extracts as nitrite replacers in meat products due to their antioxidant properties and color capacity. Kim et al. [107] conducted a study to improve the quality characteristics of pork loin cured with natural nitrite. Four treatments were tested on cured meat: nitrite free, cured meat marinated with sodium nitrite and ascorbic acid, cured meat marinated with only fermented spinach, and cured meat marinated with fermented spinach with the addition of ascorbic acid, malic acid, citric acid, and tartaric acid. Cured meat with fermented spinach presented higher redness values than sodium nitrite with ascorbic acid on cooked meat. Residual nitrite levels were lower in the presence of the added organic acids. Among various organic acid, ascorbic acid had the highest efficiency on the quality properties of cured meat [107]. In the same way, Huang et al. [108] provided a new no-added-nitrite cured meat and an innovative method for substituting nitrite with Lactobacillus fermentum and Lactobacillus plantarum as starters, beet red and Monascus color as coloring agents, and nisin as antibiotic. The results provided a good effect in the complete replace nitrite, thereby improving the quality and safety of cured meat. Thus, it is possible to ensure cured meat quality without or with low nitrite content [108].

Since organic acids have been used to improve safety and extend shelf-life by retarding food deterioration, Marcos et al. [109] determined the effect of decanoic, malic, fumaric and octanoic acid treatments on sensory characteristic of ground beef. The authors observed that the decanoic, fumaric and octanoic acid treatments increased subjective redness, reduced discoloration. In addition, there was no differences in beef flavor between control (untreated with acids) and the rest of the treatments. The use of 3% solutions containing these organic acids on beef trimmings prior to grinding may improve or maintain sensory retail display properties without affecting beef flavor.

4.4. Other Additives

Flavor enhancers are substances which magnify, increase or intensify the existing taste and/or odor of a foodstuff [12]. For example, monosodium glutamate (MSG) is a flavoring agent, basically used to develop the flavor of meat and meat-based products which, at the appropriate level, could lead to consumer acceptability of the product. It comes from

a common amino acid, glutamic acid. Although it is recognized as safe, several studies have questioned its due to its demonstrated negative health effects [110,111]. Jůzl et al. [112] examined differences in sensory evaluation of sliced cooked salami, manufactured according to several recipes. The monitoring factors were salt content (1.6% or 2.0%), presence (1.6% or 2.0%) or absence of MSG and age of the evaluators group. No significant negative result was found in the sensory evaluation; however, samples with MSG were rated as better, regardless of the age of the assessors. In their previous studies, Wang et al. [113] found that consumers prefer to have MSG replaced by extracts from natural sources that are rich in umami substances, including mushroom extract, tomato extract, kelp extract, and yeast extract. Thus, the authors replaced MSG with natural umami substances in chicken soup to enhance flavor and reduce sodium chloride. It was observed that all the tested extracts (yeast, mushroom, tomato, and kelp) in different levels (0.05%, 0.1%, 0.2%, 0.4%; except yeast extract: 0.0125%, 0.025%, 0.05%, 0.1%) exhibited an enhancement effect on the overall flavor, meaty flavor, saltiness, and umami taste [113].

Although the presence of salt (sodium chloride) in high concentrations and nitrites discourage the intention of purchase of the consumer, at the same time, these additives are key factors for the taste, color and juiciness of the final product [114]. As health strategies to reduce sodium levels (from sodium chloride) in processed foods, potassium and phosphorus from food additives may be added in meat and meat products. Sodium chloride acts to preserve and enhance the taste and texture of meat. It may be replaced by phosphorus (as potassium phosphate) and potassium (as potassium chloride, potassium lactate, and potassium phosphate) additives in sodium-reduced food [115]. The study performed by Parpia et al. [115], showed that significantly high amounts of potassium, which confer a salty taste, are being added to meat and poultry products that can negatively influence consumers with pre-existent health conditions such as chronic kidney disease. The authors argue that the quantity of the added and total potassium should be a mandatory component of foods' labeling [115].

Emulsifiers are substances added to foods to form or maintain a homogenous mixture of two or more immiscible phases, preventing the separation of the product components, ensuring consistency [12]. Emulsified meat-based products include frankfurters, chicken nuggets, bologna, spreadable sausage, surimi and other products. Actomyosin complex and, mainly, myosin protein are the most used emulsifiers, largely due to their high concentration and amphiphilic nature. The breakdown of the actomyosin complex indicates that myosin is released from the actomyosin complex and,

predictably, can act as a natural emulsifier and participate in the gelation process, crucial for the fat and water stabilization in emulsified meat based products [116]. Phosphates as food additives are used in many processed foods as stabilizers and emulsifiers. They maintain the moisture content, reduce the cooking loss and improve the textural properties and flavor protection. Furthermore, phosphates accelerate the formation of cured meat color as well as having antioxidant and antimicrobial effects [117,118]. Phosphates may be added to meat products as sodium or potassium salts of phosphoric acid. However, an avoidable risk to health arises from the increased use of phosphates as food additives. Thereby, the use of an alternative natural additive, as a polysaccharide, with potential to substitute phosphates was discussed by Câmara et al. [119]. The authors evaluated the functional properties of chia (*Salvia hispanica* L.) mucilage in powder and gel format as a phosphate replacer in low-fat Bologna sausages. Chia gel provided a better emulsion stability and texture parameters (closer to the control) than chia powder. Chia mucilage gel at 2% in the total absence of phosphates and with 50% fat reduction was effective (except for the color attribute) and has proven to be a feasible strategy to substitute phosphate in low-fat Bologna sausages [119].

Glorieux et al. [116] also tested the possibility of phosphate reduction on cooked pork sausages, by testing the effect of seven different phosphate types (mono-, di- and trisodium phosphate; tetrasodium di- or pyrophosphate; sodium acid pyrophosphate; sodium tripolyphosphate; and sodium hexametaphosphate). The most promising phosphate type was obtained from tetrasodium pyrophosphate and sodium tripolyphosphate which caused an increase in pH, improved sausages' structural properties, presented the highest emulsion stability and lowest cooking loss and had a very low effect on textural properties. The authors calculated, based on the structural properties results, that 0.06% of tetra sodium pyrophosphate is sufficient to obtain a quality product [116].

Humectants are substances added to foods to help retain moisture and improving food softness [12]. Among several types of humectants, glycerol and sorbitol are usually used in meat products. In the study carried by Sorapukdee et al. [120], glycerol and sorbitol, at the concentration of 0, 10, and 15%, were tested to develop a jerky product using spent hen meat. In addition, a roasting process was also applied to enhance quality of jerky product. All jerky samples showed microbial counts in undetectable levels. Moreover, the jerky containing 15% of glycerol showed better quality, indicated by low activity water, soft and springy texture than the sorbitol and control samples (no humectants added) [120]. Regardless effect of humectant, a roasting process following

the drying process could improve the jerky' quality by increasing color, appearance, and intense flavor, leading to a positive sensory reception. Triyannanto and Lee [121] tested the application of honey and rice syrup, natural humectants, in the replacement of sorbitol for the production of restructured duck jerky. Rice syrup (consisting of dextrin, maltose, maltotriose, and a minor amount of glucose) and honey, which is a natural humectant in a concentrated sugar form, were mixed separately, at 3%, 6%, and 10% (w/w) concentrations, with the marinating solution. The use of these humectants had a positive effect in the chemical properties of duck jerky, especially when used in higher concentrations. In addition, duck jerky samples treated with 10% honey showed the highest scores for the sensory parameters evaluated, while samples treated with rice syrup were comparable with those of samples treated with sorbitol. Therefore, honey shows a great potential to be used as a natural humectant in restricted duck jerky, replacing sorbitol [121]. The addition of humectant, such as hydrocolloids, can improve the sensory properties, tenderness and decrease water activity of jerky. Effects of curing solution prepared using different combinations of humectants konjac and collagen was investigated in the study carried out by Kim et al. [122]. The authors observed that the use of combinations of collagen and konjac, 60/40 in duck jerky processing results in the best quality characteristics of the jerky.

The substances which are added to foods to thicken (increase the viscosity) or improve texture are commonly called binders. Gums, such as carrageenan, are commonly used as binders in meat due to their high hydration ability. Beyond them, the non-meat proteins as whey protein and soy protein also could be added as binders to stabilize moisture and fat and improve their moisture binding capacity in meat products [123]. Thus, the combination of carrageenan and soy protein isolate was evaluated for the functional properties of chopped low-fat pork batters during heat-induced gelation by Gao et al. [124].

Similarly, in other research lead by Zouari et al. [125] the effect of whey powder, ι-carrageenan and fat ratio variation sand their interactions on sensory and texture properties of mechanically separated turkey meat sausages, was evaluated. Whey powder had a more notable influence than ι-carrageenan on all of the texture parameters. Sensory evaluation also indicated that whey powder increased the flavor, the firmness and the sliceability. Low-fat sausage processed with 8 g of whey powder/100 g sausage was the best evaluated[125]. The study carried out by Jin et al. [123] tested different binders (isolated soy protein, sodium caseinate, egg white powder and pork plasma protein, at 0, 1, 1.5% concentration levels) on physicochemical and sensory

properties of pork sausages. It was revealed that the sensory profiles with all treatments were indifferent, except for 1% pork plasma protein with a slightly lower overall acceptability score. The inclusion of binders lowered the pH values and cooking loss in pork sausages, besides not show any remarkable results in the textural properties. In addition, lower redness and higher yellowness values were found in pork sausages that did not contain binders [123].

5. Active Packaging

Due to the growing consumer's demand for nutritious, healthy and safe meat and meat products, along with the ever-increasing retailers demand for cost-effective technologies to extend products' shelf-life, the food packaging industry has evolved to meet and satisfy these expectations [126]. The main goals, when packaging fresh meat, are the delay of natural spoilage, avoid cross contamination, and allow some enzymatic activity to improve tenderness, decrease weight loss and preserve color and aroma. On the other hand, when it comes to package processed meat products, the main goals are to avoid or delay different phenomena, such as lipid oxidation, dehydration, loss of aroma and discoloration [127,128]. Meat quality and safety are highly dependent on the applied packaging materials and technologies.

Currently, several meat packaging systems are available, each of them with different characteristics and applicability. These include overwrap packaging for a short-term chilled storage; a variety of specific MAP systems for longer-term chilled storage and/or display; and a combination between vacuum packaging with MAP systems with 100% carbon dioxide for long-term chilled storage [129]. Recently, different new packaging technologies and materials have been developed to ensure food safety and quality, extend shelf life, minimize environmental impact and highlight appealing aspects of the packaged product to retailers and consumers.

In this sense, and due to the diversity of characteristics of the product to be packaged and basic applications of meat packaging, a packaging technology, that can offer a better control over product quality in a more economically and diversified way, is desirable. Therefore, the interest in the use of active packaging systems for meat and meat products has increased in recent years. When a package, in addition to presenting an inert barrier to the external environment, also has any other functions, can be called active [130]. Active packaging is a system that promotes positive interaction between the product, package and environment, extending food's shelf life and maintaining or even improving food's quality, safety or sensory properties [131,132]. According to the

Regulations No. 1935/2004/EC [11] and No. 450/2009/EC [60], active packaging comprises packaging systems that "*deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food*". Active packaging systems can be divided into two types: active scavenging systems (absorbers) and active-releasing systems (emitters) [133].

Although release packaging systems, such as antioxidant and antimicrobial active packaging, are the main goal of this chapter, oxygen scavengers, carbon dioxide and moisture scavengers will also be mentioned.

5.1. Oxygen Scavengers

High levels of oxygen in food packages can be an enabler for microbial growth, promote the off-flavors and off-odors development, color change, and nutritional losses. All these factors can induce significant reductions in the foods' shelf-life. In fresh meat, the presence of oxygen allows myoglobin oxygenation, which provides the characteristic red color [134,135]. High levels of oxygen promote the oxidation of muscle lipids, which has detrimental effects on the color of fresh meat [136]. The decreasing of oxygen within the package helps to increase meat's shelf-life by preventing the growth of fungi and aerobic bacteria [137,138]. Therefore, to limit the rate of these deteriorative and spoilage reactions in foods, it is important to control oxygen levels inside food packages.

One of the main active packaging technologies, which aims to remove any residual oxygen present in the food package, is the application of oxygen scavengers (OS) [133,139]. The development of oxygen scavenging systems includes the use of silica gel, natural clays (e.g., montmorillonite), calcium oxide, calcium chloride, molecular sieves, and modified starch, or other moisture-absorbing substances [137]. The development of oxygen-scavenging systems mostly includes the use of self-adhesive labels or loose sachets in the packaging containers. Besides, the oxygen-scavenging systems are also based on the design of active substances to be included in the packaging material itself using monolayer or multilayer materials [140]. Oxygen scavenging technologies have been successfully used in the meat industry, alone or in combination with MAP [134].

Commercially, it is more frequent to remove most of the atmospheric oxygen by MAP and then use a relatively small and inexpensive scavenger to eliminate the remaining oxygen within the food package, namely, the oxygen which permeates through the packaging film or is trapped within the meat or between meat slices [134]. Oxygen scavenging compounds react with oxygen to reduce the concentration of this inside the

package [141]. Nowadays, the existing technologies use one or more of the following concepts: iron powder oxidation, ascorbic acid oxidation, photosensitive dye oxidation, enzymatic oxidation (e.g. alcohol oxidase and glucose oxidase), and immobilized yeast on a solid substrate [142,143]. Ferrous oxide is the most widely used scavenger. Currently, the majority of commercially available oxygen scavengers are based on the principle of iron oxidation Figure 1.1[144].

The iron kept in the small sachet is oxidized to iron oxide. For the sachet to be effective, the sachet material is highly permeable to oxygen and, in some cases, to water vapor. The initial oxygen level in the package, the amount of dissolved oxygen present, the permeability of the packaging material, the water activity (size, shape, weight, etc.) and the nature of the food are several factors that determine the type and amount of absorbent that needs to be used in the sachet [137]. This oxygen scavenging system was developed and introduced to the food packaging market by the Mitsubishi Gas Chemical Company, known as Ageless [134].

5.2. Carbon Dioxide Scavengers and Emitters

To slow down the microbial growth on meat and poultry surfaces, high levels of CO_2 are desired (60 to 80%) to prolong the shelf-life of packaged food [135,137]. Since the permeability of CO_2 is 3 to 5 times higher than that of O_2 in most plastic films, it must be continuously produced to keep the desired concentration within the package [137]. Therefore, CO_2 generating system can be viewed as a technique complementary to oxygen scavenging by the impregnation of a packaging structure with a CO_2 generating system or the addition of the latter in the form of a sachet [145]. These sachets can contain sodium hydroxide and calcium hydroxide or potassium hydroxide; or silica gel and calcium oxide [132]. It should be noted that an oxygen-free environment alone is insufficient to retard the growth of *S. aureus*, *Vibrio* spp., *E. coli*, *Bacillus cereus*, and *Enterococcus faecalis* at room temperatures [135]. Also, an O_2 and CO_2 absorber can inhibit the growth of *Clostridium sporogenes* [146].

Carbon dioxide emitting sachets or labels can also be used. This innovating package consists of a standard MAP tray that has a porous sachet containing sodium bicarbonate/ascorbate under a perforated false bottom. So, CO_2 is emitted when juice exudates from the packaged meat drip onto the sachet, substituting any CO_2 absorbed by the meat and preventing package collapse [134].

5.3. Moisture Scavenger (Absorbers)

To suppress microbial growth and prevent foggy film formation, it is important to lower the water activity of the product, which means control the excess moisture in food packages. The water accumulation inside the package is more pronounced if the package has a low permeability to water vapor [134,137].

Several factors contribute to the excess water development inside a food package, such as temperature fluctuations in high relative humidity food packages, the respiration of a fresh product or leak of tissue fluid from cut meats and poultry [130]. The accumulation of excess water inside the package promotes bacterial and mold growth, resulting in quality loss and shelf-life reductions [137].

The use of a moisture scavenger is a useful way of controlling excess water accumulation in a food package with a high barrier to water vapor. Moisture absorbents are commonly used as pads, sheets, or blankets for liquid water control in foods with a high water activity, like meat and fish [141]. They consist of two layers, a microporous nonwoven plastic film, like polyethylene or polypropylene, between superabsorbent polymers that is capable of absorbing moister up to 500 times its weight. Typical superabsorbent polymers include carboxymethyl cellulose (CMC), polyacrylate salts, and starch copolymers, due to their strong affinity to water. Moisture drip absorber pads are commonly placed under packaged fresh meats and fish to absorb unsighted tissue drip exudates [141].

5.4. Antimicrobial Active Packaging

For the meat industry, antimicrobial packaging is one of the most important features to guarantee food quality once meat provides excellent nutrients for the potential growth of microorganisms. Therefore, to provide safe and healthy meat and meat products to consumers, special attention is needed to minimize microbial proliferation [129]. There are spoilage microorganisms, such as bacteria, molds, yeast, and pathogenic microorganisms (such as *Salmonella* spp., *L. monocytogenes*, *S. aureus*, *Clostridium botulinum*, *Clostridium perfringens*, and *E. coli* O157: H7) leading to quality deterioration and food safety issues on meat [147]. In this sense, the use of antimicrobial packaging is a way to extend foods' shelf-life and to assure the food safety of meat and meat products. According to Appendini & Hotchkiss [148] and Coma [149], the antimicrobial packaging can be classified into four categories:

1. Inside the package, antimicrobial substances are incorporated into sachets/pads, which, during storage, are released [150].

2. Direct incorporation of the antimicrobial compounds into the packaging film, by co-extrusion or by non-heating methods such as electrospinning, solvent compound, and casting. In both cases, the antimicrobial compounds will be gradually released from the packaging films to the packaging headspace or food surface [151].
3. Packaging coating with a matrix with a plastic film or any food derivative material, such as wax or polysaccharides. This matrix performs the transport of the antimicrobial agents, so they can be released onto the surface of food through evaporation into the headspace (volatile substances) or migration into the food (non-volatile substances) through diffusion [129].
4. The use of polymers, such as chitosan and poly-L-lysine, in food packaging and coatings, which are inherently antimicrobial. These polymers cause cellular death of microorganisms, once the charged amines of the polymers interact with negative charges on the microorganism cell membrane which causes leakage of intracellular constituents [152].

A large number of antimicrobial agents, including ethanol, CO_2, chlorine dioxide, silver ions, bacteriocins, antibiotics, organic acids, spices, and essential oils, are being used to inhibit the microorganisms growth in foods [153]. Table 1.2 summarizes several studies where different active packaging systems were applied. This Table compiles the changes in the physical, barrier and mechanical properties, as also the antimicrobial and/or antioxidant properties of the active film.

There is a wide variety of food packaging incorporated with antimicrobial active compounds. These indirect additives are responsible for the increasing or maintaining the quality of products. In the work of Konuk Takma and Korel [154], active polyethylene films, assembled with antimicrobial chitosan and alginate coatings, incorporated with black cumin oil were used as packaging for chicken breast meat. These films improved the quality and shelf-life of chicken meat during refrigerated storage, exhibiting more effective against total aerobic mesophilic bacteria in comparison to psychrotrophic bacteria [154]. In another study, CMC – PVA films incorporated with citric acid as cross linked and aloe vera as the active component delayed bacterial growth of packaged minced chicken meat and thus, extended the shelf-life of product [155].

Clarke et al. [156] applied two antimicrobials, sodium octanoate and auranta FV (a commercial antimicrobial composed of bioflavonoids, citric, malic, lactic, and caprylic acids) into polyethylene – polyamide packaging materials for coating elaboration. Then, these packaging materials were used to vacuum pack beef subprimal cuts and stored at

4 °C. Both films increased meat' shelf-life, however, sodium octanoate reduced microbial counts for all the tested bacteria (total viable counts, psychrotrophic bacteria, total anaerobic bacteria, Lactic acid bacteria, total coliforms and *E. coli*) [156]. Marcous et al. [157] evaluated the anti-bacterial effects of low-density polyethylene (LDPE) films coated with titanium dioxide (TiO_2), zinc oxide (ZnO) and mixed TiO_2–ZnO nanoparticles. The authors concluded that the TiO_2 -ZnO nanoparticle-coated LDPE films are not a suitable option to inhibit *E. coli* growth and reproduction. However, ZnO nanoparticle-coated LDPE films were identified to have improved the shelf-life and prevent E. coli growth in fresh calf minced meat [157]. In another study by Mulla et al. [158], linear LDPE surface was chemically modified by chromic acid treatment and coated with clove essential oil forming, thus, an active packaging. Films exhibited high antimicrobial activity against *Salmonella* typhimurium and *L. monocytogenes* in a packed chicken sample, completely restricting their growth on the 5th day of storage and during the 21 days storage period under refrigerated [158].

At this point, it is essential to alert for important aspects that must be considered in the use of antimicrobial packaging, namely the minimal impact that these packaging systems must have on the visual and sensory properties of the packaged product, because these greatly influence the choice of the consumer. Only in this way, the application of antimicrobial active packaging to meat and meat products will be effective.

5.5. Antioxidant Active Packaging

The high levels of oxygen in meat packaging increases, besides microbial growth and color changes, meats' lipid oxidation. Lipid oxidation leads to the development of rancidity and to the potential formation of toxic aldehydes due to the degradation of PUFAs [159]. To overcome this major food industry issue, active packaging containing antioxidant compounds can be used for improving product quality and extending the shelf-life of meat and meat products, through the control of the oxygen level to which the product is exposed.

Table 1.2. Active Packaging Systems developed for meat and meat products.

Polymers	Active Compounds	Method	Physical, Barrier and Mechanical Properties	Antimicrobial/ Antioxidant Properties	References
Starch + PVA	Nisin Z (2 to 8.70 %wt.) + CNC (0 to 5 %wt.) + MA (0 to 12.56 %wt.)	The films were prepared by the dissolution of PVA into deionized water and stirred. Another solution prepared was plasticized corn starch (70 % starch and 20 % glycerol) and MA into deionized water. The solution was then mixed and stirred. CNC and NIS Z were added to the mixed solution and homogenized. The final suspension was poured into glass plates and let to dried.	The active film presented a significant improvement of the mechanical and barrier properties, with a decrease of the force to break. The compounds incorporated did not influence the swelling index and *L**, *a** and *b** color coordinates did not vary with the addition of MA, CNC, and NIS Z into starch-PVA matrices, although the *b** coordinate showed a slight yellowing in the films due to the high NIS Z addition, which is naturally yellow.	The interaction between the compounds in the active film improved the antimicrobial activity against *Listeria Monocytogenes*.	[160]
LDPE	Carvacrol (10 %wt.) + Nanomer I.28 (5 %wt.)	The films were prepared by mixing the nanofiller and the matrix to the extruder whereas the carvacrol was added to the molten polymer using a downstream port to get antimicrobial blends at a nominal 10 % content.	The addition of carvacrol to the film improved the barrier properties, through the reduction of the permeability. The mechanical properties are improved by the synergistic effects of the compounds. Carvacrol acts as a plasticizer and dispersing agent and I.28 increased strain at break value.	The antimicrobial activity of carvacrol was not affected by the presence of the Nanomer I.28, being all the indicator strains studied (*Brochotrix thermosphacta* 1R2; *Listeria innocua* ATCC 33090; *Carnobacterium* sp. 9P), sensitive to films containing carvacrol.	[161]
CMC + PVA	Clove Oil (1, 2 and 3%)	The CMC 1% solution was prepared by dissolving in distilled water at room temperature with stirring. The PVOH 5% solution was prepared in hot distilled water. The solutions of CMC and PVOH were mixed at various ratios (1:1, 1:2, and 2:1). Glycerol was added as the plasticizer in all the films. Clove oil was added to the film-forming solution at different concentrations (1%, 2%, and 3%), except the control. The film-forming mixtures were blended, and the films were	The incorporation of clove oil into the CMC + PVA film did not affect the thickness of the films, although it has a slightly yellowish color. The addition of clove oil resulted in a decreased tensile strength and puncture force, but no significant changes in water vapor transmission rate and negligible oxygen transmission rate.	The active film showed higher antimicrobial activity against *Staphylococcus aureus* than *Bacillus cereus*, but no activity against gram-negative bacteria that was tested. The active film was also effective against reducing the natural microflora of ground chicken meat and was able to extend the shelf life of ground chicken meat during refrigerated storage.	[162]

Polymers	Active Compounds	Method	Physical, Barrier and Mechanical Properties	Antimicrobial/ Antioxidant Properties	References
		prepared by the casting method. Finally, the dried films were peeled from the casting surface and preconditioned in a constant temperature humidity chamber set at 23 °C with 50% RH for 24 h.			
LDPE	Ag (0 to 1 % w/w), CuO (0 to 0.667 % w/w), and ZnO (0 to 1 % w/w) nanoparticles	The films were prepared through the method of extrusion, where NPs and LDPE pellets were put into a co-rotating twin-screw extruder. The LDPE and NPs were fed into the extruder from the feed hopper and the molten material was extruded as a string into a basin of cold water and cut into granules. Finally, the granules were added into another twin-screw extruder and the films obtained were chilled using a chilling roll system at room temperature.	The active films presented a homogenous distribution of NPs on the fracture surfaces and the NPs are not recrystallized into clusters after being incorporated in the LDPE matrix. Both tensile strength and elongation at break were improved by the incorporation of combinate NPs. LDPE-Ag presented a significant increase in tensile strength and the highest elongation at break values, which could contribute to the strength and flexibility of the films. On the other hand, films containing both ZnO and CuO and without Ag had significant effects on improving the mechanical properties of the film.	All active films presented a significant decrease of CFUs for both, *Escherichia coli* and *Staphylococcus aureus*. The containing all NPs were more effective in reducing *Escherichia coli* and *Staphylococcus aureus* compared to those containing single NP. Once again, films containing both ZnO and CuO and without Ag significantly decreased the number of colonies of both bacteria.	[163]
PP	carvacrol (4, 6 and 8 wt.%) + thymol (4, 6 and 8 wt.%)	The different formulations were obtained by melt blending and both additives were introduced in the mixer once the polymer was already in the melt state. The films were obtained by compression-molding at 190 °C in a hot press and kept between the plates at atmospheric pressure for 5 min until melting and was successively pressed under 2 MPa for 1 min, 3.5 MPa for 1 min and finally 5 MPa for	A slight modification of tensile properties and a significant decrease in elastic modulus was observed, in the result of a significant decrease in the crystallinity of the material. Also, an increase in oxygen transmission for the active film was observed. The addition of carvacrol and thymol to the polymer matrix did not significantly affect its thermal degradation profile in the inert nitrogen atmosphere.	The active films showed antioxidant activity trough differential scanning calorimetry by determining the oxidation induction parameters, i.e. oxidation onset temperature and oxidation induction time. The active film showed antimicrobial activity against *Staphylococcus aureus*, but low antimicrobial activity against *Escherichia coli*.	[164]

Polymers	Active Compounds	Method	Physical, Barrier and Mechanical Properties	Antimicrobial/ Antioxidant Properties	References
		5 min, to liberate the trapped air bubbles.			
LDPE + EVA	Clove leaf, Sweet basil, and Cinnamon bark oil (0.5, 2, and 4% wt.)	LDPE incorporated with 0%, 2.5%, 10%, 20 %wt. EVA and neat EVA were melt-compounded in a co-rotating twin-screw extruder. After the extruded were removed from the twin-screw extruder and were ground to pellet. The same procedure was performed with the active compounds.	Grainy and porous surface was found on the active films, probably due to the evaporation of EOs.	The film incorporated with Cinnamon bark oil was found to be more effective against both microorganisms than the one incorporated with Clove leaf oil. The film with Sweet basil oil did not reveal enough antimicrobial activity. The film with Clove leaf oil and Cinnamon bark oil was more active against *Escherichia coli* inhibition than *Staphylococcus aureus*.	[165]
LDPE + PP	Attapulgite and Allium sativum essence oil (1% w/w)	The formulations of bilayer films were precisely prepared. For the inner layer of the films, EVA (10% w/w) pellets were initially blended with active substances by using a hand whisk. Then, LDPE pellets (90% w/w) were added and mixed continually. The mixture was placed in the hopper of the inner layer of the film to manufacture the inner layer of bilayer films. Finally, the films were manufactured using a blown film extrusion process.	The active films have high transparency and no agglomeration phenomenon occurred, but the color of the films changed to yellow with the addition of AEO or AT+AEO. The incorporation of AEO and AT+AEO into the film did not affect the mechanical properties of the films and did not show significant superiority in barrier performances of water vapor and oxygen transmission rate.	AEO and AT delayed the growth of microbes on large yellow croaker, up to 9 days at 4 ± 1 °C. The yellow croakers with active films had significantly lower values of CFU of specific spoilage organisms than the control. The yellow croaker packaged by active films presented lower values of pH, total volatile basic nitrogen contents, K-value, and thiobarbituric acid reactive substances compared to the control group in the preservation of large yellow croaker, extending the shelf life by up to 5 days at 4 ± 1 °C.	[166]
EVA	Anthocyanin (0 to 1 % wt.); ZnO nanoparticles (0 to 1 % wt.); rosemary extract (0 to 3 % wt.) and	EVA was dissolved in chloroform to obtain a 10 wt% solution. Then, ZnO and Fe-MMT nanoparticles were added to the above solution under ultrasonic agitation for 2 min. Required amounts of anthocyanin-	All active films presented a typical behavior of flexible plastic; films containing ZnO and modified montmorillonite exhibit higher tensile strength, and Young's modulus of these films show a similar trend.	Active films showed notable increases in antioxidant activity, compared to the control film, with a film containing anthocyanin and rosemary exhibiting significant improvement. All active films showed antibacterial activity	[167]

Polymers	Active Compounds	Method	Physical, Barrier and Mechanical Properties	Antimicrobial/ Antioxidant Properties	References
	modified montmorillonite (0 to 1 % wt.)	MMT and rosemary extract were added to the resultant mixture subsequently. After all, mixtures were stirred for an extra 10 min to provide a homogeneous dispersion of materials inside the EVA solution. Finally, the prepared mixture was spread out on a smooth and cleaned glass plate and was dried at room temperature.		against both *Escherichia coli* and *Staphylococcus Ureus*, and the maximum antibacterial activity belongs to the film containing rosemary extract.	
PP	Sorbic acid (2, 4 and 6% w/w)	PP granules and SA powder were mixed well in a stainless-steel container. The mixture was introduced to a co-rotating twin-screw extruder. After the mixture of the materials melted and mixed inside the extruder as a result of the shear and pressure forces. The molten materials were left out of the extrusion in string form. They passed through cold water basin and were then cut into the granules. The granules were collected and added into another twin-screw extruder to produce the desired packaging film.	The incorporation of SA in PP matrix increases the tensile strength at the break but the elongation at break of the active films decreased by increasing the concentration of SA. Water vapor permeability was strongly influenced by the amount of SA, as it was increased significantly with higher concentrations of SA. The addition of SA to the PP matrix had no significant effect on L*, a*, and total color changes, but b*, yellowness index, and whiteness index were affected.	SA active films had a significant antimicrobial effect on *Escherichia coli* and *Staphylococcus aureus*. Increasing SA concentration in films increased the antimicrobial properties of the films. Though the inhibiting effect of SA against *Aspergillus niger* was noteworthy, the growth inhibition was more effective with the increase of the SA concentration.	[168]
LDPE	Cu nanoparticles (0.5, 1, 1.5, 2, 2.5 and 3 wt. %)	The films were prepared by using solvent evaporation. For that, LDPE polymer was dissolved in xylene at a constant temperature 110 °C using an oil bath; Cu-NPs were dispersed in LDPE/ xylene solution by temperature-controlled ultrasonic bath sonicator to achieve	Cu-NPs were uniformLy dispersed on the film, an increase in melting peak temperature was observed as Cu-NPs increase in the active films, and the mechanical properties and water vapor permeability were improved.	The active film showed antimicrobial inhibition efficiency against *Escherichia coli* and *Staphylococcus aureus.*	[169]

Polymers	Active Compounds	Method	Physical, Barrier and Mechanical Properties	Antimicrobial/ Antioxidant Properties	References
		proper dispersion. Finally, the films were fabricated on glass plates and peeled off from glass plates.			
Whey Protein Isolate	Chitosan Nanofiber + Cinnamon essential oil (3–6% dry weight of WPI)	The casting method was used to prepare the films. First of all, WPI was dissolved in distilled water and mixture stirred for 2 h to obtain a homogeneous solution and heated at 80 °C for 30 min to denature the WPI, followed by the film solution cooled to the ambient temperature. The CNF was dissolved in distilled water and homogenized in the ultra-sound bath, followed by the homogenous solution of CNF added to denatured WPI solution and homogenized to obtain homogenous distribution. Then the glycerol was added to the obtained film solution and after good dispersion poured on the 15 cm plates and dried at a temperature of 25 °C.	The incorporation of CNF caused a significantly decreased water vapor permeability, while the addition of CEO caused an insignificant increase in the water vapor permeability value. The water solubility value of the WPI+CNF film decreased significantly, such as the active film were also significantly low. The WPI+CNF presented a significant increase of the tensile strength and Young's modulus and the strain to break value decreased significantly. For the active film with CEO, the tensile strength and Young's modulus decreased significantly and the strain to break value increased significantly. The active films represented the higher L* value, whiteness index and ΔE with the coincidentally lower a*, b* value and Yellowness index, compared with WPI film.	The WPI and WPI-CNF film did not show any antimicrobial activity, against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. On the other hand, the active films containing CEO presented antimicrobial activity against all tested bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.	[170]
LDPE	Grapefruit seed extract (3 %wt.) + melanin nanoparticle (0.1 %wt.) + ZnO nanoparticles (3 %wt.)	The extrusion blowing method was used to prepare the films. The masterbatch was prepared by mixing the active compounds with LDPE using Ca-stearate as the dispersing agent. The mixture was then extruded using a twin-screw extruder. The masterbatch compounds were blown into a film through a single screw extruder.	All the films were smooth-surfaced, homogeneous, and flexible, and the active film was dark gray due to Mel. The L* value of the active film decreased, the b* value and ΔE increased significantly and the a* value did not change significantly. Both the strength and the resiliency of the LDPE film were significantly reduced after incorporation of the GSE/Mel/ZnO NPs, so was the tensile strength and the elongation at break	The active film showed strong antibacterial activity against both bacteria, *Escherichia coli,* and *Listeria Monocytogenes.* The antimicrobial activity was mainly due to the presence of GES and ZnO NPs.	[171]

Polymers	Active Compounds	Method	Physical, Barrier and Mechanical Properties	Antimicrobial/ Antioxidant Properties	References
PLA	MgO NPs (1, 2, 3, and 4 % wt.)	The films were prepared using the solvent casting method. Dried PLA was dissolved in chloroform by vigorous stirring. The MgO NPs were dispersed separately in chloroform with the help of an ultrasonicator and then poured into the dissolved PLA solution and continued to stir. The mixture of MgO NPs suspended in dissolved PLA matrix was evenly spread at room temperature on a glass plate to allow the evaporation of chloroform to produce uniform thickness films, and let to dry during 2-3 days, and then were peeled out from the plate and stored in a dry place, until further use.	The mechanical properties of the film improved significantly by incorporation of MgO NPs, good compatibility of MgO NPs and PLA matrix. Also, the incorporation of MgO NPs leads to lower thermal stability, which is directly proportional to the amount of NP added. The oxygen barrier property was significantly improved by the incorporation of MgO NPs, but the water vapor permeability increased.	The active film showed significant improvement in the bactericidal properties, specifically against *Escherichia coli.*	[172]
LDPE and PLA	Grapefruit seed extract + thermoplasticized starch	First, the TPS was prepared by mixing cornstarch with 20 % wt. of glycerol and 40 %wt. of GSE with a mixer. Then, the mixture was heated at 120 °C and cooled to room temperature by spreading in the room and powdered using a blender. The films were prepared by mixing the TPS and polymer. The LDPE based-films were prepared using an extrusion blowing method and the PLA based-films were prepared using an extrusion casting method.	All films were uniform and freestanding, and both active films presented a decrease in L* value and an increase in a* and b* value, and consequently an increase of ΔE. The LDPE film was very resilient with low strength, while the PLA film was tough and brittle. However, for both active films the tensile properties decreased significantly, and consequently so did the mechanical strength, flexibility, and stiffness. Water vapor permeability and water solubility value increased significantly for both active films, while water contact angle decreased significantly.	Both active films exhibited antimicrobial activity against *Escherichia coli* and *Listeria Monocytogenes,* mainly due to the GSE incorporated into the TPS. However, generally, the GSE incorporated films exhibited stronger antibacterial activity against *Listeria Monocytogenes* than *Escherichia coli.*	[173]

Polymers	Active Compounds	Method	Physical, Barrier and Mechanical Properties	Antimicrobial/ Antioxidant Properties	References
PLA	CNC (1 and 3% wt.) and LNP (1 and 3% wt.)	The extrusion method was used to prepare the films. Various amounts of LNP and CNC were mixed with PLA. The films were manufactured by using a twin-screw micro-extruder.	All active films showed higher tensile strength and Young's modulus mean Values and remarkable improvements in toughness. The enhancement of the effect is more from cellulose nanocrystals than lignin nanoparticles.	The active films showed antibacterial activity with a reduction in the multiplication of the bacterial plant pathogen *Pseudomonas syringae* pv. *Tomato*.	[174]
LDPE	Curcumin (1, 2, 3, 5 and 7% wt.)	The extrusion method was used to prepare the films. The extrusion of the LDPE with and without Curcumin was carried out through a three-heating zone single-screw Rheoscam extruder. The extruded filaments were subsequently pelletized and formed pellets. Finally, the films were produced by compression molding.	The active films are transparent but have a typical orange color attributed to the curcumin filler. The active film presented higher thermal stability, an increase in the elastic modulus and a decreased in the elongation at break. The water vapor transmission rate and water vapor permeability are improved by the presence of curcumin in the matrix.	The active film presented an effective antioxidant scavenging activity against the 2,2-diphenyl-1-picrylhydrazyl free radicals (DPPH), due to the presence of curcumin.	[175]

*a** – Redness value; *AEO* – Allium sativum essential oil; *Ag* – Silver; *AT* – Attapulgite; *b** – Yellowness value; *CEO* – Cinnamon essential oil; *CFUs* – Colony-forming unit; *CMC* – Carboxymethyl cellulose; *CNC* – Cellulose nanocrystals; *CNF* – Chitosan nanofiber; *Cu* – Copper; *CuO* – Copper oxide; *EOs* – Essential oils; *EVA* – Ethylene vinyl acetate; *GSE* – Grapefruit seed extract; *L** – Lightness value; *LDPE* – Low density polyethylene; *LNP* – Lignin nanoparticles; *MA* – Maleic anhydride; *MeI* – Melanin nanoparticle; *MgO* – Magnesium oxide; *NIS* – Nisin Z; *NPs* – Nanoparticles; *PLA* – Polylactic acid; *PP* – Poly propylene; *PVA* – Poly vinyl alcohol; *SA* – Sorbic acid; *TPS* – Thermoplasticized starch; *WPI* – Whey protein isolate; *ZnO* – Zinc oxide; *ΔE* – Total color changes.

There are two different forms to apply the antioxidant active packaging system, by independent antioxidant devices, or by the incorporation of the antioxidant into the packaging material. In the first case, sachets, pads or labels, which contain oxygen scavengers, can be independent devices and added to the package (Sect. 4.1) [159]. In the second case, the antioxidant active agent is incorporated in the polymeric matrix of the packaging with the aim of being released to the food or the headspace surrounding it [129]. To select the manufacturing procedure, it is important to consider the type of polymer and the characteristics of the antioxidant agents. To produce an antioxidant packaging material, there are several ways to mix the antioxidant agent (or the reactive substances which produce the agent) with the packaging polymers, namely: (i) using coating technologies, wherein an appropriate solvent is used to dissolve the antioxidant agent and the packaging material polymer(s) together, and then this solution is applied to a substrate, (ii) using extrusion technologies, in which the polymer is melted and mixed with the antioxidant agent or (iii) immobilizing the antioxidant on the film surface [176].

A new tendency in antioxidant active packaging for meat and meat products is reducing the use of synthetic additives, being replaced using natural antioxidants. The most common natural antioxidants are essential oils and plant extracts (e.g. rosemary, green tea, oregano), and tocopherols [177,178]. Another alternative is to use the natural and synthetic antioxidants together, taking advantage of the possible synergic effect between the two components. Table 1.2 summarizes several studies in different active packaging systems, including antioxidant active packaging, applied to meat and meat products.

Many studies have been carried out in this direction with positive effects using different synthetic and natural antioxidants incorporated in films. For instance, antioxidant films exhibited a great effectiveness to protect the meat against oxidation, as showed in the study performed by Ribeiro-Santos et al. [97] where a blend containing cinnamon (*Cinnamomum zeylanicum* L. and *Cinnamomum cassia* L.) and rosemary (*Rosmarinus officinalis* L.) essential oils was incorporated in a whey protein film aiming the reduction of salami lipid oxidation. The study showed that the active whey protein packaging can reduce the lipid oxidation phenomenon of meat products, extending their shelf-life while releasing natural antioxidants to their surface [97]. Alizadeh-Sani et al. [179], applied a biopolymer-based cellulose nanofiber/whey protein matrix containing titanium dioxide particles (1% of TiO_2) and essential oil droplets (2% of rosemary oil),

in lamb meat during storage which, in turn, increased its shelf-life from around 6 to 15 days [179].

In another study, shelf-life extension of fresh minced meat was achieved by wrapping the fresh minced meat in a polyethylene film incorporated with an encapsulated green tea extract [180]. Gallego et al. [181] evaluated the antioxidant potential of a freeze-dried tomato by-product in pork loin cubes. The authors found that the gelatin coating with the freeze-dried tomato by-product was able to slow the meat' lipid oxidation for 13 days. Also, the meat' quality properties, such as texture, hardness and elasticity was improved [181]. Andrade et al. [182], evaluated the packaging of salami slices with a why protein coating incorporated with an ethanolic extract of rosemary (*Rosmarinus officinalis* L.) and stored at 5 °C for 90 days. The lipid oxidation evaluation showed that the salami slices packaged with the active film present less malonaldehyde content that the salami slices packaged with the control film (without the rosemary extract) [182]. In another study from the same authors, this rosemary extract showed antimicrobial activity against *L. monocytogenes*, *S. aureus* and *Clostridium perfringens*, which makes it an additive to be taken into account to be applied in meat and meat products [183].

6. Conclusion

Meat and meat products are one of the major food categories consumed in the world. Their safety is assured, mainly, by synthetic food additives which are regulated by the European and FDA legislation. However, recent findings have questioned their use, as well as, their authorized concentration. Natural additives, such as fruit and plant extracts and essential oils, and their incorporation in food packaging to obtain releasing active systems, seems to be the answer for more natural and safer processed products. Essential oils present powerful antimicrobial activity against most of common microorganisms can be present in meat products, like *L. monocytogenes*, *E. coli* and *S. aureus*, presenting themselves as a safe and effective alternative to the use of synthetic additives, such as nitrites. Plant and fruit extracts, as well as their main bioactive compounds, present themselves as powerful antioxidants which can delay lipid oxidation. Their addition to the food packaging matrix and their gradual migration from the package to the packaged food can increase their antimicrobial and antioxidant activities.

New technologies and alternatives for food additives and active food packaging arise every day, which present a great challenge for the responsible authorities to test and develop legislation that enable and assure the safety of these new alternatives.

Chapter II

Pomegranate and grape by-products and their active compounds: Are they a valuable source for food applications?

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1. Introduction

According to Food and Agriculture Organization of the United Nations (FAO), one-third of the world's food production (1.3 billion tones) is lost or wasted [18]. Although being different concepts, food loss and food waste represent less available food along the stages of the food supply chain for human consumption. The difference between the two is that a food is lost when it gets spoilt before it reaches its final destination. On the contrary, food waste is when a food is not consumed; being discarded or left to spoil by retailers or consumers and it is still fit for human consumption. FAO's report showed that, more than 40% of the food losses in developed countries occur at retail and consumer levels [18].

As far as fruits are concerned, they often do not reach the consumer as a whole, but in the form of formulations, such as juices. For example, tomatoes are often commercialized in the form of tomato paste, juice or sauce. For these formulations, the seeds, skin and its combined fraction (pomace) must be separated, resulting in tomato by-products, which are often used as feed for cattle [184,185]. Since fruit by-products are considered food waste, their economic value is low. This, combined with their high biological activities, makes them a potential source of bioactive compounds to be included in nutraceutical and pharmaceutical compounds [186]. Although fruits by-products can represent an economic problem mostly because they are considered a waste and companies are forced to discard these products in a responsible and environmentally friendly way may increase the final cost of the fruit formulations [184].

Tropical fruits (coffee, macadamia, pomegranate, pineapple mango, papaya, among others), are known for their high content in health benefit compounds. The most common bioactive compounds found in these fruits are carotenoids, phenolic compounds and dietary fiber [184,187]. Regarding the carotenoids, they are natural pigments responsible for the occurrence of the yellow, orange and red colors in plants, fruits, and algae [188]. Their antioxidant activity and their role in intercellular communication and in the immune system are well known. It is proven that a diet poor in carotenoids is associated with visual disorders, including night blindness and corneal ulceration, and a diet rich in these natural pigments is associated with a lower incidence of cancer and cardiovascular diseases [189–191]. Present in the majority of edible plants and fruits, phenolic compounds are part of the plant's defense against external stimuli such as radiation and pathogenic organisms [21,192–196]. Dietary fiber can also be extracted from fruit by-products. These fibers may present interesting bioactive compounds such as antioxidants and antimicrobials [184,186]. In addition, they can be

used for technological purposes improving food's texture and replacing sugar applications as a bulking agent [184,197]. Plants and fruits antioxidant and antimicrobial activities are recognized for centuries and the application of some of their essential oils and extracts in foods is authorized by the Food and Drug Administration (FDA) and the European Union [13,184,198,199]. If these plants and fruits possess some powerful biological capacities, it is possible that their by-products possess equivalent or superior biological activities than the fruit or plant itself. The food industry, in order to assure food safety and improve some organoleptic characteristics, uses food additives. These additives have several functions, from the antimicrobial protection of foods to delaying lipid oxidation and confer various organoleptic characteristics to foods. Food additives can be synthetic or natural. The first ones are the most used additives for their chemical stability, easy application, and their low cost. On the other hand, the prolonged exposure to these additives has been linked with allergic reactions, promotion of carcinogenesis and the onset of neurodegenerative diseases such as Parkinson's and Alzheimer's diseases [29,177,200]. The modern world has a serious problem with food waste and byproducts. It is urgent to find a sustainable solution for these by-products so that they do not end up being considered waste, without being fully availed. In this line of thought, the main goal of this review is to summarize, through an exhaustive compilation of the scientific literature, the main active compounds of pomegranate and grape and their biological activities, assessing their potential uses and risks, mainly those related with negative impacts in human health.

2. Pomegranate and grape by-products

Pomegranates and grapes are well-known fruits with high biological activities. They are used in the food industry for several applications, including juices and jams. These formulations result in a large number of by-products which may contain bioactive compounds with interesting biological properties.

The extracts from these fruits by-products can present great differences on their content in active compounds. These differences can be explained due to the use of different cultivars and varieties of the plants, differences in the edaphoclimatic conditions in which the plant is grown and in the maturation state of the fruits and differences in the extraction procedures used. For instance, in the case of a grape skin extract obtained by the same extraction process, from the same species *Vitis vinifera* but from a different cultivar, the catechin content is very different. For the grape cultivar Muscat Alexandria, the catechin content is 628 μg/g, but for the extract of the grape cultivar Ghara Shani,

the catechin content is 945 μg/g [201].

Table II.1shows the nutritional value of grape and pomegranate according to the United States Department of Agriculture Food Composition Database (USDA Food Database). In this database, there is no data referring to the nutritional value of pomegranate and grape byproducts. According to this data, Vitis vinifera presents high sugar content (15.48 g/100 g) similar, although lower, sugar content of pomegranate (13.67 g/100 g). Grape is also high in potassium (191.00 mg/100 g), β-carotene (39.00 μg/100 g) and lutein + zeaxanthin (72.00 μg/100 g). β-carotene is a precursor of vitamin A, while lutein + zeaxanthin are important carotenoids to maintain a good eyehealth. On the other hand, pomegranate is also rich in potassium (236.00 mg/100 g) and presents a considerable fiber content (4.00 g/100 g). It also presents a good source of vitamin C (10.2 mg/100 g). According to the National Academy of Sciences [202], the Adequate Intake for Total Fiber is 38 g/day for adult men and 25 g/day for adult women, which pomegranate can contribute for this daily intake with 10.53% (for men) or 16% (for women) [203]. Pomegranate contributes also to the daily intake of vitamin C, which is set at 80 mg per day according to the Regulation n° 1924/2006 [204]. The same European Regulation sets the potassium daily intake at 2000 mg/day, of which pomegranate and grape contribute to 11.80% and 9.55%, respectively.

2.1. Pomegranate (*Punica granatum* L.)

Native of Asia, pomegranate (*Punica granatum* L.) is one of the oldest consumed fruits in the world. Its existence is traced to the Egyptian Empire where it represented prosperity and ambition and it was used for the treatment of certain infections and for the decoration of sarcophagi [205–207]. Pomegranate fruit and plant are also mentioned in other traditional medicines and texts including the traditional Indian medicine (Ayurveda), Unani and Greek documents, and it is used for the treatment of several diseases and ailments as vermifuge, bactericide, stimulant, hair dye, to alleviate the symptoms of respiratory illness (asthma, bronchitis) and fever, inflammation, bleeding disorders [208]. In the traditional Cuban medicine, hydroethanolic extract of the whole fruit is used to treat respiratory diseases [209]. Currently, pomegranate is produced worldwide and, due to its high biological activities that can present beneficial effects on human health, production has been increasing. In consequence, the amount of pomegranate by-products is also increasing [210]. Pomegranate is formed by a deciduous shrub that can grow up to 8m tall and belongs to the Lythraceae family (formerly placed in its own monogeneric family, Punicaceae) [210,211].

Table II.1. Nutritional value of pomegranate and grape according to the USDA database.

	Units	Grape *V. rotundifolia*	Grape (red or green) *V. vinifera*	Pomegranate *P. granatum*	Pomegranate juice
Water	g	84.29	80.54	77.93	85.95
Energy	kcal	57.00	69.00	83.00	54.00
Protein	g	0.81	0.72	1.67	0.15
Total Lipids	g	0.47	0.16	1.17	0.29
Carbohydrate (by difference)	g	13.93	18.10	18.70	13.13
Fiber (total dietary)	g	3.90	0.90	4.00	0.10
Sugars (total)	g	-	15.48	13.67	12.65
Calcium (Ca)	mg	37.00	10.00	10.00	11.00
Iron (Fe)	mg	0.26	0.36	0.30	0.10
Magnesium (Mg)	mg	14.00	7.00	12.00	7.00
Phosphorus (P)	mg	24.00	20.00	36.00	11.00
Potassium (K)	mg	203.00	191.00	236.00	214.00
Sodium (Na)	mg	1.00	2.00	3.00	9.00
Zinc (Zn)	mg	0.11	0.07	0.35	0.09
Copper (Cu)	mg	0.12	0.13	0.16	0.02
Selenium (Se)	µg	-	0.10	0.50	0.30
Manganese (Mn)	mg	1.97	0.07	0.12	0.10
Vitamin C (total ascorbic acid)	mg	6.50	3.20	10.20	0.10
Thiamin	mg	-	0.07	0.07	0.02
Riboflavin	mg	1.50	0.07	0.05	0.02
Niacin	mg	-	0.19	0.29	0.23
Pantothenic acid	mg	-	0.05	0.38	0.29
Vitamin B-6	mg	-	0.09	0.08	0.04
Folate (DFE)	µg	-	2.00	38.00	24.00
Vitamin A (ERA)	µg	3.00	3.00	0.00	0.00
β-Carotene	µg	39.00	39.00	0.00	0.00
α-Carotene	µg	1.00	1.00	0.00	0.00
β-Cryptoxanthin	µg	1.00	0.00	0.00	0.00
Lutein+zeaxanthin	µg	64.00	72.00	0.00	0.00
Vitamin E (α-tocopherol)	mg	-	0.19	0.60	0.38
Fatty acids (total saturated)	g	-	0.05	0.12	0.08
Fatty acids (total monounsaturated)	g	-	0.01	0.09	0.06
Fatty acids (total polyunsaturated)	g	-	0.05	0.08	0.05

Legend: DFE - Dietary Folate Equivalent; ERA - Estimated Average Requirement.

Pomegranate fruit is constituted by peel, arils, and seeds in an approximate 50:40:10 ratio, respectively. Like the majority of plants and fruits, the chemical composition of the pomegranate differs according to the edaphoclimatic conditions that the plant is exposed to and the ripen degree of the fruit at the time of harvest [212,213]. Table II.2 has a selection of pomegranate extracts and oils, as other fruits by-products, and their main active compounds. This fruit is used for several food products such as, fresh and concentrated juice, infusions, jams, among others [214].

The antioxidant capacity of pomegranate by-product extracts is shown in several studies. Özalp Özen and Soyer [215] applied an aqueous extract of pomegranate peel to mackerel (*Scomber scombrus*) in order to delay lipid oxidation of the fish fatty acids. The authors compared the antioxidant action of the extract to the antioxidant action of BHT (Butylated hydroxytoluene), a powerful synthetic antioxidant widely used in the food industry. The natural pomegranate extract showed similar results to the BHT, demonstrating that natural antioxidants can be as powerful as synthetic antioxidants [215]. The antioxidant capacity of pomegranate is linked to the presence of bioactive compounds, such as phenolic compounds. With more than 8000 known chemical structures, phenolic compounds are present in all plants and fruits, contributing to their defense against predators, radiation and pathogenic organisms [194].

In pomegranate, the most commonly encountered bioactive compounds are anthocyanins and hydrolysable tannins, namely ellagitannins, gallotannins, flavonoids, lignans, triterpenoids, phytosterols, fatty acids, organic acids and phenolic acids. Punicalagin (Figure II.1) isomers are the most common phenolic compounds, constituting up to 85% (w/w) of tannins present in the pomegranate peel [216–218].

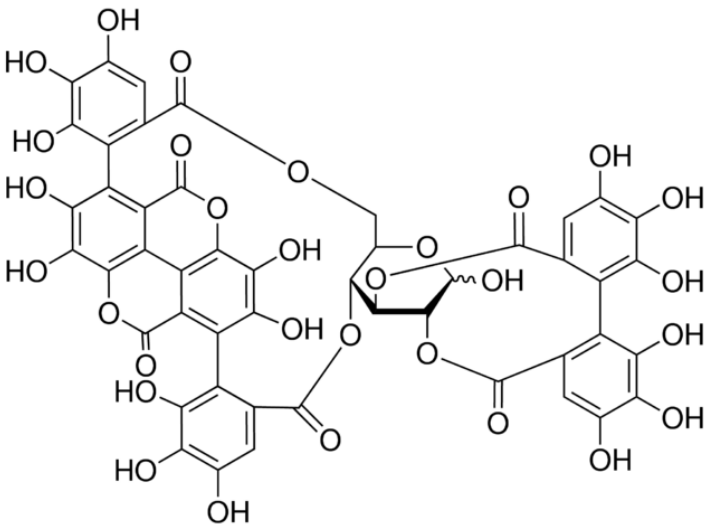

Figure II.1. Punicalagin chemical structure [219].

In addition to the antioxidant activity, pomegranate extracts, juices and oils have showed anticancer activity against breast cancer [220,221], prostate cancer [222–225] and colorectal cancer [225–227]. These bioactive activities are mainly due to the high content of ellagitannins of pomegranate. In the human digestive system, in the gastrointestinal tract, ellagitannins are hydrolyzed producing, among other metabolites, ellagic acid. Once in the presence of colon microflora, the ellagic acid is partially transformed into 3,8-dihydroxy-6H-dibenzo[b,d]-pyran-6-one (urolithin A). Then, both compounds are absorbed and transported by the blood flow to be conjugated in the liver and excreted in the urine [228–231].

Pomegranate juices and extracts from its by-products present a great potential to be applied directly or indirectly to foodstuffs in order to retard or inhibit oxidation and microorganisms. The pomegranate juice has even greater antioxidant capacity than green tea and wine, which makes it a potential candidate to be also applied in pharmaceutical formulations [214].

2.2. Grape

Grape (*Vitis vinifera*) is one of the most produced crops worldwide used mainly for wine production. According to EUROSTAT, in 2015 alone, the production of grapes by the European Union countries was 25 576. 04 T [232]. In 2017, at global level, the grape production was higher than 77,000 tones [233]. However, the grape is not used in its total for wine making, producing a great quantity of by-products (seeds, skin and pulp). Grape by-products (seeds, stem, and peels) are usually referred as grape pomace and are known to be a very high source of phenolic compounds such as epicatechin, catechin, gallic acid, procyanidins and phenolic acids and, consequently, important biological activities for the food industry such as antioxidant and antimicrobial activities [215,234,235]. Grape pomace is the solid waste after the alcoholic fermentation of wine and its high quantity of phenolic compounds present in grape by-products are the inefficiency of the extraction process of the winemaking process [236,237]. The phenolics content of the seeds is the highest (60–70%), followed by skin (28–35%) and pulp (10%) [238]. Seeds are essentially constituted by fiber, essential oil, protein and phenolic compounds, like tannins. Grape peels are an anthocyanin source with antioxidant and antimutagenic properties. Finally, stems are a great source of tannic compounds with high nutraceutical and pharmacological potential [234]. In grape pomace the most commonly found phenolic compounds are hydrobenzoic and hydrocynnamic acids, flavonols, stilbenes and anthocyanins [237].

Table II.2. Pomegranate and grape by-products and their main bioactive components.

Fruit by-product	Species/variety	Presentation form and extraction procedure	Main bioactive compounds and levels found	Main conclusions	Ref.
Pomegranate peel	*P. granatum* L., Gabsi	Peels were dried at 35 °C for 2 days, mixed with 150 mL of methanol, stirred for 4 h at 25 °C, centrifuged, filtered and concentrated.	Punicalagin b (106.93 ± 0.02 mg/g) Punicalagin a (86.85 ± 0.02 mg/g) HHDP-hexoside (45.86 ± 0.01 mg/g)	Both extracts (methanolic and aqueous) were effective in the extraction of active compounds that possess antioxidant and antimicrobial activity.	[239]
		Peels were dried at 35 °C for 2 days, mixed with 150 mL of water, stirred for 4 h at 55 °C, centrifuged, filtered and concentrated.	Punicalagin b (132.69 ± 0.01 mg/g) Punicalagin a (112.78 ± 0.00 mg/g) HHDP-hexoside (46.46 ± 0.00 mg/g)		
Pomegranate arils	*Punica granatum* L., cv. *Wonderful*	Juice (arils were manually extracted and pressurized in a nylon mesh).	Cyanidin-3-O-glucoside (70.3 ±0.1 μM cyanidin 3-*O*-glucoside) Cya-3,5-diglc (57.4 ± 0.9 μM cyanidin 3-*O*-glucoside) Delp, 3-glc (47.1 ± 0.5 μM cyanidin 3-*O*-glucoside) Ellagic acid glucoside (37.8 ± 0.2 μM EA) Delp-3,5-dilc (37.3 ± 0.9 μM cyanidin 3-*O*-glucoside) Ellagic acid hexoside (13.4 ± 0.5 μM EA) Ellagic acid deoxyhexoside (11.1 ± 0.3 μM EA) Punicalin (12.7 ± 0.4 μM EA) Ellagic acid (12.5 ± 0.8 μM EA)	In this study, pomegranate juice was extracted from 19 cultivars. The cultivars *Wonderful* and *Hizcaznar* showed the highest content in phenolic and anthocyanins, as well as the highest antioxidant and colour values.	[240]
	Punica granatum L., cv. *Hizcaznar*		Cya-3-glc (119 ± 2 μM cyanidin 3-*O*-glucoside) Cya-3,5 -diglc (100 ± 1 μM cyanidin 3-*O*-glucoside) Gallolyl-HHDP hexoside (21.9 ± 1.1 μM EA) Delp, 3-glc (15.4 ± 0.4 μM cyanidin 3-*O*-glucoside) Ellagic acid glucoside (14.6 ± 0.1 μM EA) Pg-3-glc (12.6 ± 0.1 μM cyanidin 3-*O*-glucoside) Ellagic acid deoxyhexoside (12.3 ± 0.9 μM EA)		

Fruit by-product	Species/variety	Presentation form and extraction procedure	Main bioactive compounds and levels found	Main conclusions	Ref.
Pomegranate peel	*P. granatum* L.	Extract (peel was separated, lyophilized, grounded and extracted with an aqueous solution of methanol (80 %, v/v; 0.1 % HCl) after flushing with nitrogen for 30 min. The solution was then filtered and evaporated to dryness at 30 °C)	Granatin B (5868.7 ± 351.3 mg/kg DM) Galloyl-HHDP-hex (3994.8 ± 157.5 mg/kg DM) Digalloyl-HHDP-gluc acid (3779.1 147.5 mg/kg DM) bis-HHDP-hex (3498.8 ± 156.7 mg/kg DM) Castalagin der (2936.9 ± 547.3 mg/kg DM) Lagerstannin B (2118.0 ± 71.1 mg/kg DM) Galloyl-bis HHDP-hex (2086.5 ± 138.8 mg/kg DM) Gallic acid (270.4 ± 18.5 mg/kg DM) Cya-3,5-digc (157.8 ± 7.1 mg/kg DM) Pel-3,5-diglc (145.8 ± 15.5 mg/kg DM) Pel-3-glc (56.7 ± 1.8 mg/kg DM) Cya-3-glc (41.2 ± 0.8 mg/kg DM)	The authors concluded that the processing and extraction methods clearly affect the phenolic profile of the samples. That being said, the phenolic profile of a sample can be used to distinguish pomegranate arils-based products from products made from pomegranate peels and mesocarp.	[241]
Pomegranate mesocarp	*P. granatum* L.	Extract (mesocarp was separated, lyophilized, grounded and extracted with an aqueous solution of methanol (80 %, v/v; 0.1 % HCl) after flushing with nitrogen for 30 min. The solution was then filtered and evaporated to dryness at 30 °C)	Galloyl-HHDP-gluc acid (5692.4 ± 673.1 mg/kg DM) Granatin B (2970.9 ± 41.6 mg/kg DM) Digalloyl-HHD-hex (2461.5 ± 243.7 mg/kg DM) Ellagic acid der (2288.6 ± 534.8 mg/kg DM) bis-HHDP-hex (1954.6 ± 210.3 mg/kg DM)		
Pomegranate peel	*P. granatum* L.	Pomegranate peels were dried at 40 °C, grounded and extraction though EE using 4 % of pectinase and 4 % of cellulase for 15 min. Then, extracts were submitted to a 90 °C water bath for 5 min and cooled rapidly.	Punicalagin isomer (626.62 ± 27.37 μg/g DW) bis-HHDP-glucoside isomer (145.05 ± 0.91 μg/g DW) 2-O-Galloylpunicalagin (80.39 ± 1.38 μg/g DW) Punicalin isomer (79.42 ± 4.69 μg/g DW)	The authors concluded that the Punicalagin isomer, digalloylpentoside and bis-HHDP-glucoside were the compounds better extracted with enzymes while the opposite was observed with galloyl-HHDP-glucoside. Also, they found that the extract of pomegranate peel obtained though high	[242]

Fruit by-product	Species/variety	Presentation form and extraction procedure	Main bioactive compounds and levels found	Main conclusions	Ref.
		Pomegranate peels were dried at 40 °C, grounded and extraction though HPE 300 MPa.	Punicalagin isomer (657.97 ± 13.02 μg/g DW) bis-HHDP-glucoside isomer (155.77 ± 1.57 μg/g DW) 2-O-Galloylpunicalagin (86.67 ± 4.34 μg/g DW)	pressures can be a good source of bioactive compounds.	
		Pomegranate peels were dried at 40 °C, grounded and extraction though HPE 600 MPa.	Punicalagin isomer (666.36 ± 19.23 μg/g DW) bis-HHDP-glucoside isomer (157.08 ± 1.70μg/g DW)		
		Pomegranate peels were dried at 40 °C, grounded and extraction though combined EE and HPE 300 MPa.	Punicalagin isomer (619.05 ± 8.79 μg/g DW) bis-HHDP-glucoside isomer (149.02 ± 2.28 μg/g DW) Punicalin isomer (80.75 ± 1.53 μg/g DW)		
Grape seeds	*Vitis vinifera* L., cv. Cabernet Mitos	Oil (seeds dried at 60 °C for 8 hours and then pressed with a screw extrusion press)	Gallic acid (152.9 ± 7.8 mg/kg DM) Caftaric acid (53.5 ± 6.6 mg/kg DM) Coutaric acid (22.1 ± 2.3 mg/kg DM) *p*-Coumaric acid (17.4 ± 0.1 mg/kg DM) Caffeic acid (16.9 ± 0.3 mg/kg DM)	Although the pressing process was applied without any additional temperature, the mechanical process did increase the temperature of the pomace to 60 °C. However, the grape seed oil still has a significant amount of polyphenols. In order to increase the content of polyphenols in the seed oil, a cold pressing method should be applied.	[235]
		Seeds (no extraction)	Gallic acid (278.8 ± 3.4 mg/kg DM) Caftaric acid (91.1 ± 1.6 mg/kg DM) Coutaric acid (28.3 ± 0 mg/kg DM) Fertaric acid (21.1 ± 0.3 mg/kg DM)		
	Vitis vinifera L., cv. Samtrot	Oil (seeds dried at 60 °C for 8 hours and then pressed with a screw extrusion press)	Gallic acid (353.7 ± 22.0 mg/kg DM) Caftaric acid (139.0 ± 16.5 mg/kg DM) Catechin (4.56 ± 0.08 g/kg DM) Epicatechin (2.42 ± 0.06 g/kg DM)		
		Seeds (no extraction)	Gallic acid (1116.5 ± 60.0 mg/kg DM) Caftaric acid (49.2 ± 7.2 mg/kg DM) Catechin (4.64 ± 0.78 g/kg DM) Epicatechin (3.31 ± 0.37 g/kg DM)		

Fruit by-product	Species/variety	Presentation form and extraction procedure	Main bioactive compounds and levels found	Main conclusions	Ref.
	Vitis vinifera L., cv. Kernert	Oil (seeds dried at 60 °C for 8 hours and then pressed with a screw extrusion press)	Gallic acid (133.2 ± 19.7 mg/kg DM) Caftaric acid (23.2 ± 2.3 mg/kg DM) Coutaric acid (7.7 ± 0.5 mg/kg DM) Epicatechin (1.06 ± 0.01 g/kg DM) Catechin (1.01 ± 0.10 g/kg DM)		
		Seeds (no extraction)	Gallic acid (248.6 ± 14.2 mg/kg DM) Caftaric acid (15.6 ± 3.6 mg/kg DM) Epicatechin (2.23 ± 0.43 g/kg DM) Proc B2 (1.16 ± 0.15 g/kg DM)		
Grape pomace	*V. vinifera* L., cv. Syrah	Extract (pomace was lyophilized, frozen with liquid nitrogen, grounded and homogenized with 15 mL of acidified methanol (0.1 % HCl, v/v) at 4 °C for 2 hours. The mixture was centrifuged and the supernatant was frozen. The solid pellet was re-extracted with 5 mL of acidified methanol)	Malvidin-3-acetyl glucoside (195.0 ± 16.6 mg/kg DW) Malvidin-3-coumaroyl glucoside (238.9 ± 4.7 mg/kg DW) Quercetin (93.0 ± 29.5 mg/kg DW) Quercetin-3-glucuronide (81.4 ± 3.4 mg/kg DW) Ethyl gallate (29.0 ± 4.1 mg/kg DW) (-)-Epicatechin (27.2 ± 4.0 mg/kg DW) Isoquercetin (26.5 ± 1.4 mg/kg DW) (+)-Catechin (21.8 ± 1.7 mg/kg DW) Isorhamnetin (16.1 ± 1.7 mg/kg DW) Epicatechin gallate (14.7 ± 2.8 mg/kg DW) Myricetin-3-glucoside (11.4 ± 0.7 mg/kg DW) Procyanidin dimer (10.0 ± 1.1 mg/kg DW)	The Syrah variety presented the highest anthocyanin content, which can result in a better antioxidant activity than the Merlot variety.	[236]

Fruit by-product	Species/variety	Presentation form and extraction procedure	Main bioactive compounds and levels found	Main conclusions	Ref.
	V. vinifera L., cv. Merlot		Quercetin (251.1 ± 65.6 mg/kg DW) Malvidin-3-coumaroyl glucoside (142.8 ± 31.4 mg/kg DW) (-)-Epicatechin (112.8 ± 8.2 mg/kg DW) Malvidin-3-acetyl glucoside (103.7 ± 23.5 mg/kg DW) (+)-Catechin (89.7 ± 9.6 mg/kg DW) Epicatechin gallate (45.6 ± 6.7 mg/kg DW) Kaempferol (34.2 ± 5.6 mg/kg DW) Quercetin-3-glucuronide (31.9 ± 3.6 mg/kg DW) Procyanidin dimer (24.6 ± 3.0 mg/kg DW) Isoquercetin (16.0 ± 0.8 mg/kg DW) Procyanidin dimer monogallate (13.6 ± 2.0 mg/kg DW) Isorhamnetin (12.5 ± 1.5 mg/kg DW) Ethyl gallate (50.5 ± 1.2 mg/kg DW)		
Grape pomace	*V. vinifera* L., cv. Cabernet Sauvignon	Extract (pomace was dried at 80 °C for 36 h, grounded and mixed in a solute:solvent ratio 1:50 with ethanol and water (60:40, v/v). Mixtures were shaken for 24 h at 25 °C and centrifuged)	Syringic acid (345.55 ± 5.53 μg/mL) Vanillic acid (279.70 ± 4.69 μg/mL) Gallic acid (225.35 ± 3.40 μg/mL) Caffeic acid (111.31 ± 2.30 μg/mL) Catechin (160.44 ± 2.40 μg/mL) Rutin (216.96 ± 0.17 μg/mL) Anthocyanins (65.50 ± 1.67 Cya-3-glc Equivalents μg/mL)	The authors concluded that grape pomace from the wine industry is a suitable source of bioactive compounds, such as phenolic compounds and anthocyanins. The grape pomace can be used in the development of new products.	[238]
	V. vinifera L., cv. Merlot		Syringic acid (368.73 ± 17.86 μg/mL) Gallic acid (291.23 ± 14.84 μg/mL) Vanillic acid (283.30 ± 16.64 μg/mL) Quercetin (249.23 ± 1.14 μg/mL) Catechin (153.04 ± 6.47 μg/mL) Anthocyanins (24.52 ± 0.57 Cya-3-glc Equivalents μg/mL);		

Fruit by-product	Species/variety	Presentation form and extraction procedure	Main bioactive compounds and levels found	Main conclusions	Ref.
	Vitis labrusca L., mixture of Bordeaux (65 %), Isabel (25 %) and BRS Violet (10 %)		Gallic acid (397.67 ± 1.18 μg/mL) Syringic acid (343.97 ± 0.91 μg/mL) Vanillic acid (284.09 ± 0.66 μg/mL) Catechin (275.09 ± 0.37 μg/mL) *p*-Coumaric acid (209.87 ± 2.47 μg/l) Quercetin (159.60 ± 1.04 μg/mL) Chlorogenic acid (142.89 ± 4.65 μg/mL) Caffeic acid (141.52 ± 2.10 μg/mL) Rutin (112.96 ± 1.49 μg/mL) Anthocyanins (121.90 ± 3.05 Cya-3-glc Equivalents μg/mL)		
	V. labrusca L., cv. Terci		Vanillic acid (332.38 ± 31.76 μg/mL) Syringic acid (219.15 ± 26.84 μg/mL) Gallic acid (186.18 ± 3.53 μg/mL) Catechin (153.88 ± 13.05 μg/mL) Rutin (113.13 ± 0.52 μg/mL) Quercetin (101.89 ± 0.20 μg/mL) Anthocyanins (188.77 ± 7.99 Cya-3-glc Equivalents μg/mL)		
Grape skin	*V. vinifera* L., cv. Muscat Alexandria	Extract (the crushed lyophilized skins were mixed with methanol/HCl (99/1, v/v) and submitted to an ultrasound bath at 25 °C (350 W) during 20 min. The extracts were left at room temperature for 0.5 min and filtered. The remaining solids were extracted 4 times with the same method)	Catechin (628 ± 42 μg/g) Epicatechin (323 ± 14 μg/g) Quercetin (316 ± 12 μg/g) Rutin (223 ± 9 μg/g) Gallic acid (122 ± 7 μg/g)	The authors concluded that grapes variety has a strong influence on the phenolic profile of the by-products. Iranian varieties presented higher phenolic content than the international variety (Muscat Alexandria).	[201]
	V. vinifera L., cv. Ghara Shani		Catechin (945 ± 50 μg/g) Epicatechin (482 ± 30 μg/g) Quercetin (405 ± 24 μg/g) Gallic acid (319 ± 17 μg/g) Rutin (287 ± 18 μg/g)		

Fruit by-product	Species/variety	Presentation form and extraction procedure	Main bioactive compounds and levels found	Main conclusions	Ref.
Grape seeds	vr. Pinot Noir	Extract (seeds were dried at 22 °C, grounded, mixed with methanol:water (80:20) containing 0.1 % of HCl, and then filtered.)	Gallocatechin gallate (1454.60 mg/kg DS) Catechin (270.26 mg/kg DS) Catechin 3-gallate (253.11 mg/kg DS) Epicatechin (223.08 mg/kg DS) Gallic acid (136.74 mg/kg DS) Epigallocatechin (39.29 mg/kg DS)	The authors concluded that grape seeds are characterized for the presence of flavan-3-ols.	[243]
	vr. Prokupac		Catechin (1111.66 mg/kg DS) Gallocatechin gallate (788.80 mg/kg DS) Catechin 3-gallate (296.07 mg/kg DS) Gallic acid (289.13 mg/kg DS) Epicatechin (124.10 mg/kg DS) Ellagic acid (77.17 mg/kg DS)		
	vr. Chardonnay		Gallocatechin gallate (266.43 mg/kg DS) Catechin 3-gallate (122.36 mg/kg DS) Catechin (100.06 mg/kg DS) Gallic acid (65.75 mg/kg DS) Epicatechin (50.48 mg/kg DS)		
	vr. Pinot Gris		Gallocatechin gallate (481.71 mg/kg DS) Catechin (163.02 mg/kg DS) Catechin 3-gallate (139.53 mg/kg DS) Epicatechin (86.64 mg/kg DS) Gallic acid (71.09 mg/kg DS) Epigallocatechin (39.26 mg/kg DS) Ellagic acid (33.37 mg/kg DS)		

Fruit by-product	Species/variety	Presentation form and extraction procedure	Main bioactive compounds and levels found	Main conclusions	Ref.
Grape skins	vr. Pinot Noir	Extract (skins were mixed with methanol with 0.1 % of HCl, stirred for 1 h at 22 °C, left in the dark at 4 °C for 24 h and frittered. The extract was evaporated to dryness at 40 °C.)	Myricetin (12.40 mg/kg FS) Gentisic acid (5.64 mg/kg FS) Resveratrol (5.64 mg/kg FS) Gallocatechin (2.91 mg/kg FS) Quercetin (2.61 mg/kg FS) Epigallocatechin (2.42 mg/kg FS) Gallic acid (2.42 mg/kg FS) Ferulic acid (2.12 mg/kg FS) Chlorogenic acid (2.01 mg/kg FS) *p*-Hydroxybenzoic acid (1.42 mg/kg FS)	The authors concluded that grape skins are characterized by the presence of flavonols.	[243]
	vr. Prokupac		Gallic acid (3.90 mg/kg FS) Gentisic acid (7.15 mg/kg FS) Chlorogenic acid (2.09 mg/kg FS) Ferulic acid (2.46 mg/kg FS) Aesculin (1.31 mg/kg FS) Epigallocatechin (1.98 mg/kg FS) Kaempferol (5.34 mg/kg FS) Quercetin (44.72 mg/kg FS) Rutin (1.30 mg/kg FS) Myricetin (7.70 mg/kg FS) Resveratrol (13.42 mg/kg FS)		
	vr. Chardonnay		Ferulic acid (13.95 mg/kg FS) Rutin (11.67 mg/kg FS) Gallocatechin gallate (11.60 mg/kg FS) Gentisic acid (5.87 mg/kg FS) Epicatechin (2.95 mg/kg FS) Gallic acid (2.78 mg/kg FS) Epigallocatechin gallate (2.51 mg/kg FS) Epigallocatechin (2.25 mg/kg FS) Chlorogenic acid (1.72 mg/kg FS) Aesculin (1.20 mg/kg FS)		

Fruit by-product	Species/variety	Presentation form and extraction procedure	Main bioactive compounds and levels found	Main conclusions	Ref.
	vr. Pinot Gris		Gallic acid (2.34 mg/kg FS) Gentisic acid (5.90 mg/kg FS) Chlorogenic acid (1.75 mg/kg FS) Ferulic acid (13.33 mg/kg FS) Aesculin (1.15 mg/kg FS) Gallocatechin (3.09 mg/kg FS) Epigallocatechin (2.09 mg/kg FS) Gallocatechin gallate (8.93 mg/kg FS) Epigallocatechin gallate (2.07 mg/kg FS) Kaempferol (1.66 mg/kg FS) Quercetin (21.98 mg/kg FS) Rutin (1.21 mg/kg FS)		

Legend: cv – cultivar; DM – Dry Matter; EA – Ellagic acid; SFE – Supercritical Fluid Extraction; COSE – Classical Organic Solvent Extraction; DS – Dried sample; vr – variety; FS – Frozen Sample; SPE – Solid Phase Extraction; EE - Enzymatic extraction; HPE – High Pressure Extraction; AP – apple pomace

Lingua et al. [236] studied the change of the phenolic profile of three grape varieties (Syrah, Merlot and Cabernet Sauvignon) grown in Argentina, their pomaces and wines. The results showed that in the Syrah variety, the wine extract presented higher content of phenolic compounds than the grape and pomace extracts, while grape extracts showed higher amounts of in petunidin-3-glucoside, peonidin-3-glucoside, peonidin-3-coumaroyl glucoside, malvidin-3-glucoside and malvidin-3-coumaroyl glucoside. On the other hand, in the Merlot variety, the pomace extract presented the highest phenolic content of the three extracts and the highest values of delphinidin-3-coumaroyl glucoside, petunidin-3-coumaroyl glucoside and peonidin-3-coumaroyl glucoside. Different from the other two varieties, the Cabernet Sauvignon grape extract presented the highest total phenolics content and the highest content in malvidin-3-glucoside and malvidin-3-acetyl glucoside. The authors reached to the conclusion that Syrah was the variety that showed the highest content of anthocyanins which are responsible for the highest antioxidant capacity of this variety [236].

As a major source of anthocyanins and monomeric phenolic compounds, such as (+)-catechins and (−)-epicatechin, and dimeric, trimeric and tetrameric procyanidins, grape pomace shows a great potential for applications to foodstuffs as direct or indirect additive [201].

3. Active compounds of pomegranate and grape by-products

The active compounds present in fruits and fruits by-products are extensively applied to food formulations in order to enhance or offer nutritional, sensory and active properties [244]. The application of these active compounds can be direct, by applying the active compound to the food itself, or indirect, by applying these compounds, for example, in active food packaging.

The bioactive compounds can be obtained in the form of extracts or essential oils. There are several extraction processes that can be applied to the extraction of these compounds, which are directly related to the chemical quality and the quantity of extracts and essential oils [69]. The choice of the extraction method shall be carried out in accordance with the purpose of the extract/essential oil/active compound, which are, mostly, phenolic compounds.

As previously stated, phenolic compounds are secondary metabolites produced by plants for their defense against predators, radiation and pathogenic organisms. They are characterized for possessing an aromatic ring linked to at least one hydroxyl group. They are classified in several subclasses, being flavonoids and tannins, two of the most

common and largest classes. Pomegranate and grape by-products are a great source of tannins and flavonoids, namely anthocyanins.

According to Table II.2, for grape seeds, catechin (4.64 g/kg DM) and epicatechin (3.31 g/kg DM) are the most commonly found bioactive compounds, followed by gallic acid (136.74–1116.5 mg/kg DS) and gallocatechin gallate (266.43–1454.60 mg/kg DS). For grape pomace, syringic acid (368.73 μg/mL) and gallic acid (186.18–397.64 μg/mL) are the major phenolics and for grape skins, catechin (628–945 μg/g) and epicatechin (323–482 μg/g) are the major phenolic compounds. Regarding the pomegranate by-products, galloyl-hexahydroxydiphenoylglucoside acid (5692.4 mg/kg), galloyl-hexahydroxydiphenoyl-hex (3994.8 mg/kg), digalloyl-hexahydroxydiphenoyl-gluc acid (3779.1 mg/kg), granatin B (2970.9 mg/kg) and castalagin derivative (2936.9 mg/kg) are the major phenolic compounds.

3.1. Tannins

Tannins are high weight molecules (500–3000 Da) formed due to the polymerization of polyphenols. Acting as a natural defense agent in plants, they can be classically divided into two groups: hydrolysable tannins and condensed tannins [213,245,246]. Recently, two other groups have been added to the list: complex tannins and phlorotannins [247]. Condensed tannins, also known by proanthocyanins, are chemically considered flavonoids (polymeric or oligomeric) and are composed by flavan-3-ols and/or flavan-3,4-diols. Catechins are one of the most common examples of condensed tannins [245,247]. Hydrolysable tannins are composed by polyphenolic acids and their derivatives. Under basic or acid conditions, these tannins can be fractionated though hydrolysis into basic components, being classified into two categories: gallotannins and ellagitannins. The first ones are the simple's kind of hydrolysable tannins and have the ability to generate, through the hydrolysis reaction, gallic acid [247–249]. On the other hand, ellagitannins can generate hexahydroxydiphenic acid units by hydrolysis, which turn into ellagic acid, a very well-known phenolic compound with powerful antioxidant capacity [247]. Regarding the two more recent tannin groups, complex tannins are formed by an ellagitannin or a gallotannin moiety linked by a carbon-carbon bond to a flavan-3-ol building block [247]. Finally, phlorotannins are most commonly found in brown algae (such as *Fucus vesiculosus* L.) and are composed by phloroglucinol units [247,250]. The direct and indirect use of tannins as food additives is controversial. On one hand, tannins are known to possess antioxidant and antimicrobial activities, which can help to extend the shelf-life of foodstuffs, as well as anticarcinogenic, antimutagenic,

anti-AIDS and immunomodulation activities. On the other hand, tannins possess antinutritional, carcinogenic and hepatogenic activities [245]. The undesirable activities of tannins are related to their molecular mass: the greater the molar mass, the greater its antinutritional activity. The larger molecules bind to the proteins, starch and digestive enzymes present in the gastrointestinal tract, preventing their absorption by the digestive system, acting as antinutritional compounds [251]. Gallotannins are hydrolysable tannins and, when hydrolyzed by bases, acids or enzymes, produce glucose and gallic acid. Ellagitannins are other hydrolysable tannins that contain one or more hydroxydiphenoyl [245].

3.2. Anthocyanins

Flavonoids are polyphenols (phenolic compounds with more than one hydroxyl group linked with an aromatic ring) present in most plants. Currently, there are more than 8000 flavonoids structures identified. This subclass of phenolic compounds includes flavonols, flavones, catechins, flavanones, isoflavonoids and anthocyanins [252]. Their biological activities and reactivity are determined by their properties, such as their molecular weight and solubility. Their roles in plants include the protection of the plant against microorganisms and predators, radiation (namely UV) and oxidative cell injury, as well as, the control of auxin transport and the coloration of flowers, which attracts the pollinators. On the other hand, in foods, they are responsible for the protection of enzymes, vitamins, taste and color and, avoid lipid peroxidation [253].

Anthocyanins (Figure II.2) are water-soluble pigments (flavonoids) present in several edible plants and fruits, specifically berries and pomegranate. They are responsible for red, blue and purple colors and possess strong antioxidant, anti-inflammatory and cardioprotective activities [218,254,255]. Anthocyanins, are glycosides formed by anthocyanidins (aglycones). The most commons anthocyanins found in fruits and vegetables are cyanidin (coloration reddish-purple), pelargonidin (coloration red and orange), peonidin (coloration magenta), delphinidin (blue-red-ish color), petunidin (dark red or purple color) and malvidin (purple color) [256].

Anthocyanins stability is dependent on light, pH and temperature. Due to their ionic nature, the color presented by anthocyanins is dependent on pH: at high pH, the anthocyanins hue appears to be blue while in more acid conditions, the anthocyanins hue is red. In the case of a neutral pH, they present a purple hue [256]. Due to their powerful coloration, anthocyanins can be extracted and used as food colorants, replacing synthetic additives due to their low to no toxicity [256].

4. Biological activities of fruits by-products and their extracts

The need to increase the shelf-life of foods is growing with the facility of acquisition by the consumers of any type of food, anywhere in the world. The fact that consumers do not consume only locally produced food has made the food industry search for more efficient ways of transporting, packaging and preserving foods. This may lead to the widespread use of food additives. The food additives can be used for several purposes, besides preserving foods and, can be from synthetic or natural origin. Recent discoveries are questioning the application of synthetic additives since their long-term effect on human health is unknown [66,199,257–259]. Therefore, alongside the growing preference of the consumers for more 'natural' foods, the food industry and the scientific community are searching for natural additives as effective as the synthetic ones.

As observed in this paper, the non-edible part of fruits can have higher biological activities than the edible part of the food, making their extracts and essential oils a possible alternative to the use of synthetic additives.

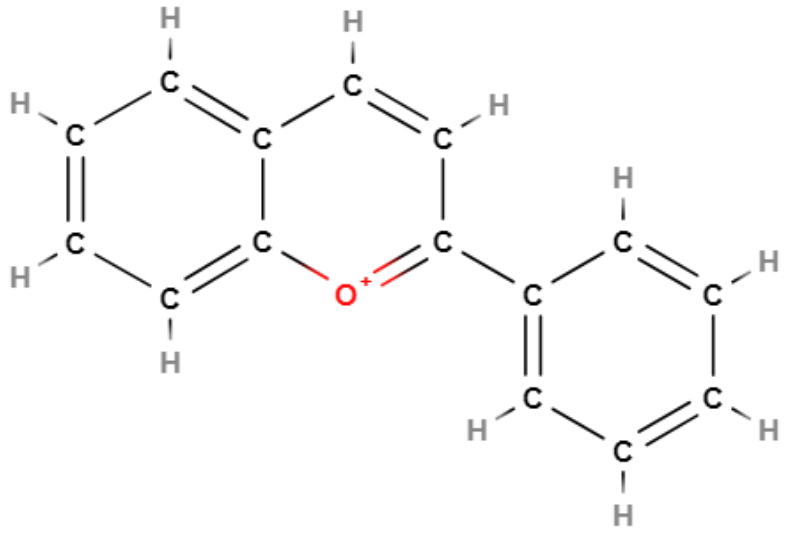

Figure II.2. Anthocyanins chemical structure [260].

4.1. Antioxidant activity

The definition of "antioxidant" is rather controversial. In the European legislation, antioxidants are defined as "*substances which prolong the shelf-life of foods by protecting them against deterioration caused by oxidation, such as fat rancidity and color changes*" [12]. On the other hand, according to the Institute of Medicine (US), "*a dietary antioxidant is a substance in foods that significantly decreases the adverse effects of reactive species, such as*

reactive oxygen and nitrogen species, on normal physiological function in humans" [261]. In the food industry, antioxidants (E300 – E399) are used to extend food' shelf-life by preventing oxidation of lipids and vitamins in foodstuffs, through the prevention of their autoxidation and, consequently, the development of rancidity or other undesirable flavors [68]. The antioxidant activity of some fruits by-products extracts can be observed in Table II.3.

Farhadi et al. [201] developed a methanolic grape skin extract that presented 94.06% of inhibition of the DPPH radical and a total phenolic content (TPC) higher than 600 mg GAE/g of DW. Also, Ćetković et al. [262] found that an apple pomace methanolic extract presented an EC_{50} of 6.33 mg/mL, which is the necessary amount for inhibiting 50% of the DPPH radical.

Andrés, Petrón, Adámez, López, and Timón [99] tested the antioxidant capacity of aqueous extracts of pomegranate and red grape byproducts. Pomegranate by-products extract presented a higher TPC (134.79 mg GAE/g of extract) and total carotene content (122.87 μglycopene/g of extract) than the red grape by-products extract (32.16 mg GAE/g of extract and 6.96 μg lycopene/g of extract). However, the red grape by-products extract presented a higher content of vitamin C (71.00 μg ascorbic acid/g of extract) and a higher IC50 of DPPH radical (0.05 mg/mL) than the pomegranate extract (64.57 μg ascorbic acid/g of extract and 0.16 mg/mL, respectively). When the authors applied the extracts to lamb patties, the grape by-products extract effectively slowed down the meat discoloration and lipid oxidation (1.28 mg malonaldehyde/kg) than the synthetic antioxidant sodium ascorbate (3.47 mg MDA/kg) at the end of 7 days [99].

Table II.3. Antioxidant capacity of some fruit by-products.

Fruit by-product	Species/variety	Extract/oil/form	Method	Value	Unit	Ref.
Pomegranate peel	*Punica granatum* L.	Pomegranate was washed in distilled water and the peels were dried at 40 °C for 48h and grinded. The extract was obtained through a high voltage electrical discharge extraction system (flow rate of materials 12 mL/min. electrodes gap distance .1 mm and liquid to solid ratio 35 mL/g).	TPC	196.7 ± 6.4	mg GAE/g of DS	[263]
Pomegranate Peel	*Punica granatum* L.	Fresh peels were cut into pieces and mixed with a combination of methanol, ethanol, and acetone. Then filtered, re-extracted with the same solvent and concentrated under vacuum at 60 °C.	TPC	249.4 ± 17.2	TAE mg/g	[209]
			TFC	59.1 ± 4.8	RE mg/g	
Pomegranate pulp	*Punica granatum* L.		TPC	24.4 ± 2.7	TAE mg/g	
			TFC	17.2 ± 3.3	RE mg/g	
Pomegranate peel	*P. granatum* L.	15 g of finely powdered peel was mixed with 100 mL of ethanol, shaken for 16 h. filtered and centrifuged. The clear extracts were filtered and concentrated.	DPPH	75.54 ± 0.37	% Inhibition	[264]
			TPC	81.15 ± 0.27	mg GAE/g	
			ABTS	8.17 ± 0.43	µmol TE/g	
			FRAP	11.47 ± 0.27	µmol TE/g	
Pomegranate Peel	*P. granatum* L.	Peels and mesocarp were separated, lyophilized and grounded. The powder was extracted with an aqueous solution of methanol (80 %. v/v; 0.1 % HCl) after flushing with nitrogen for 30 min. the solution was then filtered and evaporated to dryness at 30 °C.	FRAP	589.1 ± 34.7	mg/kg DM	[241]
			TEAC	1361.9 ± 13.7	mg/kg DM	
			TPC	101586.3 ± 12810.0	mg/kg DM	
			TEAC	2887.1 ± 6.5	mg/kg DM	
			TPC	198173.3 ± 2899.7	mg/kg DM	
Grape pomace	*Vitis vinifera* L.. vr. Cabernet sauvignon	Extract by Soxhlet Extraction	DPPH	49.5 0.3	IC_{50} (µg/mL)	[234]
			TPC	3.4 0.1	GAE/mg of extract	

Fruit by-product	Species/variety	Extract/oil/form	Method	Value	Unit	Ref.
Grape pomace	*V. vinifera* L.. vr. Syrah	Extract (pomace was lyophilized. frozen with liquid nitrogen. grounded and homogenized with 15 mL of acidified methanol (0.1 % HCl. v/v) at 4 °C for 2 hours. The mixture was centrifuged and the supernatant was frozen. The solid pellet was re-extracted with 5 mL of acidified methanol).	TPC	1013 ± 63	mg GAE/100 g DW of sample	[236]
	V. vinifera L.. vr. Merlot			2122 ± 214		
	V. vinifera L.. vr. Cabernet sauvignon			985 ± 136		
Grape skin	*V. vinifera* L.. vr. Muscat Alexandria	Extract (the crushed lyophilized skins were mixed with methanol/HCl (99/1. v/v) and submitted to an ultrasound bath at 25 °C (350 W) during 20 min. The extracts were left at room temperature for 0.5 min and filtered. The remaining solids were extracted 4 times with the same method)	TAC	0.78 ± 0.08	mg/g DW	[201]
			TPC	< 600	mg GAE/g DW	
			IC_{50}	0.62 ± 0.05	mg/mL	
			DPPH	94.06 ± 0.15	%	
	V. vinifera L.. vr. Ghara Shani		TAC	63.1 ± 6.0	mg/g DW	
			TPC	1205 ± 141	mg GAE/g DW	
			IC_{50}	0.49 ± 0.04	mg/mL	
			DPPH	95.62 ± 0.27	%	
Grape seeds	vr. Pinot Noir	Extract (seeds were dried at 22 °C. grounded, mixed with methanol:water (80:20) containing 0.1 % of HCl. and then filtered)	TPC	102.98 ± 0.58	mg GAE/g DW	[243]
			RSA	863.29 ± 10.91	µmol TE/g	
	vr. Prokupac		TPC	101.25 ± 5.23	mg GAE/g DW	
			RSA	967.90 ± 3.64	µmol TE/g	
	vr. Chardonnay		TPC	62.34 ± 0.19	mg GAE/g DW	
			RSA	873.62 ± 0.00	µmol TE/g	

Fruit by-product	Species/variety	Extract/oil/form	Method	Value	Unit	Ref.
	vr. Pinot Gris		TPC	96.89 ± 2.51	mg GAE/g DW	
			RSA	1039.92 ± 0.00	µmol TE/g	
Grape skin	vr. Pinot Noir	Extract (skins were mixed with methanol with 0.1 % of HCl. stirred for 1 h at 22 °C, left in the dark at 4 °C for 24 h and frittered. The extract was evaporated to dryness at 40 °C)	TPC	7.21 ± 0.10	mg GAE/g DW	
			RSA	95.10 ± 3.53	µmol TE/g	
	vr. Prokupac		TPC	12.32 ± 0.19	mg GAE/g DW	
			RSA	132.59 ± 4.24	µmol TE/g	
	vr. Chardonnay		TPC	2.04 ± 0.05	mg GAE/g DW	
			RSA	46.86 ± 2.40	µmol TE/g	
	vr. Pinot Gris		TPC	3.71 ± 0.09	mg GAE/g DW	
			RSA	64.07 ± 1.75	µmol TE/g	

Legend: DM – Dry Mass; S – sample; DS – Dried Sample; DW – Dry Weight; TPC – Total Phenolic Content; TFC – Total Flavonoid Content; GAE – Gallic Acid Equivalents; QE – Quercetin Equivalents; cv – cultivar; vr – variety; TE – Trolox equivalents; ABTS - 2.2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); DPPH - 2.2-diphenyl-1-picrylhydrazyl; FRAP – Ferric-Reducing Antioxidant Power; TAE - Tannic Acid Equivalents; RE – Rutin Equivalents; TEAC – Trolox Equivalent Antioxidant Capacity; TAC – Total Anthocyanin Content; RSA – Radical-Scavenging Activity; ORAC - Oxygen Radical Absorbance Capacity; CE – Catechin Equivalents; CA – Chlorogenic acid; GA – Gallic Acid; AA – Ascorbic acid; DP – Dry powder.

4.2. Antimicrobial activity

Antimicrobial resistance is one of the most severe worldwide concerns. Like antioxidant agents, antimicrobials can be synthetic or natural. The natural ones can be more efficient than or as efficient as the synthetic ones, having the advantage of being able to inhibit more than one microorganism at the time [265]. Like the antioxidant capacity of plants and fruits, the antimicrobial activity can be traced back to the presence of phenolic compounds (Table II.4).

Pomegranate peel is very rich in anthocyanins and other phenolic compounds (Table II.2). Nur Hanani, Aelma Husna, Nurul Syahida, Nor Khaizura, and Jamilah [266] tested the antimicrobial activity of a 5% pomegranate peel powder incorporated into a fish gelatin active film. The film presented inhibited *Staphylococcus aureus*, *Listeria monocytogenes* and *Escherichia coli* [267].

Panichayupakaranant, Tewtrakul, and Yuenyongsawad [268] proved the antibacterial activity of a standardized pomegranate rind extract with 13% (w/w) of ellagic acid against Gram-positive bacteria *Propionibacterium acnes*, *S. aureus* and *Staphylococcus epidermidis*, at a concentration of 2 mg/disc.

Nur Hanani et al. [266] develop a gelatin/polypropylene bilayer active film incorporated with pomegranate peel extract. The antimicrobial activity of the films was tested by disc diffusion tests against *Listeria monocytogenes*, *Bacillus cereus*, *Escherichia coli*, *Salmonella* Typhimurium. The authors also compared the results of gelatin/polypropylene bilayer active films incorporated with papaya and jackfruit peel extracts and chitosan. Only the film incorporated with pomegranate peel extract showed inhibition against all the tested bacteria, showing the antimicrobial potential of the active compounds present in the pomegranate peel extract, especially tannins [266].

Table II.4. Antimicrobial activity of fruit by-products extracts and oils.

By-product/Species and variety	Extract/oil/applied form	Concentration	Microorganism	Assays	Unit	Ref.
Pomegranate peel (*P. granatum* L.)	15 g of finely powdered peel was mixed with 100 mL of ethanol, shaken for 16 h, filtered and centrifuged. The clear extracts were filtered and concentrated.	1 mg/mL	*Listeria monocytogenes*	6.58 ± 0.36	MIC - Zone of inhibition (mm)	[264]
			Staphylococcus aureus	4.55 ± 0.36		
			Escherichia coli	8.42 ± 0.24		
			Pseudomonas aeruginosa	2.59 ± 0.47		
			Klebsiella pneumonia	8.58 ± 0.47		
			Salmonella enteritidis	10.63 ± 0.41		
Pomegranate peel (*P. granatum* L.)	Pomegranate peels were dried at 40 °C, grounded and extraction though EE using 4 % of pectinase and 4 % of cellulase for 15 min. Then, extracts were submitted to a 90 °C water bath for 5 min and cooled rapidly.	0.5 g/mL	*B. cereus* (ATCC 2599)	16 ± 1.0	Inhibition halo (mm)	[242]
			S. aureus (ATCC 25923)	17 ± 0.6		
			Methicillin-resistant Staphylococcus aureus	17 ± 0.6		
			L. monocytogenes	11 ± 0.6		
			S. enteritidis (ATCC 13076)	14 ± 1.0		
			P. aeruginosa (ATCC 10145)	29 ± 0.0		
			E. coli (ATCC 25922)	16 ± 0.6		
Pomegranate rind extract (*P. granatum* L.)	Standardized pomegranate rind extract with 13 % of ellagic acid.	200 mg/mL	*Propionibacterium acnes* (DMST 14916)	21.5 ± 1.89	Inhibition zone	[268]
			Staphylococcus epidermidis (ATCC 14990)	19.1 ± 0.49		

By-product/Species and variety	Extract/oil/applied form	Concentration	Microorganism	Assays	Unit	Ref.
	Rinds were dried at 50 °C for 24 h, and reduced to powder. 0.5 kg of powder were mixed with 2 l of methanol (10 %, v/v, water) under reflux conditions for 1 h. Extracts were then dried *in vacuo* and resuspended in 2 % aqueous acetic acid, partitioned with ethyl acetate and evaporated until dryness.		*S. aureus* (ATCC 25923)	18.6 ± 0.38	(mm ± SD)	
			S. aureus (isolated strains PSU01)	18.8 ± 0.40		
			S. aureus (isolated strains PSU02)	18.1 ± 0.42		
			S. aureus (isolated strains PSU03)	16.8 ± 0.38		
			S. aureus (isolated strains PSU04)	19.4 ± 0.30		
			S. aureus (isolated strains PSU05)	18.6 ± 0.79		
			S. aureus (isolated strains PSU06)	15.2 ± 0.60		
			S. aureus (isolated strains PSU07)	16.2 ± 0.78		
Wine by-products (*Vitis vinifera* vr. Syrah)	50 g of the freeze-dried by-products were mixed with 200 mL of methanol and the pH was adjusted to 4.0 with HCl. The mixture was agitated at 4 °C overnight, sonicated 3 min, centrifuged, filtered and concentrated with a rotary evaporator.	500 µg TAC	*E. coli* (BL21 [DE3])	9.78 ± 0.71	Inhibition diameter zone (mm)	[269]
			S. aureus (ATCC 6538)	9.08 ± 0.42		
			Bacillus subtilis (ATCC 6633)	7.00 ± 1.45		
			B. cereus (ATCC 11778)	8.43 ± 2.00		
Grape seeds (*Vitis vinifera* L., vr. Touriga Nacional)	Seeds were freeze-dried, mill-powdered, stirred for 2 h with water/ethanol (50:50) followed by a sonification for 5 min. The samples were centrifuged, and the solvent were evaporated until dryness.	100 µg/mL	*Enterococcus faecalis* (vanB2-C3735)	50	MIC (µg/mL)	[270]
			Enterococcus faecium vanA-C2302	100		
			Klebsiella pneumoniae C1370 (CTX-M-15)	50		

By-product/Species and variety	Extract/oil/applied form	Concentration	Microorganism	Assays	Unit	Ref.
			S. aureus C5932 (MRSA CC398)	50		
			S. epidermidis C3658 (linezo-R)	10		
			L. monocytogenes ATCC700302	100		
			B. cereus (ATCC1306)	10		
Grape skins (*Vitis vinifera* L., Touriga Nacional)	Skins were freeze-dried, mill-powdered, stirred for 2 h with water/ethanol (50:50) followed by a sonification for 5 min. The samples were centrifuged, and the solvent were evaporated until dryness.	100 µg/mL	*Enterococcus faecium* vanA-C2302	100	MIC (µg/mL)	
			Klebsiella pneumoniae C1370 (CTX-M-15)	100		
			S. epidermidis C3658 (linezo-R)	10		
			L. monocytogenes ATCC700302	50		
			B. cereus (ATCC1306)	100		
Grape stems (*Vitis vinifera* L., Touriga Nacional)	Stems were freeze-dried, mill-powdered, stirred for 2 h with water/ethanol (50:50) followed by a sonification for 5 min. The samples were centrifuged, and the solvent were evaporated until dryness.	100 µg/mL	*Enterococcus faecium* vanA-C2302	100	MIC (µg/mL)	
			S. aureus C5932 (MRSA CC398)	100		
			S. epidermidis C3658 (linezo-R)	75		
			L. monocytogenes ATCC700302	50		
Grape seeds (*Vitis vinifera* L., Preto Martinho)	Seeds were freeze-dried, mill-powdered, stirred for 2 h with water/ethanol (50:50) followed by a sonification for 5 min. The samples were centrifuged, and	100 µg/mL	*S. epidermidis* C3658 (linezo-R)	25	MIC (µg/mL)	
			S. aureus C5932 (MRSA CC398)	10		
			E. faecalis (vanB2-C3735)	10		

By-product/Species and variety	Extract/oil/applied form	Concentration	Microorganism	Assays	Unit	Ref.
	the solvent were evaporated until dryness.		*L. monocytogenes* ATCC700302	10		
			B. cereus (ATCC1306)	50		
			K. pneumoniae C1370 (CTX-M-15)	100		
Grape skins (*Vitis vinifera* L., Preto Martinho)	Skins were freeze-dried, mill-powdered, stirred for 2 h with water/ethanol (50:50) followed by a sonification for 5 min. The samples were centrifuged, and the solvent were evaporated until dryness.	100 µg/mL	*S. epidermidis* C3658 (linezo-R)	10	MIC (µg/mL)	
			S. aureus C5932 (MRSA CC398)	75		
			E. faecalis (vanB2-C3735)	25		
			L. monocytogenes ATCC700302	50		
Grape Stems (*Vitis vinifera* L., Preto Martinho)	Stems were freeze-dried, mill-powdered, stirred for 2 h with water/ethanol (50:50) followed by a sonification for 5 min. The samples were centrifuged, and the solvent were evaporated until dryness	100 µg/mL	*S. epidermidis* C3658 (linezo-R)	25	MIC (µg/mL)	
			E. faecalis (vanB2-C3735)	50		
			E. faecium vanA-C2302	100		
			L. monocytogenes ATCC700302	50		
			B. cereus (ATCC1306)			
			K. pneumoniae C1370 (CTX-M-15)	75		

Legend: MIC - Minimum Inhibitory Concentration; CFU - Colony-Forming Unit; EE – Enzymatic extraction; TAC – Total Anthocyanin Content

4.3. Others

The range of health-promoting effects associated with bioactive compounds is large since a diverse group of bioactive compounds is present in the by-products of the selected fruits to be considered in the present review. Thus, besides antioxidant or antimicrobial activities, other effects have also been investigated.

Pomegranate peel has a widely recognized therapeutic potential. Besides antioxidant and antimicrobial capacities, studies have demonstrated its potential to prevent some human carcinomas and other activities have been investigated such as cardiovascular protection, antiinflammatory, anti-allergic, anti-influenza, anti-malarial properties and wound healing properties [271].

Hassanpour Fard, Ghule, Bodhankar, and Dikshit [272] tested the cardioprotective effect of an aqueous whole fruit pomegranate extract against the cardiac toxicity of doxorubicin, an anthracycline antibiotic used in chemotherapy against several cancers (breast and bladder cancers, Kaposi's sarcoma lymphoma and acute lymphocytic leukemia). The authors verified the protective effect of pomegranate extract (dose: 100 mg/kg) in male Wistar albino rats against the cardio toxicity of Doxorubicin [272].

In another study, carried out by Mohan, Patankar, Ghadi, and Kasture [273], the cardioprotective effect of pomegranate juice extract (100 and 300 mg/kg) was tested against the side effects of isoproterenol (isoprenaline), a drug used for the treatment of bradycardia, heart block and asthma, in male Wistar albino rats. The extracts presented a protective action against the negative cardiac effects of the isoproterenol.

Houston, Bugert, Denyer, and Heard [274] assessed the anti-inflammatory activity of a pomegranate rind extract in ex vivo porcine skin. The authors proved that the pomegranate rind extract presented a significant anti-inflammatory effect on cyclooxygenase-2 (involved in the conversion of arachidonic acid to prostaglandin H2) expression [274].

Elzayat, Auda, Alanazi, and Al-Agamy [275] tested the healing effect of a pomegranate extract in Wistar male rats. Excision wounds were made in the rats, the pomegranate extract (10 mg/kg of body weight) was applied and the contraction of the wound were measured over 24 days and compared with a control group (rats with wounds, with the application of a base ointment). In the 4^{th} day, the wound with the pomegranate extract have contracted 41.8 ± 0.74%, superior to the contracted percentage of the control group (17.45 ± 1.51%). The authors also compared the healing effect of the pomegranate extract with henna (*Laurus nobilis* Linn.) extract, myrrh extract,

a blend of each extract and gentamycin ointment. At the 16th day, the pomegranate extract ointment presented a contraction percentage of 93.55 ± 0.58, surpassed only by the blend of the three extracts (97.30 ± 0.6%) and gentamycin ointment [275].

El-Elimat, Jarwan, Zayed, Alhusban, and Syouf [276] tested *in vitro* the anticancer activity of V. vinifera, vr. Golden Scatt, seed extract on PC3 human prostate cancer cells. The authors observed that the extract was able to inhibit the migratory potential of PC3 cells using a wound healing assay in three doses: 5, 10 and 20 μg/mL [276].

Badr El-Din, Ali, and Abou-El-Magd [277] tested the anticancer activity of grape seeds and skin extract against Ehrlich ascites carcinoma in Swiss albino mice. The extracts were applied in the mice standard diet (10%, w/w). On day 9 of the tumor cells inoculation, all the mice without the treatment of the grape seed and skin extract, exhibited palpable tumors (100% malignancy), in contrast with the mice that ate the grape extracts, which showed an inhibition in the growth of the tumors in 8 of 15 mice (53% malignancy). The other 7 mice were tumor free. Among the 8 mice that presented inhibition growth, 2 of the mice presented complete tumor regression on days 22 and 26. The study demonstrated the anticancer and anti-tumor in vivo potential of grape by-products extracts [277].

Zhang et al. [278] tested the anticarcinogenic Effect of grape seeds proanthocyanins in human colorectal carcinoma cells. The authors verified in vitro that the grape seeds proanthocyanins modulated the expression level and activation of genes and proteins that are involved in the mitochondrial apoptotic pathway, which supports the fact that the grape seeds proanthocyanins promote the colon cancer cells apoptosis (via the mitochondrial pathway) [278].

Hamza et al. [279] tested the anti-cancer property of a commercial grape seed extract against liver cancer in an *in vivo* assay with adult male Wistar rats. The rats were injected intraperitoneally with diethylnitrosamine (DEN, a single injection of 100 mg/kg of body weight), a common carcinogen found in tobacco, processed food and cosmetics, and 2-Acetil Aminofluorene (2-AAF, 30 mg/kg of body weight every week, for four weeks), a carcinogenic and mutagenic derivative of fluorene that can induces liver, bladder and kidney cancer. The grape seed extract was administrated orally in three doses: 25, 50 and 100 mg/kg. The authors also evaluated the in vivo antioxidant activity of grape seed extract for the oxidative stress markers myeloperoxidase, P.carbonyl, catalase and superoxide dismutase. In the antioxidant tests, the 50 and 100 mg/kg of the grape seed extract successfully inhibit the oxidative stress induced by DEN and 2-AAF. The grape seed extract was also able to inhibit the induced formation of pre-neoplastic

foci of altered hepatocytes (FAH), promote the cell proliferation and apoptotic cell death [279].

5. Possible toxicological effects for human health

Considering that plant extracts contain active compounds that are toxic against phytopathogens and insects, it is important to determine potential toxicological effects from human consumption and identify non-toxic doses. However, the literature available is limited. Research on pomegranate peel extracts administrated on animals identified different lethal doses (LD_{50}) uses: LD_{50} higher than 5 g/kg body weight in rats and mice for oral administration and LD_{50} of 217 mg/kg (rats) and 187 mg/kg (mice) for intraperitoneal administration [271].

On humans, the safety of a pomegranate dietary supplementation was determined after the administration of 1420 mg of ellagitannin enriched polyphenol pomegranate dry extract per day during 4 weeks in normal and obese individuals [280].

A toxicological evaluation of extracts from black grapes seeds and skins observed no acute toxic effects, in mice, and displayed oral LD_{50} values greater than 5000 mg/kg. A slight mutagenicity was also noticed at the 5 mg/plate dose (maximum tested) [281].

A recent clinical study was conducted to test the safety and tolerability of oral doses of grape seed extracts rich in proanthocyanidins. During 4 consecutive weeks, twenty-nine healthy adults were randomized into three groups and given doses of grape seed extracts (1000, 1500 or 2500 mg). Adverse events were registered and biological tests were done and the extract was considered generally safe and well tolerated when taken up to 2500 mg per day [282].

Despite the studies presented above, further research is necessary to define adequate doses for the application of these extracts on food products.

6. Conclusion and future perspectives

The content of fruits in bioactive compounds is considerable. They have been used since the beginning of human civilization to fight bacterial infections and diseases, because of their powerful biological activities such as antioxidant and antimicrobial capacities. Nowadays, in the major metropolitan areas, fruits are often consumed in the form of juices and pastes without seeds and peels that often have a greater content of these highly biological active compounds than the edible part of the fruits. The concern of consumers and food industry for the possible dangers to human health of synthetic food

additives has driven to the search for more natural and, at least, equally efficient additives. Being food waste, one of the major concerns of the modern world, fruits by-products are an 'eco-friendly' source of phenolic compounds that possess powerful antioxidant and antimicrobial activities which can be applied, not only to food products, but also to cosmetic and pharmaceutical formulations. Their extracts and essential oils can also be applied to active packaging for retarding food oxidation and inhibiting pathogenic microorganisms.

Pomegranate and grape by-products extracts are amongst the most powerful by-products extracts, presenting extremely high antioxidant and antimicrobial activities, showing a tremendous potential for their application in food products. Also, grape by-products are one of the most produced by-products in the world, making them very appealing for re-use comparing with other fruits by-products. With this extensive review, their potential for being used as additives was recognized. Although, more simple, 'greener' and economic extraction methods should be developed. Likewise, their biological activities should be compared with the capacities of the synthetic additives and their direct and indirect application to foods should be evaluated. Also, these natural extracts effects and possible toxicological effects on human health should be addressed by means of pre-clinical and clinical trials in order to assure the safety and well-being of the consumers.

Chapter III

Citrus by-products: valuable source of bioactive compounds for food applications

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1. Introduction

Citrus fruits are one of the most produced crops in the world. According to FAOSTAT data [283], its world production in 2020 was 158 490 986 tones, an increase of approximately 7.5 % compared to 2017, being oranges the most produced *Citrus* fruit (Table III.1). Around 30 million tons are used for juice production from this kind of citrus [284]. Juices, jams, concentrated formulas, pastes and other fruits formulations can easily originate tons of fruits by-products targeted to several cattle feed, bioethanol production or for the extraction of compounds with powerful antimicrobial and antioxidant activities. However, these by-products still originate tons of waste which, due to their high content in bioactive compounds, must be discarded in a responsible and eco-friendly way which, in consequence, can increase the final cost of the final product [184,285]. Every year, it is estimated that 15 million tons of Citrus by-products/waste are produced, worldwide [286].

Table III.1. Production of *Citrus* fruits in 2019, according to the Food and Agriculture Organization of the United Nations (FAO) [283].

Fruit	**Harvested area (ha)**	**Production (tonnes)**
Oranges [common, sweet orange (*Citrus sinensis*); bitter orange (*C. aurantium*)]	4 060 129	78 699 604
Tangerines, mandarins, clementines, satsumas [mandarin, tangerine (*Citrus reticulata*); clementine, satsuma (*C. unshiu*)]	2 756 887	35 444 080
Lemons and limes [lemon (*Citrus limon*); sour lime (*C. aurantifolia*); sweet lime (*C. limetta*)]	1 226 617	20 049 630
Citrus Fruits [Some minor varieties of citrus are used primarily in the preparation of perfumes and soft drinks, including bergamot (*Citrus bergamia*); citron (*C. medica* var. cedrata); chinotto (*C. myrtifolia*); kumquat (Fortunella japonica)]	1508639	14 496 484
Grapefruit (inc. pomelos) [*Citrus maxima*; *C. grandis*; *C. paradisi*]	346 191	9 289 462
Total	**9 898 463**	**157 979 260**

Citrus is a genus belonging to the Rutaceae family of trees and shrub, including oranges, lemons, grapefruits, tangerines, and limes [287]. The origin of the *Citrus* genus is a topic of debate in the scientific community since some scientists defend that the origin of the genus was in Southeast Asia and other scientists in Australia [288].

Citrus fruits by-products can be divided into peels (flavedo and albedo), seeds and pulp residue. The flavedo is the outside colorful part of the peel that contains the oil sacs and, the albedo is the white interior part of the peel, rich in pectin [288]. Peels are also rich in sugars and have a high concentration of *D*-limonene, a powerful antimicrobial compound [284,289,290]. Pulp residue is constituted by the segment wall or membranes that contain the juice [291]. Seeds are mainly composed of nitrogen-free extract, lipids, crude protein, and fiber [291]. These by-products have several active compounds with powerful bioactive activities that have a very important role in the food industry, such as antioxidant and antimicrobial capacities.

As well as every other fruit, the chemical composition of *Citrus* fruits and their by-products, varies with the edaphoclimatic conditions that the plant is exposed to. These fruits are known for their high content in vitamin C and carotenoids with pro-vitamin A, namely β-cryptoxanthin (Table III.2) [292,293]. Carotenoids are natural compounds responsible for the yellow, orange, and red colors in fruits and plants, helping the plants in photosynthesis and defense against light oxidation [189,294]. These compounds are very important for human health, acting against carcinogenesis, preventing cardiovascular and degenerative diseases, so their presence is of utmost importance in the human diet [295]. Regarding vitamin C, *Citrus* fruits can contribute to vitamin intake with 40 % of the Dietary Reference Intake (DRI) [296].

Consumers' demand for more 'natural' and high-quality products is increasing, representing an important challenge for the food industry. Generally, the food industries resort to synthetic food additives, which are economically viable, chemically stable, and easily applied, to improve or maintain foods' quality and prolong their shelf-life. However, there has been an increasing concern about the use and direct application of some of these compounds since they have been associated with allergic reactions, promotion of carcinogenesis and the appearance of neurodegenerative diseases [29,177,200,285].

In this line, the main purpose of this review is to critically discuss the potential of *Citrus* by-products based on their bioactive compounds and their applicability to foods as direct or indirect additives.

Table III.2. Chromatographic techniques for the determination and quantification of some bioactive compounds found in *Citrus* by-products extracts.

Species/ Variety	Common/ Local name	Main bioactive compounds and levels found	Chromatographic technique/ Apparatus	Chromatographic method	Ref.
Peel from *Citrus microcarpa*	Kumquat	Quercetin (0.78 ± 0.003 mg/g, db) β-cryptoxanthin (37.0 ± 1.45 μg/g, db) Lutein (36.4 ± 1.56 μg/g, db) Zeaxanthin (36.4 ± 1.57 μg/g, db) Caffeic acid (17.3 ± 1.57 μg/g, db) β-carotene (2.79 ± 0.14 μg/g, db) Diosmin (0.40 ± 0.01 mg/g, db)	Reversed phase HPLC with UV detector. Column: LiChrospher®100 RP18e, 5 μm, 4.0 mm internal diameter x 250 mm	**Flavonoids**: MPA: 2 % acetic acid (aqueous) MPB: 0.5 % acetic acid (aqueous)-acetonitrile (v/v; 50:50) Flow rate: 1 ml/min. Gradient: Time (min) / % MPA / % MPB 0 / 95 / 5 10 / 90 / 10 40 / 60 / 40 55 / 45 / 55 60 / 20 / 80 65 / 0 / 100	[297]
Peel from *Citrus reticulata* x *Citrus sinensis*	Murcott	Chlorogenic acid (339 ± 4.01 μg/g, db) Sinapic acid (178 ± 5.62 μg/g, db) Zeaxanthin (25.2 ± 0.99 μg/g, db) β-cryptoxanthin (16.9 ± 0.75 μg/g, db) Lutein (13.3 ± 0.51 μg/g, db) β-carotene (12.1 ± 0.51 μg/g, db) Naringin (23.9 ± 0.32 mg/g, db) Hesperidin (20.7 ± 0.38 mg/g, db)			
Peel from *Citrus sinensis* (L.) Osbeck	Liucheng	Sinensetin (0.42 ± 0.01, mg/g, db) β-carotene (50.2 ± 2.28 μg/g, db) Lutein (29.3 ± 1.17 μg/g, db) Zeaxanthin (27.7 ± 1.21 μg/g, db) β-cryptoxanthin (0.76 ± 0.04 μg/g, db)			
Peel from *Citrus grandis* Osbeck CV	Peiyou	Naringin (29.8 ± 0.20 mg/g, db) Caffeic acid (27.5 ± 1.74 μg/g, db)			
Peel from *Citrus tankan* Hayata	Tonkan	Hesperidin (23.4 ± 0.25 mg/g, db) β-carotene (36.9 ± 1.38 μg/g, db) Zeaxanthin (11.6 ± 0.58 μg/g, db)			

<table>
<tr><th>Species/ Variety</th><th>Common/ Local name</th><th>Main bioactive compounds and levels found</th><th>Chromatographic technique/ Apparatus</th><th>Chromatographic method</th><th>Ref.</th></tr>
<tr><td>Peel from Citrus reticulata Blanco</td><td>Ponkan</td><td>Hesperidin (29.5 ± 0.32 mg/g, db)
Kaempferol (0.38 ± 0.002 mg/g, db)
Rutin (0.29 ± 0.004 mg/g, db)
Luteolin (0.21 ± 0.01 mg/g, db)
p-Coumaric acid (346 ± 2.45 μg/g, db)
Ferulic acid (150 ± 4.89 μg/g, db)
β-carotene (69.2 ± 2.67 μg/g, db)
β-cryptoxanthin (30.5 ± 1.26 μg/g, db)</td><td></td><td>Carotenoids:
MPA: Acetonitrile
MPB: Methanol
MPC: Dichloromethane
All mobile phases contained 0.1 % BHT, 0.1 % triethylamine and 0.005 M ammonium acetate (in methanol).
Gradient:<table><tr><th>Flow (mL/min)</th><th>Time (min)</th><th>% MPA</th><th>% MPB</th><th>% MPC</th></tr><tr><td>0.6</td><td>0</td><td>100</td><td>0</td><td>0</td></tr><tr><td>1.0</td><td>25</td><td>100</td><td>0</td><td>0</td></tr><tr><td>1.0</td><td>30</td><td>60</td><td>35</td><td>5</td></tr><tr><td>1</td><td>80</td><td>60</td><td>35</td><td>5</td></tr></table></td><td></td></tr>
<tr><td>Peel from Citrus limon (L.) Bur</td><td>Lemon</td><td>Rutin (0.29 ± 0.002 mg/g, db)
Caffeic acid (80.0 ± 3.72 μg/g, db)
β-carotene (10.3 ± 0.47 μg/g, db)
Lutein (2.95 ± 0.12 μg/g, db)
Zeaxanthin (0.81 ± 0.04 μg/g, db)
β-cryptoxanthin (0.81 ± 0.04 μg/g, db)</td><td></td><td></td><td></td></tr>
<tr><td>Citrus sinensis peels</td><td>Sweet orange</td><td>Naringin (3.1 mg/100 g FW)
Hesperidin (4.6 mg/100 g FW)</td><td>HPLC-PDA
Column: reversed-phase column Microsorb-MV 100-5 C18 (25×0.46 cm; 5 μm particle size) with a precolumn (5×0.46 cm; 5 μm particle size) of the same material</td><td>Column temperature: 40 °C
MPA: water-formic acid solution (95:5)
MPB: ACN
Injection vol: 10 μL
Flow rate: 1 mL/min
Gradient:<table><tr><th>Time (min)</th><th>% MPA</th><th>%MPB</th></tr><tr><td>0</td><td>98</td><td>2</td></tr><tr><td>25</td><td>94</td><td>6</td></tr><tr><td>40</td><td>85</td><td>15</td></tr><tr><td>52</td><td>80</td><td>20</td></tr><tr><td>70</td><td>60</td><td>40</td></tr></table></td><td>[298]</td></tr>
</table>

<table>
<tr><th>Species/ Variety</th><th>Common/ Local name</th><th>Main bioactive compounds and levels found</th><th>Chromatographic technique/ Apparatus</th><th>Chromatographic method</th><th>Ref.</th></tr>
<tr><td>Juice by-products from Citrus latifolia and four cultivars from Citrus sinensis</td><td>-</td><td>Hesperidin (232.65 ± 10.47 mg/100 g DM)
Narirutin (29.34 ± 0.43 mg/100 g DM)
Ellagic acid (10.97 ± 0.08 mg/100 g DM)
Tangeretin (1.41 ± 0.04 mg/100 g DM)
Hesperetin (1.05 ± 0.04 mg/100 g DM)
Naringin (1.02 ± 0.05 mg/100 g DM)</td><td>HPLC-DAD
Column: C-18 Acclaim 120 column (Dionex, 3 µm, 4.6 x 150 mm)</td><td>Column temperature: 30 °C
MPA: 0.1 % of formic acid in water
MPB: 0.1 % of formic acid in methanol
Flow rate: 0.6 mL/min
Gradient:<table><tr><td>Time (min)</td><td>%MPA</td><td>%MPB</td></tr><tr><td>0</td><td>90</td><td>10</td></tr><tr><td>5</td><td>90</td><td>10</td></tr><tr><td>80</td><td>20</td><td>80</td></tr><tr><td>85</td><td>90</td><td>00</td></tr></table></td><td>[299]</td></tr>
<tr><td>Citrus fruits juice by-products (non-specified)</td><td>Citrus fruits juice by-products (non-specified)</td><td>Narirutin (9.6 ± 1.5 mg/100 g DM)
Hesperidin (99.7 ± 7.4 g/100 g DM)
Naringenin (22.6 ± 0.6 g/100 g DM)
Hesperetin (80.8 ± 13.7 g/100 g DM)
Tangeritin (1.7 ± 0.2 g/100 DM)
Narirutin (8.1 ± 0.9 g/100 g DM)
Hesperidin (88.3 ± 5.8 g/100 g DM)
Naringenin (21.1 ± 2.6 g/100 g DM)
Hesperetin (82.5 ± 11.9 g/100 g DM)
Tangeritin (1.6 ± 0.1 g/100 g DM)
Narirutin (50.9 ± 4.5 mg/100 g DM)
Hesperidin (228.9 ± 7.0 mg/100 g DM)
Tangeritin (1.1 ± 0.1 mg/100 g DM)
Narirutin (27.1 ± 0.2 mg/100 g DM)
Hesperidin (117.3 ± 1.6 mg/100 g DM)
Naringenin (19.5 ± 0.6 mg/100 g DM)
Hesperetin (51.8 ± 2.1 mg/100 g DM)
Tangeritin (1.3 ± 0.1 mg/100 g DM)</td><td>HPLC with an Dionex UltiMate 3000 chromatography system
Column: C18 Acclaim® 120 column (Dionex, 3 µm, 4.6×150 mm)</td><td>Column temperature: 30 °C
Detector: UV/VIS detector (DAD-3000)
Wavelength: 280 nm
MPA: water-formic acid (99.9:0.1, v/v)
MPB: methanol-formic acid (99.9:0.1, v/v)
Flow rate: 0.6 mL/min</td><td>[300]</td></tr>
</table>

<table>
<tr><th>Species/ Variety</th><th>Common/ Local name</th><th>Main bioactive compounds and levels found</th><th>Chromatographic technique/ Apparatus</th><th>Chromatographic method</th><th>Ref.</th></tr>
<tr><td>Peels from lemon</td><td>-</td><td>Hesperidin (84.44 ± 8.35 mg/100 g FW)
Eriocitrin (176.35 ± 15.39 mg/100 g FW)</td><td>HPLC with a diode-array detector
Column: C18 reverse phase column</td><td>Column temperature: 40 °C
MPA: Acidified bidistillated water (0.1% of glacial acetic acid)
MPB: Acidified acetonitrile (0.1% of glacial acetic acid)
Flow rate: 0.5 mL/min
Gradient:<table><tr><td>Time (min)</td><td>%MPA</td><td>%MPB</td></tr><tr><td>0</td><td>90</td><td>10</td></tr><tr><td>2</td><td>90</td><td>10</td></tr><tr><td>12</td><td>70</td><td>30</td></tr><tr><td>17</td><td>90</td><td>10</td></tr></table></td><td>[301]</td></tr>
<tr><td>Peel from Citrus limon</td><td>Algerian oranges</td><td>Limonene (94.427 %)
b-Myrcene (2.158 %)
Linalool (0.293 %)
Valencene (0.165 %)
Octanal (0.435 %)</td><td>GC/MS coupled with mass spectrometry (HP 6890 (II) interfaced with a HP 5973 mass spectrometer)
Column: capillary column RTX-5MS (30 m, ID 0.25 mm, film thickness 0.25 lm)</td><td>Carrier gas: Helium
Flow rate: 1 mL/min
Temperature ramp: 40 °C for 8 min; increased to 180 °C at 3 °C/min; increased to 230 °C at 20 °C/min</td><td>[302]</td></tr>
<tr><td>Citrus reticulata peels</td><td>Mandarin</td><td>Narirutin (2044.46 ± 55.48 µg/g of Sample)
Hesperidin (1346.44 ± 67.78 µg/g of Sample)
Nobiletin (218.02 ± 7.29 µg/g of Sample)
Rutin (214.50 ± 9.28 µg/g of Sample)
Taxifolin (134.36 ± 3.71 µg/g of Sample)
Sinensetin (113.82 ± 4.73 µg/g of Sample)</td><td>HPLC coupled with DAD
Column: Agilent Eclipse XDB-C18 (4.6 × 250 mm, 5 µm) reverse phase column</td><td>Column temperature: 40 °C
Injection volume: 10 µL
MPA: Acidified water (0.5 % of formic acid)
MPB: Acetonitrile
Flow rate: 0.5 mL/min
Gradient:<table><tr><td>Time (min)</td><td>%MPA</td><td>%MPB</td></tr><tr><td>0</td><td>90</td><td>10</td></tr><tr><td>30</td><td>75</td><td>25</td></tr><tr><td>40</td><td>30</td><td>70</td></tr></table></td><td>[303]</td></tr>
</table>

Species/ Variety	Common/ Local name	Main bioactive compounds and levels found	Chromatographic technique/ Apparatus	Chromatographic method			Ref.
				50	10	90	
				52	10	90	
				54	90	10	
Crude orange juice by-products extract	Orange	Hesperidin (25.62 ± 0.44 mg/g LE) Narirutin (3.96 ± 0.10 mg/g LE)	HPLC coupled with DAD Column: Acclaim® 120 C18 column (Dionex, 3 μm, 4.6 x 150 mm)	Column temperature: 30 °C MPA: Acidified water (0.1 % of formic acid) MPB: Acidified methanol (0.1 % of formic acid) Flow rate: 0.6 mL/min			[304]
Enzyme-treated orange juice by-products extract	Orange	Hesperetin (22.02 ± 0.48 mg/g LE) Hesperidin (6.08 ± 0.09 mg/g LE) Naringenin (2.23 ± 0.01 mg/g LE)					
Crude orange pectin by-products extract	Orange	Hesperidin (81.17 ± 3.01 mg/g LE) Narirutin (6.73 ± 0.21 mg/g LE) Tangerintin (1.28 ± 0.08 mg/g LE)					
Enzyme-treated orange pectin by-products extract	Orange	Hesperetin (43.70 ± 0.79 mg/g LE) Hesperidin (11.11 ± 0.39 mg/g LE) Naringenin (3.49 ± 0.10 mg/g LE)					

2. Active compounds of *Citrus* by-products

2.1. Bioactive compounds extraction

As stated before, the chemical composition of *Citrus* fruits and its by-products are directly dependent of the edaphoclimatic conditions to which the plant is exposed. Additionally, the chemical composition varies, varies mainly according to the species and cultivar. However, in terms of its essential oils (EOs) and extracts, the extraction protocol directly influences the content of the active compounds and the yield. The most common extraction method to obtain EOs and extracts are hydro and steam distillation, solvent extraction, and cold pressing [305–307]. "Greener" and recent extraction techniques, such as microwave extraction, ultrasound extraction and supercritical fluid extraction, have emerged as a more sustainable alternative to more traditional techniques since they require less use of energy and solvent(s) [308,309]. The application of *Citrus* EOs is extensive and transversal to several industries, including the food, cosmetic and pharmaceutical industries [310,311].

Steam distillation is the most used method to obtain EOs from plants. Generally, using this method for EOs extraction, around 93% yield is obtained. Briefly, the application of heat in the form of steam is responsible for the breakdown of the cell structure of the plant material and consequent release of the essential oil [312–315]. Sikdar & Baruah [316] compared the extracted essential oils from orange, sweet lime, and lemon peels obtained with steam distillation varying the applied temperature and extraction time. For all the conditions, essential oil from orange peels presented the highest yield, followed by sweet lime essential oil. The authors reach to the optimal conditions of 96 °C for a period of 60 min [316]. Hydrodistillation is often used to extract of essential oils from flowers and wood. This technique consists of the complete immersion of the plant material in water, followed by heating the mixture until boiling and the condensation of the steam and essential oil vapor to an aqueous phase. The water protects the oil from overheating, acting as a barrier [315]. Although relatively economical and easy-to-apply technique, the extraction by distillation processes presents some disadvantages such as low efficiency, loss of volatile compounds, long extraction times, and degradation of unsaturated or ester compounds as a result of the use of high temperatures [315].

Solvent extraction is a conventional technique used mostly for extracting compounds from fragile parts of plants, such as flowers. Usually, contrary to distillation techniques, it does not resort to high temperatures, protecting the active compounds that are thermo-sensitive. Several solvents can be used in this extraction, such as hexane,

ethanol, methanol, and acetone. However, the solvent' choice is directly dependent on the final use of the extract or essential oil, since it is possible that the toxic solvent may be present in the extract or essential oil [315]. The solvents and the authorized additives to be directly or indirectly used are specified in the European Commission Regulation No 231/2012 and its amendments [317].

To overcome some limitations of the solvent extraction techniques, researchers started to apply supercritical fluids as solvents in this technique. A supercritical fluid is any substance at a pressure and temperature above its end point of a phase equilibrium curve (critical point), below the pressure required to compress it into a solid, where there is no distinction between the gas and liquid phase [318]. The extraction by supercritical fluids presents a higher efficiency and a lower loss of volatile compounds than the previous extraction methods. Supercritical carbon dioxide is one of the most used fluids in this type of extraction. Besides being eco-friendly, the use of carbon dioxide allows the extraction process to occur at relatively low temperatures since its critical temperature is 31 °C, as well as its easy application, since at high-pressure conditions it presents a liquid form [315,319]. However, this extraction technique using carbon dioxide has disadvantages, due to its non-polar properties. Although, this can be compensated by adding other solvents such as ethanol, methanol, and water [319–321]. Menichini et al. [322] compared the essential oil extracted from *Citrus medica* L. cv. Diamante peels by three different methods: hydrodistillation, supercritical CO2 and cold pressing. Limonene was the major compound found in the essential oils extracted by hydrodistillation and cold pressing followed by γ-Terpinene, while in the essential oil obtained with the supercritical CO_2 extraction, the major compound was citropen (84.5 %), followed by 2,3-Dihydrobenzofuran (2.9 %) [322]. Also, the authors found that the essential oil obtained by supercritical CO2 presented no anti-inflammatory activity, while the essential oils obtained by hydrodistillation and cold-pressing presented anti-inflammatory activity [322]. Sicari & Poiana [323] compared the EOs extracted through hydrodistillation, solvent extraction by Soxhlet with pentane and supercritical CO_2 extraction from kumquat (*Fortunella margarita* Swingle) peels. All three essential oils presented almost the same content in limonene (around 96 %) and their chemical composition was not significantly different. However, the EO obtained with supercritical CO_2 presented a slightly higher content in esters and sesquiterpenes, which improved the essential oil aroma [323].

Having emerged in the 20th century, microwave extraction, or microwave-assisted extraction (MAE), is one of the most applied extraction techniques. Microwaves,

located between the higher infrared frequencies and the lower radio frequencies, are non-ionizing electromagnetic waves [324]. In the MAE, microwaves act as energy vectors which, when applied to a certain material it will absorbs the electromagnetic energy and transforms it into heat [325,326]. The transformation of electromagnetic energy into heat relies on two mechanisms, that can occur simultaneously in both the sample and the solvent: the ionic conduction and the dipole rotation [325,327]. This guarantees that the system heating takes place at the same time, meaning, that the heating of both the solvent and the solid matrix occurs at the same time, unlike other extraction techniques where the heating occurs from the outside to the inside of the matrix and the mass transference occurs from the inside to the outside [327]. When compared with the more conventional/traditional extraction techniques, MAE presents several advantages such as, the use of lower quantities of solvent and lower human exposure to the used solvent, significant reduction in the extraction time, higher selectivity of the extracted compounds and the possibility of a solvent-free extraction [325,327–329]. However, not all are advantages regarding MAE. Method optimization is one of them. Several parameters must be considered when implementing/developing an MAE method, such as applied power, extraction time, solvent:matrix ratio and matrix composition [48]. The choosing of the solvent is particularly important. Although both polar and non-polar solvents can be used, the choice must consider the solvent' dielectric properties: a low dissipation factor translates into less dissipated heat, originated by the absorption of the microwave energy [325,327]. For instance, the water has a very low dissipation factor, which can lead to superheating that for the extraction of some thermo-sensitive compounds is not advised [325]. Ferhat et al. [330] compared the extraction of EOs from fresh lemon (*Citrus limon* L.) peels by microwave accelerated distillation (or microwave 'dry' distillation) with the conventional techniques of cold pressing and hydrodistillation. The microwave extraction resulted in a higher yield with a lower extraction time period. Also, the oxygenated fraction in the OE extracted with microwaves was 10 % higher than the essential oil extracted with hydrodistillation and 40 % higher than the OE extracted by cold pressing [330]. Bustamante et al. [331] also compared MAE of EOs from orange peels with hydrodistillation extraction, stating that MAE EO possessed slightly higher quantities of monoterpenes (0.78% higher), including *D*-Limonene, α-pinene, β-pinene and γ-Terpinene [331].

Usually applied to liquid and semi-solid foods, Pulsed Electric Field (PEF) extraction is one of the most recent extraction techniques applied in the food industry. Usually applied to liquid and semi-solid foods, consists in applying short pulses, micro-

or milliseconds, of high voltage between 10 to 80 kV/cm, to the food placed between two electrodes [332,333]. The application of short high voltage pulses increases the cell membrane conductivity and permeability due to the incensement of the transmembrane potential [333,334]. PEF is largely applied in the food industry to assure food microbiological safety since it has the advantage of inactivating pathogenic microorganisms without having to apply high temperatures, maintaining the original sensorial (texture, flavor, color) and nutritional value of unprocessed foods [332]. Coupled with other extraction techniques, such as solvent extraction, PEF can be used as a tool to improve the extraction or recovery of valuable compounds, such as phytochemicals. For instance, Hwang et al. [335] applied PEF to subcritical water extraction in *Citrus unshiu* peels improving the hesperidin content from 38.45 mg/g to 46.96 mg/g. Also, Kantar et al. [336] applied PEF in the extraction of polyphenols with ethanol extraction of from orange pomelo and lemon. The authors found that the application of the PEF treatment increased the polyphenol content of ethanolic extracts by 50%. In addition, it can also increase the efficiency of juice extraction and increase the yield, from fruits by-products and plants, of bioactive compounds, extracts and essential oils [333,334]. Luengo et al. [298] used PEF applying 1, 3, 5 and 7 kV/cm to sweet orange (*C. sinensis*) peels, increasing the orange peels' antioxidant capacity extract by 51%, 94%, 148% and 192%, respectively. The authors also concluded that the total polyphenol extraction yield increased by 20%, 129%, 153% and 159% for the respective applied high voltages and, for the extract obtained with the 5 kV/cm, the content of naringin from 1 to 3.1 mg/100 g of fresh weight (FW) of peel and hesperidin from 1.3 to 4.6 mg/100 g FW of peel [298]. In another study led by El Kantar et al. [336], PEF was applied to orange, pomelo and lemon fruits in aqueous media at 3 kV/cm. The authors found that the applied current increased the juice yield by 25 % for oranges, 37 % for the pomelo and 59 % for lemon [336]. In a more recent study, led by Peiró et al. [301], an electric field of 7 kV/cm was applied to lemon peels, which increased the polyphenol extraction by 300 %, with astonishing contents of hesperidin (84 mg/100 g FW) and eriocitrin (176 mg/100 FW).

2.2. Active compounds of *Citrus* fruits by-products

Citrus fruits and their by-products present a large spectrum of phytochemical compounds. Phenolic compounds (namely flavonoids), terpenoids, carotenoids, vitamins, fatty acids, and aromatic compounds are among them [287,289,337]. Some of the active compounds that can be found in extracts and essential oils from *Citrus* fruits by-products

can be observed in Table III.2, as well as the chromatographic methods used for their determination.

Beyond the edaphoclimatic conditions, the composition of the extracts and EOs obtained from *Citrus* by-products is also dependent on the processing of the fruit itself (for example, to obtain the juice) and the extraction method applied to the by-products (temperature conditions, solvent, time of the extraction, among others) [337].

Peels are the major by-product of *Citrus* fruits' industrial processing and are responsible for most of the commercialized *Citrus* EOs. The oils composition may vary but, approximately, 90 % is composed by *D*-limonene [289]. Linalool, β-myrcene and α-pinene can also be found in high amounts [286,302,338,339]. Flavonoids are another class of compounds that can be easily found in Citrus fruits by-products, being neoeriocitrin, neohesperidin and naringin the main flavanones in lemon (*Citrus limon*), orange (*Citrus aurantium*) and bergamot (*Citrus bergamia* Fantastico) peels [297].

Citrus fruits are rich in carotenoids, specially α-carotene, β-carotene, lutein, zeaxanthin and β-cryptoxanthin [340]. Carotenoids are well-known for their antioxidant activity, and their moderated consumption is related to the reduction of the incidence of cancer, arteriosclerosis and arthritis and the promotion of immune functions and 0.1 to 0.5 % of the dry weight of *Citrus* peels is composed by carotenoids [64,65]. β-cryptoxanthin can be easily found in orange, tangerines and mandarins, representing a very important role in human nutrition since it has a powerful antioxidant capacity and provitamin A activity [341].

Limonene (*D*-limonene, *L*-limonene) is the major compound present in *Citrus* by-products extracts and EOs. Is a monocyclic monoterpene with low toxicity (oral LD_{50} values 5-6 g/kg), registered as Generally Recognized as Safe (GRAS) in the Code of Federal Regulations (CFR) for its use as synthetic flavoring [342,343]. Limonene is widely used in the pharmaceutical, food, and cosmetic industries as a fragrance in perfumes, soaps and in household cleaning products. Also, it can be found in some pesticides and insect repellents [344]. Limonene has several clinical applications and is recognized for its anticancer, anti-asthmatic, and anti-microbial activities [342,343]. Due to its ability to dissolve cholesterol, *D*-limonene has been clinically used to dissolve gallstones containing cholesterol. It is also used to neutralize heartburn due to its action on gastric acid [343,344].

Although not considered an active compound or a phytochemical, pectin is a major compound in *Citrus* peels, representing, generally, 20-30 % of the dry weight of peels [340]. Present in the peels (flavedo and albedo), central column and juice sac of

Citrus fruits, limes, lemons, grapefruits, and oranges are the fruits with a higher pectin content. It is highly used in the food industry as a thickener and stabilizer for jams and juices [340]. Pectin enhances gastric motility and nutrient absorption, and has shown preventative and therapeutical effects on cancer, diabetes, high blood pressure and obesity [345]. The pharmaceutical industry uses pectin in the production of plasma and hemostatic agents and laxatives [340]. Barbosa et al. [299] extracted polyphenols from industrial *Citrus* juice by-products (*Citrus latifolia* and four cultivars from *Citrus sinensis*) and the remaining residue from the pectin extraction of those by-products. The authors found that, in total, the extract from the juice by-products presented a lower content on polyphenols than the extract from the pectin by-products. Extract from the pectin by-products presented a higher content in hesperidin (314.44 mg/100 g DM vs 232.65 mg/100 g DM), naringin (3.11 mg/100 g DM vs 1.02 mg/100 g DM) and tangeretin (6.07 mg/100 g DM vs 1.41 mg/100 g DM). However, the extract from the juice by-products obtained a higher content in narirutin (29.34 mg/100 g DM vs 17.50 mg/100 g DM) and in ellagic acid (10.97 mg/100 g DM vs 0.27 mg/100 g DM). Also, the authors found that the antioxidant activity of the juice by-products extract was higher than the antioxidant activity of the pectin by-products extract [299].

2.3. Biological activity of *Citrus* fruits by-products

As described earlier, *Citrus* fruits and their by-products are known for their health benefits. These benefits are due to their biological activities, like antioxidant, anticarcinogenic, anti-tumor, antimicrobial, anti-inflammatory properties. EOs, the main product of the extraction of *Citrus* peels, have powerful biological activities, specially the antimicrobial potential [307]. *Citrus* EOs are used for their germicidal, antioxidant and anticarcinogenic properties [305].

In the study led by Han et al. [346], 57 participants were exposed to bergamot essential oil, which improved the participants' positive feelings by 17 % compared with the control group. Another study, led by Matsumoto et al. [347], proved that the Japanese citrus fruit yuzu (*Citrus junos* Tanaka) EO has an anti-stress effect and eases premenstrual emotional symptoms, namely tension–anxiety, anger–hostility, and fatigue—common. Mazloomi et al. [348] concluded that orange seed protein concentrate could reduce blood pressure and help diabetes management. Menezes Barbosa et al. [349] proved the antimicrobial activity of *Citrus* by-products from juice and pectin extraction of *Citrus latifolia* and four cultivars of *Citrus sinensis* ('Hamlin', 'Valência', 'Pêra Rio', and 'Pêra Natal'), against *Bacillus cereus*, *Staphylococcus aureus*,

Listeria monocytogenes, *Escherichia coli*, and *Salmonella Typhimurium*. Ruviaro et al. [304] research showed the vasorelaxation potential of hesperetin, a flavanone common present in *Citrus* by-product extracts. The authors also improved the extraction of this compound by resorting to enzyme-assisted extraction of pectin *Citrus* by-products (Table III.2). Yue Liu et al. [303] observed the antifungal activity of *Citrus reticulata* (Mandarin) peel ethanolic extract against *Aspergillus flavus*, a major producer of aflatoxins which present a serious health risk.

3. Direct application of *Citrus* by-products, their EOs and extracts to foods

3.1. Prolonging foods' shelf-life

Due to their high nutrient and phytochemical content, *Citrus* by-products are used in several ways, for different and distinct purposes. Lately, studies have been made to evaluate the benefits of application of *Citrus* by-products and their extracts and EOs to foods [350–352].

Microbial growth in foods is one of the major concerns in the food industry. Usually, the manufacturers resort to several antimicrobial compounds to inhibit those microorganisms and prolong foods' shelf-life. These additives are regulated in the European Union through the Regulation No 1333/2008 and all of its amendments [353]. Benzoic acid and its derivatives (E210-219, E928, and E1519), nitrates and nitrites (E240-E259), sorbic acid and its derivatives (E200 and E202) are the most common used antimicrobial additives. However, their safety for human consumption has been brought to question, so finding their safe substitutes has become a priority [200,257,362–364,354–361]. Lipid oxidation is, also, one of the major concerns of the food industry, being one of the major causes of food spoilage [365,366]. Antioxidant compounds can be used to stop or delay this chemical process, prolonging foods' shelf-life and preventing the occurrence of off-flavors.

Fernández-López et al. [367] studied the ability of lemon and orange extracts to inhibit the bacterial growth and to extend the storage shelf-life of cooked meatballs, as well as their antioxidant activity. Although both extracts showed significantly low malonaldehyde (MDA) values, indicating antioxidant activity, meatballs with orange extracts showed lower MDA values than those with lemon extracts at the end of 12 days of storage. Also, during the storage time, in the meatballs with *Citrus* extracts, lactic acid bacteria were not detected, which suggested that the extracts could be more

effective to control lactic acid bacteria growth during storage time [367]. This could be due to the high fiber content extracts, which have high water absorption, and, consequently, reduce microbial growth [367–369]. Devatkal, Narsaiah, & Borah [370] and Devatkal & Naveena [371] studied the antioxidant properties of kinnow rind powder extracts. They used it as a natural antioxidant instead of a synthetic one, in goat meat. In both cases, the use of the extract was successful as the lipid oxidation was significantly reduced, during refrigerated storage. This was corroborated by the low MDA values after refrigerated storage [370,371].

Spinelli et al. [372] enriched the nutritional quality of fish burgers with micro-encapsulated extract from orange epicarp and then evaluate the bio-accessibility of phenolic, flavonoid and carotenoid compounds. They observe an increase in bio-accessibility of the bioactive compounds, concluding that enrich fish burgers with micro-encapsulated extract from orange epicarp is beneficial since it increases the quality of the food [372].

Bambeni et al. [373] applied an extract obtained from orange (*C. reticulata*) pomace to beef patties and compared the lipid oxidation and the microbial growth with beef patties with no treatment and with beef patties treated with synthetic additive sodium metabisulphite (SMB). Although the beef patties with SMB presented lower MDA and an inferior microbiological growth than the patties treated with the orange extract, SMB contain sulphites which have been associated with the occurrence of asthma and allergic responses [373–375]. However, it is noteworthy that the orange extract presented lower MDA content and lower microbiological growth than the control patties, showing an antioxidant and antimicrobial effect [373].

Tayengwa et al. [376] feed Angus steers with dried *Citrus* pulp, consisted of comprised seeds, pulp and peels. The authors found that the α-tocopherol content of the Angus feed with the citrus pulp was tree times higher than the control group and the MDA content of the beef was also significantly lower than the control group. Regarding the antimicrobial analysis, the group fed with the citrus pulp showed a reduction of coliforms than the control group [376]. This study shows the importance of the active compounds biological activities and reinforces the use of *Citrus* by-products in the animal and food industry, reinforcing the existence of a circular economy and waste management regarding the fruit industry. Following this line of though, Wu et al. [377] investigated the antifungal potential of golden finger citron (*Citrus medica* L. var. sarcodactylis) flowers, fruits, and leaves EO in Chinese steamed bread. The EO obtained from the leaves prolonged the Chinese steam bread shelf-life for longer periods (11 to

13 days) than the EOs from the flowers (4 to 5 days) and fruits (3 to 5 days). The authors also observed a significantly higher antifungal activity of the leave EO than the antifungal activity of the synthetic preservative potassium sorbate [377].

Shehata et al. [103] evaluated the potential of an orange peel extract in inhibiting the lipid oxidation of vegetable oil and compared to the synthetic additive butylated hydroxytoluene (BHT). The authors found that the vegetable oil with the orange peel extract presented lower peroxide values than the control and the oil with BHT, showing that the orange peel extract is more effective against the oils' lipid oxidation than the synthetic additive [378]. Nishad et al. [379] incorporated grounded goat meat with a citrus peel extract and evaluated its lipid oxidation after three and six months of storage. The authors found that the meat with the citrus peel extract exhibit significantly lower MDA values and peroxide values than the control meat [379].

3.2. Foods' quality improvement through *Citrus* by-products

This section indicates the quality improvement of foods by using *Citrus* phytochemicals, such as texture and color. Additionally, to the phytochemicals with powerful biological activities, *Citrus* by-products are also a great source of dietary fiber. This dietary fiber is preferable to the other sources, such as cereals, due to their high content in bioactive compounds [368,369,380]. Dietary fiber, known for its water and fat-biding properties, is widely used in meat and meat products to improve cooking yield and texture [287,306,337,368,369,380,381]. Fernandez-Gines et al. [381] studied the influence of the addition of *Citrus* by-products fiber and the storage condition of bologna sausage. They manufactured the bologna sausage with different concentration of citrus fiber (0.5, 1, 1.5 and 2%). The bologna sausages with added *Citrus* fiber showed a significant decrease in residual nitrite level. The addition of *Citrus* fiber, significantly, altered the color parameters and the textural characteristics. Specifically, lightness values were increased on the sausage with *Citrus* fiber, but no differences were found between fiber concentrations. On the other hand, aspect, saltiness, fatness, residual taste, and pH levels were not significantly affected by *Citrus* fiber addition. Also, no microbial growth was observed in the sausage with *Citrus* fiber. MDA values were higher in the sausage stored under lighting conditions, when compared with those stored under darkness, for all citrus fiber concentrations [381]. Fernández-López et al. [382] also studied the benefits of fiber by incorporating *Citrus* by-products, specifically lemon albedo, and orange dietary fiber powder, into cooked and dry-cured sausage. The study concluded that, in both cases, the nitrite levels produced were significantly lower. Furthermore, the color

parameters were altered and TBA values were higher in the sausage stored under lighting conditions than those stored under darkness [382].

Pectin is a very common dietary fiber obtained from the *Citrus* peel. Pectin is mostly used as thickener, stabilizer, and emulsifier. Thus, it is used to produce jams, jellies, marmalade, fruit juice, confectionary products, and bakery fillings. It is also used for the stabilization of acidified milk drinks and yogurts [306,337].

There are several examples of the direct application of *Citrus* by-products EOs and extracts and their antimicrobial and antioxidant potential. However, similar to the concerns with the synthetic additives, the safety ingestion limits and their long-term effects in human health are still unknown and there are several variables to be explored. Nevertheless, active food packaging can be a suitable short-term solution for the reduction of the concentration of synthetic additives and the application of the natural additives.

4. Application of *Citrus* by-products to active food packaging

With the technology advances, new concepts and materials began to emerge. From a traditional/conventional perspective, the main purpose of packaging is the protection of foods from external factors without interacting with the food's matrix. In an attempt to overcome the shortcomings of conventional food packaging, intelligent and active food packaging have emerged. Regarding the active packaging systems, their objective is to directly interact with the packaged food in order to extend foods shelf-life. According to the European Legislation, an active package can "change the composition or the organoleptic properties of the food only if the changes comply with the Community provisions applicable to food, such as the provisions of Directive 89/107/EEC (4) on food additives" [383]. There are two kinds of active packaging: absorbent packaging and releasing packaging. The first type is designed to interact with foods absorbing compounds from the packaged food or the headspace of the package, without having the active substance(s) or component(s) migrating to foods. The most common, within this type, in the market are moisture and oxygen absorbents. Regarding the releasing packages, the polymeric matrix is loaded with active compounds that will migrate gradually into the packaged food to increase food's shelf life, through the delay of the phenomena responsible for food deterioration such as inhibition of microorganisms and/or lipid oxidation. These packages can also be used for maintaining, enhancing or improving food's organoleptic characteristics [384].

The most common material used in food packaging is plastic, which raises an enormous environmental concern. Several biopolymers are being proposed to replace conventional plastics due to the massive environmental concern raised by non-biodegradable plastics and plastics obtained from non-renewable resources. For instance, Kraft paper is widely used for packaging, but it has several disadvantages such as high permeability to gas and moisture [385], which can be improved with *Citrus* by-products and their extracts and EOs. Kasaai & Moosavi [386] successfully enhanced water and gas barrier of Kraft paper using mandarin peel and hydrophobic leaf extracts. Due to their high content in carbohydrates, *Citrus* by-products are being used for the production of bacterial cellulose (BC) and polyhydroxyalkanoate (PHA) [387]. BC, described for the first time in 1988, is a polymer of β-1,4-linked glucose produced by aerobic bacteria [388,389]. BC has several interesting properties such as high crystallinity, high cellulose purity, high tensile strength, and water-holding capacity. It is used in the food industry to produce fruit cocktails and jellies [390]. Mostly, the BC is produced using coconut water as a medium for the bacteria (generally *Gluconacetobacter xylinum*), which is a limited source since coconuts only grow in tropical areas. To overcome this problem, Cao et al. [390] resorted to the *Citrus* pulp water, resulting from the juice extraction of *Citrus* fruits, to produce BC, and compared the production of BC through coconut water. The authors concluded that the BC grown in the *Citrus* pulp water almost reached industrial levels. The medium promoted the growth of BC with different physicochemical features (higher water holding capacity and low hardness) [390]. Güzel & Akpınar [391] produced BC from *Komagataeibacter hansenii* GA2016, using peels from lemon, mandarin, orange and grapefruit, with a yield between 2.06 to 3.92 % with a higher water holding capacity than the BC usually produced, high crystallinity and thermal stability, with a thin fiber diameter.

Arrieta et al. [392] studied the influence of the incorporation of *D*-limonene in PLA and poly-hydroxybutyrate (PHB), a biodegradable thermoplastic obtained from microorganisms under physiological stress. The authors manage to obtain five different formulations. As expected, the PLA presented a colorless and transparent appearance, while the PHB presented an amber color with a light transparency. Visually, regarding the films' transparency and color, there were no apparent differences between the films without *D*-limonene and the films with *D*-limonene. In conclusion, the film with PLA:PHB, on a ratio of 75:25, incorporated with *D*-limonene, can offer transparent and flexible films, with water resistant properties and enhance oxygen barrier, suitable for biodegradable food applications [392].

Muñoz-Labrador et al. [393] transformed industrial pectin obtained from *Citrus* fruits by-products (lemons and limes peels) in coatings that were able to increase the quality of strawberries for 5 days, when compared with stored strawberries with no treatment.

Wu et al. [394] resorted to pomelo peels to produce a biodegradable film, using the dried and ground pomelo peels with sodium alginate and glycerol. The authors also incorporated tea polyphenols, rich in catechins, to increase the antimicrobial and antioxidant properties of the active film. The films were applied to soybean oil for a maximum storage time period of 30 days. The authors obtained flexible and transparent films suitable for oil packaging. The incorporation of the tea polyphenols increase the film' antioxidant and antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* [394]. In a study led by Kaanin-Boudraa et al. [395], Citrus x paradisi extract was obtained through MAE with 40 % ethanol, and incorporated in a multilayer LDPE-PET active packaging. The multilayer film with 10 % of extract presented the highest antioxidant activity, followed by the film with 5 % extract [395].

Li et al. [396] successfully developed a new packaging film made from pectin from orange peels, sodium alginate and pterostilbene, with low water vapor permeability, good barrier properties, and antioxidant activity. A pectin extracted from citrus by-products-based film incorporated with green propolis extract, with antioxidant activity, was also developed by Marangoni Júnior et al. [397].

Nanoparticles can be use in food packaging to reinforce the polymeric matrix. Gao et al. [398] compared commercial ZnO nanoparticles with ZnO nanoparticles synthetized with *Citrus sinensis* peel extract. They also incorporated the nanoparticles in carboxymethylcellulose (CMC) in order to obtain a coating to be applied in strawberries. The authors found that the ZnO synthesized with the *Citrus* extract presented a higher antimicrobial activity, similar cytotoxicity and similar crystallinity when compared with the commercial ZnO nanoparticles [398].

Yanjie Li et al. [396] incorporated orange peels EO, by casting method, into fish (*Cynoglossus semilaevis*) skin gelatin and chitosan, at different percentages (0.25, 0.5 and 1.0 %, v/v). The authors observed that the addition of the EO increase the films' thickness from 43.29 μm (in the control film) to 86.95 μm (in the film with 1.0 % of EO). The EO addition decreased the water vapor permeability to 0.86 x 10-11 g m^{-1} s^{-1} Pa^{-1} (in the film with 0.5 % of EO) and increased the elongation at break and films' opacity. Also, the antioxidant activity increased with the continuous addition of the orange EO, from 14.80 % DPPH free radical inhibition and 4.67 % ABTS free radical inhibition, to 49.38 % and

57.71 %, respectively. The active films also presented antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* [396].

In another research, carried out by Roy and Rhim [399], grapefruit seed extract was incorporated into a poly(vinyl alcohol) to form an active film. The addition of the grapefruit seed extract increased the films' thickness from 72.9 to 75.1 μm, the tensile strength from 28.6 MPa to 31.1 MPa, the water vapor permeability 4.18 x 10-10 $g \cdot m/m^2 \cdot Pa \cdot s$ to 4.43 x 10-10 $g \cdot m/m^2 \cdot Pa \cdot s$, and the elongation at break from 148.0 % to 158.0 %. However, the elongation modulus decreased from 0.68 GPa to 0.44 GPa. Regarding the films' biological activities, the addition of the grapefruit seed extract significantly increased the antioxidant percentage in the DPPH free radical scavenging assay from 0.7 to 50.3 % and in the ABTS free radical scavenging assay, from 2.9 % to 90.2 %. Also, the active film also presented antimicrobial activity against E. coli and a remarkable antimicrobial activity against *L. monocytogenes* [399].

Evangelho et al. [400] incorporated *Citrus sinensis* peels EO, at different quantities (0.3, 0.5 and 0.7 μL/g), in corn starch films by casting and evaluated its antimicrobial activity and its properties. All films showed antimicrobial activity against *S. aureus* and *L. monocytogenes*. This antimicrobial activity increases with the increasing of the EO content. The films' thickness and opacity decreased with the addition of the EO, being the active film with 0.5 μL/g of EO the most thicken, with 0.142 μm, and the active film with 0.7 μL/g of EO the opaquest (16.24 %). The films' tensile strength decreased with the addition of the EO, from 5.11 MPa (control film) to 2.40 MPa (film with 0.7 μL/g) [400].

EOs and extracts, as well as other compounds extracted from *Citrus* by-products, can be used as the active additive in the food packaging polymeric matrix to prevent or delay food spoilage, but also, can be used to extract pectin or to produce BC or BHA as a substitute of the conventional polymeric matrix of the food package itself.

5. Conclusion and Future perspectives

Being *Citrus* fruits one of the most consumed fruits in the world, they are also one of the most significant sources of food waste among fruits. These by-products are rich in a wide variety of active components which could be applied in several industries for numerous purposes. With the technological advances, new and more efficient extraction methods, such as the microwave assisted extraction or the pulsed electric field extraction, using less aggressive/toxic solvents and less energy, obtaining higher yields

without compromising the extracts and EOs quality. However, most of the studies evaluate the individual by-products or by-products produced on a small scale. Therefore, there it is necessary to better characterize industrial *Citrus* by-products and to standardize its EOs and extracts in order to guarantee their quality and effectiveness.

Citrus by-products have enormous industrial potential, from polymers for plastic-like food coatings and packaging to active compounds with antioxidant and antimicrobial activities. For instance, pectin can be the base polymer for a new form of food packaging or coating, being a possible substitute for plastic. *Citrus* by-products can also be used for nanoparticles' stabilization and used as a direct or indirect food additive. Nevertheless, the toxicity of these bioactive compounds remains unknown and its use in food must be extensively studied as well as the future effects on human and environmental health.

Chapter IV

Novel Active Food Packaging Films based on Whey Protein incorporated with Seaweed Extract: Development, Characterization, and Application in Fresh Poultry Meat

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1. Introduction

Since the early stages of the human civilization, seaweeds are part of the traditional gastronomies of several Asian countries. Nutritionally, these type of plants are characterized for having a low fat content and a mineral content higher than the terrestrial plants, being a good matrix to be used for nutritional supplements in order to overcome some nutritional deficiencies [401–404]. Human consumption of seaweeds and algae is very common in the Asian culture, especially in Japan. In the Asian gastronomy, several species are consumed as seasonings and vegetables, which contribute for a diversification on their diet. On the other hand, recently, seaweeds and algae in Europe are used for supplementation or for industrial applications such as gelling agents [405,406].

Terrestrial and aquatic plants, for their defense against UV radiation, pathogenic organisms, predators and parasites, naturally produce secondary metabolites known as phenolic compounds. These compounds are also responsible for the plants' coloration and organoleptic properties [194,407–410]. Moreover, the bioactivity of plants, namely antioxidant and antimicrobial properties, are related to their content in phenolic compounds. The content in phenolic compounds depends on the plant's edaphoclimatic characteristics, such as the soil, the geographical location, the moment of harvest, among others [194,259,411,412]. These active compounds can be obtained in the form of essential oil or extract through several extraction methods that can be applied to fresh, dried or freeze-dried plants and fruits.

Fucus vesiculosus is a brown edible seaweed (Class Phaeophyceae), known for its high iodine content and can be easily found in the tempered and cold zones of the North Atlantic [413–415]. The deficiency of iodine is a very serious issue once it can lead to serious conditions. Iodine is a micronutrient essential during the gestation period and for the proper functioning of the thyroid. This deficiency can cause neonatal death, decrease of intellectual development in children and can lead to infertility. The World Health Organization (WHO) recommends a diary intake of 150 μg/day for adults and 200-300 μg/day during the gestation period [416–418]. Although it is mainly under control in the first world countries, iodine deficiency still represents an important issue in undeveloped countries besides being the major preventable cause of brain damage in the world. In this vision, the WHO has set a goal to end iodine deficiency by 2020 [417,418] high content in iodine and phytosterols, *F. vesiculosus* was recommended for obesity treatment. Although, this recommendation is no longer valid because of the secondary effects of iodine on the thyroid activity when treatment stops [419].

Food packaging main goal is to delay the foods' natural degradation process, maintaining their organoleptic properties, protecting the packaged food during transportation and storage. With the advancement of technology, new packaging systems have emerged, such as active food packaging. This type of packaging, by direct interaction with the packaged food throughout the absorption or releasing systems, aims to prolong foods' shelf-life maintaining or improving its organoleptic properties [1,384,420,421]. The releasing systems, as the name implies, release active compounds or substances to the packaged food with biological activities, such as antimicrobial or antioxidant [97,384]. Extracts and essential oils from aromatic plants, fruits by-products and seaweeds have powerful antioxidant activities due to their high content in phenolic compounds [422].

Till this day, lipid oxidation is one of the major causes of food spoilage and, consequently, originates high amount of food loss [423–425]. This natural occurring phenomenon can lead to molecular changes in foods, changes in foods' nutritional value and formation of unpleasant tastes and/or aromas which, subsequently will reduce foods' shelf-life [182,423,425]. Active food packaging incorporated with antioxidant substances can help to minimize this problem, which will gradually migrate to the food' surface during storage time [182,426–430].

The objective of this study was to evaluate the antioxidant activity of ten hydroethanolic extracts obtained from *F. vesiculosus* L., and to incorporate the extract with the highest antioxidant capacity into a whey protein film as an active packaging to control lipid oxidation of chicken breasts.

2. Materials and Methods

2.1. Extract Production

F. vesiculosus was acquired from the Portuguese company, ALGAPlus™, Ílhavo, Portugal. The seaweed was washed three times in cold running tap water to removed most of the salt and possible impurities. One part of the seaweed was frozen and freeze-dried in a freeze drier (Heto PowerDry PL 9000, Thermo Fisher Scientific™, Waltham, MA, USA) and the other part was dried in an oven at 30 °C for 10 days, in the dark.

Five hydroethanolic solvents were used: 100% ethanol, 75% ethanol, 50% ethanol, 25% ethanol and 100% water. For the extraction process, the method described by Andrade et al. [183] was used. Briefly, to 5 g of the freeze-dried or the dried seaweed, 50 mL of solvent were added and homogenized for 30 min in a compact stirrer (Edmund

Bühler™ Shaker KS 15 A, Hechingen, Germany) at 350 rpm. Then, the mixtures were centrifuged for 10 min at 2000 g (10,000 rpm) (Eppendorf AG 5804R, Hamburg, Germany). The supernatant was removed to an evaporation amber pear-shaped flask and ethanol was evaporated at 35 °C. The extracts with water were then frozen and freeze-dried.

2.2. Antioxidant Capacity Assays

For the antioxidant capacity assays, all the extracts were tested at 5 mg/mL with the exception of the 100% ethanolic extracts that were tested at 1 mg/mL due to their low yield (Table IV.1).

2.2.1. DPPH Radical Scavenging Assay

The antioxidant capacity of the ten hydroethanolic extracts was evaluated through the DPPH radical scavenging assay by the method described by Andrade et al. [37]. To 50 μL of sample, 2 mL of a methanolic solution of the DPPH radical (14.2 μg/mL) were added. The samples were homogenized and kept in the dark, for 30 min, at room temperature (23 ± 1 °C). The absorbance of the samples was measured at 515 nm in a Thermo Scientific Evolution 300 LC spectrophotometer. For the control samples 50 μL of the used solvent were used. The Inhibition Percentage (IP) was calculated by the Equation (1).

$$IP\ (\%) = \frac{AC-AA}{AC} \times 100 \quad (1)$$

In which, *AC* stands for the absorbance of the control samples and the *AS* stands for the absorbance of the samples.

2.2.2. β-Carotene Bleaching Assay

The method applied in this study was initial described by Miller [431] and adapted by Andrade et al. [183] prepare the β-carotene and linoleic acid emulsion, 1 mL of a β-carotene in chloroform solution (2 mg/mL), 20 mg of linoleic acid and 200 mg of Tween®40 were mixed in an amber evaporation flask. Then, the chloroform was evaporated in a rotary evaporator at 40 °C, for 5 min, and 50 mL of ultra-pure water, obtained from a MilliQ™ filter system, were added and the solution was strongly shaken. Then, 5 mL of the emulsion were added to 200 μL of sample. For the control assays, 200 μL of solvent were used and the absorbance of the controls was measured at 470

nm. Then the controls and the samples were submitted at 50 °C for 2 h. At the end of this period, the absorbance of the samples and the controls were measured at 470 nm. The Antioxidant Activity Coefficient (AAC) was measured through the Equation (2).

$$AAC = \frac{AS - AC2}{AC0 - AC2} \times 1000 \quad (2)$$

wherein, *AS* stands for the absorbance of the sample, *AC0* stands for the absorbance of the control before heating and *AC2* stands for the absorbance of the control after heating.

2.3. Total Phenolic Compounds (TPC)

The total content on Phenolic Compounds was determined through the method described by Wang et al. [432]. Briefly, to 1 mL of sample, 5 mL of an aqueous solution of Folin-Ciocalteu (10%, v/v) was added. The mixture was homogenized and, after 5 min, 4 mL of an aqueous solution of sodium carbonate (7.5%, w/v) were added. The mixtures were homogenized and kept in the dark for 2 h, at room temperature (23 ± 1 °C). The samples absorbance was measured at 725 nm. The results are expressed in mg equivalents of phloroglucinol per g of extract.

2.4. Whey Protein Film Production

The active film was produced by casting, applying the method described by Andrade et al. [183] and is composed by 8% of concentrated whey protein, 8% of glycerol, 1% of *F. vesiculosus* L. extract and 83% of water. The whey protein concentrated was acquired from MyProtein® (Chicago, USA). Briefly, the whey protein was mixed with water using an Ultra-Turrax (IKA DI 25 basic, Werke GmbH & Co, Staufen im Breisgau, Germany) and then submitted to 80 °C for 30 min in a thermostatic bath. Then, the mixture was rapidly cooled in ice for 15 min. Glycerol and *F. vesiculosus* L. extract were added, homogenized with the Ultra-Turrax and casted in an aluminum foil surface. The film was left to dry for 3 to 4 days, at room temperature.

2.5. Film Mechanical Properties and Thickness

The films thicknesses was measured with a digital micrometer (0.001 mm, Mitutoyo, Japan) on ten randomly points of each sample, and the results were expressed by the means ± standard deviation.

The film mechanical properties (tensile strength, elastic modulus and elongation at break) were determined using the universal testing machine Shimadzu Autoghaph (Shimadzu, Australia), equipped with a 0.5 kN load cell, in accordance with ASTM D882–12 [433]. Five specimens of each repetition/treatment with dimensions of 150 × 25 mm2 were evaluated using the grip separation speed of 50 mm/min and an initial gauge length of 50 mm. The mechanical parameters were calculated from the stress x strain curves, where the tensile strength (TS) corresponds to the maximum stress before breaking (i.e., the force divided by the area in which the traction is applied), the elastic modulus (EM) represents the slope of the straight-line portion of a stress-strain curve and the percentage of elongation at break (EAB) is the ratio between the stroke before breakage (maximum elongation) and the initial distance between grips [434].

2.6. Water Vapor Permeability (WVP)

Water vapor permeability (WVP) (mol/m·s·Pa) was obtained, at 30 °C, using the gravimetric method described by Ferreira et al. [435]. Samples of each film were sealed on top of glass cells (45 mm diameter) containing 8 mL of supersaturated NaCl solution (relative humidity (RH = 76.9%). The cells were then placed inside desiccators containing supersaturated CH3COOK solution (RH = 22.5%) and equipped with a fan to promote air circulation and keep the driving force constant throughout the test. Prior to the test, the films were equilibrated in a desiccator containing supersaturated NaCl solution (RH = 76.9%) at 30 °C. The relative humidity and temperature of the inside air of the desiccators were monitored throughout the test using a thermo-hygrometer (Vaisala, Finland). The flow of water vapor was determined by weighing the cells at regular time intervals for approximately 22 h, and the WVP was calculated using Equation (3):

$$WPV = \frac{N_W \, X \, \delta}{\Delta P_{w.eff}} \quad (3)$$

where *Nw* (mol/m^2·s) corresponds to the flow of water vapor, δ (m) to the thickness of the film and *ΔPw.eff* (Pa) to the effective driving force. The results were expressed as the mean ± standard deviation of three replicates.

2.7. Film Optical Properties

The optical properties were evaluated by measuring the CIELab parameters *L*, *a**, *b**, using the Konica Minolta colorimeter (CR 400/410, Tokyo, Japan) with D 65 light source,

and 10° visual angle. The analysis was conducted according to the CIELab color scale, where *L* is the measure of luminosity, ranging from zero (black) to 100 (white); *a** the chromaticity of green (−60) to red (+60); and *b** the chromaticity of blue (−60) to yellow (+60). From the *L*, *a** and *b** coordinates, the hue angle (*hue**) and chromaticity were calculated using Equations (4) and (5) respectively.

$$hue^* = arctan\left(\frac{b^*}{a^*}\right) x \frac{180}{\pi} + 180\,,\ if\ a^* < 0 \quad (4)$$

$$chromaticity = (a^{*2} + b^{*2})^{1/2} \quad (5)$$

2.8. Evaluation of the Lipid Oxidation

The chosen model food for the evaluation of the efficacy of the active package against lipid oxidation was chicken breast, which were kindly supplied by the Portuguese company, Lusiaves® (Leiria, Portugal). The chicken breasts were packaged with the control (without the extract) and the active films, respectively, and stored at 4 ± 1 °C. To maximize the contact surface between the chicken breast and the active package, the samples were vacuum packaged (Figure IV.1). The chicken breasts were analyzed at the end of 3, 6, 8, 11, 15, 21 and 25 days of storage.

Thiobarbituric Reactive Substances Assay (TBARS)

The TBARS assay was performed according to the method described by Rosmini et al. [436] with minor changes. In brief, 10 g of minced chicken breasts were mixed with 20 mL of trichloroacetic acid (7.5%, w/v) and homogenized in a compact stirrer at 400−450 rpm. After 1 h, the solutions were filtered through a Whatman n° 1 paper filter. Then, 5 mL of the filtered samples were mixed with 5 mL of an aqueous solution of thiobarbituric acid (2.88 mg/mL, w/v). The solutions were homogenized and submitted to 95 °C for 30 min. Then, they were rapidly cooled in ice for 15 min and their absorbance was measured at 530 nm. The results are present in mg of malonaldehyde equivalents per kg of meat.

2.9. Statistical Analysis

All experiments were conducted using three replications. Statistical analysis of data was performed through a one-way analysis of variance (ANOVA) using the Software OriginLab, version 8.5 (OriginLab Corporation, Northampton, USA). The differences

among mean values were processed by the Tukey test. All requirements necessary to carry out the ANOVA (namely, normality of data and homogeneity of variances) have been validated. Significance was defined at $p < 0.05$. Results are expressed as the means of the replicants ± standard deviation.

3. Results

3.1. Antioxidant Assays

The results of the antioxidant assays can be observed in Table IV.1. All the obtained extracts from the freeze-dried seaweed presented a significantly better antioxidant capacity results than the extracts obtained from the dried seaweed, probably because the freeze-drying process protected the seaweed bioactive compounds [437,438].

As expected, the extracts obtained with a higher ethanol percentage, presented the lowest yields. Additionally, the extracts obtained from the dried seaweed, presented higher yields than the extracts obtained from the freeze-dried seaweeds. The extract obtained with the 75% ethanol presented the highest inhibition of the DPPH radical percentage, followed by the extract obtained with 25% ethanol. On the β-Carotene Bleaching Assay, the 100% aqueous extract presented the highest antioxidant activity coefficient, followed by the 75% ethanol extract. Regarding the total phenolic compounds content, the value obtained for the 100% ethanol extract was the highest, followed by the extract obtained by ethanol at 75%.

Based on extraction yields and antioxidant assays results, the extract obtained from the freeze-dried *F. vesiculosus* L. with 75% ethanol was chosen to be incorporated into the whey protein concentrate film.

Table IV.1. Seaweed extracts antioxidant properties characterization. Results are the means ± standard.

Solvent	**Yield (%)**		**IP (%)**		**β-Carotene Assay (AAC)**		**TPC Assay (mg PGE/g of extract)**	
	FD	**D**	**FD**	**D**	**FD**	**D**	**FD**	**D**
100 % H_20	18.63	26.66	62.51 ± 0.17 ^{Ca}	0.00 ^{Cb}	842.67 ± 5.55 ^{Aa}	575.10 ± 10.64 ^{Bb}	26.18 ± 0.11 ^{Ea}	19.03 ± 0.10 ^{Cb}
25 % Ethanol	20.02	21.38	66.69 ± 0.33 ^{Ba}	17.88 ± 0.79 ^{Bb}	356.07 ± 6.42 ^{Da}	250.76 ± 5.31 ^{Db}	27.05 ± 0.31 ^{Da}	16.18 ± 0.09 ^{Db}
50 % Ethanol	13.09	22.30	57.00 ± 0.47 ^{Da}	16.33 ± 0.86 ^{Bb}	564.59 ± 11.06 ^{Ca}	383.24 ± 7.07 ^{Cb}	31.12 ± 0.26 ^{Ca}	12.42 ± 0.11 ^{Eb}
75 % Ethanol	4.71	18.77	78.26 ± 0.21 ^{Aa}	57.44 ± 0.99 ^{Ab}	636.22 ± 5.16 ^{Ba}	611.91 ± 8.09 ^{Ab}	45.21 ± 0.21 ^{Ba}	21.06 ± 0.12 ^{Bb}
100 % Ethanol	2.71	3.86	44.65 ± 0.79 ^{Ea}	0.86 ± 0.03 ^{Cb}	237.82 ± 5.03 ^{Ea}	125.36 ± 11.90 ^{Eb}	194.65 ± 0.87 ^{Aa}	70.97 ± 0.74 ^{Ab}

Legend: IP – Inhibition Percentage; AAC – Antioxidant Activity Coefficient; PGE – Phloroglucinol equivalents.
(A-E): Within each parameter, values in the same column not sharing upper case superscript letters indicate statistically significant differences among solvents ($p < 0.05$); (a–b): Within each parameter, values in the same line not sharing lower case superscript letters indicate statistically significant differences among formulations ($p < 0.05$).

3.2. Active Whey Protein Film

The control and the active film can be observed above a paper sheet with the National Institute of Health Doutor Ricardo Jorge logo and applied to the chicken breasts (Figure IV.1).

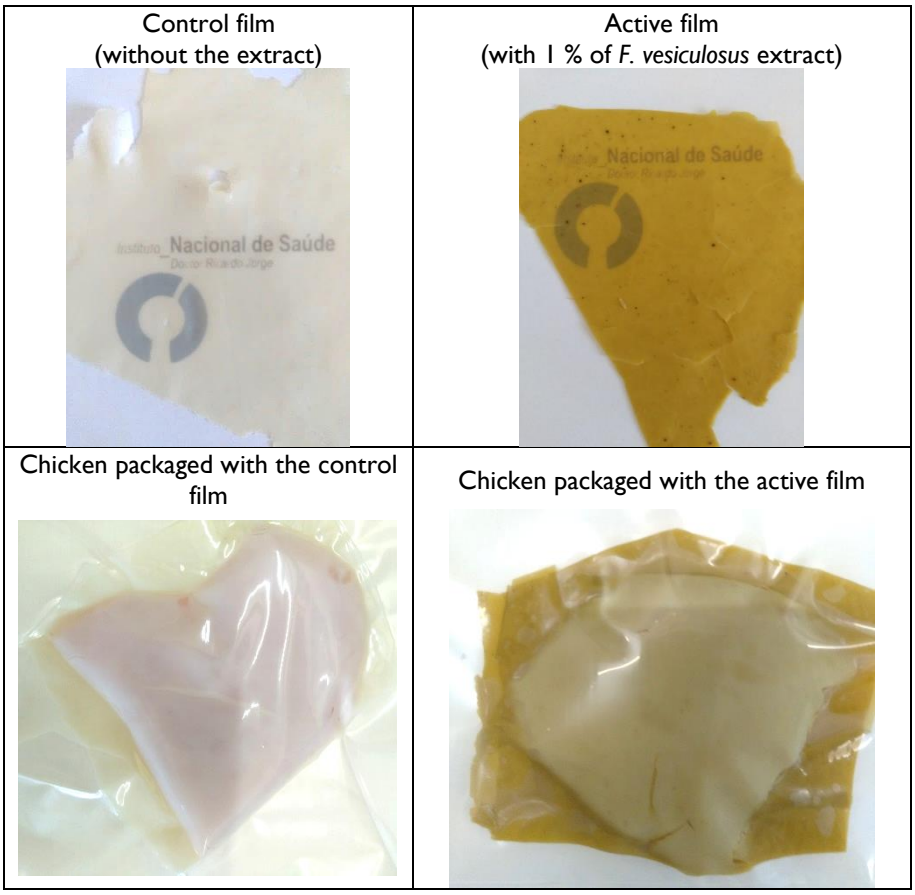

Figure IV.1. Control film (left) and active film with 1% of *F. vesiculosus* extract (right).

3.2.1. Thickness, Mechanical Properties, Optical Properties and WVP

The addition of the *F. vesiculosus* extract seems to significantly increase the thickness of the control film in, approximately, 12% ($p < 0.05$), which is coherent with the greater solid content per surface unit (Table IV.2). Regarding the other parameters, there were no significant differences, nevertheless, the addition of the *F. vesiculosus* extract seems to slightly reinforce some of the film' physical properties. When compared to the control film (Table IV.2), the active film presented a higher value of tensile strength and elastic modulus which, in consequence, decreased the elongation at break (elasticity of the film). However, the extract addition seemed to increase the WVP of the film, since the active film collapsed in the first hours of the essay, which indicates that the extract addition made the films more hydrophilic. Fernandes et al. [439] also found a similar WVP value

for a whey protein film with 30% of glycerol. The addition of glycerol appears to increase the WVP of whey protein films. Ribeiro-Santos et al. [97] used the same percentage of glycerol and found similar results for the control film. Once the samples with seaweed extract collapsed during the test, it is impossible to conclude whether it enhanced or diminished the film's WVP, however as previously mentioned it probably enhanced the WVP, which corroborates with observed in Ribeiro-Santos et al. [97] with the incorporation of a mixture of essential oils to whey protein films. Gounga et al. [440] studied the influence of different whey protein and glycerol ratios on films' WVP. The authors concluded that a higher percentage of whey protein increase the WVP and also, the higher the glycerol percentage (plasticizing effect), the higher the WVP [440].

Table IV.2. Film characterization. Results are the means ± standard deviation of the replicants.

Parameter	**Control film**	**Active film**
Thickness (μm)	248.8 ± 6.7^{sig**}	280.7 ± 7.5^{sig}
Tensile strength (MPa)	0.184 ± 0.022^{ns}	0.203 ± 0.055 ^{ns}
Elongation at break (%)	11.7 ± 2.5 ^{ns}	8.7 ± 1.9 ^{ns}
Elastic Modulus (MPa)	1.468 ± 0.617 ^{ns}	3.364 ± 1.300 ^{ns}
WVP (10^{-10} mol/m·s·Pa)	1.178 ± 0.21	*Sample collapsed*
*L** (lightness)	84.51 ± 0.35 ^{sig}	60.72 ± 0.19 ^{sig}
*a** [red (+a) or green (−a)]	-2.89 ± 0.02 ^{sig}	-1.33 ± 0.02 ^{sig}
*b** [yellow (+b) or blue (−b)]	13.14 ± 0.66 ^{sig}	23.50 ± 0.03 ^{sig}
*Hue**	102.42 ± 0,67 ^{sig}	93.25 ± 0.05 ^{sig}
Chromaticity	13.46 ± 0.64 ^{sig}	23.54 ± 0.03 ^{sig}

** Means followed by sig superscripts are statistically significant different ($p < 0.05$); ns (not significant).

Comparing with the results obtained by Ribeiro-Santos et al. [97], the whey protein film presented the same behavior, in terms of thickness and elongation at break, in the presence of an additive. In both studies, the addition of extract and essential oils seemed to thicken the film, as well as increase the tensile strength. Abdalrazeq et al. [441] studied the application of different percentages (40 and 50%, w/w) of glycerol into isolated whey protein based films. These films showed a higher tensile strength and were less thick than the control film in the present study, which can be explained by the higher percentage of added glycerol. Moreover, the elongation at break was higher in the film with 50% of glycerol and higher than the values found for the control and active films

conceived in this study. However, the film with 40% of glycerol showed a lower elongation at break value (3.6%) than the control film [441] (Table IV.2).

The produced films were predominant yellowish, and the incorporation of the best active extract interfered on this optical parameter, which can be visually observed (Figure IV.2) and confirmed by the measured CIELab coordinates (Table IV.2). Films incorporated with the extract got darker (increased *L*, $p < 0.05$) with a stronger yellow appearance (higher *b**, $p < 0.05$). A similar behavior was also observed in chitosan films incorporated with hydroalcoholic extracts from different plants (food seasoning herbs and tea) [442] or essential oil [443]. The extracts contribute to add color to the films due to their intrinsic color, and the ability of their active compounds to structurally bind with the polymer, changing the film's optical properties [442]. The active film presented a hue angle around 90°, which corresponds to the yellow color. On the other hand, the control films presented a superior angle (around 102), which indicates a tendency to the green color (Table IV.2). These results suggest that the extract potentialize the yellow color in the active films when compared to the control films. Regarding the chromaticity, the extract significantly ($p < 0.05$) increased the films' color saturation, which means that the active film present more vivid colors than the control film [442]. The optical values of the control film were higher than the values of the whey protein film produced by Ribeiro-Santos et al. [97], indicating that the film produced in this study was more shining (*L*), green (−*a*) and yellow (+*b*).

3.2.2. TBARS Assay

Lipid oxidation is a natural complex phenomenon. It's initiated by the formation of free peroxyl radicals, which will react with unsaturated fatty acids, forming lipid hydroperoxides. Antioxidant compounds may inhibit lipid oxidation of the unsaturated fats by prevent the formation of free peroxyl radicals and the formation of lipid hydroperoxides [444–446].

The TBARS assay is one of the most common methods to evaluate foods' lipid oxidation by measuring the malonaldehyde (MDA) content. MDA is an aldehyde formed during the polyunsaturated fatty acids peroxidation, being a product of the primary oxidation of lipids [426,445–447]. However, this assay only measures substances reactive to the thiobarbituric acid which, during lipid oxidation, will continue to be degraded [426,448]. The results of the TBARS assay are present in Figure IV.2. For this assay, a calibration curve ($y = 308.7x - 0.0071$) was obtained using 1,1,3,3-tetramethoxypropane as standard. The determination coefficient of the calibration curve

was 0.9995, in a working range of 0.1−3 μg/mL, indicating linearity and correlation. As can be observed in Figure IV.2, the chicken packaged with the active film for 15, 21 and 25 days of storage, presented a significantly lower MDA value than the chicken packaged with the control film. The decrease of the MDA content on the 8th day of storage seems to suggest that the oxidative status of the chicken breasts reached an oxidation peak between the 6th and the 8th day of storage. Shahbazi and Shavisi [449] also registered the same TBARS behaviour in rainbow trout fillets on the 10th day of storage. This can also be explained by the chemical reactions between MDA and the meat compounds, which may lead to a decrease in the MDA amount [449,450].

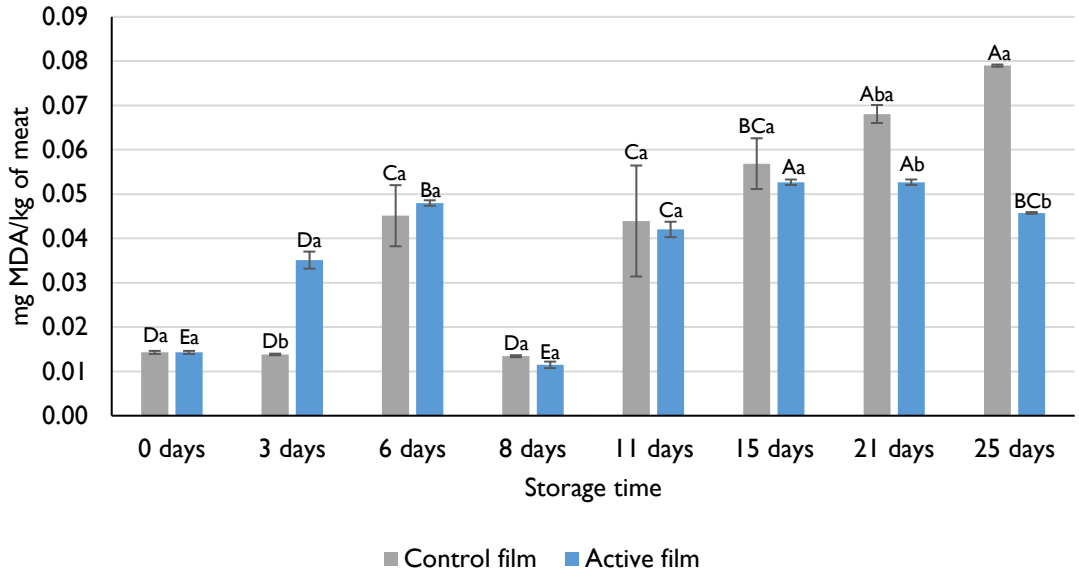

Figure IV.2. Results of the TBARS assay of the chicken breasts packaged for 25 days with the control and the active films. The results are expressed in mg of malonaldehyde equivalents per kg of meat, as means ± standard deviation of the replicants. (A−E): Within each treatment, values not sharing uppercase superscript letters indicate statistically significant differences among days ($p < 0.05$); (a–b): Within each storage day, values not sharing lower case superscript letters indicate statistically significant differences among treatment ($p < 0.05$).

Although not conclusively, the study lead by Reboleira et al. [451] showed some inhibition of chicken patties' lipid oxidation when in contact with *Porphyra dioica* extract for 96 h (4 days). Gonçalves et al. [452] evaluated the effect of microalgae biopeptides nanofibers in chicken breasts. The authors found that the chicken with the microalgae nanofibers presented a lower MDA value (1.0 ± 0.0 mg MDA/kg) than the control breast chicken (2.6 ± 0.0 mg MDA/kg). The values found in the study of Gonçalves et al. [452] are much higher than the values calculated for the chicken packaging with the whey protein film. The authors studied the influence of polycaprolactone (PCL) nanofibers incorporated with microalgae biopeptides on chicken lipid oxidation. At the end of 12

days of storage, the MDA values were much higher than the values found in this study. However, the MDA content of the chicken meat at day 0 was also higher that the MDA value of the chicken breasts, indicating that the chicken evaluated on this study was 'fresher' [452].

4. Conclusions

A whey protein edible film incorporated with an ethanolic extract of *Fucus vesiculosus* L. was successfully prepared by casting method. The addition of the *F. vesiculosus* extract seems to reinforce some of the whey protein film physical characteristics, such as thickness, tensile strength, and elastic modulus. However, the addition of the extract made the film more permeable to water vapor. The new film was able to inhibit lipid oxidation of packaged poultry meat, at least for 25 days of storage compared with the control whey protein film. One of the studies limitations was the inability to perform other lipid oxidation assays, such as the determination and quantification of the aldehyde hexanal, responsible for the rancid smell and taste. The determination of the peroxide value is also another lipid oxidation evaluation assay, but the authors were not able to perform this assay in the laboratory. Moreover, the obtained results encourage more assays, namely microbiological assays, and sensorial analysis, in order to evaluate the effect of this new film in the extension of poultry meat shelf-life.

Chapter V

Industrial multi-fruits juices by-products: total antioxidant capacity and phenolics profile by LC–MS/MS to ascertain their reuse potential

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Introduction

Fruits and vegetables are known to be a great source of nutrients and vitamins, contributing to a balanced and healthy diet. They also represent 45% of the world's food losses (≈1.3 × 106 tons/year) [18,453]. Fruits by-products have a great industrial and economic potential since they are considered as an excellent source of bioactive compounds that can be used as additives in food, cosmetic and pharmaceutical industries [186].

Additives are used in food formulations to extend shelf-life and ensure food safety. Recently, the application of synthetic additives has been questioned since some studies have associated their consumption with carcinogenesis promotion and the emergence of neurodegenerative diseases [29,454]. In addition to all these risks, the consumers demand increase for natural products, have encouraged the scientific community and food industry to search for a suitable and natural replacement of these synthetic additives.

Phenolic compounds are metabolites produced by plants for their defense against natural predators and aggressions, such as radiation, drought periods and pathogenic organisms [183]. These compounds found in the plant materials are responsible for important biological activities such as antioxidants and antimicrobial activities, which are crucial for extending the shelf life, quality and safety of foods.

Apples (*Malus domestica*), one of the most consumed fruits in the world, are an important source of bioactive compounds such as, pectin, dietary fibers, vitamins and phenolic compounds [262,455]. Carrots (*Daucus carota* L.), a vital source of carotenoids namely carotenes, are one of the most produced and consumed vegetables in the world, contributing to a balanced and healthy diet [456]. They can be used in several food formulations, such as juices and concentrates that produce a significant amount of food waste [457]. According to the USDA Food Database, carrots present a high content in pro-vitamin A carotenoids, namely β-carotene and α-carotene, with recognized high antioxidant activity [295,458].

Beetroot (*Beta vulgaris* L.) is a vegetable rich in water soluble pigments (betanin, betacyanins and betaxanthins), carotenoids, flavonoids, and vitamins [459]. The carotenoids and polyphenols present in beetroot have been reported to have antioxidant and anticarcinogenic activities [460]. Due to its powerful and vibrant color, beet extracts and juice are used as a natural food coloring [459].

Ginger (*Zingiber officinale* Roscoe), a safe ingredient with proven antioxidant, antimicrobial and anticarcinogenic activities, is used in several gastronomies, mostly in

Asian and African countries [318,461]. In its composition a great variety of bioactive compounds such as phenolic compounds, volatile oils, proteolytic enzymes and vitamins can be found.

If fruits by-products are a low economic product with powerful antioxidant and antimicrobial capacities which are very important in multiple industries, then it should be possible to extract the phenolic compounds responsible for the powerful antioxidant activities for them to be applied in the food industry. As what concerns to authors' knowledge, several studies have been performed to evaluate the antioxidant capacity and quantified the phenolic compounds of fruits by-products, but most of these studies are not performed in industrial by-products [190]. By evaluating these parameters in industrial by-products, this study reinforces the importance and potential of circular economy and, contribute to demonstrate how it can be applied to fruits by-products.

The aim of this study is to obtain ethanolic extracts from fresh and the freeze-dried industrial by-products from two juice formulations: formulation 1 made up of the by-products of the juice prepared with apple and ginger (50:50, w/w) and formulation 2 made up of the by-products of the juice prepared with apple, carrot, beet and ginger (50:29:20:1, w/w). The antioxidant capacity if the extracts will be evaluated, and the main phenolic compounds of the industrial by-products will be determined by HPLC–DAD and HPLC–MS/MS. Regarding the by-products, the choice was made according to the company's availability at the time of sampling. This availability depends on the season of the year and consumers' preferences, which dictate the volume produced of each juice.

Materials and methods

Multi-fruits by-products

Multi-fruits by-products of two industrial juices formulations were kindly supplied by Frubaça (CRL, Portugal). The first formulation (F1) was composed by apple and ginger (50:50, w/w). The second (F2) was composed by apple, carrot, beet and ginger (50:29:20:1, w/w). The by-products were grinded and kept at – 20 °C. Apple varieties used in the juices were green (Granny Smith) and red (Jonagold) apples.

Determination of the antioxidant capacity, total phenolics and total flavonoids

Reagents and materials

Ethanol, chloroform, methanol, sodium carbonate anhydrous, sodium nitrite, gallic acid and sodium hydroxide were acquired to Merck (Darmstadt, Germany). The 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 to Sigma-Aldrich (Madrid, Spain). Also, a Heto PowerDry PL 9000 freeze drier (Thermo Fisher Scientific™), a compact stirrer Edmund Bühler™ Shaker KS 15 A (Hechingen, Germany), an Eppendorf AG 5804R centrifuge (Hamburg, Germany), 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.

Extraction procedure

Samples were frozen in the same day they were produced in the fruit juices' company. The concentrated ethanolic extracts were obtained from the fresh and the freeze-dried (FD) by-products. The freeze samples were placed in the freeze dried for 1 week. The extraction procedure was carried out accordingly with the method used by Andrade et al. [183]. Briefly, 50 mL of ethanol were added to 5 g of each sample and mixed using the compact stirrer, at room temperature (≈23 °C) for 30 min. Then, the mixture was centrifuged at 11,952 *g*, for 15 min at 15 °C. The supernatant was removed to a pear-shaped flask and evaporated until dryness at 35 °C. The extracts were removed and stored under vacuum conditions, protected from the light. The yield (%) of the extracts was calculated thorough the Eq. 1.

$$Y = \frac{Extm}{Sm} \times 100 \quad (1)$$

Extm represents the mass (g) of the obtained extract and *Sm* represents the mass (g) of the sample (freeze-dried or fresh).

Antioxidant capacity

Samples concentrations

For the antioxidant capacity assays, ethanolic solutions of each concentrated extract, were prepared with a final concentration of 5 mg/mL.

Free radical DPPH inhibition system

The applied method was described by Andrade et al. [183]. Briefly, to 50 µl of each sample, 2 mL of a DPPH methanolic solution (14.2 µg/mL) were added and kept in the dark for 30 min. Then, the absorbance was read at 515 nm and the Inhibition Percentage (IP) was calculated by the Eq. 2. A calibration curve using trolox was drawn and the results are expressed in µg of trolox equivalents by g of dry weight (µg TE/g of DW) [183].

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

AC represents the absorbance of the control and *AS* stands for the absorbance of the samples.

β-Carotene bleaching assay

The applied method was described by Andrade et al. [183]. For the emulsion, 20 mg of linoleic acid and 200 mg of Tween ®40, 1 mL of a β-carotene in chloroform solution (2 mg/mL) was added. Chloroform was evaporated in the rotary evaporator at 40 °C. Afterwards, 50 mL of oxygenated ultrapure water were added, and the mixture was vigorously shaken. Then, 5 mL of the emulsion were added to 200 µL of each sample. All the samples and the control assays were then submitted to 50 °C for 2 h, in the heating block. The absorbances were measured at 470 nm after 2 h. The control assays absorbances were measured before and after the heating period. The antioxidant activity coefficient (AAC) was calculated with the Eq. 3 [431].

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

AS represents the absorbance of the sample, *AC* is the control absorbance and *AC2* is the control absorbance after 2 h.

Total phenolic compounds content

The determination of the total phenolic compounds content was performed according to the method described by Erkan et al. [462]. To 1 mL of each sample, 7.5 mL of Folin−Ciocalteu reagent (10%, v/v) were added and homogenized. After 5 min, 7.5 mL of an aqueous solution of sodium carbonate (60 mg/mL) were added. Samples were kept in the dark at room temperature for 2 h and the absorbance was measured at 725 nm. A gallic acid calibration curve was drawn and the results are expressed in mg gallic acid equivalents per g of dry sample (mg GAE/g of DS) [462].

Total flavonoids content

The determination of the total flavonoids content was performed in accordance to the method described by Yoo et al. [463]. To 1 mL of each sample, 4 mL of ultrapure water and 300 μL of sodium nitrite (5%, w/v) were added and the mixture was homogenized. After 5 min, 600 μL of aluminum chloride (10%, w/v) were added and the mixture was re-homogenized. After 6 min, 2 mL of sodium hydroxide (1 M) and 2.1 mL of water were added. The mixture was homogenized, and the sample absorbance was read at 510 nm. A calibration curve using epicatechin as standard was drawn and the results are expressed in mg epicatechin equivalents per g of dry sample (mg ECE/g of DS) [463].

Quantification and identification of bioactive phenolic compounds

Standard solutions

Standards of catechin, epicatechin, epigallocatechin, epigallocatechin gallate, rutin, isoquercitrin, myricetin, naringenin quercitrin, *trans*-resveratrol, *trans*-piceid, piceatannol, morin, kaempferol, stilben, apigenin, pterostilbene and protocatechuic, caffeic, *p*-coumaric, benzoic, m-coumaric, rosmarinic, ferulic, synaptic and gentistic acids were purchased from Sigma-Aldrich; quercetin and gallic, vanillic, chlorogenic, syringic and salicylic acids were supplied by Fluka Chemie AG (Buchs, Switzerland); procyanidin B1 was provided by Extrasynthese (Genay, France) and homovanillic and *p*-hydroxybenzoic acids were supplied by Alfa Aesar (Karlsruhe, Germany). All standards had a purity ≥ 95%, except quercitrin (78%). Methanol and acetone for chromatography were supplied by Merck and the acetic acid by Sigma-Aldrich. The purified water used for all solutions was obtained from a purification system (Wasserlab, Navarra, Spain).

Preparation of extracts for HPLC analysis

The FD by-products were extracted according to Ferraces-Casais et al. [464] procedure with a slight modification. Briefly, 0.5 g were crushed and sieved (opening 0.2 mm), mixed with 6 mL of methanol/water/acetic acid (30:69:1, v/v/v) and homogenized using vortex mixer, protected from the light, at room temperature for 60 min. Then, the mixture was centrifuged at 1506 g for 4 min at 23 °C. Supernatant was collected, and the residue was re-extracted with 6 mL of acetone/water (70:30, v/v) under the same conditions. The supernatants were pooled, filtered and volumes adjusted to 10 mL with acetone/water (70:30, v/v). For the analysis, 1 mL was evaporated to dryness and then re-dissolved in purified water. The samples were filtered with 0.22 μm PTFE filter and injected in HPLC [465].

HPLC-DAD

Chromatographic analysis was performed on an Agilent HPLC system 1200 (Waldbronn, Germany), equipped with a quaternary pump, a degassing device, an autosampler, a column thermostat system, a diode array detector (DAD) and controlled by Agilent ChemStation for LC and ChemStation for LC 3D Systems software. Chromatographic separation of compounds was carried out on a Kinetex® EVO C18 100 Å column (150 × 3 mm i.d., 5 μm particle size) (Phenomenex, Torrance, CA, USA). The solvents constituting the mobile phase were purified water with 0.1% acetic acid (solvent A) and methanol with 0.1% acetic acid (solvent B). The gradient program was as follows: 0–3 min, 90% A; 10 min, 80% A; 18 min, 70% A; 25 min, 30% A; 33 min, 0% A; 33–40 min, 0% A and 100% B; finally, the column was washed and reconditioned with 95% A (40–46 min). The mobile phase flow rate was 0.6 mL/min during the entire analytical run, the column temperature was set at 30 °C, and the sample injection volume was 20 μL. A scan in the range of 200−400 nm was performed during 40 min of run time, by DAD. Individual phenolic compounds were identified by comparing their retention time and their UV spectrum with those obtained by injecting standards in the same HPLC conditions. Phenolic compounds were monitored and quantified at 205, 278, 300 and 360 nm.

HPLC-ESI-MS/MS

To confirm the presence of the phenolic compounds in the samples an HPLC–ESI–MS/MS (Thermo Fisher Scientific, San José, CA, USA), equipped with an Accela quaternary pump, degasser, autosampler, column oven and a triple quadrupole mass

spectrometer TSQ Quantum Access max and electrospray ionization source (ESI) working in positive (quercetin) and negative (all other compounds) mode, was used. The chromatographic conditions were the same as in HPLC–DAD analysis. Nitrogen (purity > 99.98%) was used as a sheath gas, ion sweep gas, and auxiliary gas. The MS conditions were as follows: electrospray voltage: 2500 V, vaporizer temperature: 340 °C, sheath gas pressure: 25 psi, auxiliary gas pressure: 5 arbitrary units, and capillary temperature: 350 °C. Argon was the collision gas and the detection was performed by SRM. The compounds were characterized by their retention time relative to an external standard, and the precursor and product ions obtained.

Statistical analysis

All the assays were conducted in triplicate and the results are expressed in means of ± standard deviation (SD). The obtained values were treated on IBM® SPSS® Statistics Version 25, applying an ANOVA and Tukey HSD to determine the differences between means with a significance defined at $p < 0.05$.

Results and discussion

Antioxidant capacity

The yields of the FD F1 and F2 concentrated extracts were 13.08% and 15.70%, respectively. The calculated calibration curve for DPPH· assay was y = 0.5896 x + 0.5691 (range: 25−150 μg/mL; $R^2 = 0.996$), for the TPC assay was y = 6.4655 x + 0.1252 (range: 0.025−0.2 μg/mL; $R^2 = 0.9993$) and for the TFC assay was y = 3.2412 x + 0.0203 (range: 0.025 to 0.175 μg/mL; $R^2 = 0.9991$).

In the antioxidant capacity assays, the extract of the byproducts from the juice F1 presented a higher antioxidant capacity than the F2 (Table V.1). Both extracts obtained from the FD by-products presented a higher antioxidant capacity than the extracts from the fresh by-products in all assays, with the exception of TFC assay were the extract from F2 presented 6.15 mg ECE/g DW extract, while the extract from the fresh F2 presented 6.87 mg ECE/g DW extract (Table V.1).

Table V.1. Results of the antioxidant capacity assays.

Samples	Inhibition Percentage (%)	mg TE/g extract DW	AAC	mg GAE/g extract DW	mg ECE/g extract DW
F1	36.55^{a} + 0.99	14.92 ± 0.41^{a}	291.0 ± 10.53^{a}	32.71 ± 0.24^{a}	23.86 ± 1.11^{a}
F1 Freeze-dried	50.32^{b} + 0.58	21.22 ± 0.25^{b}	385.5 ± 4.90^{b}	52.95 ± 0.18^{b}	54.72 ± 1.28^{b}
F2	13.37^{c} + 0.25	5.17 ± 0.10^{c}	223.1 ± 2.08^{c}	15.01 ± 0.06^{c}	6.87 ± 0.30^{c}
F2 Freeze-dried	14.77^{c} + 0.30	5.56 ± 0.12^{c}	235.7 ± 9.35^{c}	15.56 ± 0.08^{d}	6.15 ± 0.23^{c}

The results are expressed in triplicate means ± standard deviation. Different letters within the same column (assay) indicate significant differences ($P < 0.05$) among the by-products
DW dry weight, *TE* trolox equivalents, *AAC* antioxidant activity coefficient, *GAE* gallic acid equivalents, *ECE* -(-) epicatequin equivalents, *F1* formulation 1 (apple + ginger, 50:50, w/w), *F2* formulation 2 (apple + carrot + beet + ginger, 50:29:20:1, w/w)

Panzella et al. [466] showed inhibition percentages of the DPPH radical from 17 to 65% in apple extracts from different cultivars. Ferrentino et al. [467], using supercritical fluid extraction (SFE) on apple peels and pulp using CO_2 and ethanol, showed that, for the same SFE conditions, the FD by-products presented a higher antioxidant capacity as well as higher yield and total phenol content than the extract from the fresh apple peels and pulp.

Apple presented the second highest phenolic content when compared with 10 common fruits (cranberry, red grape, pineapple, lemon, banana, strawberry, peach, pear, orange and grapefruit) [468]. The authors determined a value of 296.3 mg GAE/100 g fresh weight of apple edible parts, which is a lower value than TPC value of the extracts obtained in the present study (between 4 and 14 times higher).

Ali et al. [469] prepared ginger extracts with petroleum ether and a mixture of chloroform and methanol (1:1, v/v). The higher phenolic compounds content was observed in the ginger rhizome extracted with the chloroform and methanol mixture (60.34 mg GAE/g), which presented a DPPH radical inhibition percentage of 86.7%.

Isabelle et al. [470] determined the total phenolics content of beet, carrot and ginger extracts. The extracts were obtained through a solid–liquid extraction process using a mixture of acetone, water and acetic acid (70:29.5:0.5, v/v). The ginger extract presented the highest phenolic content (1.46 mg GAE/g fresh weight) of the three extracts, followed by beet (0.89 mg GAE/g fresh weight) and carrot (0.16 mg GAE/g fresh weight). Given that ginger has higher phenolic content than beet and carrot, F1 presented higher antioxidant capacity, TPC and TPC than F2.

Phenolic compounds profile by HPLC–DAD and HPLC–ESI–MS/MS

The target analytes were chosen based on an extensive bibliographic review on the main phenolic compounds present in fruits by-products [471–474]. Thirty-five standards commercially available were selected as being representative of these products (item 2.4.1).

HPLC analysis revealed that the FD by-products of two juices formulations (F1 and F2) are a complex mixture of phenolics. Several phenolic compounds, representative of diverse structural types, were identified. The chromatographic separation of the phenolic compounds present in the extracts is shown in Figure V.1.

For the HPLC–ESI–MS/MS method, standard solutions of phenolic compounds were individually and directly infused into the MS–MS detector and the signal was acquired between 50 and 1000 m/z. The characteristic ion precursor was selected for each compound and different collision energies were applied to obtain the typical ion products for each phenolic compound.

As seen above, of the 35 phenolic compounds investigated initially in the extracts obtained from the multi-fruits by-products, 10 were successfully separated by HPLC–DAD (Figure V.1), and 14 were identified by HPLC–ESI–MS/MS (Table V.2). As expected, these match to characteristic compounds of apple, carrot, beet and ginger, such as phenolic acids, flavan-3-ols, flavonols and tannins [471,472,474–476].

The phenolic acids identified in the extracts were divided into two groups: benzoic acid derivates and cinnamic acids. The first one was made of protocatechuic acid (peak 1) (λmax 300 nm). The cinnamic acids identified at λmax 300 nm were caffeic acid (peak 4), p-coumaric acid (peak 6) in F1 and the rosmarinic acid (peak 10) in F2 (Figure V.1).

The flavonols isoquercitrin (peak 7), rutin (peak 8) and quercitrin (peak 9) were detected at λmax 360 nm (Figure V.1); while myricetin and quercetin with [M–H]− at m/z 317.2 and 274.2, respectively, were detected only by HPLC–ESI–MS/MS (Figure V.2). Rice-Evans et al. [477] reported quercetin as a predominant component of onions and apples.

Peaks 3 and 5 (Figure V.1) (λ max 278 nm) correspond to catechin and epicatechin, both compounds showed the same [M–H]− at m/z 289.0 and product ions at m/z 244.9 and 203.0, characteristic of flavan-3-ol compounds [478]. In addition, epigallocatechin gallate with [M–H]− at m/z 457.1 and product ions at m/z 168.8 and 124.8 were identified (Figure V.2).

The by-products also contain procyanidin B1 (peak 2), which belongs to the subclass of condensed tannins, the compound was detected at λ max 278 nm (Figure V.1) and the flavanone naringenin was only detected by HPLC–ESI–MS/MS with [M–H]− at m/z 271.2 and product ions at m/z 150.9 and 119.0 (Figure V.2).

Grigoras et al. [473] found that the major phenolics in apples were benzoic acids (gallic acid), hydroxycinnamic acids (chlorogenic acid), flavanols (catechin), flavonols (rutin) and chalcones (phloridzin); the analyses were performed by HPLC–UV–ELSD and HPLC–MS. Of these, catechin and rutin were found in samples F1 and F2 (Figure V.1).

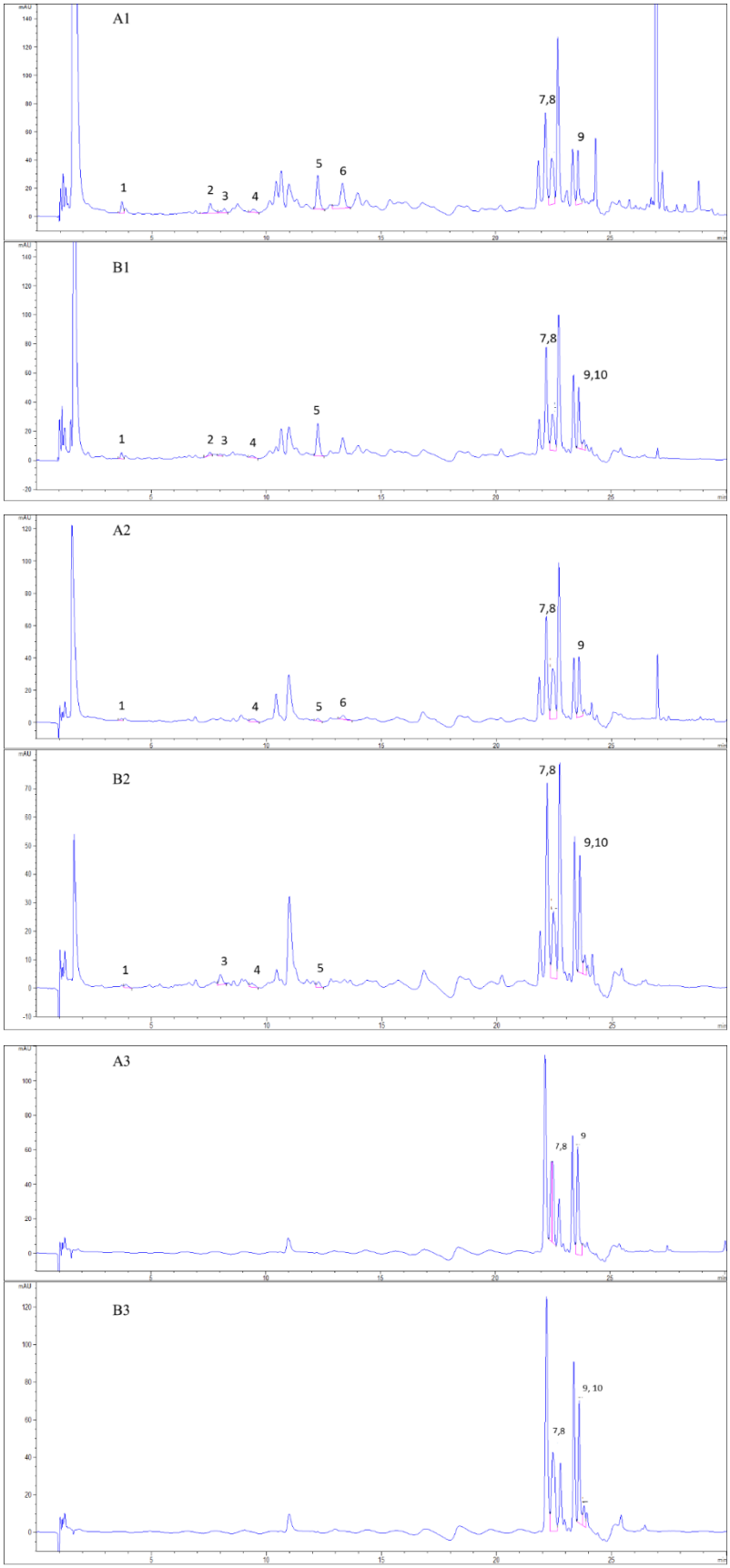

Figure V.1. HPLC chromatogram of the juices by-products F1 (a) and F2 (b) monitored at 278 (1), 300 (2) and 360 (3) nm. Peak 1—protocatechuic acid; peak 2—procyanidin B; peak 3—catechin; peak 4—caffeic acid; peak 5—epicatechin; peak 6—p-coumaric acid; peak 7— Isoquercitrin; peak 8—rutin; peak 9—quercitrin; peak 10—rosmarinic acid.

Table V.2. Identification of the phenolic compounds in the by-products of two juices formulations by HPLC–DAD and HPLC–ESI–MS/MS.

	Compound	t_r (min)	λ_{max} (nm)	Molecular formula	$[M–H]^-$ m/z	Main products m/z	Collision energy (V)	Structural subclass
1	Protocatechuic acid	4.1	260 294	$C_7H_6O_4$	153.0	109.0 108.0	-16 -26	Benzoic acid derivates
2	Procyanidin B1	8.7	280	$C_{30}H_{26}O_{12}$	577.0	407.0 288.7	-24 -25	Condensed tannins
3	Catechin	8.2	278	$C_{15}H_{14}O_6$	289.0	244.9 203.0	-17 -23	Flavan-3-ol
4	Caffeic acid	9.4	322	$C_9H_8O_4$	179.3	135.0 134.0	-65 -29	Cinnamic acids
5	Epicatechin	12.2	278	$C_{15}H_{14}O_6$	289.0	244.9 203.0	-18 -24	Flavan-3-ol
6	Epigallocatechin Gallate	12.2	274	$C_{22}H_{18}O_{11}$	457.1	168.8 124.8	-20 -42	Flavan-3-ol
7	*p*-coumaric	13.3	310	$C_9H_8O_3$	163.0	118.7 93.1	-16 -37	Cinnamic acids
8	Isoquercitrin	23.1	256 356	$C_{21}H_{20}O_{12}$	463.0	299.9 270.6	-27 -53	Flavonols
9	Rutin	22.4	256 356	$C_{27}H_{30}O_{16}$	609.1	299.8 270.7	-39 -58	Flavonols
10	Myricetin	23.2	372	$C_{15}H_{10}O_8$	317.2	151.0 178.8	-26 -22	Flavonols
11	Quercetrin	23.5	256 350	$C_{21}H_{20}O_{11}$	447.0	330.8 299.8	-25 -28	Flavonols
12	Rosmarinic acid	23.9	330	$C_{18}H_{16}O_8$	358.8	160.9 196.9	-20 -20	Cinnamic acids
13	Naringenin	24.7	290	$C_{15}H_{12}O_5$	271.2	150.9 119.0	-21 -31	Flavanones
14	Quercetin	24.8	256 372	$C_{15}H_{10}O_7$	274.2	88.1 70.2	+23 +26	Flavonols

t_r – retention time; λ_{max}– maximum absorption wavelengths; $[M–H]^-$ – molecular ions

In the work conducted by Tohma et al. [472], HPLC–MS/MS analysis showed that different phenolic acids were found in ginger, including pyrogallol, p-hydroxybenzoic acid, ferulic acid and p-coumaric acid. The *p*-coumaric acid was detected in sample F1 (Figure V.1), which is in higher proportion in ginger.

Char [471] stated that carrots contain mainly hydroxycinnamic acids, with chlorogenic acid as the main phenolic compound and the major flavonoids identified in orange carrots are quercetin, luteolin, kaempferol, and myricetin. Quercetin and myricetin were found in the analyzed samples (Figure V.2).

Beet contains coumaric and ferulic acids, as well as betalains and betacyanins, which are responsible for the red pigmentation in the plant [479]. However, in the extract F2, which contained beet, coumaric and ferulic acids were not detected.

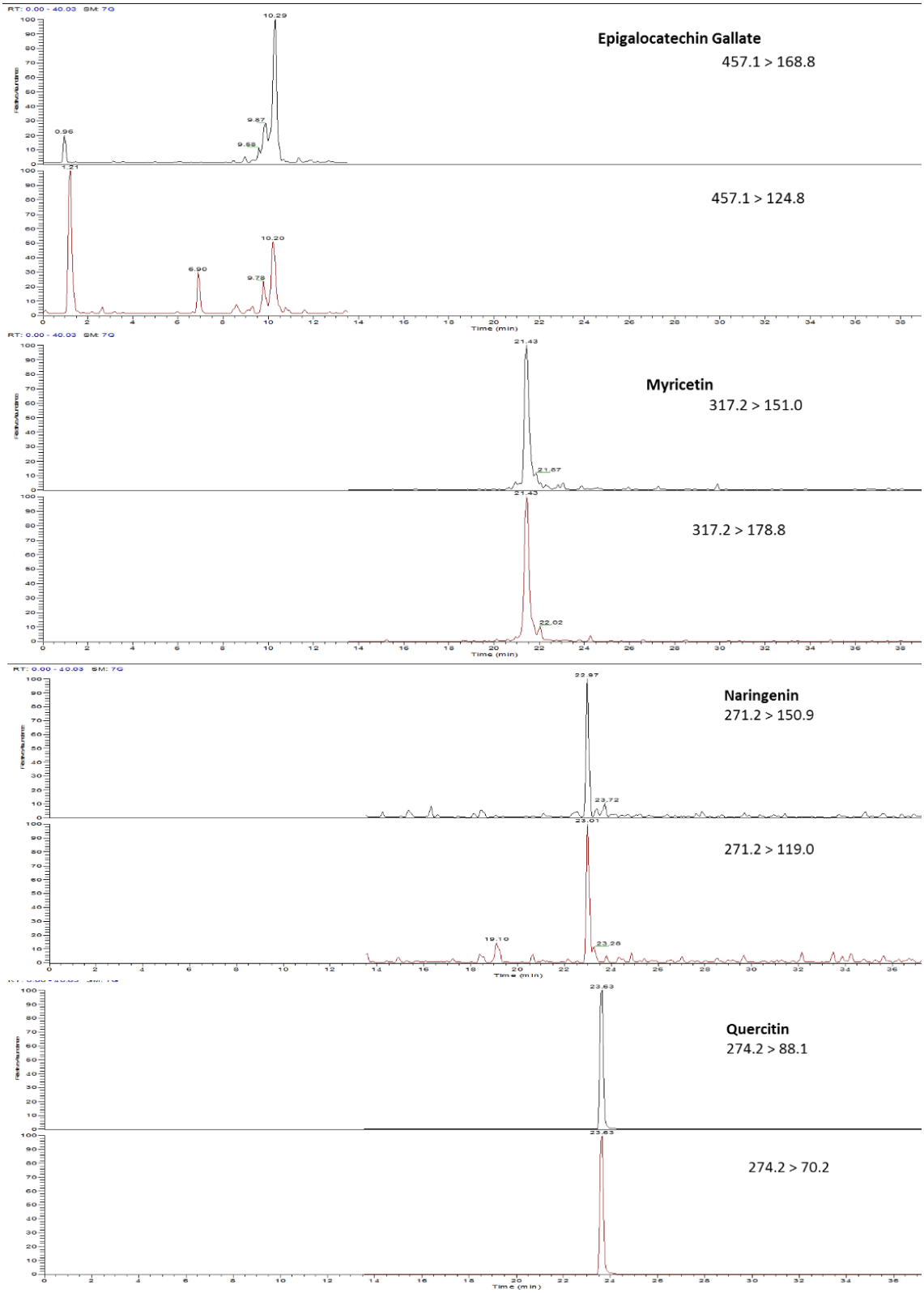

Figure V.2. Phenolic compounds in the by-product of F2 juice formulation detected by HPLC–ESI–MS/MS.

Individual phenolic compounds content

Limits of detection (LOD) and of quantification (LOQ), defined as a signal three and ten times higher than the noise level, respectively, were calculated in accordance with the Analytical Chemicals Subcommittee Guidelines (1980). LOD, LOQ, linearity and detection maximum absorption wavelengths of the 10 compounds identified in the samples by HPLC–DAD are presented in Table V.2.

Regarding the phenolic compound contents in the juice by-products, ten were quantified using HPLC–DAD, by the external standard method (Table V.3; Figure V.1) and the other four compounds that could not be quantified due to matrix interferences, were confirmed and quantified by HPLC–ESI–MS/MS (Table V.3;Figure V.1) by external calibration. Main phenolic compounds present in samples were flavonols. Isoquercitrin was found at a concentration range of 96–118 μg/g and quercitrin and rutin at a concentration range of 98–107 and 96 and 65 μg/g, respectively. All compounds were found in samples F1 and F2 (Table V.3), being flavanols the most representative group (around 50% of the total phenolic compounds). Within flavonols group, myricetin and quercetin were also detected in both samples. The flavan-3-ols was the second most prevalent phenolic subclass, with epicatechin at a concentration of 238 μg/g sample (F1) and 206 μg/g sample (F2); catechin at a concentration of 26 μg/g sample (F1) and 23 μg/g sample (F2) (Table V.3). The results of flavan-3-ols are close to those found for apple pomace from juice industry by Schieber et al. [480] who reported values of 140–190 μg/g for epicatechin and 9–14 μg/g for catechin. Epigallocatechin gallate was detected by HPLCESI– M/–MS only in the F2 sample at concentration of 0.0229 μg/g (Table V.3).

According to Rabetafika et al. [481], the apple by-products contain minor phenolic compounds with valuable antioxidant properties. In apple pomace, for example, the major phenolic compounds belong to the class of dihydrochalcones (phloridzin), flavanols (catechin and epicatechin) and hydroxycinnamic acid (chlorogenic acid). Flavonols, i.e. hyperin, quercetin and quercetin glycosides, are present in apple pomace and peels. Anthocyanins (cyaniding-3-galactoside) are mainly present in peels. Variation in the phenolic content can be due to the differences of cultivars, environmental factors, growth region, year of harvesting, and period and conditions of storage [482,483].

The condensed tannins (procyanidin B1) were found in greater proportions in FD F2 (105 μg/g) when compared to FD F1 (52 μg/g) (Table V.4). Schieber et al. [480] characterized several procyanidins from apple pomace by NMR spectroscopy and mass spectrometry, and only traces of procyanidin B1 and B5 were found in apple pomace and juice by mass spectrometry. However, Hellström et al. [484] did not detect in carrot

and beet proanthocyanidin. Therefore, it is assumed that the procyanidins present in extracts is probably from apples, which are bound to polysaccharides of the cell wall, according Rabetafika et al. [481]. Some phenolic constituents of apple pomace, especially the procyanidins and quercetin glycosides, have been shown to exert strong antioxidant capacity in vitro [485]. Tarko et al. [483] found that the high antioxidant capacity of apples is mostly connected with procyanidins and epicatechin.

Phenolic acids were present in smaller amounts. Among the benzoic acid derivates, the protocatechuic acid was present at concentrations of 17 and 7 μg/g, in the F1 and F2 extracts, respectively. Between the cinnamic acid derivates, the caffeic acid was present at concentrations of 6 and 5 μg/g, in the F1 and F2 extracts, respectively; the p-coumaric acid was present only in the F1 extract at concentrations of 6 μg/g and the rosmarinic acid was present only in the F2 extract at a concentration of 16 μg/g (Table V.4).

The sum of phenolic compounds concentrations determined by HPLC was similar in the two by-products: 646 (F1) and 600 μg/g (F2) (Table V.4).

Among the compounds of the flavon-3-ol subclass, catechin and epicatechin were present in higher quantities (16%) in the FD F1, which also had higher amounts of flavonols (isoquercitrin and rutin) (13%) and benzoic acid derivates (protocatechuic acid) (56%). The *p*-coumaric acid, a cinnamic acid derivative, was present only in the F1 extract. All these results suggest a higher antioxidant capacity of the F1 extract confirming previous results (Table V.1), which may be associated with the presence of quercetin glycosides [485] and epicatechin [483], exercising strong antioxidant capacity.

To better compare phenolic compounds content in the freeze-dried by-products by HPLC, the values of TPC and TFC were converted to mg GAE/g of freeze-dried by-product (mg GAE/g BP) and mg ECE/g of freeze-dried by product (mg ECE/g BP). The F1 by products presented 5.49 mg GAE/g BP and 5.67 mg ECE/g BP and the F2 by-products presented 2.04 mg GAE/g BP and 0.88 mg ECE/g BP. The quantification of the individual phenolic compounds corresponds to, approximately, 12% of the total phenolic compounds determined by the Folin−Ciocalteu method in the F1 freeze-dried by-products and 29% in the F2 freeze-dried by-products. Regarding the flavonoids, the quantification of the individual flavonoids corresponds to 10% of the TFC values for the F1 by-products and 56.25% of the TFC for the F2 by-products. These differences can be attributed to the non-specific method of determination of total phenolic compounds and moreover, because many of the peaks were not identified in the chromatography study.

Table V.3. Parameters of detection maximum absorption wavelengths (λ_{max}), linearity, LOD and LOQ from the phenolic compounds studied.

Phenolic Compound	**Detection λ_{max}(nm)**	**Equation**	**Concentration range (µg/mL)**	**r^2**	**LOD (µg/g sample)**	**LOQ (µg/g sample)**
Protocatechuic acid	300	y = 46.038x - 6.1504	0.05 – 20	0.9993	2.8	3.0
Procyanidin B1	278	y = 16.167x - 1.8538	0.05 – 20	0.9995	2.3	2.4
Catechin	278	y = 23.105x - 3.1991	0.10 – 20	0.9997	2.2	2.3
Caffeic acid	300	y = 115.13x - 10.003	0.05 – 20	0.9998	1.7	1.7
Epicatechin	278	y = 21.384x - 2.4887	0.05 – 20	0.9996	2.3	2.3
p-coumaric acid	300	y = 243.03x - 26.746	0.05 – 20	0.9996	2.2	2.2
Isoquercitrin	360	y = 52.545x - 5.218	0.05 – 20	0.9994	2.0	2.0
Rutin	360	y = 55.267x - 6.6429	0.05 – 20	0.9995	2.4	2.4
Quercetrin	360	y = 67.762x - 4.6164	0.05 – 20	0.9998	1.4	1.4
Rosmarinic acid	300	y = 117.46x - 21.473	0.05 – 20	0.999	3.7	3.7

r^2 – Coefficient of determination; *LOD* – Limit of determination; *LOQ* – Limit of quantification.

Table V.4. Total and individual phenolic compounds (μg/g dry basis) in the by-products of two juices formulations detected by HPLC–DAD and HPLC–ESI–MS/MS (approximate quantification).

Phenolic Compounds (μg/g dry basis)	**F1**	**F2**
*Benzoic acid derivates**	16.73 ±2.71	7.44 ± 0.94
Protocatechuic acid	16.73 ± 2.71[a]	7.44 ± 0.94[b]
*Cinnamic acid derivates**	11.98 ± 1.18	19.93 ± 1.77
Caffeic acid	5.51 ± 0.67[c]	4.62 ± 0.81[d]
p-coumaric acid	6.47 ± 1.34	nd
Rosmarinic acid	nd	15.53 ± 1.91
*Flavan-3-ols**	264.28 ± 32.63	221.58 ± 17.46
Catechin	26.11 ± 4.24	22.96 ± 1.57
Epicatechin	238.17 ± 31.65[e]	206.25 ± 12.87[f]
Epigalocatechin Gallate#	nd	0.0229
*Tannins**	52.46 ± 6.47	104.86 ± 17.15
Procyanidin B1	52.46 ± 6.47	104.86 ± 17.15
*Flavonols**	318.09 ± 14.87	272.90 ± 21.17
Isoquercitrin	117.86 ± 4.64[g]	95.57 ± 8.76[h]
Rutin	95.84 ± 7.91[i]	65.38 ± 5.14[j]
Quercitrin	97.84 ± 7.35	107.17± 11.44
Myricetin#	0.0216	0.2324
Quercetin#	6.535	2.67
Flavanones#	0.0722	0.04456
Naringenin#	0.0722	0.0456
Flavonoids total	*617.42 ± 42.55*	*553.17 ± 99.43*
Total	*646.13 ± 42.14*	*599.92 ± 75.56*

Results expressed as mean ± standard deviation or as mean. Values with different letters present statistical differences ($P < 0.05$). *F1* formulation 1 (apple + ginger, 50:50, w/w), *F2* formulation 2 (apple + carrot + beet + ginger, 50:29:20:1, w/w). *nd* not determined
* Sum of the compounds
# Determined by HPLC–ESI–MS/MS

Conclusion

With a fast and simple method, extracts with powerful antioxidant capacities were obtained from industrial fruits byproducts. The extracts obtained from the F1 FD by-products (50% ginger + 50% apple) presented the highest antioxidant capacity and the highest phenolic content. Fruits by-products have been the focus of several studies for their potential applications. However, only few studies have been carried out on industrial by-products. The industrial by-products obtained from the waste of fruit juices contain several phenolic compounds with vital and powerful biological properties and, as such, can and should be reused. However, precautions during their storage/transport must be taken in order to avoid their degradation and, subsequently, spoilage and waste. Further studies should be carried out to compare the by-products analyzed with those from other companies and with other formulations.

Chapter VI

Industrial Fruits By-Products and Their Antioxidant Profile: Can They Be Exploited for Industrial Food Applications?

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1. Introduction

Fruits are often presented to consumers in several forms, such as jams, juices, concentrates, and pastes. The manufacturing processes of these formulations do not use the fruit entirely, originating a large quantity of fruit by-products that, in turn, have to be discarded in a responsible and sustainable way, which may imply a significant increase in the final price of the product [184,285]. Additionally, fruit by-products have a low economic value and have proven biological activities derived from the presence of phenolic compounds, vitamins, carotenoids, among other active compounds. Phenolic compounds, chemically characterized for having at least one phenol unit, are present in most terrestrial plants, and they are responsible for the plant's defense against external stimuli such as radiation, predators and microorganisms [183,486]. The presence of these compounds is directly linked to the occurrence of antioxidant and antimicrobial activities, making these compounds of major interest for the food, cosmetic and pharmaceutical industries.

Apple is a well-known fruit of the genus *Malus* (family Rosaceae) [262] and one of the most-consumed fruits all over the world [455]. According to the Food and Agriculture Organization of the United Nations, the global production of apples was over 85 million tons in 2019 [487]. Although the majority is consumed as a fresh fruit, 25–30% are converted into processed products, with apple juice being the main product [488]. Apples represent an important source of bioactive compounds like pectins, dietary fibers, vitamins, oligosaccharides, triterpenic acids and phenolic compounds, such as flavonols, monomeric and oligomeric flavanols, dihydrochalcones, anthocyanidins, p-hydroxycinnamic and p-hydroxybenzoic acids [262,455]. Apples with a higher content in phenolic compounds tend to have a higher antioxidant capacity. The content in phenolic compounds varies with edaphoclimatic conditions (such as weather and water availability), cultivation practices, harvesting, storage conditions, and apple cultivars, the apple cultivar being the main factor in determining the content on bioactive compounds [455,489]. Furthermore, differences can also be found among the different parts of the apple, since the peel contains a higher content in phenolic compounds than the flesh [262]. Apple pomace, the mixture of peel, core, seed, calyx, stem and soft tissue resulting from apple juice production, is the main by-product generated, accounting for close to 25% of the fresh apple weight [473,488] and has approximately 20–30% of dried matter [490].

Lemons and oranges are other well-known fruits, belonging to the genus *Citrus*, with a production of more than 95 million tones worldwide, in 2019 [487]. Lemon is

mostly consumed as juice, originating a large quantity of lemon by-products, which are a very good source of dietary fiber, pectin, flavonoids, limonoids, coumarins and carotenoids [491]. Lemon essential oil can be obtained from lemon peels, which has proven antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* [492,493]. Oranges are also largely consumed in juice form, leaving a large trail of by-products. The orange peel, similar to lemon peel, is a good source of dietary fiber, pectin, phenolic acids and flavonoids, including polymethoxylated flavones and flavonols [494,495]. Dietary fiber is an important resource used in the prevention of cardiovascular diseases, diabetes, cancer and gastrointestinal disorders [496].

To delay the natural degradation of foods, the food industry resorts to antioxidant and antimicrobial additives, normally from a synthetic origin. The indirect and unaware consumption of these compounds has been associated with the promotion of carcinogenesis and their effects on human health due to long exposure are still unknown [29,177,200,285]. Therefore, it is important to find alternatives to these additives not associated with adverse health effects, such as extracts, and essential oils obtained from fruit by-products. Fruits are a good source of antioxidants, with important health benefits. Their by-products (peel, stems, and seeds) are also an excellent source of antioxidants [285,497]. However, there are not many studies on industrial fruit by-products. The majority of the studies are with specific parts of the by-products, such as peel, stems or seeds. Furthermore, there is not a method that can measure the antioxidant capacity precisely, therefore, different assays should be performed to obtain a more accurate result [498,499].

The main objective of this study was to obtain and determine three food-grade extracts from apple, lemon, and orange by-products and determine their antioxidant capacity. Moreover, the three extracts were chemically characterized, and their main compounds were quantified by UHPLC-ESI-MS/MS.

2. Materials and Methods

2.1. Fruits By-Product Extraction

The by-products of lemon, orange and apple were kindly supplied by the Portuguese juice company, Frubaça-Cooperativa de Hortofruticultores. Absolute ethanol was the chosen solvent for the production of the extracts since the main goal of the extract is to be applied directly or indirectly (through an active packaging) in foods. Ethanol is authorized by the Directive 2009/32/EC [500] in the extractions of bioactive compounds

to be applied in foods. The samples were first grinded and freeze-dried, followed by the extraction process. Briefly, 5 g of sample 50 mL of absolute ethanol was added, the mixture was agitated on a compact shaker (Edmund Bühler GmbH model KS-15, Hechingen, Germany) at 450 rpm for 30 min at room temperature (23 °C), protected from the light. Then, the mixture was centrifuged (Heraeus Multifuge X3 FR, Thermo Scientific, Langenbold, Germany) at 6000 rpm at 10 °C for 10 min. After that, the supernatant was removed to an amber pear-shaped flask and the ethanol was completely evaporated on a rotary evaporator (Büchi model R-210 Labortechnik, Switzerland) at 35 °C. The extract was removed with an aid of a spatula, held at 20 °C, protected from the light, until further use. To evaluate the antioxidant capacities of the different extracts, free radical DPPH inhibition and β-carotene bleaching assays were performed. In addition, the Total Phenolic Compounds (TPC) and the Total Flavonoid Content (TFC) were determined. To perform the antioxidant activity assays, the extracts obtained were dissolved in absolute ethanol, at a concentration of 3 mg/mL.

2.2. Antioxidant Activity

2.2.1. Free Radical DPPH Inhibition Assay

For the free radical DPPH inhibition assay, the method described by Moure et al. [501] and modified by Andrade et al. [183], was applied. Briefly, 2 mL of a DPPH methanolic solution (14.2 μg/mL) were added to 50 μL of the sample. The mixture was homogenized and kept in the dark for 30 min, at room temperature (23 °C). Absorbance was then measured at 515 nm using a spectrophotometer Evolution 300 UV-Vis (ThermoScientific™, England). A control assay was performed with the solvent in which the sample was dissolved. The inhibition percentage (IP) of DPPH was calculated according to the following Equation (1):

$$IP\ (\%) = \frac{AC - AA}{AC} \times 100 \quad (1)$$

where *Ac* is the absorbance of the control and *As* is the absorbance of the sample.

Furthermore, a calibration curve using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as a standard was drawn with a working range of 10–175 μg/mL.

2.2.2. β-Carotene Bleaching Assay

The β-carotene bleaching assay was performed according to the described by Miller [431] and modified by Andrade et al. [183] Firstly, a solution with 20 mg of linoleic acid, 200 mg of Tween®40 and 1 mL of β-carotene in chloroform (0.2 mg/mL) was prepared. The chloroform was evaporated on a rotary evaporator at 40 °C. Then, 50 mL of MilliQ™water was added, and vigorously agitated, until an emulsion was formed. Finally, to 200 μL of the sample, 5 mL of the emulsion was added. Afterwards, the absorbance of the control was measured at 470 nm and the mixtures were subjected to 50 °C for 120 min. The antioxidant activity coefficient (AAC) was calculated according to the Equation (2):

$$AAC = \frac{AA-AC2}{AC0-AC2} \times 1000 \quad (2)$$

where, *As120* is the absorbance of the sample after 120 min, *Ac120* is the absorbance of the control after 120 min and *Ac0* is the absorbance of the control at 0 min.

2.3. Total Phenolic Compounds Content (TPC)

The determination of the Total Phenolic Compounds Content was carried out according to the Erkan et al. [462] method. According to the method, 7.5 mL of an aqueous solution of Folin-Cioucalteu (10%, v/v) was added to 1 mL of sample. After 5 min, 7.5 mL of an aqueous solution of sodium carbonate (60 mg/mL, w/v) was added. Then, the samples were kept in the dark for 120 min, and the absorbance was measured at 725 nm. Gallic acid was used as a standard for the calibration curve, with a working range between 5–150 μg/mL.

2.4. Total Flavonoid Compounds (TFC)

The Total Flavonoid Content method was performed according to the Yoo et al. (2008) [463]method. To 1 mL of sample, 4 mL of MilliQ water and 0.3 mL of aqueous solution of sodium nitrite (5%, w/v) were added, and the solution was homogenized. After 5 min, 0.6 mL of aqueous solution of aluminum chloride (10%, w/v) were added and the solution was once again homogenized. After 6 min, 2 mL of sodium hydroxide (1 M, w/v) and 2.1 mL of MilliQ™ water were added. The solution was homogenized and the absorbance was measured at 510 nm. Epicatequin was used as a standard for the calibration curve with a working range between 5–125 μg/mL.

2.5. Identification of the Polyphenolic Compounds by UHPLC-ESI-MS/MS

The identification/tentative identification of phenolic compounds in the fruit byproducts extracts was performed with a UHPLC-ESI-MS/MS (Thermo Fisher Scientific, San José, CA, USA), equipped with a degasser, Accela quaternary pump, autosampler, and column oven, coupled to a triple quadrupole mass spectrometer TSQ Quantum Access max. The instrument control and data collection and processing were performed with Xcalibur 2.1 software (Thermo Fisher Scientific, San José, CA, USA).

A reverse-phase Kinetex® EVO C18 100Å column (150 x 3 mm internal diameter, 5 μm particle size) (Phenomenex, Torrance, CA, USA) was used for phenolic compound separation at 30 °C, according to Andrade et al. [497] The injection volume was 20 μL, and the mobile phase flow rate used was 0.6 mL/min. The solvents used as mobile phase were water (solvent A) and methanol (solvent B), both acidified with formic acid at 0.1% (v/v). The gradient elution used was as follows: 95% A; 3 min, 90% A; 10 min, 80% A; 18 min, 70% A; 25 min, 30% A; 33 min, 0% A; 33–40 min, 0% A and 100% B isocratic; and finally, the column was washed and reconditioned with 95% A (40–46 min).

The mass spectrometer electrospray ionization source (ESI) operated in both negative and positive mode, according to the nature of the phenolic compound. The optimized MS/MS detector settings were as follows: spray voltage 2500 V; vaporizer and capillary temperatures were set at 340 °C and 350 °C, respectively. Nitrogen (purity > 99.98%) was used as sheath gas (pressure 35 psi) and as auxiliary gas (the pressure set 10 arbitrary units), and Argon as the collision gas (1.5 mTorr).

The MS/MS data acquisition was performed in a Single Reaction Monitoring (SRM) mode. After the first screening at MS scan range of 100–800 m/z, tentative identification of polyphenols was accomplished by comparing their precursor ion $[M-H]^{-1}$ and mass spectrometry fragmentation pattern (MS/MS) with those already described in the literature. The identification of the individual phenolic compounds was accomplished by comparison of the retention time with those obtained by injecting pure standards, when available, under the same chromatographic conditions, and with the molecular ion and productions data provided by MS/MS analysis.

2.6. Quantification of the Polyphenolic Compounds by HPLC-DAD/UV

The quantification of phenolic compounds was performed with an Agilent HPLC system 1100 (Hewlett-Packard, Waldbronn, Germany), equipped with a quaternary pump, a

degassing device, an autosampler, a column thermostat system, coupled to a diode array detector (DAD), and controlled by HP ChemStation software (version B.03.0.1). The column and chromatographic conditions used were the same described above for UHPLC-ESI-MS/MS analysis. DAD spectra acquisition was performed continuously in full scan modality during the run time ranging from 200 to 400 nm. The identification of individual phenolic compounds was achieved by comparing their retention times and the UV spectrum (λ_{max}) characteristics of the different family of phenolic compounds or with that obtained with commercial standards injected under the same chromatographic conditions, whenever available. Phenolic compounds were monitored and quantified at 230, 278, 300, 325, and 360 nm. Quantification was carried out by the external-standard method with six-point calibration curves.

2.7. Statistical Analysis

All experiments were conducted using a completely randomized design with three replications. Statistical analysis of data was performed through a one-way analysis of variance (ANOVA) using the Software IBM®SPSS® Statistics, version 26.0.0.0, and differences among mean values were processed by the Tukey test. All requirements necessary to carry out the ANOVA (namely, normality of data and homogeneity of variances) have been validated. Significance was defined at $P < 0.05$. Results are expressed as the means of the replicants ± standard deviation.

3. Results and Discussion

3.1. Antioxidant Capacity

In this study, four assays were performed for a better characterization of the antioxidant. For antioxidant potential, DPPH radical scavenging capacity and β-carotene bleaching assay were performed. Besides that, TPC and TFC were quantified for antioxidant potential assessment. For all the assays, the extracts were analyzed in the concentration of 3 mg of extract per mL of ethanol.

Table VI.1 shows the IP of DPPH and the Trolox Equivalent (TE) for all the extracts. Lemon extract presented the highest IP of DPPH radical (51.67 ± 4.61%) followed by the apple extract (39.92 ± 1.68%) and orange extract (31.20 ± 1.28%). The DPPH radical scavenging capacity assay measures the reducing capacity of antioxidants.

Different results were obtained in other studies. Albuquerque et al. [502] evaluated a water extract obtained from industrial oranges by-products. The authors found lower values (898.9 μmol Trolox/L fruit by-products water extracts) when

compared to the ethanolic extract of the orange by-products. This can be explained by the use of different extraction solvents in the two studies. Guimarães et al. [30] evaluated the antioxidant capacity of orange and lemon peel essential oils. The authors obtained good EC_{50} values for orange (95.67 ± 2.21 mg/mL) and lemon (116.25 ± 10.56 mg/mL). M'hiri et al. [491] studied the effects of different drying processes on the antioxidant activity of industrial lemon by-products. The authors concluded that all the drying processes decreased the total content of phenolic compounds, and antioxidant radical scavenging activity, supporting the room temperature extraction procedure of active compounds, such as the method used in the present study [491].

Regarding the β-carotene bleaching assay, orange extract (3 mg/mL) presented the highest AAC (237.21 ± 29.78) (Table VI.2). The β-carotene bleaching assay is also based on color change. In the absence of antioxidants, the free linoleic acid radical bonds to the β-carotene molecule and the orange color fade.

The lemon by-products extract presented the highest TPC (43.38 ± 0.84 mg GAE/g) from the studied extracts. The TPC of orange and apple were 23.32 ± 0.18 mg GAE/g and 14.02 ± 0.13 mg GAE/g, respectively (Table VI.2). Phenolic compounds are recognized for their contribution as one of the most important antioxidants in the diet [502,503]. Therefore, it is essential to quantify the TPC presented in the food and its by-products. Guimarães et al. [504] analyzed lemon and orange peel and obtained a higher value of TPC, 87.77 mg/g extract and 79.75 mg/g, respectively. Li et al. [505] analyzed lemon and orange peel too, and the results presented as fresh matter were 118.75 mg/g and 73.59 mg/g, respectively. M'hiri et al. [491] analyzed lemon by-products and for TPC the results were 5.52 g/100 g as dry matter. On the other hand, the TPC obtained for apple by-products in this study were higher than the ones of Diñeiro García et al. [482]. Raudone et al. [455] quantified the TPC in apple by-products and the result was 31.01 mg/g as dry weight. It is also important to identify the individual phenolic compounds present in the fruits' by-products.

The TFC of lemon, orange, and apple were 20.76 ± 0.61 mg ECE/g, 7.29 ± 0.32 mg ECE/g, and 24.63 ± 1.61 mg ECE/g (Table VI.2). Apple extract showed the highest TFC and orange extract showed the lowest TFC. Flavonoids are important phytonutrients too. The results obtained in this study for lemon by-products were higher than those from Guimarães et al. [504] and M'hiri et al. [491] obtained 4.35 g/100 g as dry matter for TFC. For orange by-products, there were studies with higher and lower values than those obtained in this study [495,504]. No studies with TFC for apple by-products were found in the literature.

Dissimilarities in all results can be due to different fruits' variability and their degrees of maturation. External factors such as climate, soil and fertilization applied can also affect the results. Apart from these, the results can be affected by the variability in the solvents used for the extractions and changes in the methods used [506–509].

In general, all three fruit by-products presented a good source of antioxidant compounds, able to be incorporated as dry extracts in food and in food packaging. However, from the studied industrial by-products, lemon extract was revealed to have the greatest potential as an antioxidant extract.

Table VI.1. DPPH radical scavenging capacity of different fruit by-products. The results are expressed as mean of three replicas ± SD. Different letters indicate statistical differences.

Fruits by-products	Inhibition percentage (%)	Trolox Equivalent (mg Trolox/g of extract)
Lemon	51.67 ± 4.61 [a]	33.17 ± 2.94 [d]
Orange	31.20 ± 1.28 [b]	20.13 ± 0.43 [e]
Apple	39.92 ±1.68 [c]	25.69 ± 0.56 [f]

Table VI.2. Antioxidant capacity and characterization of different fruit by-products. The results are expressed as Mean ± SD. Different letters indicate statistical differences.

Fruits by-products	β-carotene bleaching assay	TPC (mg GAE/g)	TFC (mg ECE/g)
Lemon	67.35 ± 1.96 [a]	43.38 ± 0.84 [c]	20.76 ± 0.61 [f]
Orange	237.21 ± 29.78 [b]	23.32 ± 0.18 [d]	7.29 ± 0.32 [g]
Apple	107.44 ± 23.81 [a]	14.02 ± 0.13 [e]	24.63 ± 1.61 [h]

3.2. Chromatographic Polyphenolic Profile of the Fruit By-Products

The phenolic compounds of fruit by-products identified or tentatively identified by HPLD-DAD and UHPLC-ESI-MS/MS are described in Table VI.3. The identification of phenolic compounds was based on the elemental composition data determined from accurate mass measurements in negative ionization mode and comparison with the literature and that obtained with the available standards, except for compound 25 (Quercetin), which was measured in the positive mode as described previously by Andrade et al. [497] Each compound was characterized by its retention time (Rt), maximum absorption wavelengths (λ_{max}), structural class, molecular formula, molecular ion, and main MS/MS fragments. The peak names of the Table 3 correspond to the peak labels of the chromatograms obtained at 278 nm by HPLC–DAD for each fruit by-product represented in Figure VI.1. In this work, a total of 26 compounds (19 for orange,

18 for lemon, and 16 for apple by-products) from different classes of polyphenols were identified, including phenolic acids (benzoic acid derivates, hydroxycinnamic acids derivatives, and their glycosides) and flavonoids (flavonols, flavones, flavanones, and dihydrochalcones, as well as their glycosides). The confirmation of the identity of 17 polyphenols was achieved by a comparative analysis of authentic standards based on compounds retention time, the UV–visible spectra, and MS/MS fragmentation patterns.

3.2.1. Benzoic Acid Derivates

The benzoic acid derivates identified in the fruit by-products analyzed were protocatechuic acid (compound 1) and hydroxybenzoic acid (compound 2) detected at UV λ_{max} 293 and 255 nm, respectively. The identification was performed by a comparison of their retention times and MS/MS fragmentation patterns with standards. The hydroxybenzoic acids are widely distributed in nature and have been identified by other authors in citrus and apple fruits and products [510–512]. On the other hand, protocatechuic acid has been described in apple fruits and less in citrus. Indeed, this study identifies for the first time protocatechuic acid in orange by-products.

3.2.2. Hydroxycinnamic Acids and Their Glycosides

Several compounds from the group of hydroxycinnamic acids were identified in the fruit by-products by comparison of their retention times, UV–visible typical spectra at λ_{max} 325 nm, and MS/MS fragmentation patterns with standards. Caffeic and *p*-coumaric acids (compounds 3 and 8, respectively) were identified in all fruit by-products analyzed. Chlorogenic acid (compound 4) was found in lemon and apple by-products, while ferulic acid was determined just in orange by-products. Rosmarinic acid was found for the first time in orange by-products besides apple by-products [511]. Compound 5, with $[M-H]^-$ ion at *m*/*z* 355 and the MS/MS fragment 193 *m*/*z* from ferulic acid, was tentatively identified as ferulic acid-O-hexoside. Additionally, compound 7, with $[M-H]^-$ ion at m/z 385 and the MS/MS fragment 223 m/z from sinapic acid, was identified as sinapic acid-O-hexoside. These hydroxycinnamic acid glycosides were already described for orange pulp and juices by De Ancos et al. [513]. Both compounds were considered for the first time for orange and lemon by-products in this work.

3.2.3. Flavanones Glycosides

Together with hydroxycinnamic acids, flavanone glycosides were the main group of phenolic compounds present in the by-products analyzed in this study, mainly in those obtained from citrus fruits.

Compound 24, with $[M-H]^-$ ion at *m*/*z* 271, MS/MS fragment 151 *m*/*z*, and the UV–visible spectra typical at λ_{max} 290 nm, was identified as Naringenin in all by-products analyzed (orange, lemon, and apple). Besides, compound 11, with $[M-H]^-$ ion at *m*/*z* 595 that displayed the same fragmentation pattern in negative ionization mode that results in the fragment 151 *m*/*z*, was identified as eriodyctiol-O-rutinoside (eriocitrin). For this compound, the UV–visible spectrum showed two λ_{max} at 290 and 330 nm, which are characteristic of flavanone glycosides and are usual to the following compounds identified in this group of phenolics. Naringenin-7-O-rutinoside (narirutin) (compound 13), with $[M-H]^-$ ion at *m*/*z* 579, and naringenin-7-O-glucoside (prunin) (compound 20), with $[M-H]^-$ ion at *m*/*z* 433, showed the same fragmentation pattern that results in the fragment 271 *m*/*z* of naringenin [512].

The MS/MS fragmentation of hesperidin (compound 15) and neohesperidin (compound 18), with the identical $[M-H]^-$ ion at *m*/*z* 609, results in the same fragment 286 *m*/*z* and the UV–visible spectra λ_{max} at 290 and 355. On the other hand, compound 23 was tentatively identified as isosakuranetin-7-O-rutinoside (dydimin), with $[M-H]^-$ ion at *m*/*z* 593, and MS/MS fragment 285 *m*/*z*, as described by De Ancos et al. [513] for orange.

The identity of compounds 11, 15, and 24 (eriocitrin, hesperidin, and naringenin, respectively) was confirmed by comparison with the retention time and fragmentation pattern of commercial standards. Naringenin and prunin were identified in all by-products analyzed, while neohesperidin and dydimin were found just in orange by-products. Eriocitrin, narirutin, and hesperidin were identified in citrus by-products (orange and lemon).

The identification of phenolics, for which standards were not available, was supported by recent studies found in the literature on these groups of compounds described for citrus peels (orange and lemon) [513–515], besides some studies for apple products including peels [510,512,516].

3.2.4. Flavonols and Flavonol Glycosides

The MS1 scan spectra, the UV–visible spectra typical at λ_{max} 270 nm and 360 nm, and the same fragmentation pattern in negative ionization mode that results in the fragment

301 *m/z* in negative ionization mode compared with those of authentic standards determined that compounds 17, 19, and 22 are flavonols glycosides. Quercetin-3-O-rutinoside (rutin) (compound 19) and quercitrin (compound 22) standards allowed the identification of these flavonols in all by-products analyzed (orange, lemon, and apple). These phenolics have been described in other studies for orange, lemon, and apple products/byproducts [512]. Despite isoquercetin (compound 17) being described in apple fruit by Sommella et al. [516], in this study, it was just found in orange by-product and confirmed by the standard of reference. The identification of the aglycone quercetin (compound 25) in all fruit by-products was achieved by comparing the data with that obtained from the authentic standard. On the other hand, compound 14, with a $[M-H]^-$ ion at *m/z* 593, was tentatively identified as kaempferol-3-O-rutinoside in orange and apple by-products based on the MS/MS fragment 285 *m/z*, the UV–visible spectra typical at λ_{max} 356 nm and supported by literature where this flavonol glycoside was described before for apple fruit [512].

3.2.5. Others (Flavones and Glycosides, Dihydrochalcone Glycosides and Flavan-3-ols)

Compound 10 showed UV–visible spectra typical of flavones, and the MS1 spectra revealed a high intensity $[M-H]^-$ ion at *m/z* 593. Moreover, the comparison of the relative absorbance at 270 and 340 nm allowed the identification flavone nature of the phenolic compound. Additionally, the fragment ion at *m/z* 473 described in the literature for di-C-glucoside flavanone confirmed the identification of this compound as apigenin-6,8-di-C-glucoside, more commonly identified as Vicenin-2. Apigenin-6,8-di-C-glucoside was previously identified in pulp and juices of orange and mandarins by De Ancos et al. [513], but was described for the first time in this study for orange and lemon byproducts. Compound 26, with $[M-H]^-$ ion at *m/z* 269, was identified as another flavone, the aglycone apigenin, that followed the same fragmentation pattern of the reference standard that results in the fragment 117 *m/z* in negative mode. This compound was described before for orange products such as pulp, juice, and peels [512,513]. Still, in this study, apigenin was detected for the by-products obtained from lemon and apple fruits. Phenolic compounds 12 and 16, with $[M-H]^-$ ion at *m/z* 567 and $[M-H]^-$ ion at *m/z* 435, respectively, followed the same fragmentation pattern that results in the fragment 273 m/z, were tentatively identified as dihydrochalcone glycosides. Compound 12 was, tentatively, identified as phloretin-O-apiofuranosyl-glucopyranoside, and compound 16 was identified as phloretin-20-O-beta-glucoside (phlorizin) by comparison with the

reference standard. These compounds have been described in the literature for apple pomace and were detected in the apple by-product analyzed in this study [512,517].

Finally, also exclusive for apple by-product, phenolic compound 6 with the $[M-H]^-$ ion at *m/z* 289, the fragment ions at *m/z* 245 and 203, and the UV–visible spectra typical of flavan-3-ols at λ_{max} 278 nm, was identified as epicatechin and its identity confirmed with the commercial standard. Epicatechin was also already described in the literature for apple products [512,516].

3.3. Quantitative Distribution of Polyphenolic Compounds in Fruit By-Products

The quantification of phenolic compounds, for which the standards were available, was performed by HPLC-DAD following the parameters described in Table VI.4. The results on total and 15 individual phenolic compound contents are shown in Table VI.5. The total amount of phenolic compounds varied according to the nature of the by-product as follows: lemon (20,969 μg/g) < orange (5393 μg/g) < apple (894.8 μg/g). The results were in agreement with those obtained in the spectrophotometric assays of total phenolic compounds, even though several compounds present in the extracts were not identified and quantified (see Figure VI.1). The polyphenol content determined for lemon by-products using TPC assay was 2-fold higher than orange. However, according to the determination by HPLC-DAD, the lemon content was 4-fold higher than orange, since a substantial number of polyphenols in the orange extract were not able to be quantified.

Benzoic acid derivates and flavanones glycosides were the most representative phenolics in the orange by-product, while for the lemon by-product, the most abundant were hydroxycinnamic acids, flavanone glycosides, and flavonol glycosides. The hydroxycinnamic acids and flavonol glycosides were the most relevant groups of compounds present in apple by-products, together with the flavanols glycosides and dihydrochalcone glycosides, which were exclusively present for this by-product.

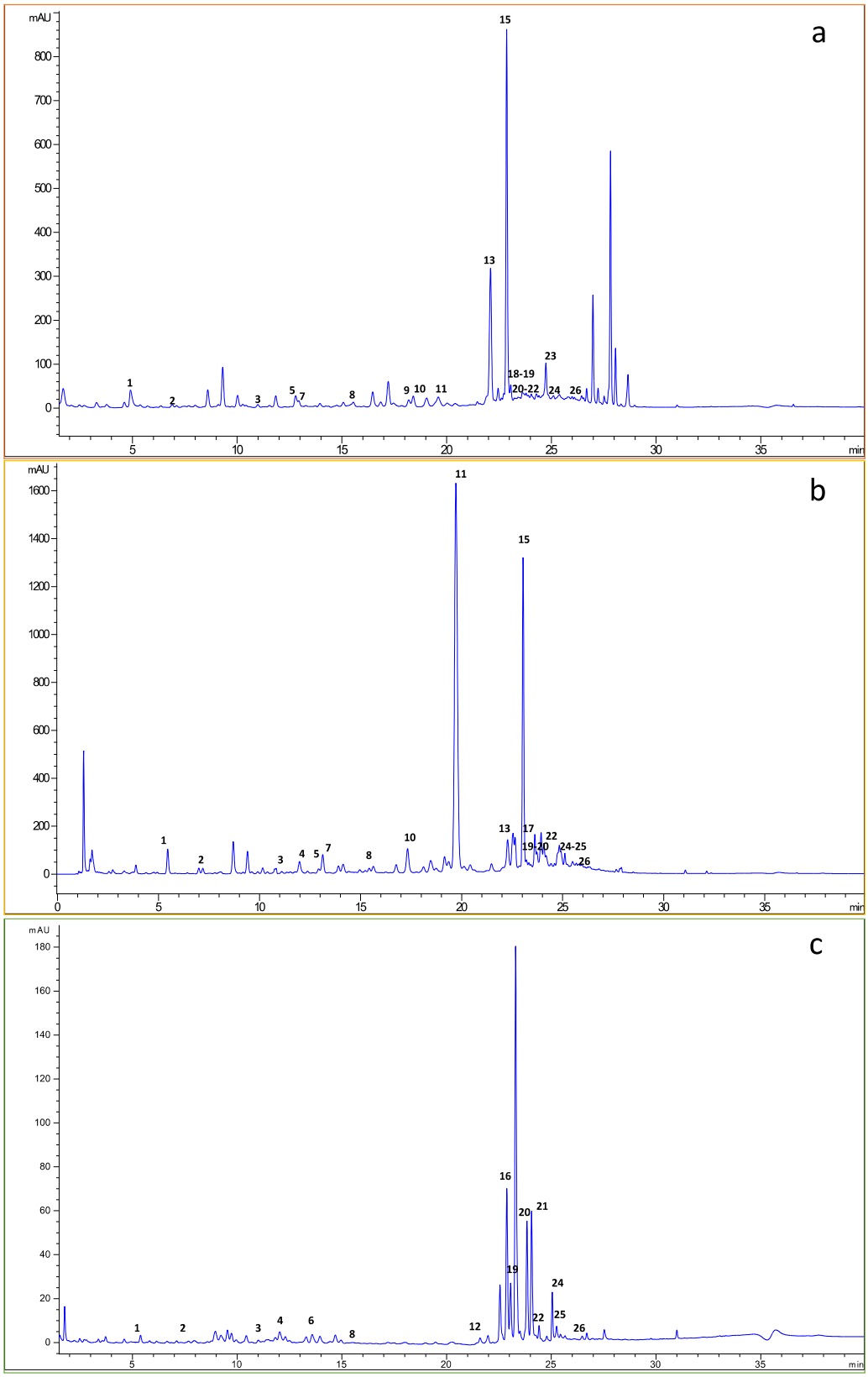


Figure VI.1. HPLC-DAD chromatograms of fruit by-products (a) orange, (b) lemon, and (c) apple, recorded at 278 nm.

Table VI.3. Phenolic compounds identified in the fruit by-products from orange, lemon and apple by HPLC-DAD and UHPLC-ESI-MS/MS.

Peak	R_t (min)	λ_{max}	[M-H] (*m*/z)	Main MS/MS fragments (*m*/z)	Molecular formula	Structural class	Tentative identification	Byproduct			Confirmation/ Ref.§
								Orange	Lemon	Apple	
1	4.31	260, 293	153	109, 108	$C_7H_6O_4$	Benzoic acid derivates	Protocatechuic acid	✓	✓	✓	38, 39, Std*
2	7.09	255	137	93, 65	$C_7H_6O_3$	Benzoic acid derivates	Hydroxybenzoic acid	✓	✓	✓	38-41, Std*
3	9.59	325	179	134, 135	$C_9H_8O_4$	Hydroxycinnamic acids	Caffeic acid	✓	✓	✓	39,40,46, Std*
4	9.99	250, 325	353	191, 173	$C_{16}H_{18}O_9$	Hydroxycinnamic acids	Chlorogenic acid		✓	✓	[38–40], Std*
5	11.42	240, 330	355	193	$C_{16}H_{20}O_9$	Hydroxycinnamic acids glycosides	Ferulic acid-*O*-hexoside	✓	✓		41
6	11.64	280	289	245, 203	$C_{15}H_{14}O_6$	Flavan-3-ols	Epicatechin			✓	[40,44], Std*
7	11.8	270, 330	385	223	$C_{17}H_{22}O_{10}$	Hydroxycinnamic acids glycosides	Sinapic acid-*O*-hexoside	✓	✓		41
8	13.39	310	163	119, 93	$C_9H_8O_3$	Hydroxycinnamic acids	p-Coumaric acid	✓	✓	✓	[38–40,46], Std*
9	15.26	325	193	134	$C_{10}H_{10}O_4$	Hydroxycinnamic acids	Ferulic acid	✓			[38–40,46], Std*
10	15.68	270, 340	593	473	$C_{26}H_{28}O_{14}$	Flavone glycosides	Apigenin-6,8-di-*C*-glucoside (Vicenin-2)	✓	✓		41
11	17.95	285, 330	595	287, 151, 135	$C_{27}H_{32}O_{15}$	Flavanone glycosides	Eriodyctiol-*O*-rutinoside (Eriocitrin)	✓	✓		[41,46,47], Std*
12	20.29	270, 350	567	273	$C_{26}H_{31}O_{14}$	Dihydrochalcone glycosides	Phloretin-*O*-apiofuranosyl-glucopyranoside			✓	40
13	20.56	290, 330	579	271	$C_{27}H_{32}O_{14}$	Flavanone glycosides	Naringenin-7-*O*-rutinoside (Narirutin)	✓	✓		40-42
14	21.00	356	593	285	$C_{27}H_{30}O_{15}$	Flavonol glycosides	Kaempferol-3-*O*-rutinoside	✓		✓	40
15	21.36	290, 355	609	300, 286, 242	$C_{28}H_{34}O_{15}$	Flavanone glycosides	Hesperetin-7-*O*-rutinoside (Hesperidin)	✓	✓		[40–43,46,47], Std*

Peak	R_t (min)	λ_{max}	[M-H] (*m*/*z*)	Main MS/MS fragments (*m*/*z*)	Molecular formula	Structural class	Tentative identification	Byproduct			Confirmation/ Ref.§
								Orange	Lemon	Apple	
16	21.37	278	435	273, 167, 123	$C_{21}H_{24}O_{10}$	Dihydrochalcone glycosides	Phloretin-2'-*O*-beta-glucoside (Phlorizin)			✓	[40], Std*
17	21.49	360	463	301, 271	$C_{21}H_{20}O_{12}$	Flavonol glycosides	Quercetin-3-*O*-glucoside (Isoquercetin)		✓		[38], Std*
18	21.52	290, 355	609	286	$C_{28}H_{34}O_{15}$	Flavanone glycosides	Hesperetin-7-*O*-neohesperidoside (Neohesperidin)	✓			42
19	21.58	255, 360	609	300, 271	$C_{27}H_{30}O_{16}$	Flavonol glycosides	Quercetin-3-*O*-rutinoside (Rutin)	✓	✓	✓	[40,41,43,44,46,47], Std*
20	22.28	270, 350	433	301, 271	$C_{21}H_{22}O_{10}$	Flavanone glycosides	Naringenin-7-*O*-glucoside (Prunin)	✓	✓	✓	40
21	22.49	250, 330	359	197, 161	$C_{18}H_{16}O_8$	Hydroxycinnamic acids	Rosmarinic acid	✓		✓	[39], Std*
22	22.51	250, 350	447	331, 300, 301	$C_{21}H_{20}O_{11}$	Flavonol glycosides	Quercetin-3-*O*-rhamnoside (Quercitrin)	✓	✓	✓	[38,40,44,45], Std*
23	23.19	285, 330	593	285	$C_{28}H_{34}O_{14}$	Flavanone glycosides	Isosakuranetin-7-*O*-rutinoside (Dydimin)	✓			41
24	23.71	295	271	151, 119	$C_{15}H_{12}O_5$	Flavanone	Naringenin	✓	✓	✓	[40–42,44,46], Std*
25	23.84	270, 360	274	70, 88	$C_{15}H_{10}O_7$	Flavonols	Quercetin	✓	✓	✓	[21,38,40,41,47], Std*
26	24.9	280, 360	269	117	$C_{15}H_{10}O_5$	Flavones	Apigenin		✓	✓	Std*

Rt - retention time; λ_{max} - maximum absorption wavelengths; $[M–H]^-$ - molecular ions; ✓ - indicates the presence of the compound identified; §Ref - references used to support tentative identification of compounds and Std* (standards available) to confirm the identification.

The by-product obtained from lemon fruits was that with higher amounts of hydroxycinnamic acids (407 μg/g of by-product), manly chlorogenic acid (386.7 μg/g), followed by the apple by-product (128.0 μg/g), which showed higher amounts of rosmarinic acid (88.62 μg/g), besides chlorogenic acid (39.41 μg/g). These amounts in apple by-products could justify the highest antioxidant capacity of apple extract rather than the orange extract described in Section 3.1 for the DPPH assay. Similar contents of chlorogenic acid were determined in lemon peels by Xi et al. [518] in different lemon varieties, but not in other parts of the lemon fruit such as the pulp or juice that were more than 10-fold lower. On the other hand, the orange by-product was that with higher amounts of caffeic, ferulic, and p-coumaric acids, but the total amount (80.53 μg/g) was the lowest among the fruit by-products analyzed. These results were in agreement with those observed in the recent study on apple and orange peels, where the total amounts of phenolic acids, and in particular chlorogenic acid, were higher for the apple rather than orange peels [510]. Still, a high amount of protocatechuic acid (317.3 μg/g) was determined, in this study, for the orange by-product. These authors observed the same tendency for benzoic acid derivates such as hydroxybenzoic acid. However, the concentrations on orange and apple peels determined by these authors were higher than those observed in the by-products analyzed in this work.

Taking into account the flavanone glycosides as the most relevant group of compounds for citrus by-products, eriocitrin, hesperidin, and naringenin were those quantified. The compound present in high quantities in the orange by-product was the hesperidin (4901 μg/g), followed by naringenin, and finally Eriocitrin, in a total amount of 4956 μg/g. The content of hesperidin was up to 3-fold higher than those described by Molina-Calle et al. (2015) [514] for orange peels from different varieties in the range of 1200 and 1800 μg/g. De Ancos et al. [513] also determine these compounds for orange pulps and juices from different varieties (49–434 μg/g). Nevertheless, the results could not be compared with the literature since the authors express the results as fresh weight. However, the amounts of hesperidin were also significantly higher than eriocitrin for pulps and juices. On the other hand, the most abundant compound in lemon by-products was the eriocitrin (17,493 μg/g), followed by hesperidin (2728 μg/g), and finally naringenin (42.12 μg/g), in a total amount of flavanone glycosides of 20,263 μg/g. The amount of hesperidin was lower than that observed for the orange by-product. The amounts were lower than those described by Gómez-Mejía et al. [515], but these authors also observed that hesperidin was 2-fold higher in orange peels than in lemon peels.

Considering the flavonols glycosides, considerable amounts were determined for lemon by-product (262.3 μg/g), followed by apple (172.7 μg/g), and finally orange (39.40 μg/g). Isoquecetin and quercitrin were the most abundant in lemon with concentrations of 111.6 μg/g and 106.0 μg/g, respectively. The main compound in apple by-product was the quercitrin (150.3 μg/g), while in orange was the rutin (31.43 μg/g). Similar results for the rutin ratio among citrus samples (lemon and orange) have been described in the literature. However, the amounts depended on the variety or part of the fruit analyzed [515,518]. The amounts of quercitrin in apple by-product (150.3 μg/g) were more than 12-fold higher than those determined by Li et al. [517] in 7 varieties of apple flesh (2.7–12.4 μg/g). These authors also determined the epicatechin content for all varieties, and the values ranged from 5.8 to 80.7 μg/g. The amount of epicatechin in the apple by-product analyzed in this study was in the range with a concentration of 31.24 μg/g. This concentration of epicatechin could be related to the high values of TFC described for apple by-products in Section 3.1 and might display a higher response to this assay. These results highlighted that the chromatographic analysis should be considered to complement and achieve a correct characterization of extracts μg/g and therefore avoid the potential lack of specificity of spectrophotometric assays.

Finally, phlorizin was the main phenolic compound quantified in apple by-product (542.0 μg/g), significantly higher than those observed in the apple fruit (11.4–40.9 μg/g). These compounds also may be related to the high antioxidant capacity of the apple byproduct on the DPPH assay.

Since the phenolic composition of the fruits and their products may change with the variety and these by-products obtained from the food industry may result from a mix of varieties, predicting the content of polyphenols and their antioxidant potential could be a complex task. Therefore, a complete characterization of the by-products by chromatography is mandatory before their application as a food ingredient or additive.

Table VI.4. Analytical parameters of detection maximum absorption wavelength (λ_{max}), linearity, LOD, and LOQ employed to determine bioactive phenolic compounds studied.

Phenolic Compound	**Detection λ_{max} (nm)**	**Slope**	**Intercept**	**R^2**	**Concentration range (µg/mL)**	**LOD (µg/g byproduct)**	**LOQ (µg/g byproduct)**
Protocatechuic acid	300	40.63	-4.334	0.9980	0.05 – 20	0.04	0.10
Hydroxybenzoic acid	278	210.0	-14.52	0.9990	0.10 – 20	0.10	0.20
Caffeic acid	325	146.6	-40.50	0.9980	0.10 – 20	0.10	0.21
Chlorogenic acid	325	61.58	-14.27	0.9989	0.10 – 20	0.10	0.20
Epicatechin	278	21.66	-2.006	0.9996	0.10 – 20	0.10	0.20
p-Coumaric acid	325	169.6	-10.05	0.9990	0.05 – 20	0.04	0.10
Ferulic acid	325	159.9	1.128	0.9999	0.05 – 20	0.05	0.20
Eriocitrin	278	19.12	-1.025	0.9999	0.05 – 20	0.04	0.10
Hesperidin	278	50.42	-2.975	0.9997	0.03 – 20	0.01	0.05
Phlorizin	278	24.78	0.1248	0.9999	0.05 – 20	0.04	0.10
Isoquercitrin	360	52.55	5.218	0.9994	0.05 – 20	0.04	0.10
Rutin	360	49.90	-3.978	0.9999	0.05 – 20	0.04	0.10
Rosmarinic acid	325	117.5	-21.47	0.9987	0.05 – 20	0.04	0.10
Quercetrin	360	67.76	-4.616	0.9998	0.05 – 20	0.05	0.10
Naringenin	300	70.09	-22.15	0.9970	0.05 – 20	0.03	0.05

R^2 - Coefficient of determination; LOD - Limit of determination; LOQ - Limit of quantification

Table VI.5. Total and individual phenolic compounds contents (g/g dry basis) in the by-products from orange, lemon and apple fruits determined by HPLC-DAD.

Phenolic compound (µg/g of byproduct)	Fruit byproduct Orange	Lemon	Apple
Benzoic acid derivates			
Protocatechuic acid	317.3 ± 7.173	16.08 ± 1.729	2.465 ± 0.0248
Hydroxybenzoic acid	<LOQ	20.27 ± 1.053	1.939 ± 0.0472
Σ	***317.3 ± 7.173***	***36.35 ± 0.7208***	***4.404 ± 0.0682***
Hydroxycinnamic acids			
Caffeic acid	26.19 ± 1.195	14.67 ± 0.4107	<LOQ
Chlorogenic acid	n.a.	386.7 ± 11.80	39.41 ± 1.016
p-Coumaric acid	18.49 ± 0.5547	6.424 ± 0.2468	<LOQ
Ferulic acid	22.88 ± 0.9469	n.a.	n.a.
Rosmarinic acid	12.97 ± 0.6995	n.a.	88.62 ± 3.606
Σ	***80.53 ± 2.915***	***407.8 ± 12.17***	***128.0 ± 4.554***
Flavan-3-ols			
Epicatechin	n.a.	n.a.	31.24 ± 0.7253
Σ			***31.24 ± 0.7253***
Flavanone glycosides			
Eriocitrin	24.63 ± 1.409	17493 ± 115.5	n.a.
Hesperidin	4901 ± 155.4	2728 ± 17.32	n.a.
Naringenin	30.09 ± 0.4647	42.12 ± 0.8605	16.33 ± 0.834
Σ	***4956 ± 156.9***	***20263 ± 131.6***	**16.33 ± 0.834**
Flavonols glycosides			
Isoquercetin	n.a.	111.6 ± 0.8220	n.a.
Rutin	31.43 ± 1.130	44.72 ± 0.5788	22.44 ± 0.977
Quercetrin	7.964 ± 0.4449	106.0 ± 0.3979	150.3 ± 3.769
Σ	***39.40 ± 0.9735***	***262.3 ± 1.793***	***172.7 ± 4.709***
Dihydrochalcone glycosides			
Phlorizin	n.a.	n.a.	542.0 ± 7.882
Σ			***542.0 ± 7.882***
Total content (µg/g)	**5393 ± 166.1**	**20969 ± 144.7**	**894.8 ± 16.22**

Results expressed as mean values (n = 3) ± standard deviation. n.a. Not applicable. The summation (Σ) of each class of phenolic compounds is highlighted in bold.

4. Conclusions

The phenolic profile and the content of polyphenols of orange, lemon, and apple fruit by-products were determined by UHPLC-ESI–MS/MS and HPLC-DAD. This methodology may be employed for the routine screening of fruit by-products and the identification and quantification of polyphenols. The phenolic compounds responsible for the high antioxidant activity of citrus by-products, in particular for lemon, were hydroxycinnamic acids, flavonols glycosides, and flavanone glycosides. Eriocitrin was the main phenolic compound (17.49 mg/g) determined in lemon by-products, while for orange by-products it was hesperidin (4.9 mg/g). On the other hand, the antioxidant capacity of the apple byproduct could be due to the high content in hydroxycinnamic acids (e.g., rosmarinic acid) and other specific compounds such as epicatechin and phlorizin.

The results encourage the valorization of the fruit by-products as powerful sources of natural antioxidants to be used as food additives or ingredients to increase the shelf life of foods and develop functional foods and active packaging, with potential health benefits creating new food market perspectives within the concept of a circular economy.

Chapter VII

LDPE and PLA active food packaging incorporated with lemon by-products extract: preparation, characterization, and effectiveness to delay lipid oxidation in almond (*Prunus dulcis*) and beef meat

1. Introduction

The extension of the foods' shelf-life is one of the most challenging goals of the food industry. To achieve a longer shelf-life and maintain the quality of food products throughout longer storage time periods, the food industry must resort to different knowledge areas and several techniques. Developing new and better packaging systems, such as active food packaging, is one alternative. Active food packaging's primary goal is to promote positive interactions between the packaging and the packaged foods [384,519–521]. One of the interaction mechanisms is through the emission of substances with bioactive properties from the packaging to the packaged food [97,148,522]. These substances have antimicrobial and/or antioxidant properties which can help to stop or inhibit microbial growth and the development of off-flavors due to the foods' oxidation [148].

Another problem of the food industry is the significant accumulation of plastic, which has turned into one of the most challenges in the modern world. Derived from oil production, polyethylene was discovered by accident in 1935 and commercialized in 1939, under the form of high pressure polyethylene, now known as low density polyethylene (LDPE) [523]. Besides being one of the cheapest synthetic polymers, it has excellent chemical resistance and can be molded in several shapes and forms, being flexible, soft, stretchable, has good clarity and can be easily sealed by heat. These characteristics are only possible due to the several long chain branches present in LDPE [523,524]. Produced by free radical polymerization of ethylene initiated by organic peroxides or other reagents, LDPE is one of the most used polymers in food packaging.

According to Geyer et al. [5], from 1950 to 2015, the global plastic production increased in 379.00 million tones. Being non-biodegradable, these polymers do not decompose and accumulate in the environment or in landfills. On the other hand, polylactic acid (PLA) is a biodegradable thermoplastic derived from renewable sources such as sugarcane and corn starch [427,524]. PLA has become a leading candidate to substitute the petrochemical-based polymers, such as LDPE, being thermoplastic and highly modular [525]. Although PLA is not an optimal candidate for food packaging, due to its oxygen barrier and water vapor permeability properties, these issues can be overcome with the incorporation of additives, such as plasticizers, nanoparticles, fillers and bioactive compounds, such as antioxidant and antimicrobial compounds [427,524]. Comparing both, LDPE and PLA, PLA presents a higher tensile strength, glass transition temperature and melting onset values [525]. Besides is widely used in 3D printing, PLA is also used in medical applications due to its unique characteristics of biocompatibility,

biodegradability and thermoplastic processability. PLA is even been used for prolonged continuous release of drugs, such as contraceptives, narcotic antagonist and vaccines, among others [525–527].

Fruits by-products are originated through the manufacture of, for example, juices, pastes, and jams, which will originate a considerable amount of by-products, often considered waste with a low economic value [285]. In addition, due to their chemical composition, these by-products have to be discarded environmentally and responsibly adding expense and effort to the company and increasing the final product value [291]. Fruits by-products are a great source of antioxidant and antimicrobial compounds, namely dietary fiber and phenolic compounds [494,518]. Phenolic compounds are present in most terrestrial plants as secondary metabolites contributing to the plants' natural defense against UV radiation, predators, and pathogenic microorganisms. They also have an important role in the plants' coloration, aroma and taste [194,409,528].

According to FAOSTAT [487], lemon (*Citrus limon*) is one of the most produced *Citrus* fruits in the world, with a production of 20.049.630 tons in 2020. Lemon by-products, specifical peels, are rich in phenolic compounds namely, flavanones (eriocitrin; hesperidin; naringin; neohesperidin; neoeriocitrin; narirutin), flavones (diosmetin 6,8-di-C-β-gluc; diosmin), flavonols (rutin, quercetin, kaempferol, limocitrol) and hydroxycinnamic acids (caffeic acid, chlorogenic acid, ferulic acid, sinapic acid, *p*-coumaric acid) [297,529–536]. These peels are also rich in dietary fiber, pectin and possess antioxidant capacity [531].

Continuing the work developed in the previous study by Mariño-Cortegoso et al. [537], the primary aim of this research was to investigate and compare the antioxidation potential of active LDPE and PLA packaging incorporated with a lemon by-products (LE) ethanolic extract, in a high fat content food. Also, the antimicrobial activity of the active PLA was determined in beef meat.

2. Materials and Methods

2.1. Materials and reagents

Methanol, absolute ethanol, petroleum ether (40-60°, ACS reagent), isooctane (spectroscopy grade Uvasol®), glacial acetic acid, chloroform, barium chloride dihydrate (ACS reagent, ≥99%) and iron(II) sulfate (ACS reagent, ≥99.0%) were acquired to Merck (Darmstadt, Germany). Trichloroacetic acid, thiobarbituric acid, and xylenol orange sodium were acquired to Sigma-Aldrich (Madrid, Spain). Acetic acid, 6-hydroxy-2,5,7,8-

tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin & Ciocalteu's phenol reagent, sodium carbonate, and the reference phenolic standards rutin, ferulic acid, rosmarinic acid, naringenin, apigenin, caffeic acid, *p*-coumaric acid, gallic acid, and eriocitrin were provided by Sigma-Aldrich (St Louis, MO, USA). Hesperidin was supplied by USP (Twinbrook Pkwy, Rockville, MD, USA), and 4-hydroxybenzoic acid by Alfa Aesar (Karlsruhe, Germany). All standards had a purity equal to or higher than 95%. Also, LDPE purchased from Polimeri Europa, Italy, and PLA (PLA-4032D) was supplied by NatureWorksTM, Minnetonka, MN). Plasticizer poly(ethylene glycol), PEG (Mw=400), was purchased from Sigma Aldrich (Italy). Ultrapure water was obtained by an automatic system of purification (Wasselab, Navarra, Spain).

Regarding the equipment, Heto PowerDry PL6000 Freeze Dryer (Thermo Fisher Scientific, United Kingdom), Edmund Bühler GmbH model KS-15 compact stirrer (Hechinger, Germany), Heraeus Multifuge X3F/X3FR (Thermo Scientific, United Kingdom), rotary evaporator Büchi model R-210 (Labortechnik, Switzerland), internal mixer (Rheomix® 600 Haake, Germany), Evolution 300 UV-Vis (ThermoScientific™, England), Grant Instruments™ QB Series Dry Block Heating System (Cambridge, England), Ox-Tran (Mocon, Model 2/20, Neuwied Germany), PermatranW3/31 (Mocon, Germany) were used.

2.2. By-products' selection and extraction

In a previous study by our group, the lemon by-products extract (LE) presented the highest DPPH free radical inhibition percentage (33.17 mg Trolox/g of extract), the highest content in total phenolic compounds (43.38 mg gallic acid equivalents/g of extract) and the highest content in total flavonoids (20.76 mg of epicatequin equivalents/g of extract) [538]. Based on these results, LE was chosen to be incorporated in active food packaging.

Lemon by-products were kindly supplied by a Portuguese juice company, Frubaça—Cooperativa de Hortofruticultores. Upon their arrival to the laboratory, the by-products were frozen and freeze-dried, grounded, and homogenized. Following the extraction method described by Andrade et al. [183], the by-products were mixed with ethanol in a 1:10 proportion, agitated in a compact stirrer for 30 min and centrifuged at 10 °C at 11952 *g*. The supernatant was moved to an evaporation amber pear-shaped flask and ethanol was evaporated until dryness at 35 °C in the rotary evaporator. The extract was removed with the help of a spatula, vacuum packaged and stored until further use, protected from the light, at -20 °C.

2.3. Incorporation of the lemon extract in LDPE and PLA

Three active films were obtained by the incorporation of the LE: LDPE with 4% (w/w) of LE (LDPE/4LE), PLA with 4% (w/w) of LE (PLA/PEG/4LE) and PLA with 6% (w/w) of LE (PLA/6LE). LE was incorporated into LDPE and PLA by direct melting, via melt mixing and hot compression. In detail, LDPE and PLA were mixed with the LE using an internal mixer (volumetric capacity of 50 cm^3) at 50 rpm for 5 min, at 170 °C and 180 °C, respectively. In the PLA with the lower percentage of LE, PEG was added (15 %, w/w) to prevent brittleness [539]. In the PLA/6LE, the higher amount of LE was enough to plasticize the polymeric matrix.

Then, the mixtures were pressed using a Collin P300P press at the same temperatures (P=50 bars for 3 min). The active films presented an average thickness of about 100-150 microns. Pristine PLA, PLA/PEG and LDPE films have been manufactured for comparison purposes by using the same specific processing conditions.

2.4. Oxygen and water permeability tests of active films

The oxygen permeability (OP) of LDPE and PLA-based films was determined by the Ox-Tran. Samples with a surface area of 5 cm^2 have been tested at 25 °C, setting the relative humidity (RH) at the downstream and upstream side of the film at 50%.

Water vapor permeability (WVP) has been determined using infrared sensor technology using the PermatranW3/31. Samples with a surface area of 5 cm^2 have been tested at 25 °C. Permeation tests have been performed by setting the relative humidity at the downstream and upstream sides of the film to 0% and 50% respectively. A flow rate of 100 mL/min of a nitrogen stream has been used. Each test was carried out in triplicate.

2.5. Migration assay of active films

Following the European Commission Regulation No. 10/2011 [540] and its amendments, the migration tests of the active films (LDPE/4LE, PLA/PEG/4LE and PLA/6LE) were carried out using ethanol 95 %, a substitute of the food simulant D2 (vegetable oil) used to mimic foods with high-fat content. Samples of films were cut in specimens of 10 cm^2 and immersed in a glass vial with 10 mL of the food simulant, 95% (v/v) of ethanol. The vials were incubated in an oven for 10 days at 40 °C to simulate the storage conditions of fatty food for 30 days at room temperature. After, 9 mL of food simulant were evaporated under nitrogen flow at 40 °C, and the residue was redissolved in ultra-purified water to reach a 10-fold concentrated extract. Finally, the films extracts were filtered through 0.45

µm PTFE hydrophilic filters for further HPLC analysis. All determinations were performed in independent triplicates.

2.5.1. HPLC-DAD analysis

An Agilent HPLC 1200 (Waldbronn, Germany) equipment, fitted out with an autosampler, a pump, a degassing system, a thermostatic column system, and a diode array detector (DAD), all controlled by HP ChemStation software, was used for the chromatographic analysis following the same conditions described by Barbosa et al. [538]. The phenolic compounds were separated on a reverse-phase Kinetex EVO C18 100Å column (150 x 3 mm internal diameter, 5 µm of particle size) (Phenomenex, Torrance, CA, USA), at 30 °C. The injection volume was 20 µL, and the mobile phase flow rate was 0.6 mL/min. The following solvents constituted the mobile phase: water (solvent A) and methanol (solvent B), both acidified at 0.1 % with acetic acid.

The gradient elution was as follows: 0 min, 95% of A, 5% of B; 3 min, 90% of A, 10% of B; 10 min, 80% of A, 20% of B; 18 min, 70% of A, 30% of B; 25 min, 30% of A, 70% of B; 33 min, 0% of A, 100% of B; 33 – 40 min, 100% of B, and finally the gradient returns to initial conditions with 95% of A; 41 – 46 min.

UV/VIS scanning was performed continuously at wavelengths between 200 nm to 400 nm. The identification of the phenolic compounds was attained by comparison with the retention times and the UV/VIS spectrum obtained by the injected standards in the same conditions. The quantification of phenolic compounds was performed at the maximum absorbance of characteristic wavelengths of the different chemical phenolic families: 278 nm for 4-hydroxybenzoic acid, 325 nm for hydroxycinnamic acids, and 360 nm for rosmarinic acid and flavonoid glycosides, except for naringenin and eriocitrin that were quantified at 300 nm, using the external linear calibration curves with determination coefficients of $r^2 > 0.998$ [537]. HPLC analyses of the individual extract were performed in a previous study led by Barbosa et al. [538].

2.5.2. Total phenolic content

The total phenolic content (TPC) of the food simulant after contact with films, was assessed according to the Folin–Ciocalteu colorimetric method adapted to a 96-well microplate by Barbosa-Pereira et al. [541]. The absorbance was recorded at 750 nm using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Waltham, MA USA). All the determinations were performed in triplicate. Quantification was carried out using a standard curve of commercial gallic acid (20-100 mg/L, R^2 = 0.9969), and the

concentration of total phenolic compounds was expressed as mg of gallic acid equivalents (GAE)/dm^2 of film.

2.5.3. Radical scavenging activity through DPPH radical assay

The antioxidant capacity of the food simulant after contact with films was determined by the 2,2'-diphenyl-1-picrylhydrazyl (DPPH•) radical scavenging assay described by von Gadow et al. [542] adapted to a 96-well microplate by Barbosa-Pereira et al. [541]. All determinations were performed in triplicate and the absorbance was measured at 520 nm using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Waltham, MA USA). The reduction of the radical DPPH absorbance, expressed as inhibition percentage (IP) was calculated using the following equation:

$$IP\ (\%) = \frac{A_{0\,min} - A_{30\,min}}{A_{30\,min}} \times 100 \quad (1)$$

Where *A0* is the absorbance at the initial time and *A30* is the absorbance after 30 minutes. A linear regression curve of Trolox was used at 12.5-300 µM (r^2 = 0.9995) to calculate the radical scavenging activity (RSA) values. Results were expressed as µmol of Trolox equivalents (TE)/dm^2 of active film.

2.6. High Fat Content Foods' selection

Three model foods, almond, pistachio and beef meat, were chosen based on their high content in unsaturated fatty acids, since these fatty acids are more prone to lipid oxidation [543]. According to the United States Department of Agriculture (USDA) [544] food database, beef has a total fat content of 19.1 g/100 g, of which 8.48 g/100 g are monounsaturated and 0.51 g/100 g are polyunsaturated fatty acids. Almond has a total fat content of 50 g/100 g, of which, 32.1 g/100 g are monounsaturated and 12.5 are polyunsaturated fatty acids. Pistachio has a total fat content of 45.3 g/100 g, of which 23.3 g/100 g are monounsaturated fatty acids and 14.4 are polyunsaturated fatty acids. In order to choose the model food which were later used to evaluate the antioxidant potential of the active films, the three foods were packaged and stored and their lipid oxidation status was measured through the Thiobarbituric Acid Reactive Substances (TBARS) assay.

Beef meat was vacuum packaged and kept for 6 days at 4 ± 1 °C, and the lipid oxidation was measured at the 0th, 3rd and 6th day. The almond and the pistachio were ground, vacuum packaged and kept at 40 °C for a maximum of 30 days. Their lipid

oxidation was measured at the 0th, 7th, 15th and 30th days. The performed TBARS method was described by Rosmini et al. [436], with minor changes (see point 2.8.1).

2.7. Sample preparation and packaging

Due to its linear lipid oxidation profile, almonds and beef were chosen as the model foods to be packaged with the active packaging. The almonds with shell were purchased in a local store in Lisbon, Portugal. The shell was removed using a hammer. For the soft peel removal, the almonds were put in hot water for a maximum time of 5 min and the peel was manually removed. Then, the samples were ground, and vacuum packaged with the LDPE/4LE, PLA/PEG/4LE and PLA/6LE. The samples were kept at 40 °C for a maximum time-period of 60 days, and their lipid oxidation status was assessed at the end of the 7th, 15th, 30th, 45th and 60th storage day.

Beef meat was kindly supplied by Talho Girassol, Lda., a local store in Lisbon, Portugal. Unlike the almond, the beef samples were kept at 4 ± 1 °C, protected from the light, for a maximum time-period of 11 days. At the end of the 1st, 4th, 6th, 8th and 11th day of storage, the lipid oxidation of the beef was measured by the TBARS assay and the microorganisms total count at 30 °C was also performed.

2.8. Lipid oxidation assays

2.8.1. TBARS assay

The TBARS assay was performed in accordance with the method described by Rosmini et al. [436], with slight changes. This assay measures malonaldehyde (MDA) equivalents, being MDA one of the major products of lipid peroxidation. Briefly, 20 mL of trichloroacetic acid aqueous solution (7.5 %, w/v) were added to 5 g of sample. The samples were agitated in the compact stirrer for 1 hour, at room temperature (23 ± 1 °C), at 350 rpm. The samples were then filtered through a Whatman paper filter No. 1 and, 5 mL of the filtered solution was mixed with 5 mL of a thiobarbituric aqueous solution (2.88 mg/mL). The samples were submitted to 95 °C in the heating block for 30 minutes, rapidly cooled in ice for 15 min and their absorbance was measured at 530 nm against the blank assay, containing 5 mL of MilliQ™ water instead of 5 mL of sample. 1,1,3,3-Tetramethoxypropane was used as standard, and the results were expressed in mg of malonaldehyde equivalents per kg of the sample (mg MDAE/kg).

2.8.2. Fat extraction

For the *p*-anisidine and peroxide value determination assays, the fat of the almond needed to be extracted. Briefly, 10 g of almond were mixed with 100 mL of petroleum ether and shaken for 1 hour in the compact stirrer. At the end of this period, the samples were filtered through a Whatman paper filter No 4, with 1 g of anhydrous sodium sulfate. The petroleum ether was then evaporated in the rotary evaporator, at 35 °C.

2.8.3. *p*-anisidine value determination

The determination of the *p*-anisidine value measures the secondary compounds formed during the lipid oxidation, mainly the aldehydes [426]. For the determination of the *p*-anisidine value, the method of *Instituto Português da Qualidade* [545] was applied. Briefly, 50 mg of almond fat, previously extracted (see 2.8.2), was mixed with 12.5 mL of isooctane and the absorbance was measured at 350 nm in a UV/VIS spectrophotometer. Then, 2.5 mL of this solution was mixed with 500 µL of *p*-anisidine diluted in glacial acid acetic (5 mg/mL), homogenized and kept in the dark for 10 minutes. At the end of this time period, the absorbance was measured against the blank at 350 nm. Then, the *p*-anisidine value (AV) was determined by equation 2.

$$AV = \frac{12.5 \times (Abs10 - Abs0)}{m} \quad (2)$$

Where *Abs10* stands for the absorbance of the solution at the end of the 10 min, *Abs0* stands for the absorbance of the solution at 0 min, and *m* stands for the sample's weight in g.

2.8.4. Peroxide value determination

For the determination of the peroxide value, the method described by Shantha & Decker [546] was applied. Firstly, to prepare the iron(II) chloride solution, an aqueous solution of barium chloride dihydrate (8 mg/mL) and an aqueous solution of iron(II) sulfate (10 mg/mL) were prepared. The barium chloride solution was slowly added to the iron(II) sulfate solution under constant stirring. Then, 2 mL of hydrochloric acid (10 N) were added, and the solution was filtered through a Whatman paper filter No. 1. This solution must be kept in the dark.

For the determination of the peroxide value, 300 mg of almond fat, previously extracted with petroleum ether, was mixed with 9.8 mL of a chloroform and methanol

solution (7:3, v/v) and the solution was briefly homogenized. Then, 50 µL of an aqueous solution of xylenol orange sodium (10 mM) was added and the solution was briefly homogenized. Then, 50 µL of the iron(II) chloride solution was added and the solution was again briefly homogenized. The absorbance was measured at 560 nm in the UV/VIS spectrophotometer. The peroxide value was calculated through equation 3.

$$PV = \frac{(As-Ab)\times m}{55.84\times m_0\times 2} \quad (3)$$

Where, *As* stands for the absorbance of the sample, *Ab* stands for the absorbance of the blank, *m* stands for the slope of the calibration curve using iron(III) chloride as standard, and m_0 stands for the mass of the sample. The 55.84 is the atomic weight of iron (the denominator gives the concentration of Fe^{2+} oxidized to Fe^{3+} in µg) and the division by factor 2 is necessary to present the results in milliequivalents of peroxide per kg of sample.

2.9. Microbiological analysis

Regarding the microbial analysis performed on beef meat packaged with the control and active PLA/6LE, the total microorganisms count at 30 °C was performed using the automated test TEMPO® Aerobic Count-AFNOR BIO 12/35-05/13. The microbial assays were only performed in meat since the normal degradation during the almonds shelf-life is not normally due to the action of microorganisms but to rancidity, which cause, mainly, organoleptic alterations.

2.10. Statistical analysis

All experiments were conducted with three replications and the statistical analysis of data was performed through a one-way analysis of variance (ANOVA) and ANOVA with Repeated Measures, using the Software SPSS® Statistics version 26.0.0.0, from IBM®. All requirements necessary to carry out the ANOVA (namely, normality of data and homogeneity of variances) have been validated. Differences among mean values were processed by the Tukey test. Significance was defined at $p < 0.05$. Results are expressed as the means of the replicants ± standard deviation.

3. Results and discussion

3.1. Oxygen and water permeability tests of active films

As can be observed in Figure VII.1, LDPE/4LE and PLA/PEG/4LE active films presented a light dark-yellowish color. The films were quite homogeneous and transparent, being the Institution' logo completely visible. The LDPE film, as expected, was the most malleable and resistant.

Figure VII.1. Active (LDPE/4LE and PLA/PEG/4LE) films over the Portuguese National Institute of Health logo.

WVP and OP results are exhibited in Figure VII.2. The presence of LE leads to an improvement of water barrier properties of LDPE-based film, which shows a reduction of about 33% in WVP values, which are 6.0×10^{-13} g/m s Pa and 4.0×10^{-13} g/m s Pa for LDPE and LDPE/4LE, respectively. In the case of the active PLA-based films, a WVP reduction of 20% and 60%, respectively for PLA/PEG/4LE and PLA/6LE can be observed. In the case of oxygen permeability, the presence of the LE does not significantly modify the performance of the films: LDPE-based films show no reduction in OP values, whereas both PLA-based films show a slight reduction of about 20% to pristine film. The polymer blend of PLA/PEG behaves as a hydrophilic polymer. It has a high permeability coefficient to water due to the presence of hydrophilic groups in the PEG component of the blend. Therefore, PLA/PEG had higher WVP than the pure PLA film (Figure VII.2). It was assumed that there was a large free volume in PLA/PE film due to the combined plasticizing effect of PEG 400 and water. However, the addition of the LE to LDPE, PLA or PLA/PEG decreases the free volume in the films resulting in reduction of the WVP. Oxygen permeability of both the PLA/PEG and PLA/PEG/4LE and PLA/6LE were lower than that

of the PLA/PEG and pure PLA film, respectively (Figure VII.2). This is due to the reduced free volume size in the films of PLA/PEG/4LE in comparison with PLA/PEG. The same pattern is observed in the films of PLA/6LE in comparison with PLA. There is a correlation between the OP and free volume size, the lower is free volume size, the lower is the OP [539]. In the case of the LDPE and LDP/4LE it is not observed a difference on the OP, probably because although LE reduced the free volume size, the reduction is not enough in order to see differences in the OP, because the free volume size in pure LDPE is very high.

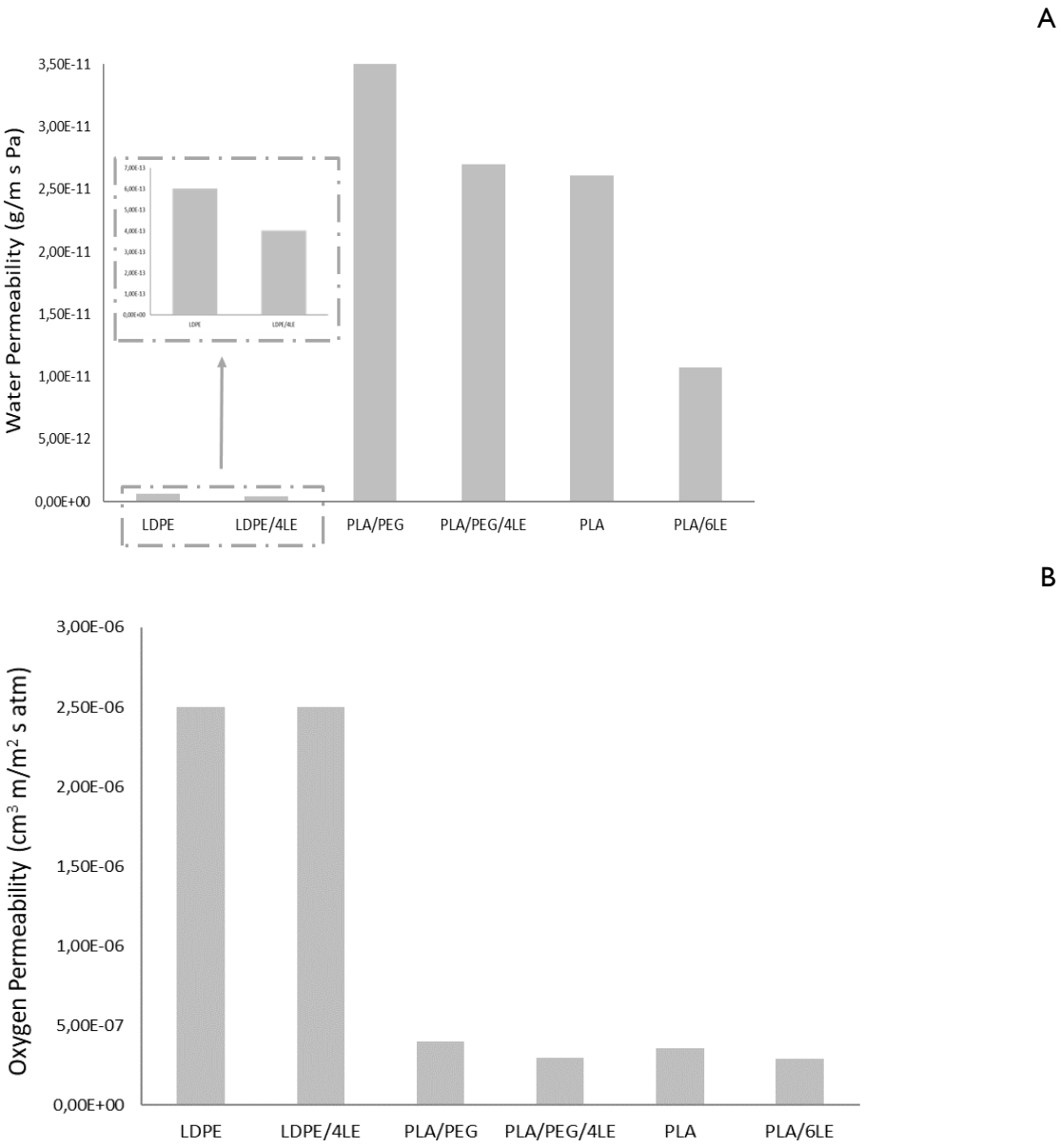


Figure VII.2. Water vapor (A) and O2 permeability (B) of the control (LDPE and PLA) and active (LDPE/4LE, PLA/4LE, PLA/6LE) films. LDPE – Low Density Polyethylene; LE – Lemon Extract; PLA – Polylactic acid; PEG - Polyethylene glycol.

3.2. Migration of polyphenols and antioxidant capacity of the active films

The results of the migration tests performed to estimate the release of the phenolic compounds from the active films to the substitute of the fat food simulant are shown in Table VII.1. From the HPLC data, the results showed that among the active films studied, PLA formulations were those that release high amounts of active compounds, up to 9-fold higher, than LDPE film ($p < 0.001$). Considering the PLA formulations added at two concentrations of lemon extract, non-significant differences were observed between them ($p > 0.05$) due to the high standard deviation observed for the replicates of PLA/PEG/4LE.

Despite the high variability observed within the PLA/PEG/4LE replicates, the results highlighted that the addition of higher amounts of extract did not result in a significant increase in the overall content of active compounds released to the food simulant. These results could be due to the possible rearrangement of the polymeric matrix that might difficult the migration process of the active compounds. Considering the polyphenols determined by chromatography, the flavonoids eriocitrin, hesperidin, naringenin, and apigenin were those found in high concentration in the food simulant in contact with PLA films. These compounds were also the most abundant in the lemon extract, as described previously by Barbosa et al. [538]. On the other hand, the polymeric matrix LDPE/4LE did not release most of these compounds, just a small quantity of naringenin was determined compared to PLA films. Indeed, in the LDPE films, 4-hydroxybenzoic acid was the phenolic acid that migrates in high amounts and is significantly higher than PLA films ($p < 0.001$). Since it was not possible to identify and quantify all the compounds in HPLC chromatograms, the Folin–Ciocalteu colorimetric assay was used to assess the total amount of phenolic compounds. Also, for this assay, the results showed that the differences were not significant among PLA films. However, considering the radical scavenging activity (RSA), a slight increase was observed ($p < 0.01$), and the antioxidant capacity was higher for the PLA films added with a higher concentration of lemon extract (PLA/6LE). This higher antioxidant capacity could be related to the migration of 4-hydroxybenzoic acid and naringenin which were those active compounds found in higher significant amounts ($p < 0.001$) than in PLA/PEG/4LE films. Considering LDPE films, both photometric assays (DPPH and TPC) were not sensible due to the low amounts of phenolics present in the food simulant after the migration test.

Table VII.1. The individual and total amount of phenolic compounds that migrate from the active films (LDPE/4LE, PLA/PEG/4LE and PLA/6LE), determined by HPLC-DAD and expressed as μg/dm² of the film; Total phenolic content (TPC) expressed as μg GAE/dm2 film; Radical scavenging activity (RSA) express as μmol TE/dm² of the film. Results of analysis of variance (ANOVA) with Duncan's test among different polymeric matrices developed with lemon extract at different concentrations (rows) and between the amounts of phenolic compounds within each film formulation (column).

Phenolic compound	**LDPE/4LE‡**	**PLA/PEG/4LE**	**PLA/6LE**	***Sig.***
4-Hydroxybenzoic acid	9.97 ± 0.32^{aA}	3.22 ± 0.83^{cdC}	6.50 ± 0.40^{eB}	***
Caffeic acid	< LOQ	3.19 ± 0.15^{cd}	< LOQ	
p-coumaric acid	2.22 ± 0.95^{c}	1.27 ± 0.20^{d}	2.06 ± 0.11^{f}	*n.s.*
Ferulic acid	< LOQ	11.4 ± 4.91^{c}	11.0 ± 0.98^{d}	*n.s.*
Eriocitrin	< LOQ	21.8 ± 3.17^{bA}	13.0 ± 0.85^{cB}	**
Hesperidin	< LOQ	49.9 ± 13.3^{aA}	26.4 ± 2.1^{aB}	*
Rutin	< LOQ	4.45 ± 0.87^{cdA}	2.95 ± 0.12^{fB}	*
Rosmarinic acid	< LOQ	4.06 ± 0.74^{cd}	3.35 ± 0.11^{f}	*n.s.*
Naringenin	3.77 ± 0.16^{bC}	22.1 ± 0.42^{bB}	27.3 ± 1.9^{aA}	***
Apigenin	< LOQ	21.4 ± 0.50^{bB}	22.2 ± 0.20^{bA}	*
Sig.	***	***	***	
∑ (μg/dm²)	**15.97 ± 1.33^{B}**	**142.7 ± 24.7^{A}**	**114.9 ± 6.66^{A}**	***
TPC (μg GAE/dm²)	n.d.	624.6 ± 4.64	673.3 ± 35.9	n.s.
RSA (μmol TE/dm²)	n.d.	1.75 ± 0.02^{B}	1.94 ± 0.09^{A}	**

GAE, gallic acid equivalents; TE, Trolox equivalents; < LOQ, below limit of quantification (HPLC); n.d. = not detected.
Values (mean ± standard deviation) followed by different lowercase super-indexes indicate significant difference at $p < 0.05$ among the different phenolic compounds identified in the same film and means followed by different uppercase super-indexes indicate significant difference at $p < 0.05$ among the different polymeric matrices.
Significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. = not significant.
‡ Data from Mariño-Cortegoso et al.[537].

3.3. Selection of model foods

Three foods were firstly analyzed in order to check their suitability to be used as model foods in the present study. As can be observed in Figure VII.3, the oxidation of the meat and the almond present a linear progression from the 0 to the 6th (in the meat case) and the 30th (almond) storage day. In the pistachio' case, the lipid oxidation progression is not linear. Hereupon, the chosen model foods for the effectiveness of the active packaging were the meat and the almond.

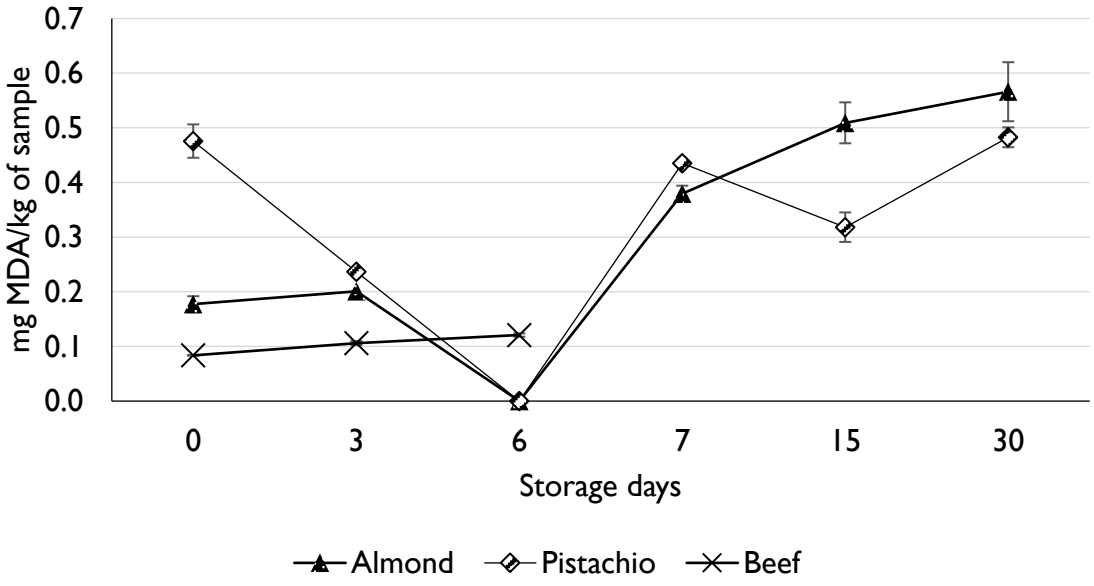


Figure VII.3. Results from the TBARS assay, evaluating the model foods beef, almond and pistachio.

3.4. Lipid oxidation evaluation of almond

The results for the assessment of almond' lipid oxidation is presented in Figure VII.4 and FigureVII.5. Both the TBARS assay and the *p*-anisidine value assay measure products resulting from the secondary lipid oxidation. The almond packaged with the, either the LDPE or the LDPE/4LE, presented lower MDA values than the almond packaged with either PLA. These values are significantly lower different on the 15th and 45th storage day. The almond packaged with PLA and the PLA/6LE presented significantly higher MDA values in all the storage days. Regarding the *p*-anisidine results, on the 7th storage day, both LDPE/4LE and PLA/PEG/4LE exhibit significantly lower *p*-anisidine values than LDPE and PLA/PEG, respectively. LDPE/4LE presented significantly lower values than LDPE on the 7th and 15th storage day. Both PLA/PEG/4LE and PLA/6LE did not present lower values than the respective controls. Both TBARS and *p*-anisidine results indicate that the LDPE active packaging is more effective in preventing almonds' lipid oxidation any of the PLA

packaging. Looking at the migration results (section 3.2), the PLA/PEG/4LE and PLA/6LE released higher amounts of active compounds that the LDPE/4LE. Combining this with the lipid oxidation results, the active PLA packaging promote the lipid oxidation of the model food since the migration of active compounds is significantly much higher than the LDPE active packaging.

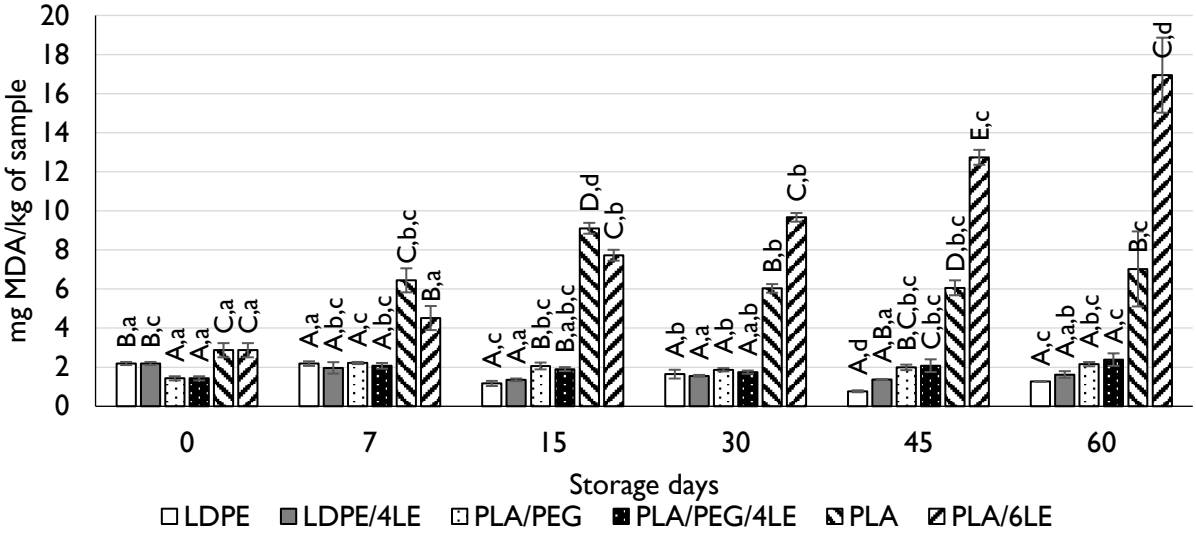
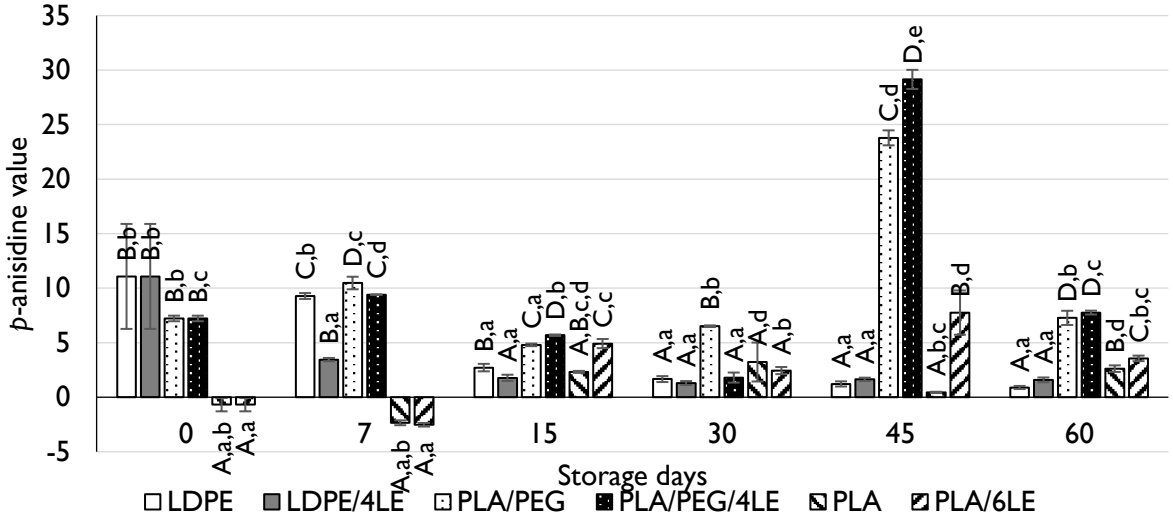


Figure VII.4. Results of the Thiobarbituric Reactive Substances from the almond packed with the control (LDPE, PLA/PEG and PLA) and active (LDPE/4LE, PLA/PEG/4, PLA/6LE) films. The uppercase letters compare samples within the same storage day. The lower letters compare samples with the same packaging over time (for instance, LDPE at 0 and 7 days present no significant differences, has noted by the lowercase "a". On the other hand, on the 7th day of storage, LDPE and LDPE/4LE present no significant differences, has identified by the uppercase "A"). Different letters, for either lowercase/uppercase comparison, represent results with significant differences.



FigureVII.5. Results of the *p*-anisidine assay from the almond packed with the control (LDPE, PLA/PEG and PLA) and active (LDPE/4LE, PLA/PEG/4, PLA/6LE) films. The uppercase letters compare samples within the same storage day. The lower letters compare samples with the same packaging over time. Different letters, for either lowercase/uppercase comparison, represent results with significant differences.

The efficiency of *Citrus* extracts was previously proven by Contini et al. [547] in reducing the MDA equivalents in cooked turkey meat packaged with a polyethylene terephthalate (PET) loaded with 0.46 mg/cm^2 of a commercial *Citrus* extract, for a 4-day period. Maru et al. [548] evaluated the possible effectiveness of lemon polyphenols incorporated in chitosan and pullulan active coatings. While chitosan loaded with lemon polyphenols (3 %) was able to slow the formation of MDA in poultry meat for 16 days of storage, the pullulan active coating with 3 % of polyphenols, accelerated the MDA formation at the 9th storage day [548].

Since the TBARS and *p*-anisidine results of the almond packaged with the PLA and PLA/6LE were not clear, the peroxide determination was performed in these samples. As can be observed in Figure VII.6, the PLA/6LE presented significantly lower peroxide content in all of the storage days, with the exception of the 7th. The peroxide value determination is one of the most used methods for the determination of oil quality and stability [549]. This assay measures the extent of lipid peroxidation through the amount of total peroxides in a sample, which are associated with the development of off-flavors, deterioration and toxic' products in fats and oils [550].

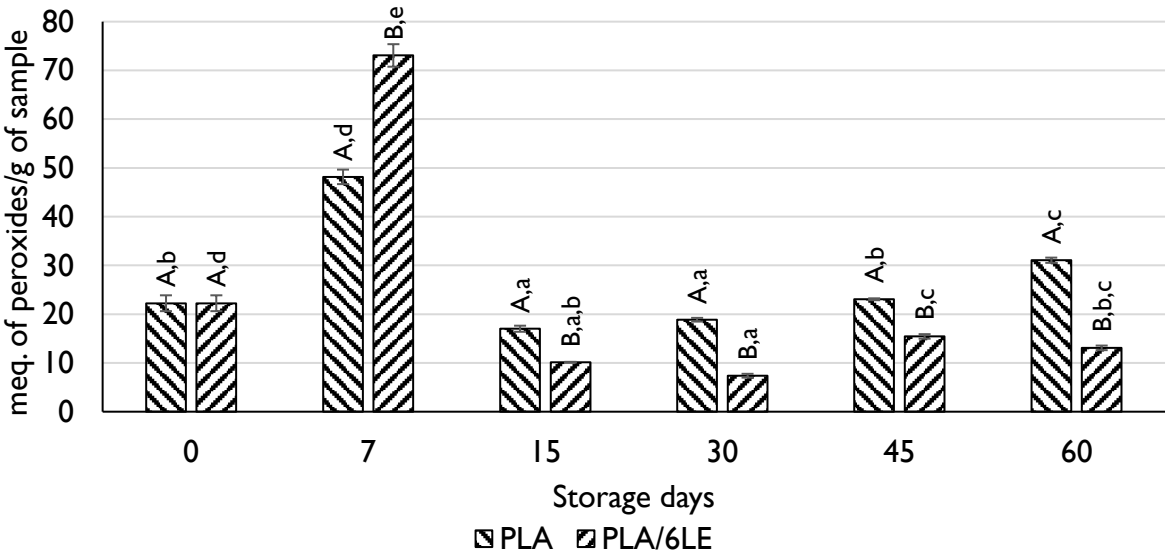


Figure VII.6. Results of the peroxide determination assay from the almond packaged with the control (PLA) and active (PLA/6LE) films. The uppercase letters compare samples within the same storage day. The lower letters compare samples with the same packaging over time. Different letters, for either lowercase/uppercase comparison, represent results with significant differences.

Lipid oxidation is a sequential process divided by three stages (initiation, propagation and termination) that occur simultaneously in a cycle, until there is no more available free-radicals [34,44,365,549]. The primary lipid oxidation continually produces

hydroperoxides that will be decomposed into secondary oxidation products such as aldehydes, ketones, hydrocarbons, volatile organic acids, among others. Thus, while TBARS and *p*-anisidine assay determine the formation of secondary oxidation products, the peroxide value assay determines the formation of primary oxidation products. Looking at the results exhibited in FigureVII.5 and Figure VII.6, they seem to indicate that the almond packaged with the PLA and PLA/6LE presents, mostly, primary oxidation products. The peak of peroxides at the end of the 7th day of storage in Figure VII.6**Erro! A origem da referência não foi encontrada.**, suggests that the active film may have a prooxidant effect in the matrix, indicating that in the subsequent storage days, the formation of secondary oxidation products will increase, as can be observed in Figure VII.4 and FigureVII.5. Also, all the storage assays were performed at temperature (40 °C) in order to accelerate the foods' oxidation and, to simulate the worst storage conditions for food intended to be stored at room temperature, in accordance with the European Commission Regulation no. 10/2011 and its amendments [540].

3.5. Lipid oxidation and microbial evaluation of the beef meat packaged with PLA/6LE

The beef meat packaged with PLA and PLA/6LE was kept at 4 °C and analyzed at the end of 1st, 4th, 6th, 8th and 11th day of storage. TBARS results are displayed in Figure VII.7 and total microorganism count at 30 °C results are displayed in Table VII.2. The PLA/6LE presented significant lower MDA equivalents at the of the 4th and 6th storage day. The results from the 8th and 11th day are also promoting the results obtained for the almond packaged with the PLA and PLA/6LE. Jiang et al. [551] described a lipid oxidation inhibition effect from grass carp collagen with chitosan encapsulated with lemon essential oil in pork meat. The authors found that the films with 30, 20 and 10 % of lemon essential oil presented the lowest peroxide value and malonaldehyde content. In this study, the authors also found that higher percentages of lemon essential oil showed higher peroxide values and malonaldehyde content [551].

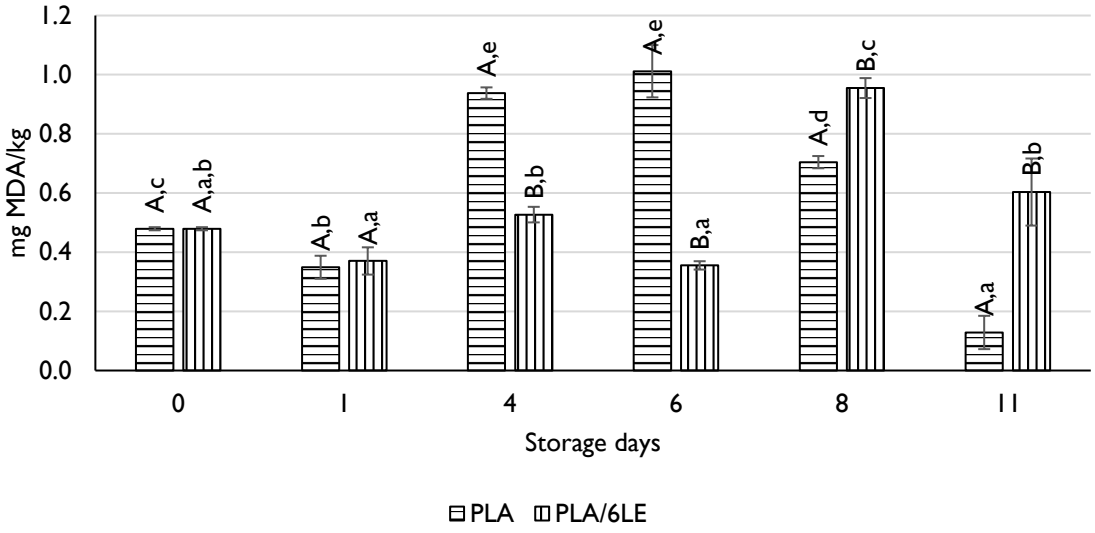


Figure VII.7. Results of the TBARS assay from the meat packed with the control (PLA) and active (PLA/6LE) films. The uppercase letters compare samples within the same storage day. The lower letters compare samples with the same packaging over time. Different letters, for either lowercase/uppercase comparison, represent results with significant differences.

Table VII.2. Results of the microbiological assays for the meat packaged with the control (PLA) and the active (PLA/6LE) films.

Storage days	**Pristine PLA (CFU/g of sample)**	**PLA with 6 % of lemon extract (CFU/g of sample)**
0	1.1×10^{6}	1.1×10^{6}
1	5.7×10^{5}	7.3×10^{5}
4	1.2×10^{7}	8.3×10^{5}
6	5.0×10^{8}	3.6×10^{6}
8	2.0×10^{7}	1.1×10^{7}
11	4.1×10^{10}	2.4×10^{7}
Legend: CFU – Colony Forming Units		

Regarding the microbiological results, the PLA/6LE was able to inhibit the total microorganisms count after the 1st day of storage until the 8th day of storage. On the 8th storage day, no differences can be observed. The significant differences between the total microbial count can be once more observed on the 11th day of storage, which shows that the microbial activity of the lemon extract still exists. Also, this effect can be due to the good water barrier property of the active film which hinders the entrance of water molecules in the packaging bag, thus creating a less humid environment that delays microbial growth. These microbiological results are in accordance with the TBARS assay results.

The antimicrobial activity of lemon by-products extracts is very well documented through the bibliography. Mexis et al. [552] demonstrated that the combined use of the *Citrus* essential oil with an oxygen scavenger was able to prolong the ground chicken meat for 2 extra days. Kadhim Hindi & Ghani Chabuk [553] showed the antimicrobial activity of aqueous extracts obtained from lemon peels against *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Moraxella catarrhalis* and *Pseudomonas aeruginosa*. Sanz-Puig et al. [554] also showed the antimicrobial activity of another aqueous extract obtained from lemon by-products against *Escherichia coli* and *Salmonella Typhimurium*. Jiang et al. [551] measured the total viable microbiological count in pork meat wrapped in grass carp collagen films with lemon essential oil encapsulated in chitosan nanoparticles. The authors found that the films with 30 and 20 % lemon essential oil showed the lowest total viable count for a storage period of 21 days, confirming the powerful antimicrobial activity of lemon by-products.

4. Conclusions

LE was successfully incorporated in different percentages in PLA and LDPE active packages, proving to be a viable additive to be incorporated into new active packages, from traditional non-biodegradable polymers, and innovative biodegradable polymers. The migration of the antioxidant compounds from the PLA/PEG/4LE and PLA/6LE to the food simulator (ethanol 95%) was higher than the migration from the LDPE/4LE. The LDPE/4LE showed a higher ability to retain the LE' phenolic compounds than the PLA films, which explains the LDPE/4LE efficiency in delaying the almonds' lipid oxidation for 30 storage days at 40 °C. The inability of the PLA/PEG/4LE and PLA/6LE to retain the active LE compounds, increased the amount of active compounds on the surface of the food since the beginning of the storage time. The higher content in phenolic compounds with high antioxidant activity promoted the lipid oxidation of the almond.

In the future, the LE extract could also be considered a viable candidate to be approved included in the list of compounds approved to be used in plastics manufacture or as a food additive, similarly to rosemary extract. Due to the variability among batches of the natural extracts, naringenin and/or 4-hydroxybenzoic acid could be proposed as active compounds to be monitored to standardize the quality of lemon by-products extracts.

Chapter VIII

Extending high fatty foods from lipid oxidation and microbiological contamination: an approach using active packaging with pomegranate extract

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1. Introduction

Fruits by-products are often discarded as waste, causing its potential applications to be lost. They are known sources of powerful bioactive compounds with different biological activities involving potential health benefits [186,285]. Phenolic compounds are among these bioactive compounds. They are secondary metabolites formed by plants for their natural defense against pathogenic organisms, predators, parasites, and UV radiation [183,194,196]. Plants' colors and organoleptic properties can be associated to the presence of certain phenolic compounds, which include flavonoids, catechins and phenolic acids. The production of these compounds depends on several factors such as the edaphoclimatic conditions to which the plant was exposed, harvest time, among others and also, their distribution in the plant is not homogenous, depending on the plant part [183,192,194–196].

Pomegranate (*Punica granatum* L.), native from Asia, is one of the oldest consumed fruits in the world. Only 40 % of the fruit is edible (arils) being the rest constituted by peels (50 %) and seeds (10 %), making it a major source of by-products. In traditional medicines is used for treatment of vermifuge, asthma, bronchitis, fever, inflammation, and bleeding disorders [208,285]. Its antioxidant and anticancer activities are highly documented in literature [194,215,220–227] The most common phenolic compounds present in the pomegranate are ellagitannins, namely punicalagin and ellagic acid [1].

Grapes are one of the most used and consumed fruits in the world, specifically in wine production. According to FAOSTAT, the grape worldwide production in 2020 was 78 034 332 tonnes and the wine worldwide production, in 2019, was 27 025 456 tonnes [283]. Grape by-products, resulting from wine production, are usually referred to as grape pomace or wort and, are rich in catechins, gallic acid, and procyanidins [215,234,235]. All of these compounds are known to possess powerful antioxidant activities. Their distribution in the grape is not homogeneous, being the seeds the part with the highest content in phenolic compounds (60-70%), followed by skin (28-35%) and pulp (10%) [285,555].

Food packaging' main function is to protect foods during transportation and storage, delaying its natural degradation, with no interaction with the packaged food. Active food packaging is a technology that came to revolutionize the concept of food packaging since its main goal is to prolong foods' shelf-life through the exchange of the package and food. This interaction could be made through the emission of antioxidant or antimicrobial compounds that, by the interaction with the food' surface, delay or even stop the natural degradation and even improve the organoleptic properties of the

packaged food. Previous works describe the incorporation of pomegranate peels and extracts, as well as grape extracts and by-products [264,556–562].

Lipid oxidation is still one of the main causes for food loss and one of the major enemies of the food industry [423–425]. The solution may lie in the gradual emission of active compounds with high antioxidant capacity from the package matrix to the food' surface, inhibiting the lipid oxidation of highly fatty foods [182,426–430,522].

This work aims to evaluate the antioxidant and antimicrobial potential of natural ethanolic extracts from pomegranate and grape by-products. Moreover, the extracts that showed more promising results in the antioxidant and antimicrobial assays where further incorporated in a polylactic acid (PLA)-based polymeric matrix. Afterwards the films were characterized in terms of chemical, mechanical and barrier properties. Additionally, the effectiveness of new active films was evaluated through the determination of the lipid oxidation status and microbial contamination of two model foods, almond and beef-meat, packaged with these films for different time periods.

2. Materials and Methods

2.1. Reagents and materials

Absolute ethanol (ACS reagent, for analysis), methanol (ACS reagent (≥99.8%) and for HPLC, ≥99.9%), chloroform (SupraSolv®, for gas chromatography ECD and FID), sodium carbonate anhydrous (ACS reagent), sodium nitrite (ACS reagent), sodium hydroxide (ACS reagent), petroleum ether (ACS reagent, bp 40-60 ° C), barium chloride dihydratate (ACS reagent), iron(II) sulfate (pro analysis), iron(III) chloride (anhydrous for synthesis), hydrochloric acid, glacial acetic acid (for HPLC, ≥99.9%), Folin-Cioucalteu's phenol reagent, were acquired to Merck (Darmstadt, Germany). n-Hexane (SupraSolv®, for gas chromatography ECD and FID) was acquired to Honeywell. Tween®40, β-carotene (≥93%), linoleic acid (analytical standard), gallic acid, 2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), epicatechin, aluminum chloride, trichloroacetic acid (ACS reagent, ≥99.0%), 2-thiobarbituric acid (≥98%), 1,1,3,3-tetramethoxypropane (97%), 2,2-diphenyl-1-picrylhydrazyl, xylenol orange sodium (spectrophotometric grade), were acquired to Sigma-Aldrich (Madrid, Spain). Standards punicalagin (A+B) and ellagic acid were acquired to MedChem Express. Ultra-pure water was obtained through a Milli-Q® purification system (Millipore Corp., Belford, USA).

Also, a compact stirrer Edmund Bühler™ Shaker KS 15 A (Hechingen, Germany), an Eppendorf AG 5804R centrifuge (Hamburg, Germany), a rotary evaporator Büchi

model R-210 (Labortechnik, Switzerland), a Thermo Scientific Evolution 300 LC spectrophotometer, a RSLAB-6PRO Vortex, Ultra-Turrax IKA® DI 25basic, a Grindomix GM 300 (Retsch) and a Grant Instruments™ QB Series Dry Block Heating System (Cambridge, England) were used

2.2. Extraction process

Pomegranate by-products were kindly supplied by Memória Silvestre Lda - Arilo Pomegranates, a Portuguese company dedicated to the production of pomegranate. The peels were manually separated from the seeds. Peels were grinded and a part was freeze-dried. The extraction protocol was applied in the natural and freeze-dried peels.

Wort (in Portuguese, mosto) are the grape by-products, after the wine process making. They were retrieved, after the wine production, in Sobreira, Idanha-a-Nova, Portugal, grinded and freezed once it arrived at the laboratory.

All the extracts were obtained using absolute ethanol and the extraction method followed the method described by Andrade et al. [183]. Briefly, the by-products were mixed with ethanol in a 1:10 ratio, homogenized for 30 min at 1000 rpm and centrifuged for 10 min at 11952 *g*. The supernatant was removed for a pear-shaped flask and the extract was evaporated in the rotary evaporator, until dryness, at 35 °C (40 mbar for 45 min). The extract was removed with the aid of a spatula, vacuum packaged and stored at -20 °C until further use. In total, three ethanolic extracts were produced: wort extract; extract from freeze-dried pomegranate peels (PPE-FD) and extract from natural pomegranate peels (PPE-N).

2.3. Antioxidant activity and total content in phenolic compounds and flavonoids

The *in vitro* antioxidant activity and the total content in phenolic compounds and flavonoids were performed in the wort, PPE-FD and PPE-N extracts.

2.3.1. DPPH radical scavenging activity

The applied method was initially described by Moure et al. [501] and modified by Andrade et al. [183]. Briefly, 50 μL of sample (1 mg/mL) were mixed with 2 mL of a DPPH• methanolic solution (14.2 μg/mL). For the control, 50 μL of ethanol were used, instead of the sample. The mixtures were left, protected from the light, for 30 minutes. The

absorbance was measured at 510 nm, in the spectrophotometer. The Inhibition Percentage (IP) of the DPPH• was calculated through the equation (1).

$$IP\ (\%) = \frac{A_b - A_s}{A_b} \times 100 \quad (1)$$

Where, *Ab* stands for the absorbance of the control and *As* stands for the absorbance of the sample. Also, a calibration curve using Trolox as a standard was drawn, opposing trolox concentration (between 25 – 175 µg/ml) *vs* the inhibition percentage. The equation was y = 0.5303x – 0.5147, with a R^2 of 0.9976. The results are expressed in mg trolox equivalents (TE)/g of sample.

2.3.2. Total Content in Phenolic Compounds

The total content in phenolic compounds was performed in accordance with the method described by Erkan et al. [462]. Briefly, 1 mL of sample was mixed with 7.5 mL of an aqueous Folin-Ciocalteu' solution (10 %, v/v), homogenized and left for 5 min. Then, 7.5 mL of an aqueous solution of sodium carbonate (60 mg/mL) were added, the samples homogenized and left to stand for 2 hours, protected from the light. The absorbance was measured at 725 nm in the spectrophotometer. Using gallic acid as a standard, a calibration curve was drawn, opposing the gallic acid concentration (between 10 – 175 µg/ml) *vs* absorbance. The equation was y = 0.0059x – 0.0704, with a R^2 of 0.9979.. The results are expressed in mg of gallic acid equivalents per g of sample (mg GAE/g).

2.3.3. Total Content in Flavonoids

The total content in flavonoids was measured applying the method described by Yoo et al. [463]. Succinctly, 1 mL of sample was homogenized with 4 mL of ultrapure water and 300 µL of a sodium nitrite aqueous solution (5 %, w/v). The samples were left to stand for 5 minutes and, 600 µL of aluminum chloride aqueous solution (10 %, w/v) was added. The mixture was, once again homogenized and left to stand for 6 min. Then, 2 mL of sodium hydroxide (1 M, w/v) and 2.1 mL of ultrapure water were added. The mixtures were homogenized, and the absorbance measured at 510 nm, in the spectrophotometer. Using epicatechin as a standard, a calibration curve was drawn, opposing the epicatechin concentration (between 5 – 175 µg/ml) vs absorbance. The equation was y = 0.0021x + 0.0391, with a R^2 of 0.9976 and the results are expressed in mg of epicatechin equivalents per g of sample (mg ECE/g).

2.4. Identification and Quantification of Punicalagin and Ellagic Acid by UHPLC-DAD

The validation, identification, and quantification of punicalagin (A+B) and ellagic acid was performed by Ultra high-performance liquid chromatography (UHPLC) in an UPLC® ACQUITY™ (Waters, Milford, MA, EUA) equipped with a DAD detector. To obtain the best separation efficiency, two columns were tested, an ACQUITY™ UPLC® BEH C18 (2.1 × 50 mm, 1.7 µm particle size) column, and an ACQUITY™ UPLC® RP18 (2.1 × 100 mm, 1.7 µm particle size). Also, different column temperatures were tested: 20; 25; 30 and 35 °C.

The best results were achieved with the ACQUITY™ UPLC® BEH C18 column, kept at 35 °C. Samples were kept at 5 °C and the injection volume was 10 µL. Flow was kept at 0.3 mL/min. Mobile phase A was ultra-pure water acidified with glacial acetic acid at 0.1 % (v/v) and mobile phase B was acetonitrile with glacial acetic acid at 0.1 % (v/v). The gradient was as follows: 0 min, 95 % A; 1 min, 90 % A; 2.5 min, 80 % A; 3 min, 75 % A; 5 min, 90 % A.

The method was validated following the parameters: specificity, working range, linearity, Limit of detection (LoD) and Limit of quantification (LoQ), precision (repeatability and intermediate precision), and accuracy (determined by recovery assays of spiked samples of pomegranate by-products).

Mixed solutions, containing 300 µg/mL of punicalagin (A+B) and 20 µg/mL of ellagic acid diluted in methanol with 0.1 % of glacial acetic acid, were made and diluted up to six times. Calibration curves were drawn by plotting the concentration (µg/mL) versus area. Linear range, LoD and LoQ were determined. Specificity was assessed by comparison of the absorption spectra of the chromatographic peaks between the analytical standards and the samples.

2.5. Incorporation of the active compounds into PLA films

PLA Inzea F18C (Nurel Biopolymers) was used to produce two films with 3 wt.% of the PPE-FD (PLA/3PPE) and another with 3 % of the powder from pomegranate peels (PLA/3PP).

Prior to processing, all materials were dried in a convection drying oven at 55 °C for 4 hours. Compositions of PLA with pomegranate by-products took place in a twin-screw extruder (Leistritz, Germany) at an average melt temperature of 170 °C, 150 rpm, a throughput of 5 kg/h, and an average residence time of 1.5 min. The extrudate filaments were air dried and ground into pellets in a knife mill for subsequent processing.

Prior to blown film extrusion the pellets were dried in a forced convection oven at 60 °C. The PLA films were prepared using a Periplast single screw extruder L/D= 25. The films were extruded with a screw speed of 50 rpm, at a temperature of 170 °C (first heating zone), 175 °C (second zone) and 180 °C (on the remain). A digital micrometer (No. 293- 340, Mitutoyo, Kanagawa, Japan) was used to measure the thickness measurement of the films that was 50 to 60 μm range.

2.6. Films' characterization

2.6.1. Fourier Transform Infrared spectroscopy (FTIR)

To access the structural characterization of the pomegranate by-products and films, Fourier Transformed Infrared Spectroscopy analysis was performed in a 4100 Jasco (Japan) spectrometer in attenuated total reflectance mode (ATR) at 64 scans, 8 cm^{-1} resolution in a wavelength range of 4000-600 cm^{-1}.

2.6.2. Scanning Electron Microscopy (SEM)

The morphology of the films was assessed by SEM analysis in an FEI Quanta 400 (FEI, Eindhoven, The Netherlands), after fractured in liquid nitrogen and coated with a thin gold-palladium (80/20) film.

2.6.3. Film Optical Properties

CIELab color space parameters (L^* – brightness, a^*-red-green and b^*- yellow-blue levels) of the control and active films were measured using a Shimadzu UV2401PC reflectance spectrophotometer operating in the range of visible light between 370 to 750 nm with a 2 nm spectral resolution. The total color difference (ΔE^*) was determined according to following formula [563]:

$$\Delta E^* = \sqrt{\left(L_f^* - L_{PLA}^*\right)^2 + \left(a_f^* - a_{PLA}^*\right)^2 + \left(b_f^* - b_{PLA}^*\right)^2} \quad (2)$$

where L_{PLA}^*, a_{PLA}^* and b_{PLA}^*, L_f^*, a_f^* and b_f^* are the parameters of PLA control and produced films with pomegranate by-products, respectively.

The haze of the film samples was measured according to ASTM D1003-00 in a XL-211 Hazegard System. Six specimens were tested for each sample. The total light and diffuse transmittance were measured to calculate haze.

2.6.4. Water Vapor Transmission (WVT)

The WVT of the film was measured according to ASTM E96/E96M-10. Calcium chloride was used as the desiccant, the tests were conducted at 23 ± 2 °C for 16 days and the samples were weighed every day.

2.6.5. Oxygen Permeability

The films oxygen permeability was obtained through the Permeameter DP-100A from Porous Materials, Inc, with the pressure increase method. Three specimens were collected from the film samples, with a 4 cm diameter. The test was carried at 23 ± 2 °C with a pressure of 1 atm for 3 hours.

2.6.6. Mechanical properties

The storage modulus and loss factor of the films were assessed by dynamic mechanical analysis (DMA) using a DMA TRITON, with 10 mm distance between grips, in tensile mode. For this test, samples with 4 mm width were used, and a load of 1 N was applied at a frequency of 1 Hz in the temperature range of 40 to 130 °C using a heating rate of 2 °C/min. The tensile tests were performed on a mechanical testing machine, INSTRON 5969, with a load cell of 50kN, at a speed of 100 mm/min, at 23 ± 2 °C, following ASTM D882 – 02. At least, five film specimens, with a length of 160 mm and a width of 25 mm were used. In both tests, samples were cut in the machine direction (MD).

2.7. Antimicrobial analysis of the pomegranate extract

The antimicrobial activity of the PPE-FD was evaluated against *Listeria monocytogenes* ATCC13922, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* NCTC 775 and *Escherichia coli* ATCC 25922. First, a Petri dish with Plate Count Agar (PCA) was flooded with the suspension (single/one culture of the inoculum of each microorganism, made from stock cultures isolated in Tryptone Soya agar (TSA)) of each individual microorganism. The excess liquid was removed, and, after 10 min, one cavity of 4 mm was made in the PCA, and 65 ± 1 mg of the extract was placed. The plates were incubated at 37 °C in aerobic conditions. The inhibition halo was measured at the end of 24 h.

2.8. Effectiveness of the active PLA films

For the antioxidant analysis of the PLA films, an accelerated migration assay was performed according to the method described by López-de-Dicastillo et al. [564], with a

few changes. Briefly, circles of 9.08 cm^2 of the PLA films were cut and submersed in 10 mL of ethanol 95 % (v/v), and kept at 40 °C for 10 days, protected from the light. At the end of this period, the DPPH• inhibition assay (see section 2.3.1), the total content in phenolic compounds and flavonoids (see section 2.3.2 and 2.3.3), and the content in punicalagin (A+B) and ellagic acid was determined.

Also, in order to total quantify the maximum the content punicalagin (A+B) and ellagic acid, the films were also submerged in 10 mL of methanol and kept protected from the light, at 25 and 40 °C, for 24h.

For evaluation of the antimicrobial activity of the PLA films, ISO 22196:2011 [565] was followed. The antimicrobial activity of the films was tested against the same microorganisms, referred in the section 2.7. Briefly, a single/one culture of the inoculum of each microorganism, made from stock cultures in isolated in TSA, was transferred with a 1 µL loop, to Brain Hearth Infusion Broth (BHI), the plates were incubated at 37 °C overnight and the obtained solutions were diluted to 10^{-4}. Squares of 5 x 5 cm of each film were placed on a Petri dish, and 0.2 mL from each dilution were applied to each film and, to keep the film hydrated, squares of sterile plastic (4 x4 cm) were placed on top of each film. The plates were incubated at 35 °C for 24h in a humid atmosphere in aerobiosis. At the end of the incubation, 10 mL of Soybean Casein Lecithin Polysorbate (SCDLP) were added to each plate and stirred for 30 s, for neutralizing the antimicrobial agents. From this, serial decimal dilutions (10^{-1} and 10^{-2}) were performed and, 1 mL was transferred to Petri dishes. Then, 15 mL of PCA at 44 – 47 °C was added to each dish, and carefully mixed by rotating. After complete solidification, the plates were incubated at 35 °C for 48 h in aerobic condition. At the end of the incubation period, counts of colonies was performed.

2.9. Packaging of the model foods

According to the Portuguese National Food Composition Table, the total lipidic content of the almond is 56 g/100 g, of which 4.7 g/100 g are saturated fat acids, 34.5 g/100 g are monounsaturated fat acids, and 14.3 g/100 g are polyunsaturated fat acids, making almond one of the richest foods in unsaturated fat acids.

Almond was acquired, still in its hard shell, in a local store in Lisbon, Portugal. The hard shell was manually separated with the help of a hammer. Then, to peel the almonds, the edible part was placed in a hot bath (approximately 80 ± 1 °C) for a maximum time of 5 min and manually peeled, grinded in a Grindomix and vacuum packaged with the control PLA and the active PLA (PLA/3PPE and PLA/3PP). The samples were stored at 40

± 1 °C and at room temperature, protected from the light. The almond samples lipid oxidation stored at 40 ± 1 °C was analyzed at the end of 2, 4, 7, 14, 21 and 30 days of storage. The almond samples lipid oxidation stored at room temperature were analyzed at the of 7, 14 and 21 days of storage.

Regarding beef meat, according to the USDA food database, the total lipidic content is 19.07 g/100 g, of which 7.29 g/100 g are saturated fat acids, 8.48 g/100 g monounsaturated fat acids and 0.51 g/100 g are polyunsaturated fat acids. Pieces of 35 ± 1 g were also vacuum packaged and stored, protected from the light, at 4 °C. The meat oxidation state and microbiological contamination was assessed at the end of 1, 4, 6, 8 and 11 days of storage.

2.10. Lipid oxidation evaluation

2.10.1. Thiobarbituric acid reactive substances assay (TBARS)

The method originally described by Rosmini et al. [436] and adapted by Andrade et al. [522] was performed for the TBARS assay. Briefly, 20 mL of an aqueous solution of trichloroacetic acid (7.5 %, w/v) were added to 5 g of sample. The samples were homogenized in a compact stirrer for 1 hour, at 400-450 rpm. Using a paper filter Whatman No. 1, the samples were filtered and 2.5 mL of the filtered were mixed with 2.5 mL of an aqueous solution of 2-thiobarbituric acid (2.88 mg/mL) and kept, at 95 °C, for 30 minutes. The samples were rapidly cooled with ice for 15 min, and their absorbance were measured at 530 nm in the spectrophotometer, against the control (2.5 mL of ultrapure water + 2.5 mL of 2-thiobarbituric acid solution). A calibration curve using 1,1,3,3-tetramethoxypropane was drawn and the results are expressed in mg of malonaldehyde equivalents per kg of sample (mg MDA/kg).

2.10.2. Fat extraction

For the determination of the peroxide value and the *p*-anisidine value, the fat of the samples had to be extracted. Briefly, 10 ± 1 g of sample was mixed with 100 mL of petroleum ether and agitated in the compact stirrer at 350-400 rpm for 1 hour. Then, the samples were filtered using a paper filter Whatman No. 4 with 1 g of sodium carbonate anhydrous. The ether was removed using the rotary evaporator at 35 °C.

2.10.3. Peroxide Value Determination

For the peroxide determination, the method described by Shantha & Decker [546] was performed. First, to prepare an iron (II) chloride solution, 50 mL of an aqueous solution of barium chloride (8 mg/mL) was slowly pored under constant stirring, in a 50 mL of an aqueous solution of iron (II) sulfate. Still under constant stirring, 2 mL of hydrochloric acid (10 N) were added, and the solution was left to stand to allow the deposition of the barium chloride. The supernatant was moved to an amber flask and stored for a maximum time period of 1 week, protected from the light. Then, 50 mg of fat were agitated with the vortex for 2-4 sec. with 9.8 mL of a chloroform-methanol solution (70-30%, v/v). Then, 50 µL of a xylenol orange solution (10 mM) were added, the solution was again homogenized in the vortex, and 50 µL of the iron(II) chloride solution were added, the solution was mixed, and, after 5 min, the absorbance was measured at 560 nm, in the spectrophotometer. The peroxide value was calculated through the equation (3) and the results are expressed in milliequivalents of oxygen per kilogram of sample (meq O_2/kg).

$$PV = \frac{(As-Ab)\times m}{55.84 \times m_0 \times 2} \quad (3)$$

Where, *As* stands for the samples' absorbance, *Ab* stands for the blanks' absorbance, *m* stands for the slope of the iron (III) calibration curve, *m0* stands for the samples' mass in g, and 55.84 is the atomic weight of iron.

2.10.4. Determination of the *p*-anisidine value

The *p*-anisidine value determination followed the method described by British Standard method BS 684-2.24 [566]. Briefly, to 50 mg of fat, 25 mL of n-hexane were added. The solution was placed in the ultrasounds for 5 min to make sure that the fat dissolved completely. The absorbance of the solution was measured at 350 nm, against n-hexane. Then, to 5 mL of sample, 1 mL of a p-anisidine in acetic acid (2.5 mg/mL) was added. The samples were placed, for 10 min, in the dark, and their absorbance was measured against the control. The *p*-anisidine value (AV) was calculated by the equation (4).

$$AV = \frac{25(1.2As-As0)}{\mathrm{m}} \quad (4)$$

Where, *As* stands for the absorbance of the sample at the end of 10 min, *AS0* stands for the absorbance of the sample at 0 min, and *m* the sample' weigh in g.

2.11. Evaluation of the microbial growth in meat with active PLA films

Antimicrobial activity of the active PLA films was evaluated in meat at the end of the same storage days. The total microorganisms count at 30 °C was performed using the automated method TEMPO® Aerobic Count-AFNOR BIO 12/35-05/13.

2.12. Statistical analyses

All the assays were performed in triplicate. The results are expressed in means ± standard deviation. The statistical analysis was performed in the software IBM® SPSS® Statistics, version 27.0.1.0, using a one-way analysis of variance (ANOVA) and ANOVA with repeated measures. The differences among mean values were processed by the Tukey test.

3. Results

3.1. Extracts' antioxidant capacity and total content in phenolic compounds and flavonoids

The antioxidant capacity of the extracts is compiled in Table VIII.1. PPE-FD presented the highest inhibition percentage (175.3 ± 0.38 mg TE/g) and content in total phenolic compounds (221.5 ± 0.62 mg GAE/g) and total flavonoids (31.39 ± 0.61 mg ECE/g). The extract obtained from wort presented a low inhibition percentage and a low content in phenolic compounds. Also, the wort extract' total content in flavonoids was not possible to be determined since the solution kept precipitating.

Based on the results from Table VIII.1, associated with the difficulty in obtaining a workable extract from wort, PPE-FD was chosen to be incorporated into the PLA active packaging.

Table VIII.1. Antioxidant capacity of the extracts.

Samples	IP (%)	mg TE/g	TPC (mg GAE/g)	TFC (mg ECE/g)
PE-FD	92.42 ± 0.2a	175.3 ± 0.38a	221.5 ± 0.62a	31.39 ± 0.61a
PE-N	88.48 ± 0.31b	167.8 ± 0.58b	151.6 ± 0.47b	17.72 ± 1.08b
Wort	4.810 ± 0.14c	10.03 ± 0.27c	15.85 ± 0.16c	*

Legend: PE-FD – Pomegranate Freeze-dried extract; PE-N – Natural pomegranate extract; IP – inhibition percentage; TE – Trolox equivalents; TPC – Total content in phenolic compounds; GAE – Gallic acid equivalents; TFC – Total content in flavonoids; ECE – Epicatechin equivalents.
*Not determined.

Comparing these results with the literature and recent review paper by Andrade et al. [285], PPE-FD presented, in general, higher antioxidant capacity and total content in phenolic compounds. The DPPH radical inhibition percentage is similar to the inhibition percentage reported by Rashid et al. [567] (97.3 %) in the pomegranate extract obtained with ultrasonic assisted extraction with ethanol 70% (v/v). However, in the other pomegranate extract obtained though maceration by the same authors, the inhibition percentage is much lower (48.7 %). Lower inhibition DPPH radical percentages were also found by in the hydroethanolic extract by Selahvarzi et al. [568] (72.11 %). El-Hadary and Taha [569] found similar inhibition percentages in pomegranate peel extract obtained with methanol 80% (v/v).

The phenolics content of both pomegranate extracts was higher than the results reported by Zago et al. [570], Rashid et al. [567], and by Selahvarzi et al. [568] (66.14 mg GAE/g; 37. 52 mg GAE/g and 2.70 mg GAE/g, respectively). However, higher content in phenolic compounds was reported by Rashid et al. [567] (277.6 mg GAE/g) in a pomegranate extract obtained with ethanol 70% (v/v) by ultrasonic assisted extraction. Other solvents, such as methanol and acetone, can be more efficient in extracting bioactive compounds than ethanol. However, these may raise safety concerns [571].

3.2. Films' characterization

3.2.1. FTIR & SEM

As can be seen in Figure VIII.1, the PLA spectrum matches the PLA spectrum presented by Yuniarto et al. [572] and Bodbodak et al. [573]. The incorporation of the PPE-FD and the pomegranate peels (PP) seemed to have no effect on the PLA structure. Bodbodak et al. [573] only reported a slight shifting in the C-OH and carbonyl groups, at 1750 – 1754 cm-1, with the addition of the pomegranate peel extract, which was not observed in this study. Also, Miletićet al. [574] and Dai et al. [575] also reported no changes in PLA-based nanofibers with pomegranate seed oil and PLA active films with pomegranate peel extract, respectively. The change in 1716 cm-1 peak intensity for both PLA/3PPE and PLA/3PP confirms the presence of pomegranate by-products into the film samples.

Observing the SEM images in Figure VIII.2, for the control and active PLA films, a clear distinction can be seen among the control PLA, the PLA/3PPE and PLA/3PP. The PLA scanning shows similar results to previous studies [575]. PLA/3PPE presents a more homogenized distribution than the PLA/3PP, which presents layers indicating a non-homogenous distribution of the pomegranate peel. Also, roughness of PLA/3PP surface is

more pronounced due to the presence of larger pomegranate peel particles embedded in the PLA matrix.

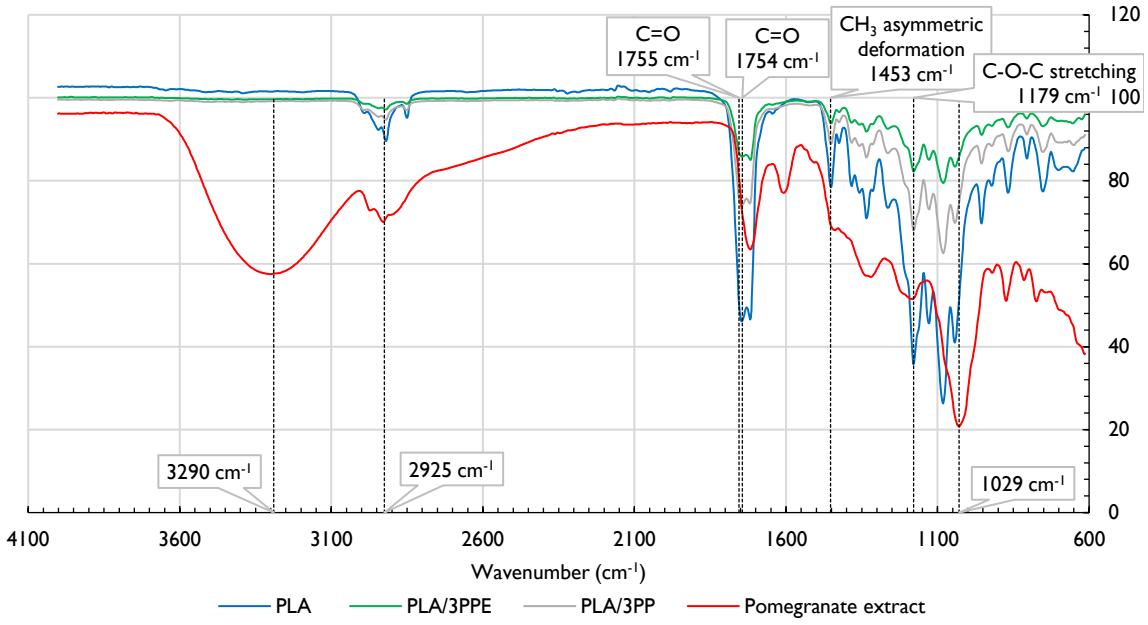


Figure VIII.1. FTIR spectrum of the pomegranate extract (PPE-FD), control (PLA) and active films (PLA/3PPE and PLA/3PP).

3.2.2. Water Vapor and Oxygen permeability and Color measurements

In terms of the films' color parameters (Table VIII.2), PLA/3PPE presented a higher variation in terms of *L**, *a** and *b** when compared to the PLA, than the PLA/3PP. The film that came closest to the color of the standard film (neat PLA) was PLA/3PP, as it can be seen by the coordinates *ΔL**, *Δa** and *Δb**. The *L** parameter (lightness) showed low values for all the samples with PLA/3PPE having the lowest value of 40.84. The *a** parameter, related to the green-red axis, showed a slight increase with the incorporation of pomegranate extract, contrary to the incorporation of pomegranate peel which maintained this parameter compared to the standard sample. The b* parameter, related to the blue-yellow axis, showed an increase from 4.65 to 8.77 with the incorporation of pomegranate extract, whereas the incorporation of pomegranate peels only increased to 6.485. These differences in the values of the Cielab coordinates do not significantly change the color of these films. The Cielab coordinates indicate that the films have a grayer color, although they visibly have a more brownish color. Overall, the change in color is more pronounced for the PLA/3PPE sample, due to an even distribution of the additive, compared to PLA/3PP.

PLA Haze, 17.5 ± 0.2 %, slightly increased with the incorporation of pomegranate extract, 19.0 ± 0.1 % and pomegranate peel 19.2 ± 0.4 %.

Table VIII.2. Optical properties of the control (PLA) and active (PLA/3PPE and PLA/3PP) films.

Samples	***L****	***a****	***b****	***ΔL****	***Δa****	***Δb****	***ΔE****
PLA	47.33	0.410	4.65	0	0	0	0
PLA/3PPE	40.84	0.600	8.77	-6.49	0.19	4.12	7.69
PLA/3PP	46.21	0.50	6.49	-1.12	0.085	1.84	2.15

Legend: PLA/3PPE – PLA with 3 % of pomegranate extract; PLA/3PP – PLA with 3 % of pomegranate peel.

Regarding barrier properties, the WVT tests were only performed for PLA and PLA/3PPE. The PLA film presented a WVT of 1.99 ± 0.57 g/m^{2}h having the film of PLA/3PPE obtained a very similar result of 1.94 ± 0.64 g/m^{2}h. The incorporation of pomegranate extract did not alter the water vapor barrier properties of the PLA film.

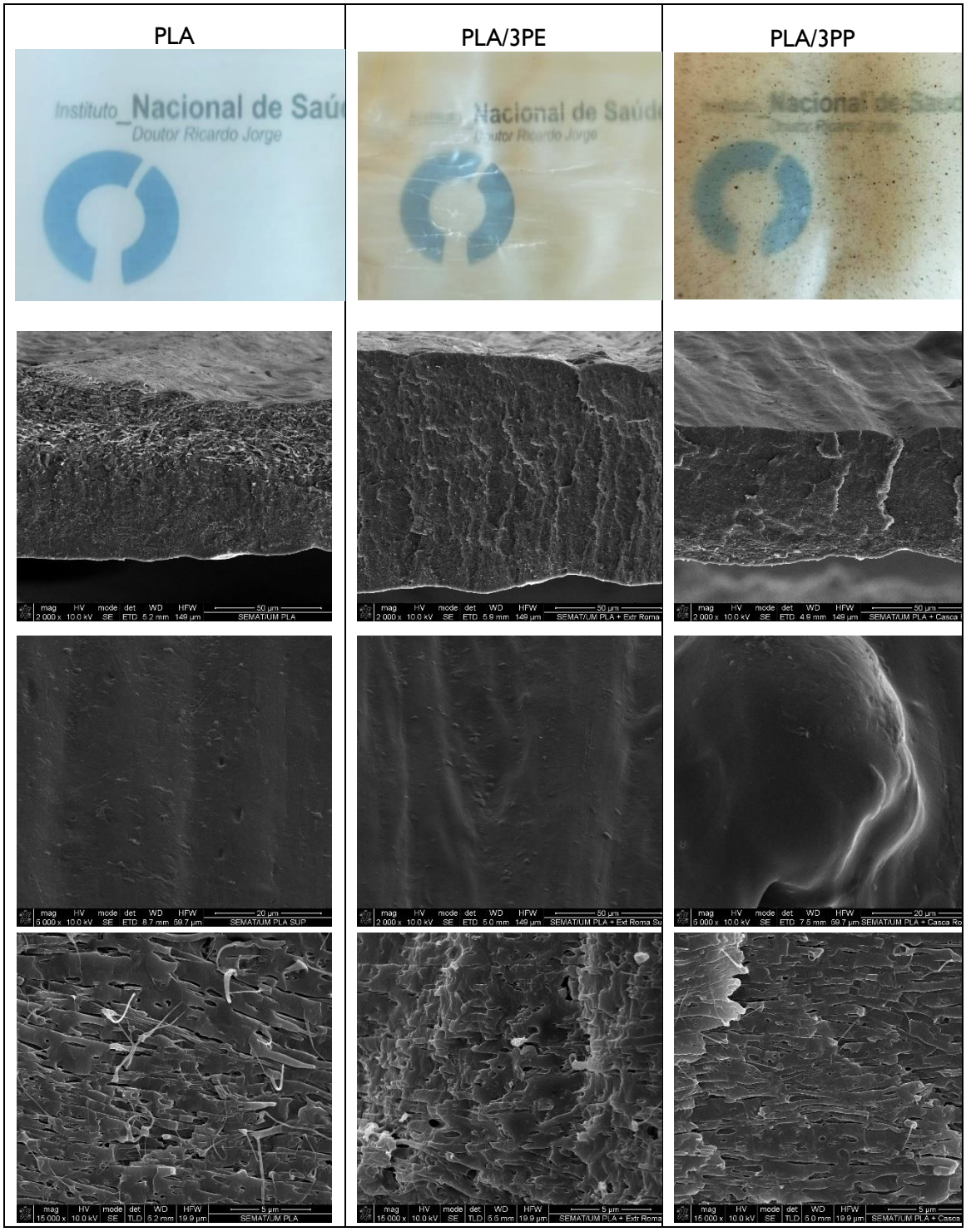


Figure VIII.2. Photographs of the control (PLA) and the active (PLA/3PE and PLA/3PP) films, and the respective SEM images.

3.2.3. Mechanical properties

The tensile test results can be seen in Figure VIII.3 in MD. The sample with the highest modulus and strength was the neat PLA. The values of the tensile test results relative to the other samples are much lower except for the percentage of deformation at break which increased considerably for the PLA/3PPE sample. It seems that the introduction of low mass molecules acts as a slip agent, allowing the polymer molecules to move, which increases the plastic region of the material. The incorporation of both pomegranate extract and peel clearly translates into a loss in stiffness.

Through dynamic mechanical analysis (Figure VIII.4) it is possible to observe that both pomegranate extract and peel have distinct effects on the polymeric matrix. DMA results show a well-defined peak around 57 °C, which is related to molecular relaxation of PLA chains (Tg). The presence of large particles (PLA/3PP) shifts the peak to higher temperature (60 °C), indicating that the peel particles restrict molecular mobility. On the other hand, pomegranate extract only increases the value of tan δ, showing that PLA/3PPE has higher loss module than PLA itself. These results are in agreement with tensile tests, where pomegranate extract has a positive effect on PLA ductile behavior, allowing PLA molecules to move more freely. Also, pomegranate by-products lower the storage modulus (E') over the temperature range, decreasing the stiffness of the polymer matrix.

3.3. Antioxidant capacity of the PLA films

The antioxidant capacity and the total content in phenolic compounds and flavonoids of the active PLA can be observed in Table VIII.3. PLA/3PE significantly presented a higher inhibition percentage and a higher content in total phenolic compounds than the PLA/3PP. PLA/3PP presented a significantly higher content in flavonoids.

Table VIII.3. Antioxidant capacity and total content in phenolic compounds and flavonoids of the active PLA films.

Samples	IP (%)	μg TE/dm^2	TPC (μg GAE/dm^2)	TFC (μg ECE/ dm^2)
PLA/3PE	13.34 ± 0.36^a	24.06 ± 0.67^a	14.64 ± 0.27^a	13.88 ± 1.04^a
PLA/3PP	7.19 ± 0.46^b	12.64 ± 0.85^b	11.59 ± 0.37^b	28.77 ± 0.77^b

Legend: PLA/3PE – PLA incorporated with 3 % of pomegranate extract; PLA/3PP – PLA incorporated with 3 % of pomegranate peel; IP – inhibition percentage; TE – Trolox equivalents; AAC – Antioxidant Activity Coefficient; TPC – Total content in Phenolic compounds; GAE – Gallic acid Equivalents; TFC – Total content in Flavonoids; ECE – Epicatechin Equivalents.

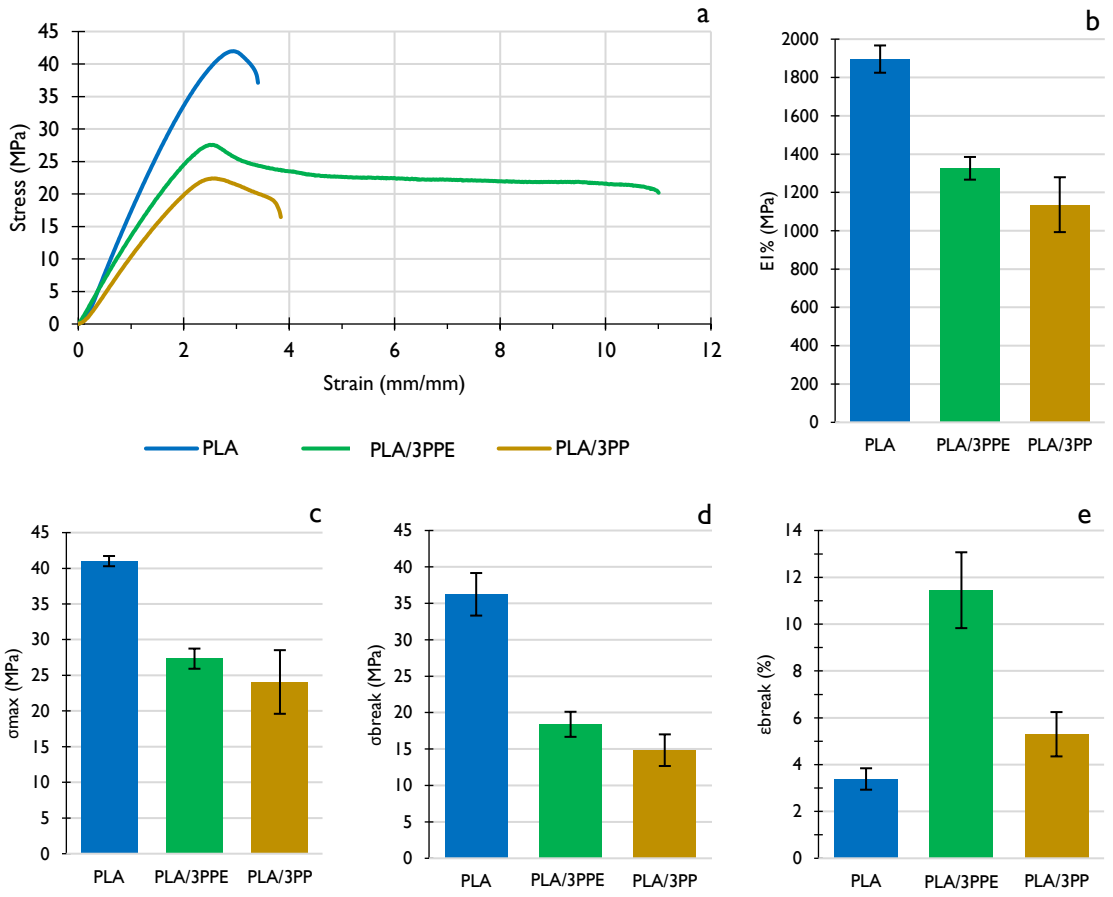


Figure VIII.3. Representative stress vs strain curves of PLA, PLA/3PPE and PLA/3PP in MD direction (VIII.3.a), and respective mechanical indexes (VIII.3.b, VIII.3.c, VIII.3.d).

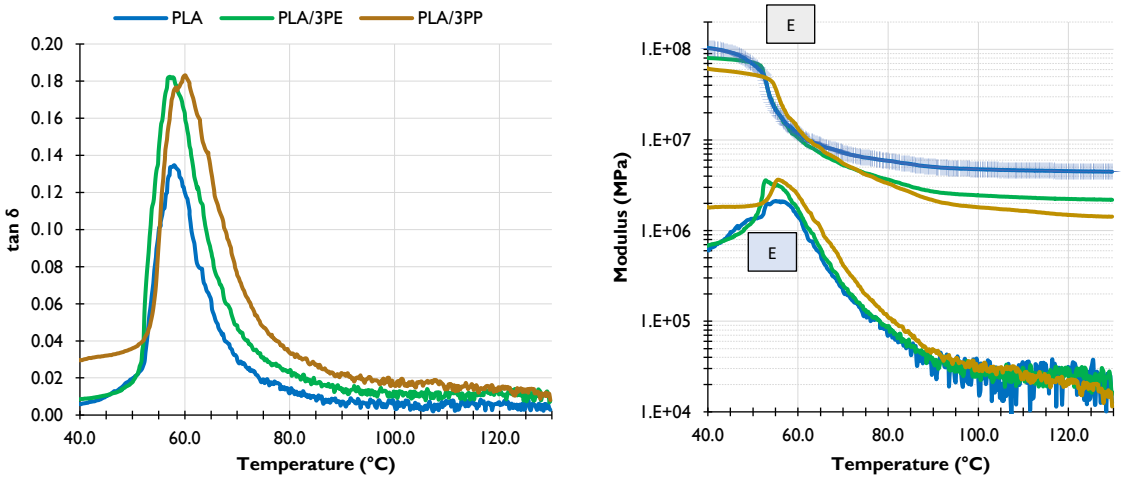


Figure VIII.4. DMA curves of PLA, PLA/3PE and PLA/3PP in MD direction: tan δ (left) and storage (E') and loss (E'') modulus (right).

Bodbodak et al. [573] developed active nanofiber based in PLA with a pomegranate peel extract by electrospinning that showed a DPPH radical inhibition percentage between 50 and 60%, in the nanofibers with 5 and 10% of extract. The inhibition percentages reported by these authors were higher than the reported in the present study, but the applied methodology for the migration assay did not follow the same procedure. Miletić et al. [574] also reported higher antioxidant activities of PLA-based nanofibers with 5 and 10% of pomegranate seed oil (43.53 and 40.94%, respectively). Dai et al. [575] also reported high antioxidant activity in PLA based films with pomegranate peel extract. PLA is a hydrophobic polymer. The introduction of polar and hydrophilic groups, such as pomegranate peels, may lead to the decrease of the PLA contact angle and, consequently, promote its hydrophilicity [573,576]. Concerning active food packaging, PLA' hydrophobicity is considered a limitation due to its low ability to encapsulating and gradually releasing antimicrobial and/or antioxidant compounds [573]. The higher release of phenolic compounds in the PLA/3PPE (Table VIII.3) suggests that the pomegranate peels have a higher compatibility with PLA than the PPE-FD.

3.4. Identification and Quantification of punicalagin (A+B) and ellagic acid by UHPLC-DAD

The chromatographic parameters of the method to identify and quantify punicalagin (A+B) and ellagic acid can be observed in Table VIII.4. Also, Table VIII.5 presents the content in punicalagin (A+B) and ellagic acid of the PPE-FD, freeze-dried pomegranate peels, pomegranate peels, and active films (PLA/3PE and PLA/3PP).

Punicalagin is one of the major phenolic compounds present in the pomegranate peels and mesocarp, with reported anti-inflammatory, antioxidant and antimicrobial activities [571,577]. Being a water-soluble compound, punicalagin can be spontaneous hydrolyzed into ellagic acid [571]. Ellagic acid is known for its antioxidant, antimutagenic and anticancer activities [578,579].

Table VIII.4. Validation parameters of the UHPLC-DAD method for the identification and quantification of punicalagin (A+B) and ellagic acid, in the pomegranate extracts and in the active films.

Analytes	Calibration curve	R^2	Linear range (µg/mL)	LoD (µg/mL)	LoQ (µg/mL)	Recovery	Repeatability	Precision
Punicalagin (A+B)	y = 27894x - 242255	0.9999	75 - 300	3.07	9.31	97.89 %	6.30 %	7.27 %
Ellagic acid	y = 120247x - 261405	0.9990	8 - 20	0.58	1.77	118.48 %	3.89 %	5.70 %

Legend: LoD – Limit of Detection; LoQ – Limit of Quantification

PPE-FD presented the highest content in punicalagin and ellagic acid, followed by the freeze-dried PP. El-Hadary and Taha [569] found lower content of ellagic acid (125.61 mg/kg) in pomegranate peel extract obtained with methanol 80 %. The authors did not quantify the punicalagin (A+B).

Table VIII.5. Quantification of punicalagin (A+B) and ellagic acid, in the pomegranate extract and by-products and in the active films.

Samples	Punicalagin (A+B)	Ellagic Acid
PPE	85.84 ± 0.15 mg/g^{a}	6.67 ± 0.01 mg/g^{a}
PP	12.15 ± 0.55 mg/g^{b}	0.77 ± 0.03 mg/g^{b}
PP-FD	44.22 ± 0.46 mg/g^{c}	1.94 ± 0.03 mg/g^{c}
PLA/3PE 40 °C, ethanol 95 %, 10 days	< LoD	0.30 ± 0.01 mg/dm$^{2\ b}$
PLA/3PP 40 °C, ethanol 95 %, 10 days	< LoD	0.31 ± 0.01 mg/dm$^{2\ b}$
PLA/3PE 40 °C, methanol, 24 h	< LoD	0.40 ± 0.03 mg/dm$^{2\ c,d}$
PLA/3PP 40 °C, methanol, 24 h	< LoD	0.43 ± 0.01 mg/dm$^{2\ d}$
PLA/3PE 25 °C, methanol, 24 h	< LoD	0.39 ± 0.00 mg/dm$^{2\ c}$
PLA/3PP 25 °C, methanol, 24 h	< LoD	0.27 ± 0.01 mg/dm$^{2\ a}$

Legend: LoD – Limit of Detection; PE – Pomegranate extract; PP – Pomegranate Peel; PP – Pomegranate Peel Freeze-Dried; PLA/3PE – PLA with 3 % of pomegranate extract; PLA/3PP – PLA with 3 % of pomegranate peel
Different letters represent results with significant differences.

Comparing the results found in the active PLA films, both PLA/3PPE and PLA/3PP presented similar content in ellagic acid. Punicalagin was not found in the food simulant for fatty foods (ethanol 95%, v/v) nor in methanol, this might be due to the high molecular weight of punicalagin (1 084.71 g/mol) [571] which prevents the migration of the molecule to the food simulant/solvent (punicalagin might be entrapped in the polymeric matrix) or due to the hydrolyzation of punicalagin into ellagic acid. Significant differences in the extraction of ellagic acid can be observed between the ethanol (95%) and methanol extraction. Methanol, although is not an approved solvent extraction for migration assays by the European Union, it is clear that this solvent can significantly extract more ellagic acid from PLA-based films, than the appointed solvent for fatty foods [540]. Also, temperature plays a significant role for the extraction of these compounds in PLA-based packaging.

3.5. Antimicrobial analysis of the extract and the active PLA films

Regarding the antimicrobial activity of the extract, the results can be observed in Figure VIII.5. Inhibition hallows can be observed for the *S. aureus* (40 mm of diameter), *E. coli* (30 mm of diameter) and *Enterococcus* spp. (12 mm of diameter). No inhibition was observed against *L. monocytogenes*.

Antimicrobial activity against *S. aureus* and *E. coli* of extracts obtained from pomegranate peels is reported in the literature [242,264,268,580]. Three of these studies also reported antimicrobial activity of pomegranate peel extracts against *L. monocytogenes*, which was not observed in this study. The main differences between the pomegranate extract from the present work and the other extracts are the extraction processes. Although the extract developed by Harini et al. [264], using 100 % ethanol as solvent, was similar to the extraction procedure, the ratio between the pomegranate peels and solvent is different as well as the extraction time (30 min for 16 h). The other pomegranate extracts were obtained with different solvents and different temperatures. These differences can explain the lack of antimicrobial activity against *L. monocytogenes*.

The results obtained for the antimicrobial activity of active PLA can be observed in Table VIII.6. The PLA/3PPE and PLA/3PP presented antimicrobial activity against *S. aureus*. Also, a decrease in the count of bacteria in the *L. monocytogenes* with PLA/3PPE seem to indicate a potential antimicrobial activity against this microorganism. However, neither the PLA/3PPE and the PLA/3PP presented antimicrobial activity against E. coli and *E. faecalis*.

Bodbodak et al. [573] and Dai et al. [575] reported antimicrobial activity of nanofibers and active films with pomegranate peel extracts against S. aureus and E. coli. Mushtaq et al. also reported antimicrobial activity of an aqueous pomegranate peel extract against, not only *E. coli* and *S. aureus*, but also against *Proteus vulgaris, Micrococcus luteus* and *E. faecalis*.

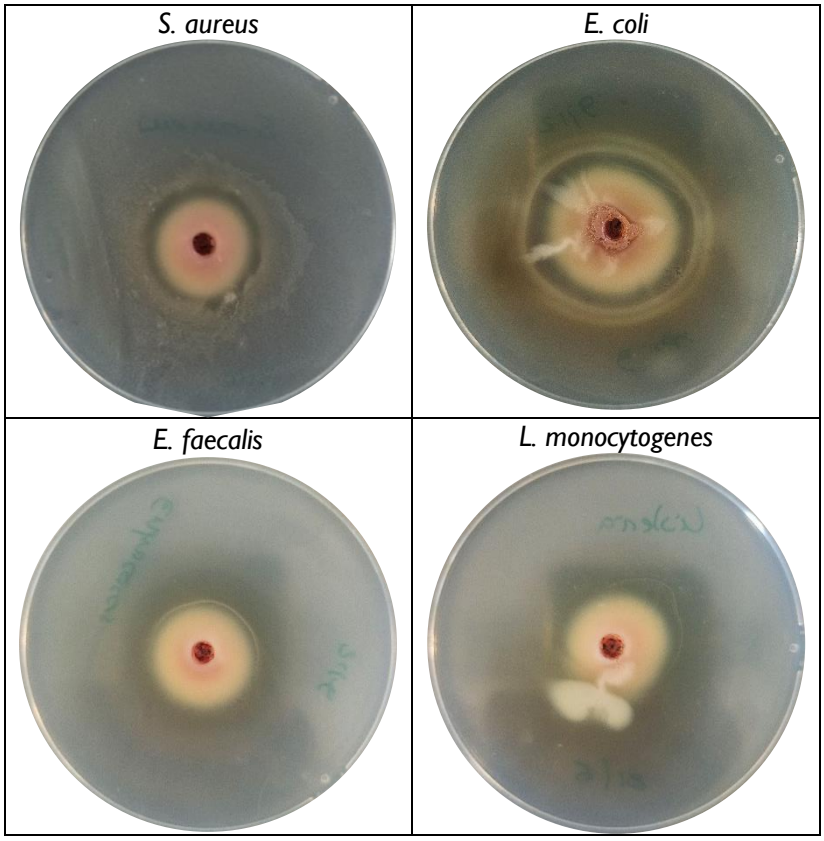


Figure VIII.5. Antimicrobial activity of the pomegranate extract.

Table VIII.6. Antimicrobial activity of the control (PLA) and active (PLA/3PE and PLA/3PP) films.

Samples	Dilution	*S. aureus*		*L. monocytogenes*		*E. coli*		*E. faecalis*	
		UFC	Log/cm²	UFC	Log/cm²	UFC	Log/cm²	UFC	Log/cm²
PLA	1	>300	-	>300	-	>300	-	>300	-
	10^{-1}	>300		>300		>300		>300	
	10^{-2}	>300		>300		>300		73	
PLA/3PE	1	15	0.97	>300	-	>300	-	>300	-
	10^{-1}	1.5		>300		>300		>300	
	10^{-2}	0		144		>300		>300	
PLA/3PP	1	4	0.49	>300	-	>300	-	>300	-
	10^{-1}	0.5		>300		>300		>300	
	10^{-2}	0.5		>300		>300		>300	

Legend: PLA/3PE – PLA with 3 % of pomegranate extract; PLA/3PP – PLA with 3 % of pomegranate peel.

3.6. Lipid oxidation of the almond packaged with the active films

The MDA eq. values (Table VIII.7 and Table VIII.8) of the almond packaged with the PLA significantly increased over time. A significant rise of the MDA eq. value occurs until the 4th day of accelerated storage, in the almonds' packaged with the PLA, PLA/3PPE

and PLA/3PP. Then, in the 4th day of accelerated storage, the almond packaged with the PLA/3PP presented a significantly higher MDA eq. value than the almond packaged with the PLA. In the 21st day of storage, the PLA/3PPE presented a significantly lower MDA eq. value than the PLA and PLA/3PP. At the 30th day of accelerated storage, only the PLA/3PP showed significantly higher MDA eq. values than the PLA and PLA/3PPE. Looking at the TBARS results from the almond stored at room temperature, no significant differences can be observed between the three types of packaging. However, the MDA eq. value significantly increased in the almond packaged with the PLA/3PP.

In the peroxide assay results (Table VIII.7 and Table VIII.8), there are no significant differences in the almond packaged with the PLA over time, either in the accelerated assay and in the assay at room temperature. However, in the accelerated assay, the active films presented significantly lower peroxide values than the control, at the 7th storage day. In the almond stored at room temperature, there are no significant differences between the control PLA and the PLA/3PPE and PLA/3PP.

In the accelerated assay, the PLA/3PPE and the PLA/3PP showed lower p-anisidine values than the PLA at the end of 2 storage days. Also, the PLA/3PP showed significant lower p-anisidine values at the end of 21 storage days. In the almond stored at room temperature, PLA/3PP showed lower *p*-anisidine value than the PLA at the end of the 7th day of storage.

The results seem to indicate that the PLA/3PP is more efficient in preventing the lipid oxidation that the PLA/3PPE. However, in the three lipid oxidation assays, both films accelerated the lipid oxidation process instead of delaying it, with the exception of the 7th day of storage in the assay at room temperature and on the 21st day of storage. These results indicate that this type of packaged is not indicated to storage almond for long time periods, since they cannot prevent the formation of primary and secondary lipid oxidation products.

Nevertheless, the *in vivo* antioxidant potential of pomegranate extracts is reported in the scientific literature. For example, El-Hadary and Taha [569] applied pomegranate peel extract to sunflower, soy bean and corn oil and submitted the oils to 70 °C for 10 days. The pomegranate peel extract, when compared to the control, significantly decreased the peroxide value content and the *p*-anisidine value of the oils in the 10-day period.

Table VIII.7. Results of the TBARS assay, determination of the peroxide value and the p-anisidine value of the almond packaged with control and active PLA, stored at 40 °C (accelerated assay).

Storage days	PLA			PLA/3PPE			PLA/3PP		
	mg MDA eq/kg	meq O_2/kg	*p*-anisidine value	mg MDA eq/kg	meq O_2/kg	*p*-anisidine value	mg MDA eq/kg	meq O_2/kg	*p*-anisidine value
0	3.53 ± 0.16^{Aa}	0.037 ± 0.01^{Aa}	35.37 ± 0.21^{Ac}	3.53 ± 0.16^{Aa}	0.04 ± 0.01^{Aab}	35.37 ± 0.21^{Ab}	3.53 ± 0.16^{Aa}	0.04 ± 0.01^{Aab}	35.37 ± 0.21^{Acd}
2	6.89 ± 0.35^{Ab}	0.041 ± 0.018^{Aa}	33.91 ± 0.37^{Ac}	6.76 ± 0.82^{Ab}	0.035 ± 0.00^{Aa}	29.02 ± 0.55^{Ba}	6.54 ± 0.79^{Ab}	0.033 ± 0.00^{Aab}	30.7 ± 0.54^{Cbc}
4	11.65 ± 0.65^{Af}	0.035 ± 0.003^{Aa}	36.57 ± 0.36^{Ac}	12.01 ± 0.39^{ABc}	0.033 ± 0.003^{Aa}	39.95 ± 3.19^{Ab}	12.92 ± 0.85^{Be}	0.03 ± 0.002^{Aa}	39.11 ± 2.54^{Ade}
7	8.89 ± 1.18^{Acd}	0.086 ± 0.001^{Ab}	34.35 ± 0.08^{Ac}	9.99 ± 1.22^{Ac}	0.062 ± 0.004^{Bc}	48.88 ± 0.2^{Bc}	8.27 ± 1.28^{Ac}	0.077 ± 0.001^{Cc}	40 ± 0.2^{Cde}
14	9.63 ± 0.31^{Ade}	0.034 ± 0.001^{Aa}	34.96 ± 2.39^{Ac}	10.78 ± 1.46^{Ac}	0.051 ± 0.004^{Bbc}	34.75 ± 4.18^{Ab}	10.96 ± 0.4^{Ad}	0.047 ± 0.002^{Bb}	44.17 ± 6.53^{Ae}
21	10.63 ± 0.62^{Aef}	0.17 ± 0.001^{Ac}	27.91 ± 0.32^{Ab}	6.21 ± 0.12^{Bb}	0.199 ± 0.006^{Bd}	28.99 ± 0.24^{Ba}	8.02 ± 0.24^{Cc}	0.188 ± 0.013^{ABd}	15.93 ± 0.25^{Ca}
30	7.76 ± 0.69^{Ac}	0.037 ± 0.003^{Aa}	21.39 ± 1.1^{Aa}	10.86 ± 2.81^{Ac}	0.037 ± 0.002^{Aab}	27.22 ± 0.94^{Ba}	11.07 ± 1.76^{Bd}	0.046 ± 0.003^{Bab}	25.59 ± 0.19^{Bb}

Legend**:** PLA – Polylactic acid; PLA/3PPE – PLA with 3 % of Pomegranate extract; PLA/3PP – PLA with 3 % of pomegranate peel
The uppercase letters compare the same packaging for different storage days. Different letters, for either lowercase/uppercase comparison, represent results with significant differences.

Table VIII.8. Results of the TBARS assay, determination of the peroxide value and the p-anisidine value of the almond packaged with control and active PLA, stored at room temperature (23 °C).

Storage days	PLA			PLA/3PPE			PLA/3PP		
	mg MDA eq/kg	meq O_2/kg	*p*-anisidine value	mg MDA eq/kg	meq O_2/kg	*p*-anisidine value	mg MDA eq/kg	meq O_2/kg	*p*-anisidine value
0	3.53 ± 0.16^{Aa}	0.037 ± 0.009^{Aa}	35.37 ± 0.21^{Ad}	3.53 ± 0.16^{Aa}	0.037 ± 0.009^{Aa}	35.37 ± 0.21^{Ab}	3.53 ± 0.16^{Aa}	0.037 ± 0.009^{Aa}	35.37 ± 0.21^{Ab}
7	6.61 ± 0.98^{Ab}	0.053 ± 0.005^{Ab}	31.28 ± 0.67^{Ac}	5.96 ± 0.45^{Ab}	0.06 ± 0.009^{Ab}	40.5 ± 0.41^{Bb}	5.73 ± 0.53^{Ab}	0.065 ± 0.007^{Ab}	29.86 ± 0.43^{Ca}
14	6.44 ± 0.04^{Ab}	0.041 ± 0.001^{Aab}	27.58 ± 1.81^{Ab}	6.73 ± 0.24^{Ab}	0.042 ± 0.002^{Aab}	37.21 ± 4.73^{Bb}	7.09 ± 0.56^{Ac}	0.039 ± 0.001^{Aa}	31.12 ± 2.27^{ABa}
21	6.29 ± 0.27^{Ab}	0.138 ± 0.004^{Ac}	24.48 ± 0.48^{Aa}	6.24 ± 0.51^{Ab}	0.144 ± 0.006^{Bc}	24.16 ± 0.57^{Aa}	6.89 ± 0.08^{Ac}	0.153 ± 0.004^{Bc}	29.53 ± 0.3^{Ba}

Legend**:** PLA – Polylactic acid; PLA/3PPE – PLA with 3 % of Pomegranate extract; PLA/3PP – PLA with 3 % of pomegranate peel.
The uppercase letters compare the same packaging for different storage days. Different letters, for either lowercase/uppercase comparison, represent results with significant differences.

3.7. Lipid oxidation and antimicrobial analysis of the meat packaged with active films

Regarding the lipid oxidation of the meat packaged with the PLA films (FigureVIII.6), both active PLA films were significant effective in delaying meats' lipid oxidation, in comparison to PLA. Between the two active films, PLA/3PP was more effective than the PLA/3PPE. Contrary to the results obtained in the almond assays, a clear decrease can be observed in the malonaldehyde equivalents over time.

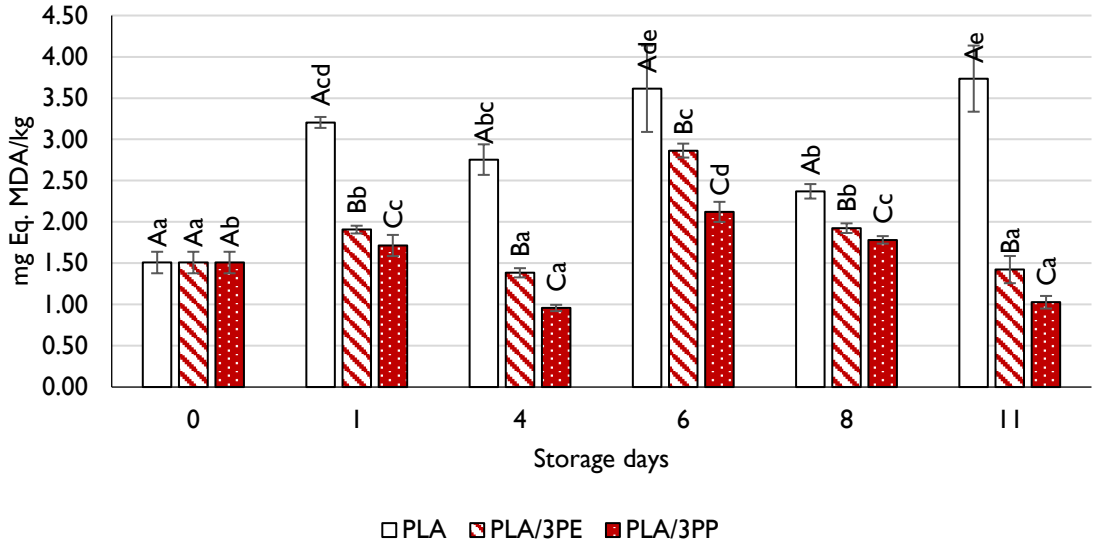
The results from the total microorganism count of the meat can be observed in Table VIII.9. The meat packaged with the PLA showed significant growth of microorganisms at the 4th storage day and again at the 11th storage day, as opposed to the meat packaged with the active PLA (PLA/3PPE and PLA/3PP). The microbiological growth in the meat packaged with the PLA presented a significant growth at the 4th and 8th storage day. The same was not observed in the meat packaged with the active PLA, indicating that the active PLA-based films possess some antimicrobial activity. The microbiological assay results support the hypothesis that both the PE and PP are viable additives to be applied to meat and meat products to delay its lipid oxidation and to assure meat and meat products safety.

The results of the present study are in agreement with the scientific literature, since the antimicrobial activity of the pomegranate by-products and its extracts is reported against both Gram positive and Gram negative bacteria [581–584].

Table VIII.9. Results of the microbiological assays regarding the meat packaged with the control (PLA) and the active (PLA/3PE and PLA/3PP) films.

Storage days	PLA (CFU/g)	PLA/3PE (CFU/g)	PLA/3PP (CFU/g)
0	2.5×10^6	2.5×10^6	2.5×10^6
1	3.0×10^6	2.2×10^6	1.4×10^7
4	9.0×10^8	3.2×10^6	8.6×10^6
6	8.1×10^8	2.8×10^6	4.2×10^6
8	3.3×10^8	1.2×10^7	1.7×10^7
11	8.3×10^9	3.3×10^7	2.9×10^7

Legend: CFU - colony forming unit; PLA – Polylactic acid; PLA/3PE – PLA with 3 % of Pomegranate extract; PLA/3PP – PLA with 3 % of pomegranate peel



FigureVIII.6. Results of the TBARS assay of the beef-meat packaged with the control (PLA) and the active (PLA/3PE and PLA/3PP) films.

4. Conclusions

A pomegranate peel extract, with high antioxidant capacity and a high content in total phenolic compounds and flavonoids, was successfully obtained with a simple and economic solvent-extraction method, using only ethanol (a food grade solvent) as an extraction solvent. On the other hand, the ethanolic extract obtained from wort did not present high antioxidant activity. A chromatographic method for the identification and quantification of punicalagin (A+B) and ellagic acid was successfully validated by UHPLC-DAD system. The components were quantified in the pomegranate peel, natural and freeze-dried, and in the pomegranate extract.

Pomegranate extract (PE) and pomegranate peel (PP) were successfully incorporated into active polylactic acid-based packages (PLA/3PPE and PLA/3PP). The addition of the PE and the PP seemed to have no significant interference in the morphologic characteristics of the PLA. It was only possible to quantify ellagic acid in the active films both in the food simulant (ethanol 95%, v/v) and extraction solvent (methanol). PE presented antimicrobial activity against strains of *S. aureus*, *E. coli* and *E. faecalis* but the active films only presented antimicrobial activity against S. aureus, suggesting that the PLA traps the antimicrobial compounds.

Regarding the lipid oxidation, the active films were extremely efficient in protecting the beef-meat against lipid oxidation and microbiological growth, when comparing to the control PLA. The same cannot be concluded in the almond assays, indicating that this new active packaging is more suitable to extend the shelf life of meat and meat products.

Chapter IX

PLA films loaded with green tea and rosemary polyphenolic extracts as an active packaging for almond and beef

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1. Introduction

Currently, food packaging is mainly based on highly durable non-biodegradable plastics, such as polyethylene and polyethylene terephthalate [585,586]. Their high versatility and wide application promote an annual production of more than 300 million tons of plastic [586]. Being a non-biodegradable product, its accumulation in the environment causes serious concerns and imbalances [586]. Finding suitable substitutes for these materials has become a priority for the scientific and industry communities.

One of the most promising candidates to substitute conventional polymers in food packaging is Polylactic acid (PLA) due to its high biocompatibility and biodegradability [427,524]. Indeed, this compound is already widely used in medical applications [587–589]. Moreover, PLA is produced through the polymerization of lactic acid of natural sources such as corn and sugarcane, can be manufactured by most polymer processing equipment, and is easily molded. Several additives, such as nanoparticles, plasticizers, and bioactive compounds, allows the improvement of PLA oxygen barrier and water vapor permeability [427,524,590].

The main function of food packaging is to protect food against several external factors responsible for accelerating food spoilage, such as radiation, microorganisms contamination, oxygen and humidity [2,384]. Conventional food packaging cannot interact with the packed food, meaning, they should be inert and migration from the package constituents to the packaged food should not occur [11,428]. On the opposite, active food packaging is intentionally manufactured to positively interact with the packed foods, by absorbing or releasing compounds from/into the food matrix [11,591].

The interest in PLA-based active food packaging has been growing, either incorporated with antioxidant and antimicrobial compounds or with nanoparticles to improve its mechanical and barrier properties [590,592–594]. Rojas et al. [593] and Velásquez et al. [595] recently review the incorporation of active agents, in the form of plant extracts and/or essential oils, in PLA to for active food packaging materials. The authors clearly stated that the addition of active agents decrease PLA' elastic modulus and increase PLA' elongation at break. Also, the addition of active agents in low percentages translates into higher microbiological growth inhibition [593]. Another important parameter is the chosen technique for the active agents' incorporation in the PLA matrix. Melting-based technics are the most common processes but, when dealing with thermosensitive agents, such as essential oils or plant extracts, other techniques must be considered such as casting and electrospinning [595].

Lipid oxidation is one of the main causes of food spoilage, especially in meat and meat products [365,366]. Unsaturated fatty acids are very susceptible to oxidization, reacting with molecular oxygen, producing hydroperoxides, (primary oxidation) which are highly unstable and will decompose resulting in secondary compounds, such as hydrocarbons, aldehydes, ketones, alcohols, esters and acids [36,366]. One process of protecting food against lipid oxidation, is through the addition and presence of antioxidant compounds, such as phenolic compounds from natural sources.

Rosemary (*Rosmarinus oficinallis* L.) is an aromatic plant from the Lamiaceae family with proven antioxidant and antimicrobial activities, through their main bioactive compounds rosmarinic acid, carnosic acid and carnosol [183,555]. Rosmarinic acid is commonly found in the Lamiaceae family and has proven to have antioxidant, antibacterial, anti-inflammatory, and antiviral activities [596–600]. Carnosic acid and carnosol, an oxidative derivative of carnosic acid, have demonstrated antioxidant and antibacterial activities [408,601–605]. The application of the rosemary extract as a food additive is approved by the Regulation (EU) nº 231/2012 from the European Commission and its subsequent amendments [317]. Green tea, obtained from *Camellia sinensis* L. leaves, is a rich source of catechins, flavonoids and phenolic acids, powerful antioxidants [590]. Catechins, the most abundant flavonoids in green tea, have shown antioxidant, anti-inflammatory and anti-viral activities [606,607], even against SARS-CoV-2 [608]. Among catechins, epigallocatechin gallate (EGCG) is the most abundant in green tea leaves and is known for its health benefits, such as neuroprotective effects against neurodegenerative diseases like Alzheimer's and Parkinson's [609]. According to the extensive review by Hu et al. [610], the adults' safe intake level of EGCG is 338 mg/day.

In the literature, there are several examples of the use of green tea extracts to delay or inhibit lipid oxidation and to inhibit pathogens growth. Castro et al. [426] incorporated a commercial green tea extract into a whey protein-based coating and successfully inhibited the lipid oxidation of salmon for 14 days of storage, when compared with the control. Also, Robalo et al. [611] incorporated a green tea extract into a whey protein-based film, which successfully delayed the lipid oxidation of goat cheese and inhibited the microbiological load. Martins et al. [590] delayed the lipid oxidation of smoked salmon for 60 days with a based PLA active packaging loaded with a commercial green tea extract. Vilarinho et al. [594] developed a PLA active packaging with green tea extract and cellulose nanocrystals to inhibit the lipid oxidation of salami slices. Additionally, Zeid et al. [612] proved the efficiency of a PLA-based active food packaging with rosemary essential oil on preserving fresh rainbow trout for 6 days. Darie-Niță [613]

developed a PLA-based active packaging with an ethanolic rosemary extract. The incorporation of the extract improved the elongation at break, rheological properties and antimicrobial activity against *Bacillus cereus*, *Salmonella* Typhimurium and *Escherichia coli* [613]. The present study resorts to ethanolic extracts of rosemary and green tea extracts and studies a possible synergic effect between the two extracts. Therefore, the main objective of this study was to evaluate the capacities and properties of ethanolic extracts obtained from rosemary and green tea, incorporated into PLA films, and to study their capacity to extend the shelf-life of almonds and beef.

2. Materials and Methods

2.1. Reagents and materials

Absolute ethanol (ACS grade reagent, for analysis), methanol (ACS grade reagent (≥99.8%) and for HPLC, ≥99.9%), chloroform (SupraSolv®, for gas chromatography ECD and FID), sodium carbonate anhydrous (ACS grade reagent), sodium nitrite (ACS grade reagent), sodium hydroxide (ACS grade reagent), petroleum ether (ACS grade reagent, bp 40-60 °C), barium chloride dihydrate (ACS gradde reagent), iron(II) sulfate (pro analysis), iron(III) chloride (anhydrous for synthesis), hydrochloric acid, glacial acetic acid (for HPLC, ≥99.9%), Folin-Cioucalteu's phenol reagent, were acquired from Merck (Darmstadt, Germany). *n*-Hexane (SupraSolv®, for gas chromatography ECD and FID) was acquired from Honeywell. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Tween®40, β-carotene (≥93%), linoleic acid (analytical standard), aluminum chloride, trichloroacetic acid (ACS grade reagent, ≥99.0%), 2-thiobarbituric acid (≥98%), 1,1,3,3-tetramethoxypropane (97%), 2,2-diphenyl-1-picrylhydrazyl, xylenol orange sodium (spectrophotometric grade), 2,4-diphenilhydrazin, hexanal (98%), were acquired from Sigma-Aldrich (Madrid, Spain). Ultra-pure water was obtained through a Milli-Q® purification system (Millipore Corp., Belford, USA).

Concerning the used equipment, a compact stirrer Edmund Bühler™ Shaker KS 15 A (Hechingen, Germany), an Eppendorf AG 5804R centrifuge (Hamburg, Germany), a rotary evaporator Büchi model R-210 (Labortechnik, Switzerland), a Thermo Scientific Evolution 300 LC spectrophotometer, a RSLAB-6PRO Vortex, Ultra-Turrax IKA® DI 25basic, a Grindomix GM 300 (Retsch) and a Grant Instruments™ QB Series Dry Block Heating System (Cambridge, England) were used.

2.2. Rosemary and Green tea extraction

Rosemary (*Rosmarinus officinalis* L.) dried leaves were obtained on a local store in Lisbon, Portugal. Dried leaves of green tea (*Camellia sinensis* L.), variety "Encosta de Bruma", were purchased from Gorreana, Azores, Portugal. As soon as the plants arrived to the laboratory, the leaves were ground in the Grindomix and extraction was made according to the method described by Andrade et al. [183]. Briefly, the powder was mixed with absolute ethanol in a 1:10 ratio. Then, the mixtures were agitated in the compact stirrer for 30 minutes, centrifuged for 15 minutes at 4025 g, at 10 °C, and the supernatant was evaporated until dryness in a rotary evaporator at 35 °C. The extract was removed with the help of a spatula, vacuum packaged and stored at –20 °C, protected from the light, until further use.

2.3. Antioxidant assays

For the evaluation of the antioxidant activity of the extracts, both rosemary extract (RE) and green tea extract (GTE) were diluted in absolute ethanol at 1.0 mg/mL and 0.1 mg/mL. All the assays were performed in triplicate. Also, to evaluate possible synergic effects, 3 solutions containing both extracts at 0.1 mg/mL were made: S1 containing 50 % of each extract (RE and GTE); S2 containing 75 % of the rosemary extract (RE) and 25 % of green tea extract (GTE); and S3 containing 75 % of GTE extract and 25 % of RE.

2.3.1. DPPH radical scavenging assay

The original method was described by Moure et al. [501] and adapted by Andrade et al. [183]. In short, 50 μL of sample (for the blanks, 50 μL of absolute ethanol) were mixed with 2 mL of a methanolic DPPH• solution (14.2 μg/mL). The samples were left for 30 minutes, protected from the light and, at the end of this period, the absorbance was measured in the spectrophotometer at 515 nm. The DPPH• Inhibition Percentage (IP) was calculated according to the equation 1.

$$IP\ (\%) = \frac{Ab - As}{Ab} \times 100 \quad (1)$$

where, *Ab* stands for the absorbance of the blank and *As* stands for the absorbance of the sample. A calibration curve, using trolox as standard was drawn, and the results are also expressed in mg of trolox equivalents per g of sample (mg TE/g).

2.3.2. Beta-Carotene Bleaching Assay

The performed method was originally described by Miller [431] and adapted by Andrade et al. [183]. Briefly, a solution of β-carotene in chloroform was prepared (2 mg/mL). Then, for the β-carotene:linoleic acid emulsion, 20 mg of linoleic acid were mixed with 200 mg of Tween®40 and 1 mL of the β-carotene solution. The chloroform was evaporated under a constant flow of nitrogen and 50 mL of ultrapure water were added. The emulsion was vigorously agitated. To 200 µL of sample, 5 mL of the emulsion were added. For the blanks, 200 µL of ethanol were used and the absorbance was immediately read, in the spectrophotometer at 470 nm. The samples and the blanks were kept at 50 °C in the heating block for 2 hours and the absorbances were measured, at 470 nm, while warm. The Antioxidant Activity Coefficient (AAC) was calculated according to the equation 2.

$$AAC = \frac{As - Ab2}{Ab0 - Ab2} \times 1000 \quad (2)$$

where, *As* strands for the absorbance of the samples, *Ab0* stands for the initial absorbance of the blanks and *Ab2* stands for the absorbance of the blanks at the end of 2 hours.

2.4. Total Content in Phenolic Compounds (TPC) and Flavonoids (TFC)

The method used to quantify the total content of the extracts in phenolic compounds was described by Erkan et al. [462]. In sum, 7.5 mL of Folin-Ciocalteu (10 %, v/v) were added to 1 mL of sample and the mixture was homogenized. After 5 minutes, 7.5 mL of sodium carbonate aqueous solution (60 mg/mL) were added, the mixtures were once again homogenized and kept in the dark for 2 hours. At the end of this period, the absorbance was measured at 725 nm. A standard calibration curve using gallic acid in ethanol was drawn and the results are expressed in mg of gallic acid equivalents per mL (mg GAE/g).

For the total quantification of flavonoids, the method described by Yoo et al. [463] (2008) was performed. Succinctly, 4 mL of ultrapure water and 300 µL of an aqueous solution of sodium nitrite (5 %, w/v) were added to 1 mL of sample and the mixture was homogenized. At the end of 5 minutes, 600 µL of aluminum chloride (10 %, w/v) were added and the mixture was homogenized. At the end of 6 minutes, 2 mL of an aqueous solution of sodium hydroxide (1 M, w/v), and 2.1 mL of ultrapure water were added. The mixture was homogenized, and the absorbance was measured in the spectrophotometer at 510 nm. Epicatechin in ethanol was used as a standard for the calibration curve. The results are expressed in mg of epicatechin equivalents per mL (mg ECE/g).

2.5. Determination and quantification of individual phenolic compounds and catechins

Identification and quantification of individual phenolic compounds and catechins was performed in a UPLC® ACQUITY™ (Waters, Milford, MA, EUA) equipped with a DAD detector, using an ACQUITY™ UPLC® BEH C18 (2.1 × 150 mm, 1.7 µm particle size) column. Column was kept at 35 °C and samples at 5 °C and the injection volume was 20 µL. Mobile phase A was ultra-pure water acidified with glacial acetic acid at 0.1 % (v/v) and mobile phase B was acetonitrile with glacial acetic acid at 0.1 % (v/v) with a flow of 0.5 mL/min. A standard curve using a mix of 14 phenolic compounds diluted in methanol acidified with glacial acetic acid at 0.1 % (v/v) was drawn. The gradient was as follows: 0 min, 99 % A; 2 min, 99 % A; 2.5 min, 98 % A; 3 min, 95 % A, 15 min, 80 % A; 18 min, 70 % A, 20 min, 60 % A; 23 min, 10 % A; 25 min, 99 % A. The 14 individual phenolic compounds were gallic acid, protocatechuic acid, gentisic acid, chlorogenic acid, caffeic acid, p-coumaric acid, rutin, rosmarinic acid, quercetin, apigenin, kaempferol, hesperetin, carnosol and carnosic acid.

For the identification of individual catechins, same column and mobile phases were used. The gradient was as follows: 0-2 min, 99 % A; 2.5 min, 98 % A; 3 min, 95 % A; 15 min, 80 % A; 17 min, 99 % A. The flow was kept at 0.3 mL/min, the column at 35 °C and samples at 5 °C. The injection volume was 20 µL. A standard curve using a mix of 5 catechins and gallic acid diluted in methanol acidified with glacial acetic acid at 0.1 % (v/v) was drawn. The identified catechins were gallocatechin (GC), catechin, epicatechin (EC), epigallocatechin gallate (EGCG), gallocatechin gallate (GCG). The calibration curve equations, detection and quantification limits, the coefficient of determination (r2) and range of concentrations are present in the supplementary material.

2.6. Preparation of rosemary and green tea PLA films

Five different active PLA films were prepared, first by melt mixing 2 and 4 % of GTE (PLA/2GT and PLA/4GT), 2 and 4 % of RE (PLA/2R and PLA/4R), and, to evaluate the potential synergistic effect of the two extracts, 2 % of RE and 2 % of GTE (PLA/2GT/2R) in a PLA matrix, this was performed in a twin screw extruder, Leistritz AG LSM 34 6L, using a constant barrel temperature of 170 ºC, a screw speed of 125 rpm and feed rate of 4 $kg.h^{-1}$. All the compounds were dried in a vacuum oven at 60 ºC, overnight prior to blown film extrusion. Monolayer films were prepared using a Periplast single screw extruder. The thickness of the films was in 50 to 60 µm range. The processing conditions were constant, with a screw speed of 50 rpm, 170 ºC on the first heating zone, 175 ºC

on the second zone and 180 ºC on the remaining zones. A digital micrometer (No. 293-340, Mitutoyo, Kanagawa, Japan) was used to measure the thickness measurement of the films.

2.6.1. Fourier Transform Infrared spectroscopy (FTIR)

FTIR spectroscopy of the films was performed using a Bruker FT-IR VERTEX 80/80v (Boston, USA) in Attenuated Total Reflectance mode (ATR) with a platinum crystal was used to obtain the FTIR spectra. The measurements were recorded from 4000 to 400 cm^{-1} wavenumber, at a resolution of 4 cm^{-1} and 64 scans. All the analyses were performed in duplicate.

2.6.2. Scanning electron microscopy (SEM)

The cross section of the films was examined by SEM (Quanta FEG 650, FEI, USA). Films were broken with nitrogen liquid and cross section of films were affixed on carbon adhesive tape and sputter-coated with gold. An acceleration voltage of 5 kV at different magnifications was applied to obtain the images.

2.6.3. Water Vapor Permeability assays

Water vapor permeability (WVP) was determined gravimetrically according to the ASTM E96/E96M – 10 standard method using Elcometer 5100 Payne Permeability Cups. Films were sealed on cups with distilled water and put into a controlled environmental chamber at 21 ± 1 °C and 0 ± 2 % relative humidity (RH) with silica gel. Cups were weighed one time for day and weight loss was measured over time until steady state was reached (for 10 days). Water vapor transmission rate (WVTR) was calculated by dividing the slope of a linear regression of weight loss *versus* time by film area. The WVP ($g.m^{-1}.s^{-1}.Pa^{-1}$) was determined by equation 3:

$$WVP = \frac{(WVTR\ x\ L)}{\Delta P} \quad (3)$$

where L is the film thickness (m) and ΔP is the water vapor partial pressure difference (Pa) across the two sides of the film. At least two replicates were made for each film sample.

2.6.4. Opacity

The opacity can be described as the ratio between the absorbance and the material thickness, meaning that the higher the opacity level, the less transparent the material is [614]. A Minolta colorimeter (CR 400; Minolta, Japan) was used to determine the film opacity, through the relationship of the film opacity on a black standard and a white standard. Three replicates were carried out with ten measurements for each sample.

2.6.5. Oxygen Permeability

The oxygen permeability was determined using a Gas Diffusion Permeameter DP-100A (Porous Materials, Inc. Ithaca, New York, USA) with the pressure increase method, at a temperature of 23 ± 2 °C with a pressure of 760 torr for 3 hours. Samples with a diameter of 4 cm were placed in the holder of the permeameter chamber and oxygen permeability was measured. Before analyses, the samples were stored at 23 ± 2 °C. Three measurements were performed for each film.

2.6.6. Mechanical properties

The tensile properties were determined based on the ISO 527-1/-3 standard, using an INSTRON 5969 mechanical testing machine, with a 50 kN load cell. The tests were carried out at a speed of 200 mm/min and a temperature of 23 °C. Five specimens of type 2 (160 mm x 25 mm) of the ISO 527-3 standard were tested in the longitudinal (LD) and transversal (TD) direction. Tensile strength (σ_{max}, MPa), elongation-at-break (ε_{max}, %), and Young's modulus ($E_{1\%}$, MPa) were determined from stress-strain curves. The Young Modulus was determined by secant modulus at 1% strain and an initial distance of 50 mm was used.

2.6.7. Antioxidant capacity and quantification of the total phenolics and flavonoids of loaded PLA films

In order to evaluate the potential antioxidant capacity and total content in phenolic compounds and flavonoids of the PLA incorporated with the extracts, an accelerated migration assay was performed according to the method described by López-de-Dicastillo [564] with a few changes. Circles with 6 cm^2 of the PLA films were cut and, the surface intended to be in contact with the food was placed in migration cells and in contact with 10 mL ethanol 95 % (v/v), as a simulant of fatty food. The migration cells were stored in an oven at 40 ± 1 °C, protected from the light, for 10 days. At the end of this period, the

antioxidant capacity assays (see section 2.3) and the assays to determine the total content in phenolic compounds and flavonoids (see section 2.4) were performed.

2.7. Packaging of the foodstuffs

Two model foodstuffs, almond and beef, due to their high content in unsaturated fatty acids. Almond was acquired in a local store in Lisbon, Portugal, still in the hard shell. As soon as it arrived at the laboratory, the hard shell was manually separated and, for the peel removal, the almond was placed in hot water (80 ± 1 °C) for 5 minutes, and manually peeled. Then, the almonds were ground in a Grindomix and, 35 ± 1 g were immediately vacuum packaged and sealed, in 10 x 10 cm squares, in a vacuum chamber machine (MULTIVAC Model C70) with the active PLA films. The packaged almond was stored at room temperature (21 ± 1 °C) and in an oven at 40 ± 1 °C (accelerated assay), protected from the light, for a maximum time period of 60 and 30 days, respectively. The almond at room temperature was analyzed at the end of 7, 14, 21, 30, 45 and 60 days of storage and the almond stored at 40 °C was analyzed at the end of 2, 4, 7, 14, 21 and 30 days of storage.

The beef from the cow's neck was acquired from a local butcher shop in Lisbon, Portugal, in 35 ± 2 g pieces. As soon as it arrived at the laboratory, the meat was vacuum packaged and sealed in a vacuum chamber machine (MULTIVAC Model C70) with the active PLA films and stored at 4 ± 1 °C, for a maximum time period of 11 days, protected from the light. The lipid oxidation as well as the antimicrobial assays were performed at the end of 1, 4, 6, 8 and 11 days of storage.

2.8. Lipid oxidation evaluation

2.8.1. Thiobarbituric acid reactive substances (TBARS)

The TBARS assay, one of the most common assays performed to evaluate lipid oxidation status, measures the malonaldehyde (MDA) content, which is an aldehyde formed during the primary oxidation being a result of the peroxidation of polyunsaturated fatty acids [426,445–447]. The applied method was initial described by Rosmini et al. [436] and altered/adapted by Andrade et al. [522]. Briefly, 5 g of sample were mixed with 20 mL of an aqueous solution of trichloroacetic acid (7.5 %, w/v). The mixture was homogenized in the compact stirrer, for 1 hour, at 400-450 rpm, and filtered using a paper filter Whatman No. 1. Then, 2.5 mL of the filtered liquid were mixed with 2.5 mL of an aqueous solution of thiobarbituric acid (2.88 mg/mL), homogenized and kept at 95 °C for 30 minutes, following by a rapid cooling in ice for 15 minutes. The absorbance of the samples was

measured in a spectrophotometer at 530 nm, against the blank (2.5 mL of water with 2.5 mL of thiobarbituric acid solution). The calibration curve was drawn using 1,1,3,3-tetramethoxypropane as standard. The results are expressed in mg of MDA/kg of sample.

2.8.2. Peroxide Value Determination

The samples' fat was extracted using the method described by Vilarinho et al. [427]. Briefly, 10 ± 1 g of sample were mixed with 100 mL of petroleum ether and mixed for 1 hour in the compact stirrer at 350-400 rpm. The solutions were filtered through a paper filter Whatman No. 4 with 1 g of sodium carbonate anhydrous. The petroleum ether was evaporated in the rotary evaporator at 35 °C.

The determination of the peroxide value was performed according to the method described by Shanthat & Decker [546]. In the first place, a solution of iron(II) chloride was prepared: 50 mL of an aqueous solution of barium chloride dihydrate (8 mg/mL) were slowly added to 50 mL of an aqueous solution of iron(II) sulfate solution, under constant stirring. Then, 2 mL of hydrochloric acid (10 N) were added, and the solution was left to rest until the barium precipitate was formed. Then, the clear supernatant was removed to an amber glass and stored, protected from the light, for a maximum time period of 1 week.

A mixture of 50 mg of fat and 9.8 mL of a chloroform-methanol solution (70:30, v/v) was prepared and mixed for 2 to 4 seconds in a vortex. . Then, 50 µL of xylenol orange sodium (10 mM) were added and the solution was again mixed, for 2 to 4 seconds. Then, 50 µL of the iron(II) solution were added, the solution was mixed and, after 5 minutes, the absorbance was measured at 560 nm. The blank contained all the reagents with the exception of the sample. A calibration curve was drawn using iron(III) chloride as standard. The peroxide values were calculated through the equation 4 and are expressed in milliequivalents of oxygen per kilogram of sample (meq O_2/kg).

$$PV = \frac{(A_s - A_b) \times m}{55.84 \times m_0} \quad (4)$$

where, *As* stands for the absorbance of the sample, *Ab* stands for the absorbance of the blank, *m* stands for the slope of the calibration curve, m_0 stands for the samples mass in grams and 55.84 is the atomic weight of iron.

2.9. Microbiological Assays

Regarding the microbiologic analysis performed on samples of beef meat packaged with the control and active PLA films, the enumeration of microorganisms that are able to grow after aerobic incubation at 30 °C was performed, according to the automated test TEMPO®AC-Validated by AFNOR with certificate N° BIO 12/35-05/13. The microbiological examinations were only performed in meat samples, since the normal degradation during the almonds shelf-life is not usually due to the action of microorganisms but to lipid oxidation (rancidity), which cause, mainly, organoleptic alterations.

2.10. Statistical Analyses

All the assays were performed in triplicate and the results are expressed as means ± standard deviation. The statistical analysis was performed using a one-way analysis of variance (ANOVA) and ANOVA with Repeated Measures, performed in the Software IBM®SPSS® Statistics, version 27.0.1.0, and differences among mean values were processed by the Tukey test.

3. Results and Discussion

3.1. Antioxidant capacity and total content in phenolic compounds and flavonoids

The results of the antioxidant capacity assays and of the total content in phenolic compounds and total flavonoids are presented in Table IX.1. GTE presented an EC_{50} of 0.14 mg/mL, lower than the EC_{50} presented by the RE (0.53 mg/mL). This results are close to the results found by Castro et al. [426]. However, RE presented significantly a higher antioxidant capacity coefficient and higher content in total phenolic compounds than GTE. Comparing with the results found by Castro et al. [426], the values obtained for the TPC and TFC in GTE were similar, but the obtained RE presented higher content in phenolic compounds and flavonoids. Since the extraction method used by Castro et al. [426] was the same, the differences detected could be due to edaphoclimatic conditions during plant development. Also, the isolated extracts presented higher values than the solutions combining both extracts, therefore no synergic effect was detected (Table IX.1).

Table IX.1. Antioxidant assays and total content of the extracts in phenolic compounds and flavonoids.

Samples	DPPH· assay (Inhibition %)	TE (mg TE/g)	EC_{50} (mg/mL)	β-Carotene assay (AAC)	TPC (GAE mg/g)	TFC (ECE mg/g)
RE (1 mg/mL)	92.34 ± 0.28^{a}	161.64 ± 0.49	0.53	817.86 ± 18.74^{p}	857.14 ± 18.16*,^{g}	611.93 ± 5.55*,^{m}
GTE (1 mg/mL)	90.07 ± 0.14^{b}	157.75 ± 0.24	0.14	478.20 ± 42.85^{q}	474.39 ± 11.05*,^{h}	174.25 ± 2.24^{n}
S1	31.60 ± 0.27^{c}	57.19 ± 0.47	-	n.d.	339.55 ± 5.89^{i}	59.22 ± 8.99^{l}
S2	22.06 ± 0.29^{d}	40.77 ± 0.50	-	n.d.	267.07 ± 20.64^{j}	73.47 ± 13.03^{l}
S3	38.21 ± 1.17^{f}	68.55 ± 2.00	-	n.d.	388.52 ± 8.37^{k}	194.97 ± 6.82^{o}

* Due to their high content in phenolic compounds and flavonoids, these values are for the extracts at 0.1 mg/mL. Different letters in the same column mean significant differences ($p<0.05$).
Legend: S1 – mixture of 50% GTE and 50% RE; S2 – 75% of RE and 25% of GTE; S3 – 25% of RE and 75% of GTE; TE – Trolox equivalents; AAC – Antioxidant Activity Coefficient; TPC – Total Phenolic Content; TFC – Total Flavonoids Content; GAE – Gallic Acid Equivalents; ECE – Epicatechin Equivalents; GTE – Green Tea Extract; RE – Rosemary Extract; n.d. – not detected.

3.2. Quantification of individual phenolic compounds and catechins

Table IX.2 presents the content of individual compounds in ethanolic extracts of RE and GTE. Carnosol (35.81 mg/g), carnosic acid (104.49 mg/g) and rosmarinic acid (25.60 mg/g) were identified in the rosemary extract. Catechin (C) was also identified in the rosemary extract, but below the quantification limit. All the five catechins were identified on the GTE, but only catechin (321.28 mg/g), epicatechin (EC) (31.30 mg/g), and EGCG (148.11 mg/g) were quantified. GTE also presented gallic acid (4.49 mg/g) and rutin (15.55 mg/g). The analyzed GTE exhibited higher contents in C, EC, EGCG and rutin than the green tea extracts developed and analyzed by Bae et al., Das & Eun and Vilarinho et al. [594,609,615]. Bae et al. [609] and Das & Eun [615] managed to quantify GCG (59.0 ± 08 mg/g; between 6.0 and 11.9 mg/g, respectively) that, although present in the GTE, was below the limit of quantification (12.9 μg/mL). Das & Eun [615] also quantified GC (between 0.5 and 3.6 mg/g) that was not identified in the GTE. The commercial green tea extract analyzed by Vilarinho et al. [594] presented higher content in gallic acid (6.64 mg/g) and the authors were able to identify chlorogenic acid (2.65 mg/g).

The differences between the extracts' phenolic content can be mainly assigned to the different extraction methods used. The green tea extract processed at high temperature (HTP-GTE) developed by [609] and the different extracts from Das & Eun [615] were obtained with heat application, which can affect some compounds, especially EGCG, due to its thermosensitive. Within extraction methods' selection, the extraction

solvent of major importance. The use of water or an ethanol-water solution can be beneficial for the extraction of some compounds but, in general, water is less efficient than organic solvents for compounds extraction. In addition, the use of water can delay the concentration of the extract, that is the evaporation of the extraction [616].

Table IX.2. Individual phenolic compounds identified in the rosemary (RE) and green tea (GTE) extracts.

Phenolic group	**Compound**	**Wavelength (nm)**	**RE (mg/g)**	**GTE (mg/g)**
Flavan-3-ols	(-)-Gallocatechin	280	n.d.	< LoQ
	(+)-Catechin	280	< LoQ	321.28 ± 5.15[a]
	(-)-Epicatechin	280	n.d.	31.30 ± 0.44[b]
	(-)-Epigallocatechin gallate	280	n.d.	148.11 ± 1.60[c]
	(-)-Gallocatechin gallate	280	n.d.	< LoQ
Benzoic acids derivatives	Gallic acid	270	n.d.	4.49 ± 0.07
	Protocatechuic acid	260	n.d.	n.d.
	Gentisic acid	325	n.d.	n.d.
Terpenes	Carnosol	280	35.81 ± 0.35	n.d.
	Carnosic acid	280	104.49 ± 5.23	n.d.
Flavanones	Hesperetin	280	n.d.	n.d.
Cinnamic acids derivatives	*p*-coumaric acid	320	n.d.	n.d.
	Ferulic acid	320	n.d.	n.d.
	Chlorogenic acid	325	n.d.	n.d.
	Caffeic acid	325	n.d.	n.d.
	Rosmarinic acid	330	25.60 ± 2.14	n.d.
Flavones	Apigenin	330	n.d.	n.d.
	Kaempferol	360	n.d.	n.d.
Flavonols	Rutin	360	n.d.	15.55 ± 0.60
	Quercetin	360	n.d.	n.d.
	Total content		**165.34**	**478.19**

The analyzed RE showed higher content in carnosic acid than the extracts analyzed by Gonçalves et al. and Lefebvre et al. [617,618]. However, the different extracts developed by Lefebvre et al. [618] presented higher concentrations of carnosol (between 9 and 43 mg/g) and rosmarinic acid (78 mg/g). the work developed by Gonçalves et al. [617] employed a simple extraction method, using only water at room temperature. No carnosol or carnosic acid were identified, but the authors managed to quantify other phenolic compounds such as yunnaneic acid F (5.6 mg/g), luteolin-O-glucuronide (3.9 mg/g) and sagerinic acid isomer (2.44 mg/g). Also, the RE presented higher content in carnosol, carnosic acid and rosmarinic acid than the extract developed by Kanakidi et al. [619]. These authors produced rosemary extracts with and without ultrasonic assisted extraction, using acetone, water, and a mixture of both.

Lefebvre et al. [618] quantified different compounds in several fractions of rosemary extracts obtained with supercritical fluid extraction with CO_2 and different percentages of ethanol and water. Although these authors managed to obtain an extract with higher amounts of rosmarinic acid and carnosol, this methodology is more expensive, and the apparatus and materials are difficult to find in a common laboratory. Nevertheless, the content in rosmarinic acid and carnosol is not that distinct to what was obtained in this study.

3.3. Films Characterization

3.3.1. FTIR & SEM

FTIR analysis (Figure IX.1) do not show any differences between PLA film and the active PLA films (with extracts), meaning that the PLA structure was not altered with the addition of the GTE and the RE. The spectrum of the PLA matches the PLA spectrum described by Jiang et al. [620]. These authors developed a PLA with ginger and angelica essential oils which did not affect the structure of the PLA [620]. FigureIX.2 presents the SEM images for the control and active PLA films. No visual significant differences were found between the active and control PLA.

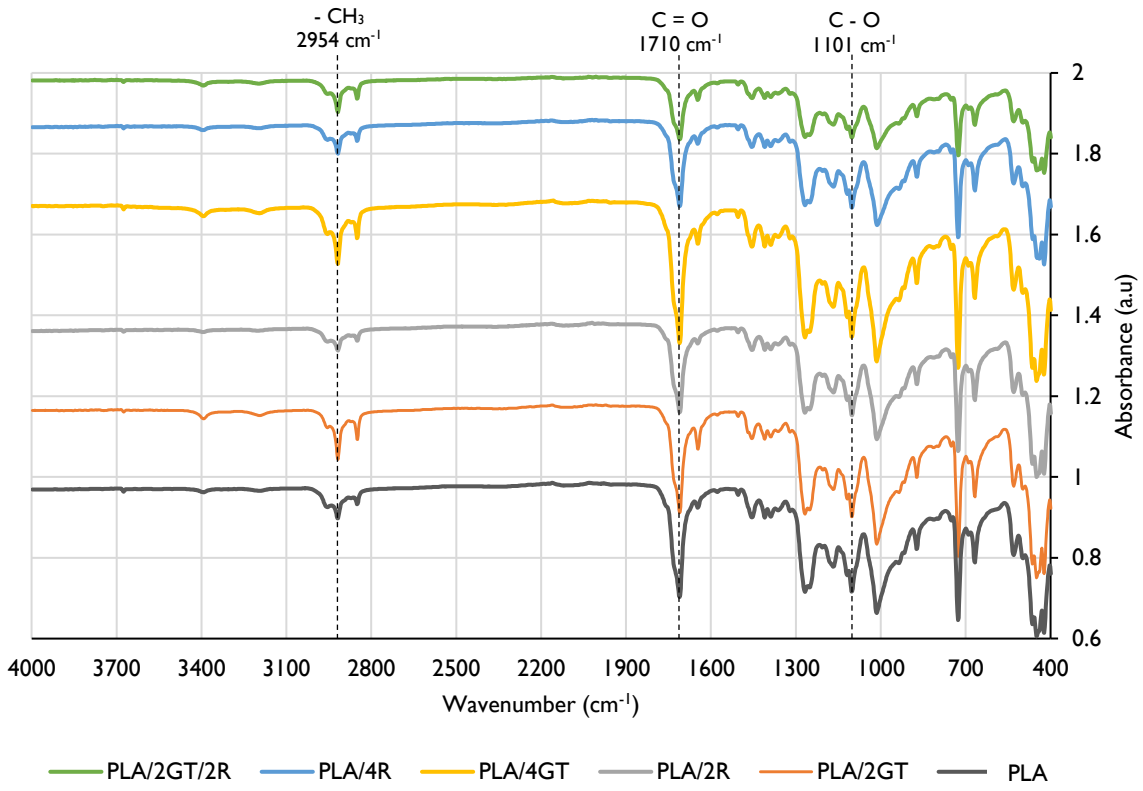


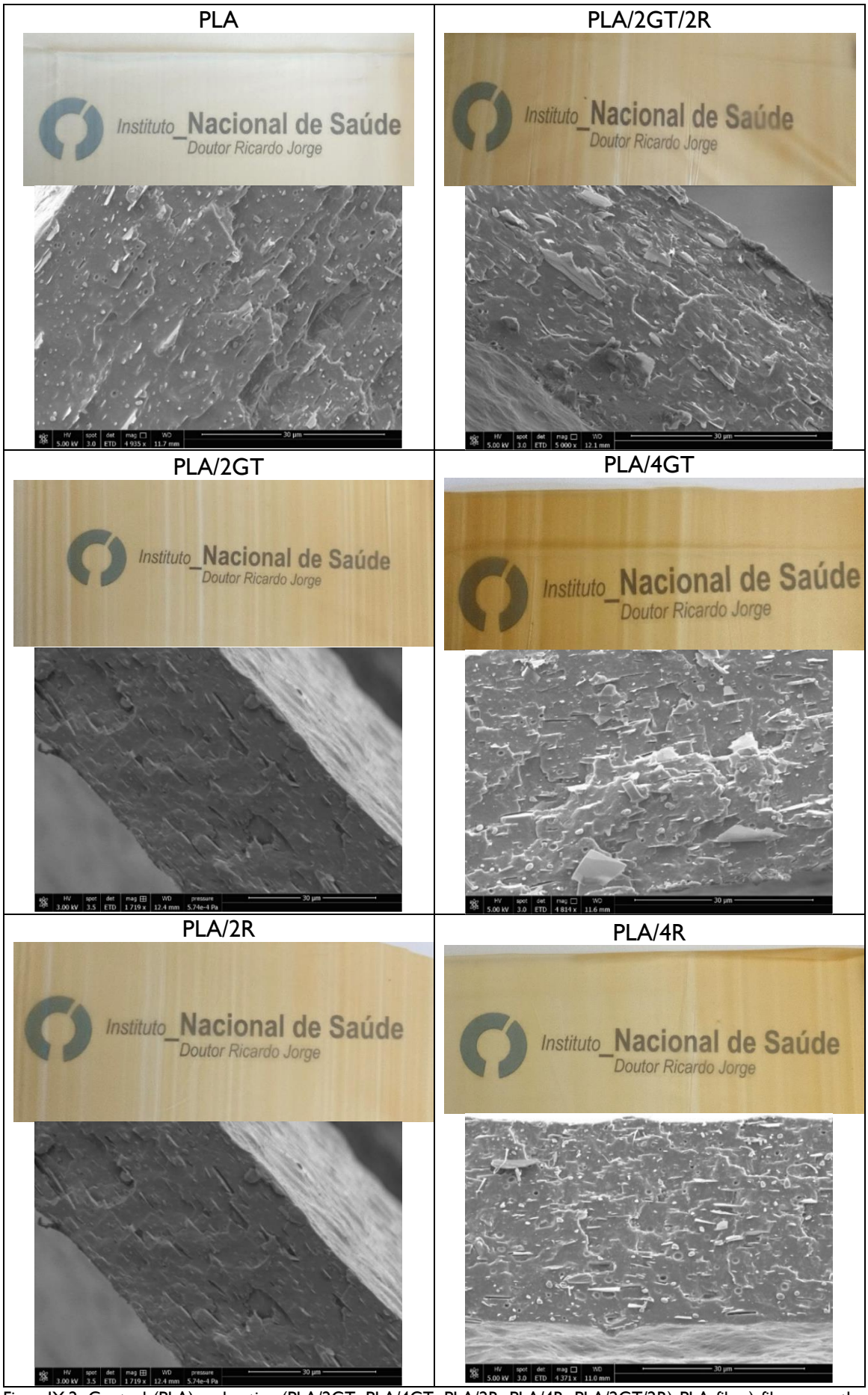
Figure IX.1. FTIR spectrum of the control (PLA) and active (PLA/2GT, PLA/4GT, PLA/2R, PLA/4R, PLA/2GT/2R) PLA films.

3.3.2. Water vapor and oxygen permeability, and opacity

The WVP of films seems to be unaffected by the addition of the extracts with the exception of the PLA/4GT, which resulted an increase ($p > 0.05$) of WVP values (Table IX.3). However, O_2 permeability was only increased with the PLA/4R film. Comparing with the literature, the WVP values for neat PLA were lower than the ones reported by Radusin et al. [621] (9.21×10^{-11} g m/Pa/s) and Vilarinho et al. [594] (approximately 1.8×10^{-11} g/m s Pa). Radusin et al. [621] revealed that the addition of the active substance, a garlic extract, decreased the WVP values (6.86×10^{-11} g m/Pa/s). Vilarinho et al. [594] also incorporated a commercial GTE in PLA films, which decreases the PLA WVP values with the exception of the PLA with 1 % of GTE.

Based on the results from Table IX.3, PLA with RE (2 and 4 %) seems to be the best candidates for food packaging, since they have the lowest WVP. However, the PLA/4R presented the highest O_2 permeability values.

Regarding the films' opacity, significant differences between the control and active PLA can be found with the addition of the extracts with exception of PLA/4GT. The addition of the extracts made the PLA less opaque (Table IX.3). Images of the control and the active PLA films can be observed in FigureIX.2.



FigureIX.2. Control (PLA) and active (PLA/2GT, PLA/4GT, PLA/2R, PLA/4R, PLA/2GT/2R) PLA films) films over the Portuguese National Institute of Health logo and cross-section images of control (PLA) and active (PLA/2GT, PLA/4GT, PLA/2R, PLA/4R, PLA/2GT/2R) PLA films

Table IX.3. Water vapor permeability (WVP), opacity and O2 permeability (O2P) of the control (PLA) and active (PLA/2GT, PLA/4GT, PLA/2R, PLA/4R, PLA/2GT/2R) PLA films.

Samples	WVP x 10^{-12} (g m^{-1} Pa^{-1} s^{-1})	Opacity (%)	O_2P x 10^{-14} (mL.cm/(Pa.s.cm^2)
PLA	5.54 ± 1.11[a]	24.21 ± 1.70[a]	4.16 ± 0.25[a]
PLA/2GT	5.83 ± 2.14[a]	22.66 ± 1.23[b]	4.87 ± 0.07[a,b]
PLA/4GT	9.78 ± 1.24[b]	26.62 ± 1.06[a]	5.27 ± 0.75[a,b,c]
PLA/2R	3.60 ± 0.16[a]	21.82 ± 0.27[b]	5.73 ± 0.18[b,c]
PLA/4R	5.03 ± 2.11[a]	20.90 ± 1.12[b]	6.54 ± 0.75[c]
PLA/2GT/2R	4.94 ± 1.93[a]	20.96 ± 2.03[b]	6.43 ± 0.42[c]

Different letters stand for significant differences.
Legend: GT – Green Tea extract; R – Rosemary extract

3.3.3. Mechanical properties

The results for the mechanical properties of the PLA films are indicated in Table IX.4. Observing the results from the transversal direction, the addition of the extracts decreased the tensile strength, elongation at break and σ_{rut} values when compared with PLA without extracts, especially in the PLA/4GT and PLA/2GT/2R. Observing the results of the longitudinal direction, the addition of the extracts seems to have no effect on the tensile strength. By decreasing the tensile strength, elongation at break and σ_{rut} the addition of the extracts weakened the PLA. An increase on the Young's modulus can be observed with the addition of the RE and 2% of GTE.

Martins et al. [590] evaluated the tensile strength through the longitudinal direction. The authors developed a control PLA film with a higher tensile strength value (40.2 MPa), which decreased with the addition of 2 % of green tea extract (35.4 MPa).

Table IX.4. Mechanical properties of the control (PLA) and active (PLA/2GT, PLA/4GT, PLA/2R, PLA/4R, PLA/2GT/2R) PLA films. Different letters stand for significant differences. Uppercase letters indicate the significant differences between the LD cuts, and the lowercase letters indicate differences for the TD cut.

Samples	Young's modulus ($E_{1\%}$, MPa)	Tensile strength (σ_{max}, MPa)	Elongation at break ε_{max} (%)
PLA TD	124.89 ± 25.49^{a}	12.5 ± 0.42^{a}	260.1 ± 37.91^{a}
PLA LD	184.2 ± 45.01^{A}	15.6 ± 1.23^{A}	432.9 ± 29.42^{A}
PLA/2R TD	145.0 ± 30.0^{b}	10.9 ± 0.84^{b}	245.8 ± 84.35^{b}
PLA/2R LD	224.6 ± 23^{B}	16.1 ± 2.3^{B}	442.2 ± 17.22^{B}
PLA/4R TD	156.7 ± 36.21^{c}	10.1 ± 0.43^{c}	129.3 ± 28.25^{c}
PLA/4R LD	173.8 ± 61.41^{C}	15.6 ± 1.02^{C}	435.5 ± 25.09^{C}
PLA2GT TD	133.9 ± 24.29^{d}	10.3 ± 0.16^{d}	121.0 ± 2.910^{d}
PLA/2GT LD	196.6 ± 25.27^{D}	15.6 ± 0.53^{D}	437.4 ± 24.25^{D}
PLA/4GT TD	114.9 ± 48.2^{e}	9.17 ± 0.23^{e}	76.40 ± 17.05^{e}
PLA/4GT LD	228.8 ± 22.72^{E}	15.76 ± 0.97^{E}	435.9 ± 26.01^{E}
PLA/2GT/2R TD	109.1 ± 28.24^{f}	9.43 ± 0.43^{f}	126.8 ± 32.84^{f}
PLA/2GT/2R LD	182.0 ± 47.9^{F}	13.5 ± 1.39^{F}	263.2 ± 53.95^{F}
Legend: GT – Green tea extract; R – Rosemary extract; Longitudinal (LD) and transversal (TD) direction			

3.3.4. Antioxidant evaluation of the active films

Table IX.5 shows the antioxidant capacity (DPPH and β-carotene assay, TPC and TFC) of the active PLA films after 10 days at 40 °C in contact with ethanol 95% (v/v). PLA/4GT presented the highest DPPH$^{\bullet}$ inhibition and the highest content in total phenolic compounds and flavonoids. In sum, the PLA with GTE presented the best antioxidant results in all assays with the exception of the β-carotene bleaching assay, in which PLA/4R presented the highest value, followed by the PLA/2R.

Table IX.5. Antioxidant capacity and total content in phenolic compounds and total content on flavonoids from of the fatty food simulant after contact with the control (PLA) and active (PLA/2GT, PLA/4GT, PLA/2R, PLA/4R, PLA/2GT/2R) PLA films during 10 days at 40 °C

Samples	DPPH$^{\bullet}$ assay (Inhibition %)	β-Carotene assay (AAC)	TPC (GAE µg/mL)	TFC (ECE µg/mL)
PLA/2GT	18.48 ± 0.44^{a}	85.86 ± 8.46^{f}	19.39 ± 0.33^{k}	15.20 ± 0.78^{n,o}
PLA/4GT	42.69 ± 2.22^{b}	75.28 ± 2.80^{f}	42.39 ± 0.56^{l}	26.40 ± 0.16^{q}
PLA/2R	5.80 ± 0.25^{c}	160.66 ± 5.03^{h}	11.72 ± 0.37^{j}	18.71 ± 0.89^{p}
PLA/4R	1.65 ± 0.44^{d}	173.22 ± 13.69^{h}	12.63 ± 0.36^{j}	14.21 ± 0.55^{n}
PLA/2GT/2R	10.15 ± 0.38^{e}	177.33 ± 37.76^{g}	18.24 ± 0.42^{m}	16.20 ± 0.99^{n,o}
Legend: n.d. – non detected; AAC – Antioxidant Activity Coefficient; TPC – Total Phenolic Content; TFC – Total Flavonoids Content; GAE – Gallic Acid Equivalents; ECE – Epicatechin Equivalents; GT – Green Tea Extract; R – Rosemary Extract				

3.4. Lipid oxidation evaluation of almond

TBARS results of the almond, packaged at different temperatures, can be seen in Figure IX.3 and Figure IX.4 . The MDA equivalent (MDA eq.) values of the almond packaged with PLA and stored at room temperature were significantly higher, over the 60 days of storage, than the almond packaged with PLA/2R, PLA/4R and PLA/2GT/2R. The PLA/2GT and PLA/4GT only presented significantly lower MDA eq. values until the 14th day of storage, suggesting that the RE has a better performance in delaying the lipid oxidation of almond. Also, the PLA/4R' almond presented lower MDA eq. values than the PLA' almond by 30, 40 and 50%, at 30, 45 and 60th day of storage, respectively. Similar results can be seen in the accelerated assay (at 40 °C), in which the almond packaged with PLA/4R presented significantly lower MDA eq. values than the PLA packaged almond, for 21 days of storage.

In the study led by Castro et al. [426] a commercial GTE was used to delay salmon' lipid oxidation in a whey protein-based coating. The results showed that the GTE presented lower MDA eq. values for 5, 7, 14 and 17 days of storage than the control [426]. However, in the study performed by Andrade et al. [182], the salami slices packaged with a whey protein-based coating with RE exhibited lower MDA eq. values at the 7th, 15th, 60th and 90th day of storage than the salami packaged with the control whey-protein package. Martins et al. [590] reported that a PLA package with 1 and 2 % of a GTE delayed the smoked salmon' lipid oxidation for 45 days of storage, when compared with the control PLA. On the other hand, Vilarinho et al. [594] presented higher MDA eq. values, for salami slices packaged with a PLA with only 1 % of a GTE, than the control PLA. The results found in the literature suggest that, although the GT extracts have higher content in phenolic compounds and higher antioxidant activity, they seem to accelerate foods' lipid oxidation process. On the contrary, the rosemary extracts seem to effectively prolong foods' shelf-life, by reducing the MDA eq. values.

Peroxide results from the almond packaged with the PLA films and stored at room temperature can be observed in Figure IX.5. At the 21st, 30th, 45th and 60th day of storage, the almond packaged with the PLA/4R presented lower peroxides values than the almond packaged with the control PLA and the other active films, supporting the PLA/4R TBARS results. Hexanal was not detected in any of the samples.

The *in vitro* antioxidant assays (see section 3.1 and 3.3.4) and the O_2 permeability (see section 3.3.2), pointed that PLA incorporated with GTE was the better film for inhibiting a fatty foods' lipid oxidation. However, the *in vivo* results suggest that the GTE has a

prooxidant effect in food, whereas the RE delays it.3.5. Lipid oxidation and microbiological analysis for meat

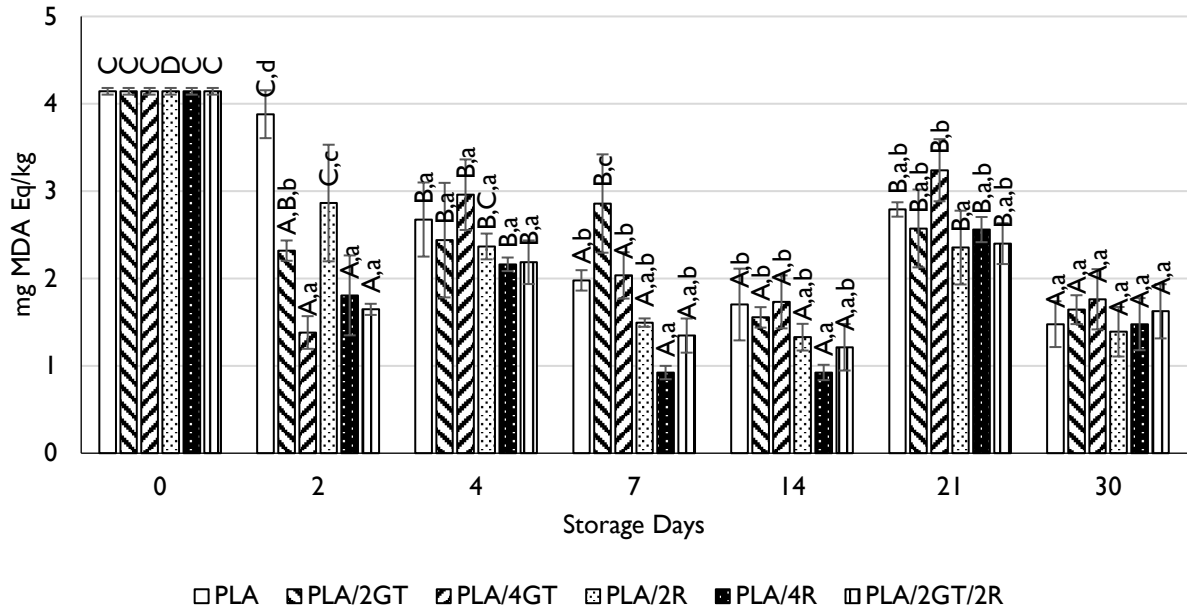


Figure IX.3. TBARS assay results for almond packaged with the active and control (PLA) and active (PLA/2GT, PLA/4GT, PLA/2R, PLA/4R, PLA/2GT/2R) PLA films stored at 40 °C. The lowercase letters compare samples within the same storage day. The uppercase letters compare the same packaging for different storage days. Different letters, for either lowercase/uppercase comparison, represent results with significant differences.

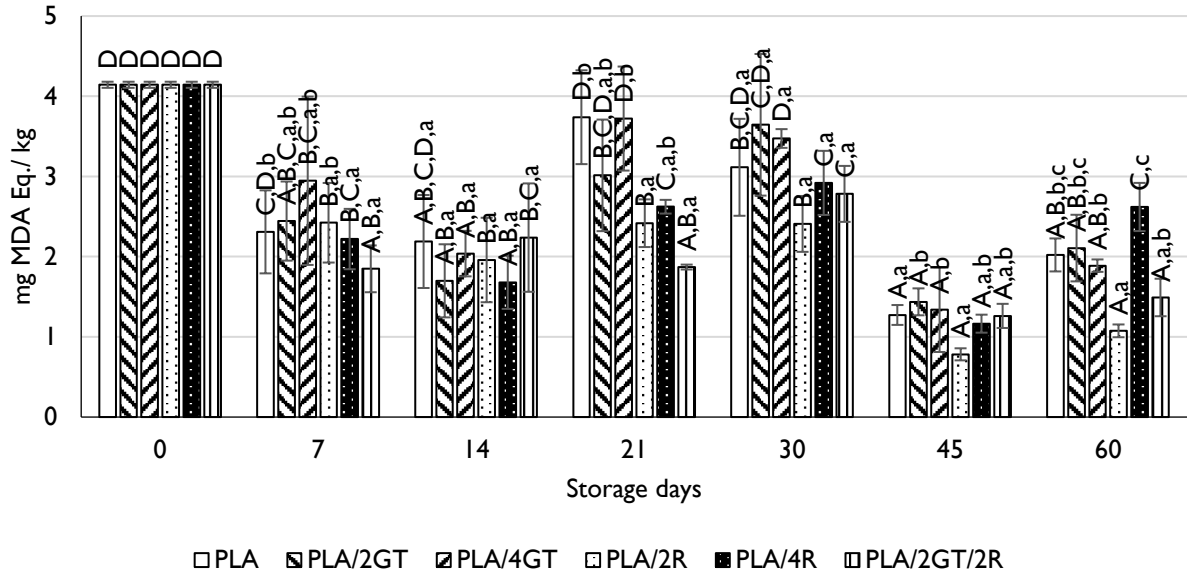


Figure IX.4. TBARS assay results from the almond packaged with the control (PLA) and active (PLA/2GT, PLA/4GT, PLA/2R, PLA/4R, PLA/2GT/2R) PLA films stored at 23 °C. The lowercase letters compare samples within the same storage day. The uppercase letters compare the same packaging for different storage days. Different letters, for either lowercase/uppercase comparison, represent results with significant differences.

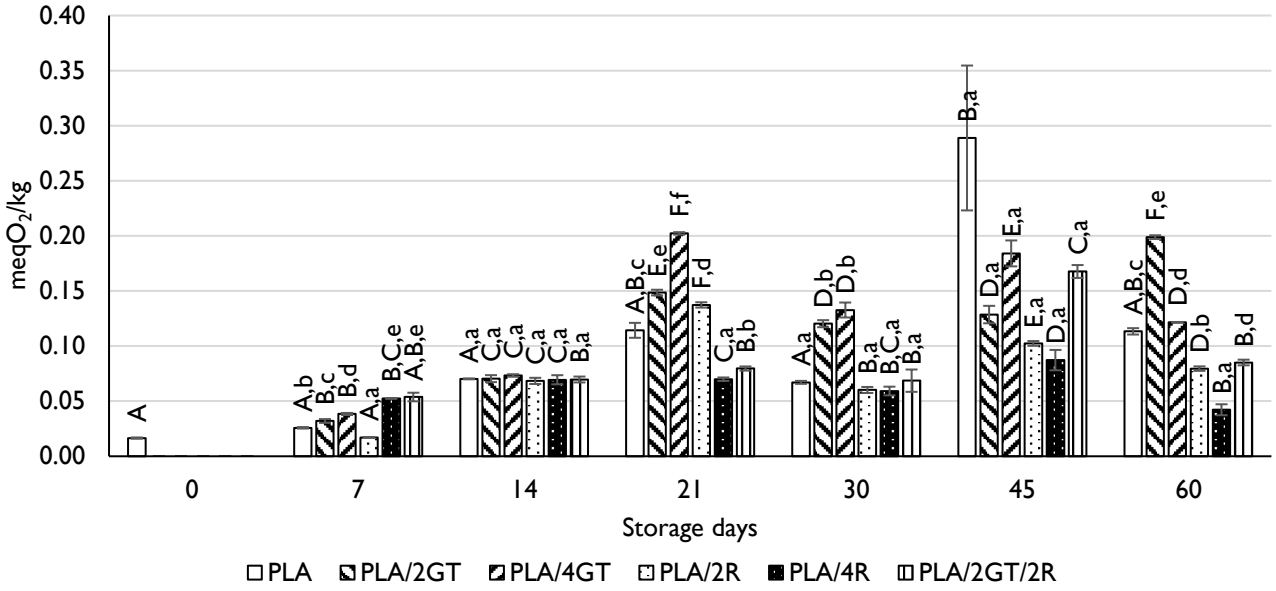


Figure IX.5. Peroxide value results for almond packed with the control (PLA) and active (PLA/2GT, PLA/4GT, PLA/2R, PLA/4R, PLA/2GT/2R) PLA films stored at 23 °C. The lowercase letters compare samples within the same storage day. The uppercase letters compare the same packaging for different storage days. Different letters, for either lowercase/uppercase comparison, represent results with significant differences.

3.5. Lipid oxidation and microbial analysis of meat

The TBARS results of the meat are presented in Figure IX.6. The MDA values are lower than the ones obtained in the almond assay. Also, for the total of the storage days, all the active films presented a lower MDA value than the meat packaged with the control PLA. At the end of the 8th and 11th day, PLA/4GT and PLA/2GT/2R presented the lower MDA values, suggesting that the PLA with the GTE presents a higher lipid oxidation inhibition for shorter times.

Fiore et al. [622] studied the incorporation of chitosan and rosemary essential oil in PLA packages in minced chicken breasts. The authors found that the incorporation of only 1 and 2 % of rosemary essential oil protects the chicken meat against lipid oxidation until the 14th day of storage, with MDA values below 0.5 mg/kg, similar to the results in this study for the RE and GTE. On the other hand, Yoon et al. [623] demonstrated that the direct addition of rosemary and green tea extracts seems to increase the MDA value of pork sausages when compared to the control. However, the MDA values presented by these authors were lower (between 0.094 and 0.142 mg/kg) than the MDA values present in this study [623]. Zhang et al. [624] developed a gelatin film incorporated with different percentages of one of the most abundant bioactive compound of rosemary extract, rosmarinic acid. The authors found that active films inhibit the MDA formation in Chinese bacon for 60 days, when compared to the control sample.

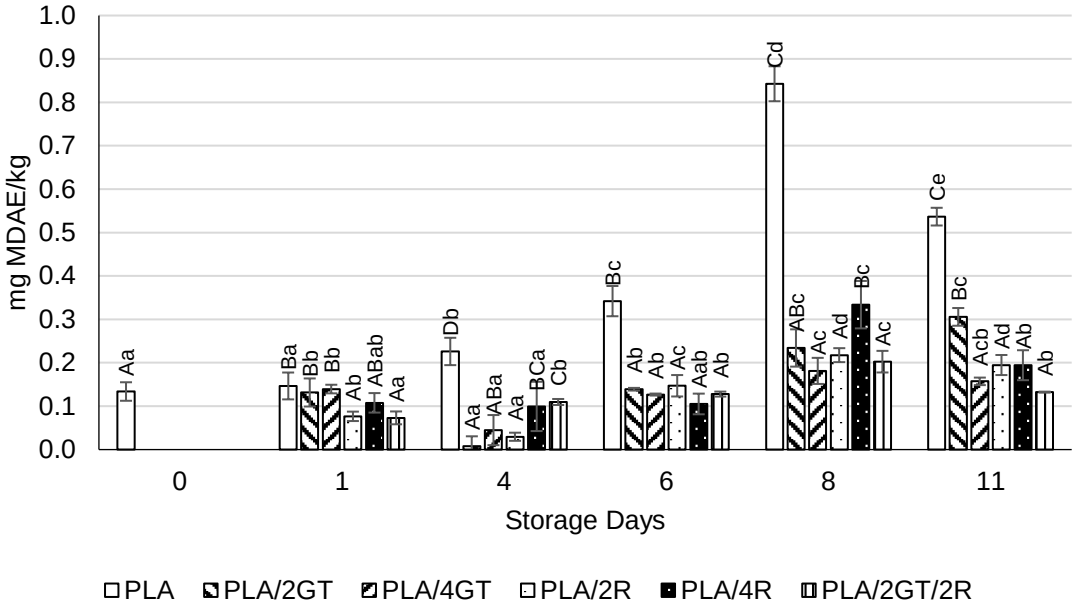


Figure IX.6. Results of the Thiobarbituric Reactive Substances from the meat packed with the control (PLA) and active (PLA/2GT, PLA/4GT, PLA/2R, PLA/4R, PLA/2GT/2R) PLA films. The lowercase letters compare samples within the same storage day. The lowercase letters compare the same packaging for different storage days (for instance, PLA at 0 and 1 day present no significant differences, has noted by the lowercase "a". On the other hand, at 1 day of storage, PLA/2R and PLA/4R present no significant differences, has identified by the uppercase "A"). Different letters, for either lowercase/uppercase comparison, represent results with significant differences.

Regarding the microbiological assay (Table IX.6), a significant inhibition of the microbial growth was observed between the 6th and 8th storage day on the meat packaged with PLA/2R and PLA/4R films, and between the 8th and 11th day of storage on the meat packaged with PLA/2GT. Andrade et al. [183] showed that a similar rosemary extract presented antimicrobial activity against *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium perfringens*, but not against *Escherichia coli*. The antimicrobial activity of rosemary extract against *L. monocytogenes* and *S. aureus* was also described by Gonçalves et al. [617] and Gazwi et al. [625], respectively.

Macroscopically, it was not possible to identify differences (e.g. microbial growth, green color) between the samples over the storage time or between the samples wrapped with the different PLA packages. It should also be highlighted that, the meat did not present any odor change.

Table IX.6. Aerobic colony average count at 30 °C, for the beef meat packaged with the control (PLA) and active (PLA/2GT, PLA/4GT, PLA/2R, PLA/4R, PLA/2GT/2R) PLA films.

Storage time (days)	PLA	PLA/2GT	PLA/4GT	PLA/2R	PLA/4R	PLA/2GT/2R
			(Log CFU/mL)			
0	4.32	4.32	4.32	4.32	4.32	4.32
1	4.57	4.57	3.86	4.32	4.64	4.38
4	6.28	6.04	5.00	5.23	5.52	5.76
6	6.63	7.41	6.72	6.92	7.08	6.48
8	6.98	7.78	7.46	6.76	6.41	7.15
11	7.49	7.65	7.49	6.98	7.34	7.36
CFU – Colony Forming Units						

4. Conclusions

Food grade extracts from dried green tea and rosemary leaves were successfully obtained through an easy and simple method. Both extracts presented high antioxidant capacity and a high content in total phenolic compounds and total flavonoids. GTE stands out for its high content in EGCG and RE for its high content in carnosic acid. The extracts were incorporated into PLA at different percentages, resulting in resistant, visually attractive and homogenous packages. PLA/2R and PLA/4R presented interesting results on inhibiting almond's lipid oxidation for longer periods (45 to 60 days) at room temperature and 21 to 30 days at 40 °C. On the other hand, PLA/2GT and PLA/4GT seemed to act as a pro-oxidation agent in the almond test. However, PLA/4GT showed promising results on inhibiting beef's lipid oxidation for short times and lower storage temperature (until 11 days at 4 °C).

It is of utmost importance to standardize the plant based active extracts used in the development active packaging in order to better control their quality and composition and consequently the effectiveness of the new packaging materials. Further studies are needed in these PLA active packages, in particular, sensorial analysis to evaluate the possible organoleptic changes that can occur with the migration of the volatile active compounds present in the extracts; selective antimicrobial assays to determine the fully antimicrobial activity spectrum of these extracts and the application of these active packages to other food matrices either with high fat content or prone to microbiological spoilage.

Chapter X

General Conclusions and Future Perspectives

General conclusions

This PhD thesis focused on the development of active food packaging with natural extracts incorporated into the packaging matrix, with the main goal of delaying the natural lipid oxidation of high fatty foods.

First, an extensive literature review was carried out. Chapter I reviewed the state of the art in the application of active food packaging in meat and meat products, as well as the direct application of food additives. This Chapter also reviewed the lipid oxidation process, particular in meat and meat products and the legislation applied to food additives and food packaging. Consumers are asking for more natural products with less synthetic additives and, in consequent, the research on natural additives, extracted from fruits by-products and aromatic plants has grown. These additives can be directly or indirectly applied to foods. In the second case, the additive can be incorporated into the packaging matrix and gradually migrate to the food surface. By applying this technique, the amount of additive added is lower and, consequently, the amount of the additive ingestion by the consumer is lower. Among the tested extracts and EOs, rosemary, oregano and thyme showed high potential to reduce foods' lipid oxidation through their antioxidant capacity and antimicrobial activity against some of the most common pathogenic microorganisms, such as *L. monocytogenes*, *S. aureus* and *E. coli*. Another common example and largely applied of active food packaging is MAP, to prevent microbial growth and food loss by replacing oxygen within the food packaging. This technique is largely applied to raw meat and meat products.

Chapters II and III carry on the scientific literature review however, reviewing the latest applicabilities of fruits by-products. Chapter II focus on pomegranate and grape by-products most common active compounds and their potential risks for their consumption. Tannins and anthocyanins are the most common compounds in these by-products. Ellagitannins and their derivatives are the most common compounds in pomegranate by-products, and catechins and tannins in grape by-products. Although the consumption of the extracts obtained from these by-products was considered safe in some studies, further and thorough toxicological research is necessary to fully address their health effects.

Chapter III reviews the main compounds of *Citrus* by-products, as well as, their main extraction process and biological activities, and their application in active food packaging. It is estimated that the generation of *Citrus* by-products is, approximately, 15 million tones every year. Bioactive compounds, such as *D*-limonene, hesperetin, narirutin, rutin, linalool, several carotenoids, among others, can be extracted from these by-products through several extraction processes. The most common method is steam

distillation, generally used in the EOs production. Solvent extraction with supercritical fluids, is also an alternative with lower loss of volatile compounds and higher efficiency. More recently, microwave-assisted extraction and Pulsed Electric Field assisted extraction are two other extraction methods that also present good outcomes. Such as every other plant, the chemical composition of *Citrus* by-products extracts and EOs, is directly dependent of the edaphoclimatic conditions to which the plant is exposed to and the chosen extraction method. Among all the *Citrus* by-products, peels are the major by-product and the most used for the EOs extraction. *D*-limonene is the major compound in the *Citrus* EOs but linalool, β-myrcene and α-pinene can be easily found in major quantities as well. Pectin, although not being a bioactive compound, is also a component of *Citrus* by-products that can be applied in the food and pharmaceutical industries. The use of *Citrus* by-products extracts and EOs is widely studied in the scientific community, either as antioxidants or as antimicrobials. These extracts and EOs have also been incorporated into polymers like PLA, BC, alginate and LDPE to test its applicability in the active food packaging field. The potential and chemical composition of *Citrus* by-products extracts is addressed in Chapters VI and VII.

Besides fruits by-products, seaweeds are also an important source of phenolic compounds with antioxidant activities. In Chapter IV the antioxidant potential of five hydroethanolic extracts of *F. vesiculosus* L. was addressed and the extract obtained using 75% ethanol was incorporated into a whey protein-based coating, which was applied to chicken meat. The new active coating was able to inhibit the lipid oxidation of chicken meat for 25 days of storage and, the extract incorporation in the polymeric matrix significantly increased thickness, tensile strength, and elastic modulus of the whey protein-based coating.

Chapter V analyses four different extracts obtained from by-products originated from the production of two juices formulations. The higher antioxidant capacity and the higher content in phenolic compounds was observed in the extracts obtained from the freeze-dried by-products from the F1, which contained equal parts of apple and ginger. The F1 major identified compounds were epicatechin (238.17 μg/g), isoquercitrin (117.86 μg/g), quercitrin (97.84 μg/g), rutin (95.84 μg/g) and procyanidin B1 (52.46 μg/g). In the case of F2, which contained by-products from apple, ginger, carrot and beet, epicatechin was also the major compound (206.25 μg/g), followed by quercetin (107.17 μg/g), procyanidin B1 (104.86 μg/g), isoquercitrin (95.57 μg/g) and rutin (65.38 μg/g).

Following the study on Chapter V, Chapter VI determined the antioxidant activity and the phenolic composition of extracts obtained from industrial by-products of apple,

lemon and orange juices. The lemon extract presented the highest antioxidant activity and the highest content in phenolic compounds, being eriocitrin (17 493 μg/g) the major phenolic compound, followed by hesperidin (2 728 μg/g), chlorogenic acid (386.7 μg/g), isoquercetin (111.6 μg/g) and quercetin (106.0 μg/g). The extract from orange juice by-products major identified compound was hesperidin (4 901 μg/g), followed by protocatechuic acid (317.3 μg/g), rutin (31.43 μg/g) and naringenin (30.09 μg/g).

As a result of Chapter VI, Chapter VII applies the ethanolic extract from lemon juice by-products incorporated in PLA and LDPE based active packaging. The incorporation of the lemon extract improved the water barrier properties of both PLA and LDPE packaging, however, the lemon extract decreased the oxygen permeability of the PLA-based packaging. Comparing the migration of the LDPE and PLA packaging into food simulants, the PLA was less able to retain the active compounds than the LDPE. Naringenin, apigenin, ferulic acid, eriocitrin, hesperidin and 4-hydrobenzoic acid were identified in the PLA packaging, but only 4-hydrobenzoic acid, naringenin and *p*-coumaric acid were identified in the LDPE packaging. Looking on the active films ability to delay the lipid oxidation of grounded almonds, LDPE/4LE was able to delay the lipid oxidation for 30 storage days, at 40 °C. PLA/6LE was only able to significantly reduce the lipid oxidation of raw meat and successfully inhibit the microbial growth in raw meat until the 8th storage day.

Following the revision of Chapter II, and since 50 % of pomegranate is constituted by peels and seeds, pomegranate by-products present an enormous potential due to their powerful biological activities. In Chapter VIII, grape and pomegranate by-products and their extracts antioxidant potential is addressed. Pomegranate by-products and its extract presented the highest antioxidant capacity and the highest content in phenolic compounds. Two PLA-based active packaging were produced, by incorporating the extract and the dried peels of pomegranate. The active packages were mostly efficient in delaying meats' lipid oxidation and microbial growth. Also, a UHPLC method for the identification and quantification of ellagic acid and punicalagin (A+B) was validated. The both the pomegranate peels and the pomegranate extract presented interesting contents in punicalagin (A+B) and ellagic acid, but only the ellagic acid was identified in the active packaging.

Since aromatic plants have major content in phenolic compounds with antioxidant and antimicrobial activities, in Chapter IX, the antioxidant capacity of green tea and rosemary extracts applied into PLA active packaging is address. Catechin, epicatechin, epigallocatechin gallate, rutin and gallic acid were successful quantified in the GTE, whereas

in the RE only carnosol, carnosic acid and rosmarinic acid were quantifies. The incorporation of the extracts seemed to have no significant influence of the structure of the PLA, as indicated by FTIR spectrum and SEM images. The delay of lipid oxidation was significantly visible in the meat assays with PLA/4GT the best results in inhibition of the meats' lipid oxidation until the 11th storage day. In the almonds case, PLA with rosemary extract was more effective in preventing almonds lipid oxidation for the total storage time.

Concluding remarks

Overall, this PhD thesis analyses 17 different natural extracts obtained with food-grade solvents, from seaweeds, fruits by-products and aromatic plants. The extracts from pomegranate by-products and rosemary showed higher antioxidant capacity. The rosemary extract presented interesting content in carnosic acid (104.49 mg/g), carnosol (35.81 mg/g), and rosmarinic acid (25.60 mg/g), while the pomegranate extract presented punicalagin (A+B) (85.84 mg/g) and ellagic acid (6.67 mg/g).

Regarding the active food packaging, 11 active food packages were produced with natural extracts, of which 10 were based on biodegradable polymers (whey protein or PLA). In terms of the appearance of the active films, the most resistant, homogenous, and visually attractive packaging was the LDPE with lemon by-products, followed by PLA with green tea and rosemary extracts, and the PLA with pomegranate by-products extract. The PLA with lemon by-product extract, regardless of the extract percentage, presented itself brittle and very hard to work with. On the contrary, the LDPE with the lemon extract presented itself flexible, homogenous, and very resistant. In the comparison of an active PLA film with lemon extract and LDPE with lemon extract, the LDPE showed more efficiency in reducing almond lipid oxidation.

In all the case studies, all the active packaging with different natural extracts were effective in reducing lipid oxidation, especially when applied to raw beef, an ideal model food for *in vivo* evaluation of antioxidant and antimicrobial properties of active food packaging with natural extracts.

Future perspectives

With the consumers' increasing demand for more 'natural' products, active food packaging presents a great alternative for the decrease of additives application, through the use of gas and moisture scavengers, modified atmospheres, and antimicrobial or antioxidant emitters. The application of natural extracts, from fruits by-products, plants and seaweed,

is a clear alternative for the use of synthetic additives. Regarding the natural extracts, additional studies comparing them with the synthetic antimicrobial and antioxidant additives should be conducted, as well as toxicological and health effects must be addressed to clearly define a safety limit for their application. Once these limits are established, the direct application of the extracts to the foods should be evaluated. In addition, taking into account all the potentially beneficial properties to health, bioavailability studies of these extracts should also be carried out. Moreover, since the literature indicates several biological activities, studies regarding extracts' antiviral and antifungal activities should be accomplished. Mycotoxins presence in some foods is also a problem in the food industry. The ability of the extracts to inhibit or prevent these toxins should be further investigated.

Nowadays, even in industrialized countries, foodborne diseases are a major health public concern. The antimicrobial potential of these natural extracts, produced with food-grade and 'green' solvents, must be applied in order to increase foods' shelf-life and decrease food waste and food loss.

Since these extracts have an intense smell and taste, further research addressing the organoleptic impacts on active packaging in real foods would provide an overview of the consumers' acceptability regarding active packaging with natural extracts. Also, the incorporation of these extracts in polymer matrix still represents a challenge. One solution is their encapsulation, which allows a more gradual migration of the active compounds and, consequently, extending their action over time.

Given that the chemical composition of plants and fruits are directly dependent on the edaphoclimatic conditions to which the plant is exposed to, the chemical instability of these extracts is a problem. To overcome this issue, the scientific community should search for more collaborations with the food industry, to characterize and standardize the extracts obtained from industrial plants and fruits by-products. The major scientific studies in this field analyze principally non-industrialized by-products, existing only a few papers addressing the full potential of the industrial by-products. These collaborations will contribute to an increasingly circular and sustainable economy, by taking advantage of this resource which is mostly seen as waste. Also, the application of biodegradable polymers should be addressed together with the packaging industries to fully evaluate the polymers prospective to be applied in the real market.

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