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PREPARATION AND EVALUATION OF POLYLACTIC ACID ANTIOXIDANT PACKAGING FILMS CONTAINING THYME, ROSEMARY AND OREGANO ESSENTIAL OILS

A Thesis Submitted to

The Food Chemistry Master's Program

In partial fulfilment of the requirements for

The degree of Master of Science

By:

Amr Zeid

Under the supervision of:

Dr. Michail Kontominas

Department of Chemistry, The American University in Cairo (AUC)

II

The American University in Cairo

Preparation And Evaluation Of Poly Lactic Acid Antioxidant Packaging Films Containing Thyme, Rosemary And Oregano Essential Oils.

A Thesis Submitted by

Amr Mostafa Ibrahim Zeid

To the Food Chemistry Graduate Program

May, 2015

In partial fulfillment of the requirements for

The degree of Master of Science

Has been approved by

Thesis Committee Supervisor/Chair

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Affiliation _____

Thesis Committee Reader/Examiner: _____

Affiliation _____

Thesis Committee Reader/External Examiner

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Affiliation: _____

_____ _____ _____ _____

Dept. Chair/Director   Date   Dean   Date

DEDICATION

To my family…

My big old family, and my small new family to be…

ACKNOWLEDGMENT

ABSTRACT

Protecting foods from oxidation for appreciable amounts of time is becoming a major challenge for the food industry. Lipid containing foods are the most susceptible types of food to oxidation. In the present research study, thyme, rosemary and oregano essential oils were incorporated into PLA to generate antioxidant active packaging films based on the antioxidant activity of the used essential oils. Antioxidant activity of pure essential oils as well as film extracts were assessed by the DPPH method to ensure their efficiency. The antioxidant effect of prepared active films was also evaluated in contact with minced trout fish meat by using the TBA method. Mechanical properties, oxygen permeability and migration properties of the films were tested. Thyme essential oil showed a Radical Scavenging Activity (RSA) of 84.57% in the DPPH test, while rosemary and oregano had a RSA of 87.92% and 87.73% respectively at 10000 ppm concentration. Antioxidant activity of methanol extracts from thyme, rosemary and oregano films were between 4% and 6% lower than that shown by pure essential oils at the same concentration. TBA test results showed a decrease in degree of oxidation of minced fish packaged in thyme active film by 10.8% as compared to that packaged in control film. While Rosemary active film reduced oxidation by 20.3%. Oregano active film caused the highest decrease in oxidation by 47.9%. PLA films prepared by the solvent casting method containing thyme, rosemary or oregano essential oils may be used as antioxidant active packaging materials.

Keywords: Antioxidant, packaging, poly lactic acid, thyme, rosemary, oregano essential oil, DPPH test, TBA test.

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LIST OF ABBREVIATIONS

AP: Ascorbyl Palmitate

AT: α-Tocopherol

BHA: Butylated Hydroxyanisol

BHT: Butylated Hydroxytoluene

DG: Dodecyl Gallate

EVOH: Ethylene Vinyl Alcohol

GTE: Green Tea Extract

HDPE: High-Density Polyethylene

ISO: International Organization for Standardization

LDPE: Low-Density Polyethylene

OEO: Oregano Essential Oil

OG: Octyl Gallate

OTR: Oxygen Transmission Rate

PEG: Poly Ethylene Glycol

PG: Propyl Gallate

PLA: Polylactic Acid

PP: Polypropylene REO:

Rosemary Essential Oil

TBHQ: Tertbutylhydroquinone

TEO: Thyme Essential Oil

TVC: Total Viable Count

1. Introduction and background

One of the major causes for food deterioration is oxidation. Protecting foods from oxidation for appreciable amounts of time is becoming a major challenge among food producers. Lipid containing foods are the most sensitive types of food to oxidation. Fatty foods are negatively affected by oxidation in many ways; including quality and sensory (color, odor and texture) changes, in addition to food safety issues resulting from the increased food toxicity of oxidized lipids. (Byun, Kim et al. 2010)

Generally, the mechanism of oxidation occurs through the formation of hydro-peroxides which are colorless, tasteless and odorless. They are further broken down into carbonyl compounds that cause rancid flavor and odor. Many solutions have been proposed and applied to overcome the problem of food oxidation and to prolong the shelf life of lipid containing foods. Natural (e.g. tocopherols and ascorbic acid) and synthetic (e.g. BHT & BHA) antioxidants have been added directly to foods to slow down the auto-oxidation process by donating protons to stop peroxyl radicals from reacting with unsaturated lipids. This results in an increase in the shelf life of sensitive foods like vegetable oils, animal fats, meat, poultry and fish products.(Gómez-Estaca et al. 2014)

In the past, packaging served as a passive barrier to delay the effects of the environment on packaged foods or beverages. Nowadays, through the concept of active packaging the packaging material interacts with the environment and with the packaged food or beverage playing an active role in product preservation and shelf life extension. The definition of active packaging adopted for the European FAIR-project CT 98-4170 is: *"a*

type of packaging that changes the conditions of normal food packaging to extend shelf-life or improve safety or sensory properties while maintaining the quality of the food". (Vermeiren et al. 1999)

Antioxidant active packaging is used for extending shelf life and minimizing deterioration of food by oxidation, especially in oxygen sensitive types like fatty foods. Many natural (e.g. Carvacol, Alpha-Tocopherol, Flavonoids, Catechin and Quercetin) and synthetic (BHA, BHT) antioxidant compounds have been introduced to polymeric packaging films in order to be used for this purpose. The most preferred option by consumers is the incorporation of natural additives into packaging and avoiding synthetic additives due to their potential health risk.(Byun et al. 2010, López de Dicastillo et al. 2011)

Poly Lactic Acid (PLA) or more precisely Poly Lactide is a thermo-plastic aliphatic polyester that is produced from natural sources such as corn starch, sugar cane, tapioca roots and potato starch. It is commonly made from alpha-hydroxy acids i.e. poly glycolic acids and poly mandelic acids (2-Hydroxy-2-phenylacetic acid). It is a biodegradable and compostable polymer. It degrades without the need of enzymes through simple hydrolysis of the ester. PLA properties such as high strength and high elastic modulus in addition to its biodegradability gives PLA a good opportunity to replace conventional polymers. PLA is also characterized by good processability using standard processes and equipment to produce films (i.e. extrusion), fibers and molded structures.(Garlotta 2001, Martin and Avérous 2001, Amorati et al. 2013)

This study aims at investigating the feasibility of preparing functional antioxidant films containing natural essential oils using the solvent casting method. It has been designed in such a way so as to provide an initial insight on the feasibility of incorporating essential oils into PLA to generate biodegradable antioxidant packaging films that can be used to improve the shelf stability of fatty fish by reducing the oxidation of fish lipids.

2. **Literature review:**

The literature review will be divided into the following subchapters:

2.1) Food packaging, 2.2) PLA, 2.3) Foods and their oxidation potential, 2.4) Mechanism of action of essential oils as antioxidant agents. 2.5) Examples of methods of measuring antioxidant activity, 2.6) Essential Oils and their antioxidant activity, 2.7) Legislative aspects

2.1 **Food Packaging**

Food packaging is one of the pillars of the food industry. Before commercializing any food, it is an essential step to develop the proper packaging. The main objectives behind food packaging include: containing the foods, setting the portion size (single serve or multi-serve), supplying physical protection against surroundings and environmental factors like compression, shock and acting as a barrier that keeps microorganisms, contaminants, light, moisture, oxygen, etc. away from the product. Packaging has gained additional importance as a food marketing tool through labeling with information and artwork that attract potential buyers to the food product. Also, packaging allows more convenience in storage, handling, displaying and consumption of food.(Young and McEneny 2001)

In the past, food packaging has supplied these needs in a "passive" way. Packaging acted as a stand-alone system to protect the food, with minimal or no interaction with the food itself and the environment. Recently however, the concept of active packaging has been gaining substantial attention by food scientists as well as manufacturers, due to its great potential as a replacement for passive packaging with more benefits to consumers and

manufacturers. Active packaging tends to interact both with the packaged food and the environment to supply better conditions for storage, which accordingly maintain food quality and extend product shelf life. (Vermeiren et al. 1999)

2.1.1 Conventional vs. biodegradable food packaging

The production and consumption of plastic products for packaging applications has been expanding dramatically over the past years. In line with this increase, the environmental awareness of the community is increasing, and people are becoming more aware of the concerns about using petroleum derived plastics. Most of plastic materials are manufactured from petroleum based hydrocarbons, which is a non-renewable resource. The separation and recycling processes of petroleum based plastics are difficult and costly processes. As a result, a small percentage of the consumed plastics is recycled. Another concern regarding petroleum based plastics is their non-biodegradability and their negative effect on the environment. These concerns led to an increased attention to 'green' plastics. Plastics are considered "green" if they are biodegradable or compostable; also if they are produced from renewable resources with an environmental friendly process. PLA is an example of a biodegradable and renewable polymers used to replace petroleum based plastics like polyethylene (PE), polyethylene terephthalate (PET), polystyrene (PS), polypropylene (PP), etc. (Camo et al. 2008)

The increased awareness of manufacturers and consumers regarding the health and environment protection has pushed both to pay more attention to biodegradable versus conventional (petroleum based) food packaging. Biodegradable packaging is much more

environmentally friendly than conventional packaging materials, because it decomposes by bacteria or other living organisms within months or a few years. There are two general types of biodegradable plastics, the first type includes "bioplastics", whose constituents are produced from renewable resources. The second includes conventional plastics formed from petrochemical products, such as PE, PP, PS, etc., containing biodegradable additives such as starch that increase the rate of plastic biodegradation. (Fairlamb and Cerami 1992)

There are several types of biodegradable plastic materials available on the market. Polyvinyl alcohol (PVA), poly anhydrides, starch derived plastics (Polyethylene-starch), cellulose esters, chitosan, etc. Another very important type of biodegradable plastics is aliphatic polyesters. They are unlike aromatic polyesters, widely known for their biodegradability resulting from the hydrolysable ester bonds. Examples for aliphatic polyesters include i) synthetic polyesters like polybutylene succinate (PBS) and polycaprolactone (PCL), ii) naturally produced polyesters like polyhydroxyalkanoates (PHAs), polyhydroxybutyrate (PHB), polyhydroxyhexanoate (PHH) and polyhydroxyvalerate (PHV) and iii) polyesters derived from natural and renewable resources like poly lactate (PLA). (Ortiz-Vazquez et al. 2011)

Bioplastics have become a potential environmental friendly replacement for conventional plastics produced from petrochemicals. According to market research Bioplastics' market potential of expansion and growth is outstanding. Prices of bioplastics have been decreasing in accordance with the developments in production equipment and technology advancements that allow larger production scale. The price drop and improved technical

specifications besides the higher production capacities are paving the road for bioplastics to compete with conventional plastics. According to market research (Bioplastics, 3rd Edition (2011)), food packaging is the largest and the fastest growing market for bioplastic polymers.

2.1.2 Antioxidant food packaging using conventional materials:

Active packaging involves mainly antioxidant and antimicrobial packaging. This subchapter will focus on studies related to development of antioxidant active packaging materials from conventional polymers.

Antioxidant active packaging films were produced using melt blending followed by compression molding methods by Ramos et al. (2014a) by incorporating carvacol and thymol in polypropylene (PP) films. Antioxidant was added to each film in a concentration of 80 g/kg (8%). The antioxidant activity of the produced films was studied in contact with food simulants in terms of DPPH scavenging activity. Water, ethanol (10%), acetic acid, ethanol (95%) and isooctane were used as food simulants to extract the films. Carvacol and thymol extracted in acetic acid showed no antioxidant activity. This is believed to be the result of the acidity of the food simulant (acetic acid) that inhibits DPPH radical quenching by the antioxidant compounds.

Other extracts including water, ethanol and isooctane showed significant antioxidant activity against DPPH. Thymol showed stronger antioxidant activity, with the best performance being shown with isooctane (42.2%). The result showed that thymol had a significantly higher antioxidant activity than carvacol, regardless of the used food simulant for extraction. This result was attributed to the greater stearic hindrance of the

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thymol phenolic group as suggested by Abdellatief and Welt (2013) because compounds having a stearically hindered hydroxyl group have shown a higher antioxidant activity as also reported by Jamshidian et al. (2012).

The study carried out by Ramos et al. (2014a) showed that it is possible to use poly propylene (PP) films as antioxidant active packaging films to extend shelf life of foods, because a significant amount of the added antioxidant is retained in the film matrix after processing. The results of the study are as shown in Figure 1. It was also shown that thymol and carvacol can be used by PP manufacturers as an antioxidant that prevents oxidative degradation during PP production.
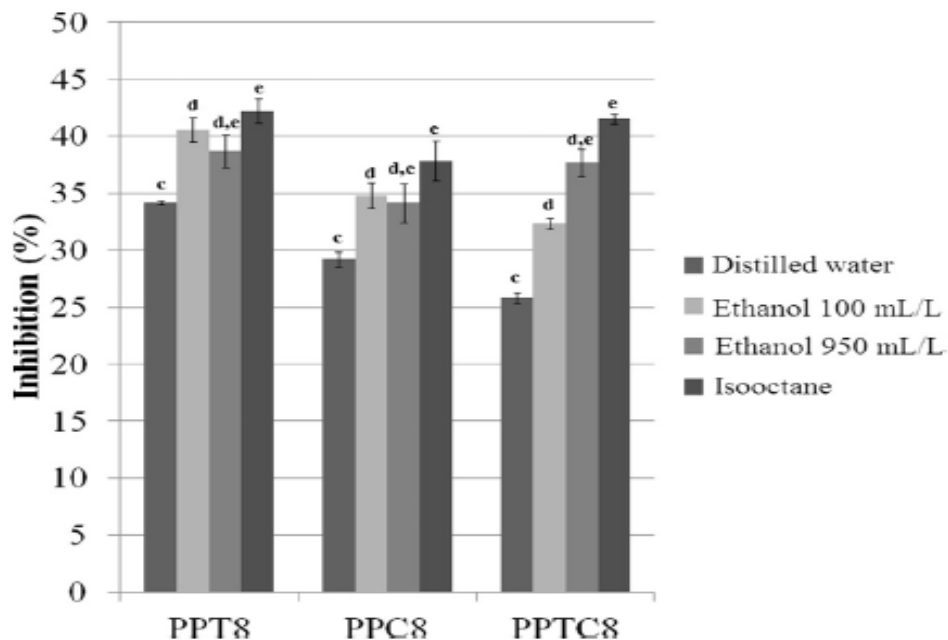
Figure 1. % RSA of thymol and carvacol extracted in different solvents.(Ramos et al. 2014a)

Citrus extract efficiency as an antioxidant agent in active packaging was assessed by Contini et al. (2012) through coating a tray made of polyethylene terephthalate (PET)

with citrus extract by spraying. The antioxidant efficiency of the developed packaging system was assessed in contact with cooked turkey meat using the 2-thiobarbituric acid reactive substances (TBARS) test. Quality parameters and sensory characteristics of meat were also assessed. The values of lipid oxidation were significantly higher in meat stored in uncoated trays on day 2, and day 4 of storage. For sensory testing, a paired-forced preference test was carried out between the meat slices stored in citrus extract- coated tray versus the meat stored in the uncoated tray. The meat stored in citrus extract coated PET trays received the highest preference with significantly higher sensory scores.

The conclusion from this study was that antioxidant active packaging generated by coating a plastic container with citrus extract significantly reduced lipid oxidation of cooked turkey meat. This, in turn, improved meat tenderness, through pH reduction, which altered muscle structure.

Aiming at producing antioxidant active films, ethylene vinyl alcohol (EVOH) films containing green tea extract (GTE) have been produced by López de Dicastillo et al. (2011) using extrusion. The extruded films were characterized by some darkness of color (transparent brown), and decreased permeability for both water vapor and oxygen at low relative humidity (RH) conditions. Also the film sensitivity to water increased at higher RH values, and thermal resistance of films improved. The produced films were analyzed by HPLC and the results indicated that GTE suffered some decomposition during the extrusion process when catechin gallates were decomposed to free gallic acid.

The antioxidant activity of GTE was assessed through measuring the radical scavenging

activity (RSA). The ABTS$^{\bullet+}$ method was used for aqueous simulants and the DPPH method was used for ethanol.(López de Dicastillo et al. 2011)

Figure 2. shows antioxidant activity induced by GTE containing films when brought in contact with different food simulants expressed as ascorbic acid equivalents. All food simulants had similar profiles, as all of them showed exponential growth until reaching a plateau. However the type of food simulant proved to be a very important part of the system, as ethanol 95% had 10 times higher antioxidant activity compared to other food simulants. This is attributed to the higher solubility of active components of GTE in ethanol, e.g. chatechins (the most effective component of GTE) are soluble in ethanol and only sparingly soluble in water.

2.1.3   Antioxidant food packaging using biodegradable materials

Recently, research on biodegradable plastics as a replacement for petroleum based plastics has been boosted by consumer demands for environmentally friendly packaging. This has driven scientists to work on the development of antioxidant active packaging from many natural sources.

In this chapter, studies on different types of biodegradable antioxidant active packaging films in literature will be reviewed.
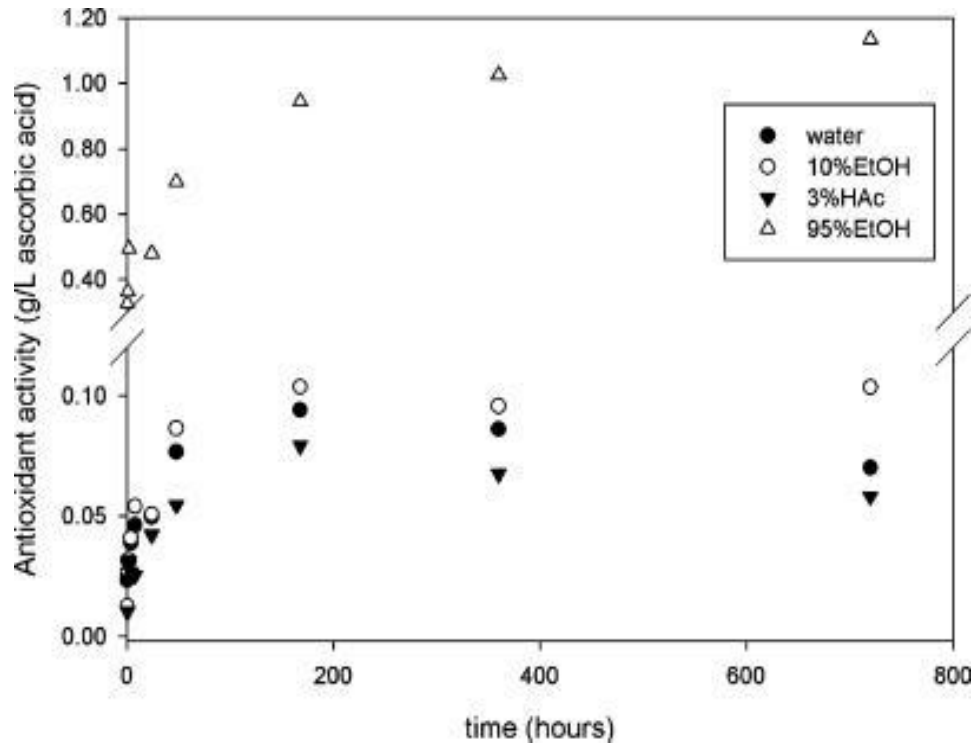
Figure 2. Antioxidant activity of different food simulants subjected to GTE containing films. Tests are done with different techniques. Results are expressed in equivalent ascorbic acid concentration. (López de Dicastillo et al. 2011)

Jouki et al. (2014) worked on developing a packaging film from quince seed mucilage (hydrolyzed quince seed gum). They investigated the possibility of incorporating thyme essential oil to prepare an active antioxidant packaging film that is edible and biodegradable. Film antioxidant activity was evaluated by the DPPH method before and after thyme incorporation into the film at concentrations ranging from 0% to 2%. The results shown in Figure 3 demonstrate that the radical scavenging activity of the film without thyme addition was 18.39 %. After adding thyme essential oil at concentrations of 1%, 1.5% and 2%, the radical scavenging activity was 30.11%, 37.29%, and 43.14% respectively.

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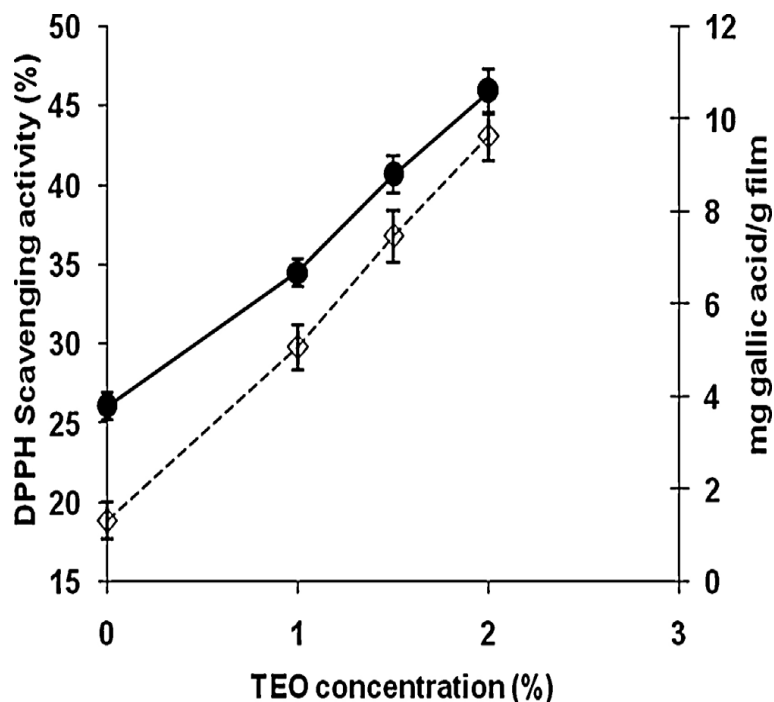
***Figure 3. DPPH radical scavenging activity and total phenolic content versus
concentration of essential oil in film (Jouki et al. 2014)***

Biologically active biomolecules like chitosan have been recently perceived by scientists

as high potential raw materials for the preparation of active packaging films and

application in the food industry. Chitosan is used with multiple objectives for shelf life

extension based on antioxidant and antimicrobial activity. Chitosan is characterized by

its biodegradability, non-toxicity and biocompatibility with human body tissues. Chitosan

has proven antimicrobial activity against pathogenic and spoilage microorganisms

including bacteria (gram positive and gram negative) as well as fungi. (Aider, 2010)

Siripatrawan and Harte (2010) developed a chitosan based active film and incorporated

GTE into it at concentrations of 2%, 5%, 10% and 20%. Physical properties of the film

were evaluated, antioxidant activity and total phenolic content were measured.

Incorporation of GTE helped to improve the mechanical properties as well as water vapor barrier properties. Also the film was shown to possess antioxidant activity. The results of DPPH scavenging activity test showed that blank chitosan film possesses little antioxidant activity. The antioxidant activity of films increased significantly with the increase of GTE concentration (Figure 4). Films containing GTE showed antioxidant activity up to 15 times higher than that shown by blank chitosan film.

2.1.4 Antimicrobial packaging

Del Nobile et al. (2009) conducted a study on preparing environmentally friendly antimicrobial active packaging films by melt extrusion of PLA, polycaprolactone (PCL) and LDPE. The first two are biodegradable and the third is recyclable polymer. Thymol, lemon extract and lysozyme were incorporated into the three polymers with three different concentrations, generating 9 active films.

The results showed that the produced films were negatively affected by the high processing temperatures of PLA and LDPE. They still possessed some antimicrobial activity, but the PCL that was processed at lower temperature showed higher antimicrobial activity.

Soy protein edible films were developed by Emiroğlu et al. (2010) as antimicrobial active packaging film by incorporating 1, 2, 3, 4 and 5% of thyme and oregano essential oils as well as a mixture of oregano and thyme essential oils.
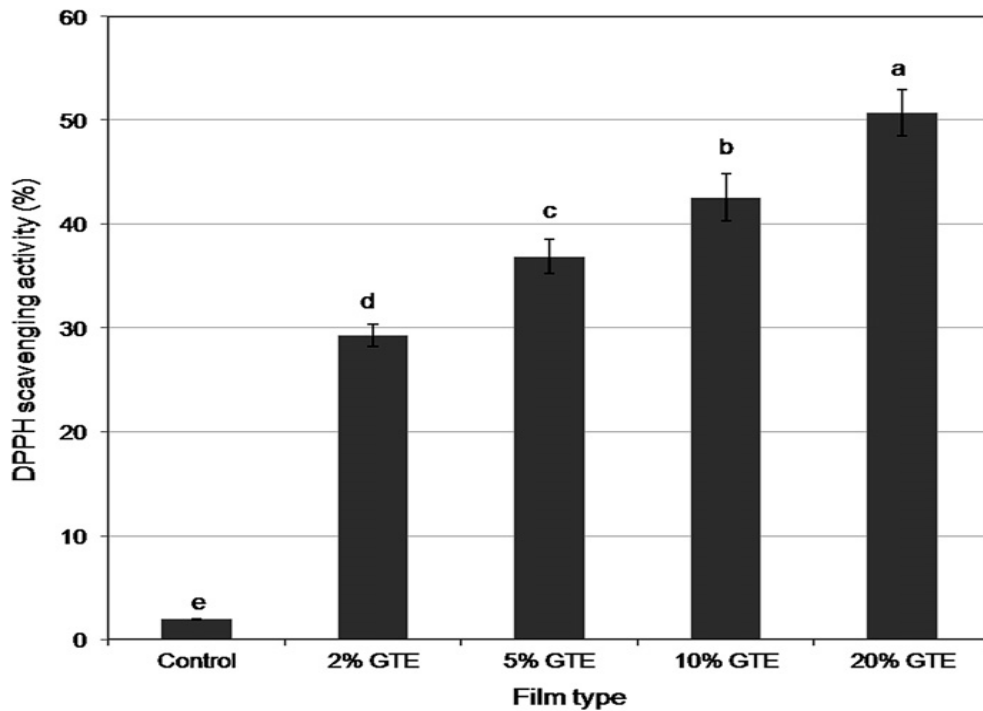
Figure 4. DPPH scavenging activity of chitosan films containing GTE. The mentioned figures represent means (±) standard deviation. Letters express significant difference as (p ≤ 0.05) Siripatrawan and Harte (2010)

The activity of the films against microorganisms *E. coli* O157:H7, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Lactobacillus plantarum* were assessed by the microbial growth inhibition zone test. Films containing oregano, thyme and mixture of both showed efficient antimicrobial activity towards all types of bacteria. *E.coli* O157:H7, *S. aureus. L. plantarum* and *P. aeruginosa* were less affected by the films' antimicrobial activity. The developed films proved to be ineffective against total viable count (TVC) and lactic acid bacteria.

2.2 **PLA**

PLA is a glossy, colorless, and stiff material. It is soluble in most organic solvents like benzene, dioxane, acetonitrile and chloroform. It is an aliphatic biodegradable and compostable polyester, belonging to the bioplastics family. PLA is produced from hydroxy acids like polyglycolic acid and poly mandelic acid. It is considered as a high strength, and high modulus polymer with versatile applications in packaging materials as well as medical devices and other uses. PLA is degraded through the hydrolysis of the ester bond without the need for enzymes or catalysts, making it a biodegradable polymer. PLA degrades at temperatures exceeding 200 ^{o}C through a mechanism that depends on time, temperature and low molecular weight impurities as well as the presence and concentration of catalyst. Melting temperature of PLA is 175 ^{o}C and the glass transition temperature is 55°C.

PLA degrades naturally into natural products with no environmental consequences. It takes from six months to two years to decompose, which is considered as a very short time as compared to non-biodegradable plastics that need a period of approximately five hundred years to degrade (i.e. polyethylene, polystyrene, etc.).(Garlotta 2001)

PLA is a very versatile polymer. It is processed by several conventional processing techniques with minimal problems. It has good mechanical properties helping it to fit several purposes including food packaging. It has been used in many studies to produce antimicrobial and antioxidant active films by the solvent casting method. It has proven very efficient in conventional extrusion as well.

One of the reported problems of PLA as a packaging material is its stiffness at room temperature. This can be overcome by adding a plasticizer to improve flexibility. Another

weakness of PLA films is their poor water vapor barrier properties.(Byun et al. 2010)

2.2.1 PLA Synthesis

PLA polymer is made up of lactic acid monomer. Lactic acid was first isolated from sour milk in Sweden in 1780 by the scientist Scheele. It is produced by carbohydrate fermentation (e.g. maize starch fermentation) or chemical synthesis.

PLA polymer (high molecular weight) is manufactured by polymerization of two types of monomers: cyclic diester lactide and lactic acid. The first monomer is more common in PLA synthesis.

PLA is commonly manufactured through a ring opening polymerization process of lactide in the presence of a metal catalyst. This method causes racemization of PLA, changing its stereo orientation with respect to the starting monomers.

As shown in Figure 5, during the ring opening polymerization, initial polymerization yields low molecular weight polymer (pre-polymer) with water condensation. De-polymerization process follows to yield an intermediate cyclic lactide (lactic acid dimer). The third step is the ring opening that yields high molecular weight PLA by internal transesterification.

Catalytic de-polymerization into oligomers of low molecular weight, and internal transesterification steps can be performed by L-lactic acid, D-lactic acid or a mixture of both. (Garlotta 2001, Auras et al. 2004, Marcos et al. 2014)

Another method of PLA production is using lactic acid monomers that are subjected to a direct condensation reaction. This reaction is carried out in three successive steps. The first step involves polymerization of lactic acid monomers to produce oligomers (low
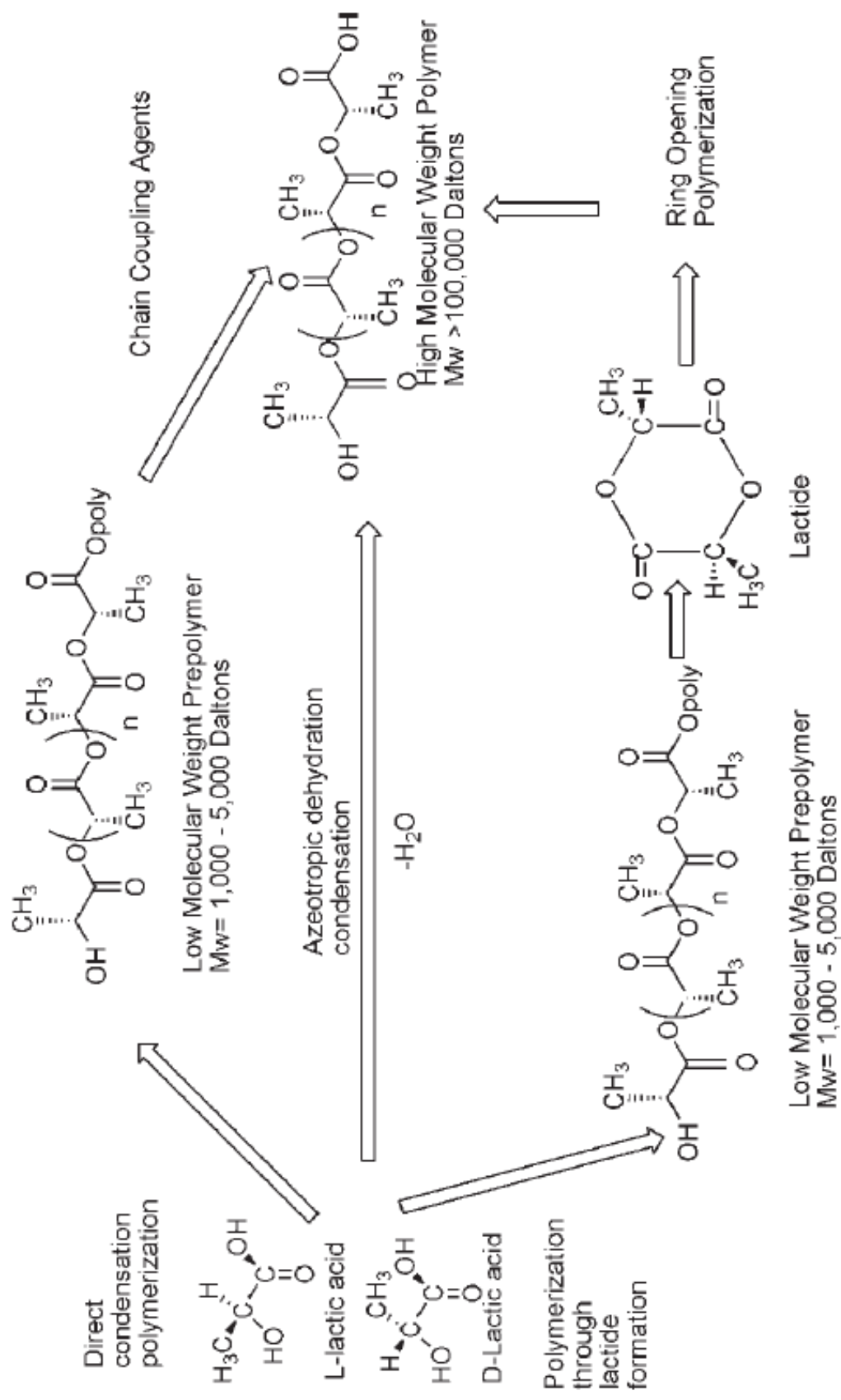
Figure 5. Methods of PLA (high molecular weight) synthesis by (Auras, Harte et al. 2004)

molecular weight), followed by a poly-condensation process that combines the oligomers into high molecular weight polymers of PLA. The third step is water elimination by applying vacuum to push the PLA to undergo poly-condensation instead of transesterification.

The direct condensation method produces water as a by-product of the esterification reaction. The water generated, causes chain transfer, resulting in lower molecular weight PLA. This is the reason behind not favoring this method as compared to the ring opening polymerization method.(Garlotta 2001, Södergård and Stolt 2010)

2.2.2 Antioxidant films based on PLA

Jamshidian et al. (2013) used PLA to prepare antioxidant films by the solvent casting technique. Efficiency of both natural and synthetic antioxidant compounds was evaluated. The release of antioxidant molecules was assessed to investigate the feasibility to create antioxidant films by incorporating antioxidants in solvent casted PLA films. Ascorbylpalmitate (AP) and α-tocopherol (AT) were used as natural antioxidant additives. Butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), propylgallate (PG), and tertbutylhydroquinone (TBHQ) were used as synthetic antioxidants. Films with incorporated antioxidants were extracted with ethanol at different concentrations (10%, 50% and 95%) at different temperatures (20°C and 40°C). Antioxidants released from the films were determined by high-performance liquid chromatography (HPLC).

Antioxidant losses during film preparation especially during solvent evaporation and drying step were monitored by nuclear magnetic resonance (NMR) analysis. AP was decomposed during the film preparation process and showed the highest loss because of its sensitivity to heat and light, making it unsuitable for the preparation of antioxidant packaging films. The rest of the antioxidants tested, suffered much lower losses, below 10%. Hence, all antioxidants tested can be used to produce films by solvent casting of PLA except AP. The more volatile the antioxidant, the more suitable it is for film preparation for food preservation because it evaporates faster from the film surface to the target food.

On the other hand, the more volatile the antioxidant, the harder it is to be retained in sufficient amounts within the film matrix during the evaporation/drying step of the solvent casting method. Solvent casting cannot be used to produce films on commercial scale however it is suitable for lab scale experiments. (Byun et al. 2010)

Extrusion is another way of producing active antioxidant films for food packaging. Synthetic phenolic antioxidants were incorporated in extruded PLA films by Jamshidian et. al., (2012) to assess their antioxidant activity and efficiency as active packaging materials. Films were extruded after mixing PLA granules with antioxidant at a concentration of 2% (w/w). Synthetic antioxidants used, included BHA, BHT, PG, and TBHQ. The process of extrusion was carried out by using a pilot plant extruder. The films were extracted with ethanol of different concentrations (10%, 50% and 90%) at different temperatures (20 ^{o}C and 40 ^{o}C). The synthetic antioxidant release was determined by HPLC over a period of 60 days.

The produced films were tested by 1H-NMR to evaluate antioxidant loss during the extrusion process. TBHQ showed the highest loss of 45%, followed by PG (loss of 18.8%), BHA (loss of 14.3%) and finally BHA (loss of 10.5%). These high losses occurred as a result of the extrusion process under a high temperature of 185 ^{o}C as well as the high extrusion pressure. Antioxidant losses depend on molecule heat stability and volatility.

According to Fick's second law, the release process is a result of diffusion of antioxidant from film to food or food simulant until the concentration of the antioxidant reaches equilibrium. The antioxidant diffuses through the polymer matrix until it reaches the film/food interface, and then migrates into the food/simulant until the concentration in both is equal. During the migration process, the release rate of migrant depends on several factors, including size and shape of migrant molecule as well as polymer macro molecules. It also depends on the number and size of gaps in the polymer matrix, because the migrant passes through these gaps during the migration process. The number of gaps depends mainly on the degree of branching of the polymer and crosslinking between polymer molecules. PG showed the highest release rate at all simulant concentrations and temperatures owing to its higher polarity compared to other antioxidants. TBHQ was decomposed to TBBQ during extrusion. BHT was the slowest released antioxidant, showing almost no migration when in contact with 10% ethanol because it is a non-polar molecule. (Jamshidian et al. 2012)

Releasing of antioxidants from PLA films into ethanol based food simulant is affected by several factors. 1) The properties of the antioxidant molecule like its solubility in the food simulant, polarity, molecular weight. 2) simulant polarity 3) thickness of PLA film, 4)

temperature. 5) agitation. 6) polymer degradation during the process, etc.(Jamshidian et al. 2012)

Arrieta et al. (2013) incorporated D-limonene in PLA films at concentrations of 15% and 20%. D-Limonene is the main component of citrus oils that contributes to the citrus flavor. Limonene is known to possess antioxidant and antimicrobial activity (Singh et al. 2010). Hence, limonene is a possible additive for PLA to develop an antioxidant active packaging film.

The obtained films were assessed for residual limonene after processing by pyrolysis gas chromatography (PY-GC), for mechanical properties, barrier properties and color. The color slightly changed towards transparent yellow. Glass transition temperature decreased after adding limonene to PLA. Mechanical properties of the films were improved by the addition of limonene, which indicates the possibility of using limonene as a plasticizer for PLA. Also the water adsorption of the developed films was reduced after adding limonene.

The conclusion is that limonene is a suitable additive for PLA films acting as an efficient natural plasticizer. It is also possible that it possesses some antioxidant activity that should be further evaluated (Arrieta et al. 2013)

Arrieta et al. (2014) developed an antioxidant active packaging material by melt blending of PLA and polyhydroxybutyrate (PHB). Catechin was incorporated in the produced film as an antioxidant agent. Addition of chatechins to the developed films increased its rigidity, as a result of hydrogen bond formation between the hydroxyl groups of

chatechins and the carbonyl groups of PLA and PHB. Accordingly, Acetyltributyl citrate (ATBC) was added as a plasticizer to improve the release of chatechin and to decrease the film rigidity caused by the addition of chatechins. Chatechins release in fatty food simulant was quantified and antioxidant activity was assessed. This study showed the effectiveness of PLA-PHB chatechin polymers as antioxidant active packaging films. Figure 6. shows the DPPH test results in gallic acid equivalent units (ppm).

A combination between high pressure treatment of foods and application of PLA-AP antioxidant active packaging film was tested by Bolumar et al. (2011) for antioxidant activity in contact with chicken minced thigh and breast meat to extend product shelf life. The minced meat was packaged in the test film, then exposed to a pressure of 800 MPa for 10 minutes at 5 °C. Lipid oxidation of meat was measured throughout a period of 25 days.

Lipid oxidation was found to be higher on the surface of meat in absence of antioxidant active packaging. In the presence of antioxidant active packaging, the surface oxidation was delayed for 25 days. The obtained results showed that there is a good market potential for using high pressure processed meats in combination with active packaging technique to preserve meat products.

Byun et al. (2010) developed antioxidant active PLA films containing α-tocopherol, BHT and Polyethylene Glycol 400 (PEG 400) as a plasticizer by the cast extrusion
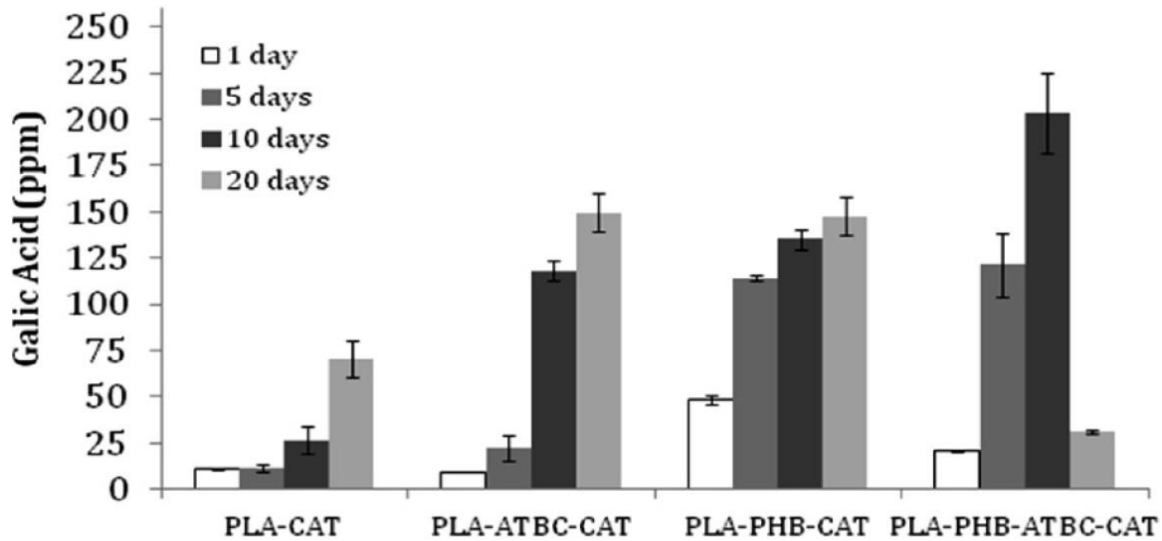
Figure 6. DPPH test results for chatechins in different matrices expressed as gallic acid equivalent concentration (ppm) (Rhim et al. 2009)

technique. Antioxidant activity of the produced films was assessed though radical scavenging activity evaluation by the DPPH method. The first film containing, α-tocopherol (1%) and BHT (0.01) showed a high antioxidant activity of 90%. BHT was added to prevent polymer degradation during extrusion. The second film containing only BHT (0.01%) showed a lower radical scavenging activity of 14%. Plain PLA film showed no radical scavenging activity at all.

Despite the fact that synthetic antioxidants (e.g. BHT) are more efficient and less expensive than natural ones (e.g. α-tocopherol) they are considered less attractive from a safety and marketing point of view. This study clarified that it is possible to replace synthetic antioxidants with natural ones in developing antioxidant active packaging films to serve food packaging purposes.

Limm and Hollifield (1996) and Jamshidian et al. (2012) reviewed the factors affecting

the release of antioxidants from PLA films. They linked migration to polymer properties (e.g. crystallinity, molecular weight, morphology, density, and orientation of monomers) since all these parameters impact the size and shape of polymer micro-cavities and their distribution. They also linked the release of antioxidants to the antioxidant molecule related factors (e.g molecular size, density, shape, polarity and solubility). Interaction between the polymer and the antioxidant agent (e.g. plasticizing and anti-plasticizing effects) were considered. Other factors including temperature were noted (e.g. glass transition temperature and melting temperature of polymer). They also considered the potential food binding effect as a factor affecting the release of antioxidants from PLA active films. The factors affecting antioxidant release from film to food were summarized by Södergård and Stolt (2010). as the polymer type, antioxidant type, affinity of antioxidant to polymer, food nature, and environmental conditions (i.e. temperature).

2.3   Foods and their oxidation potential

2.3.1   Lipid auto-oxidation

The oxidation mechanism of lipids (Figure 7.1) is initiated by a radical species ($R^\bullet$) that forms by the removal of a hydrogen atom (in α-position relative to the double bond) from an unsaturated fatty acid of the form RH. The formed radical reacts with oxygen to form peroxyl radical $ROO^\bullet$. The formed radical reacts with another fatty acid RH and abstracts another hydrogen atom to form a hydroperoxide (ROOH) leaving the $R^\bullet$ to restart the reaction again. This continuous reaction is called oxidation chain reaction, and the number of cycles occurring between the initiation and termination is called reaction chain length. The reaction chain continues until the termination step occurs when two radicals

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react together and quench one another or an antioxidant quenches the free radical. (Valgimigli and Pratt 2012, Jamshidian et al. 2013). Figure 7.2 shows the mechanism of auto-oxidation of unsaturated fatty acids.

Initiation Step:
$$RH \rightarrow R^{\bullet}$$

Propagation step:
$$R^{\bullet} + O2 \rightarrow ROO^{\bullet}$$
$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$$

Termination step:
$$R^{\bullet} + R^{\bullet} \rightarrow R\text{-}R$$
$$ROO^{\bullet} + ROO^{\bullet} \rightarrow 2\,ROO^{\bullet} \rightarrow ROOR + O2$$
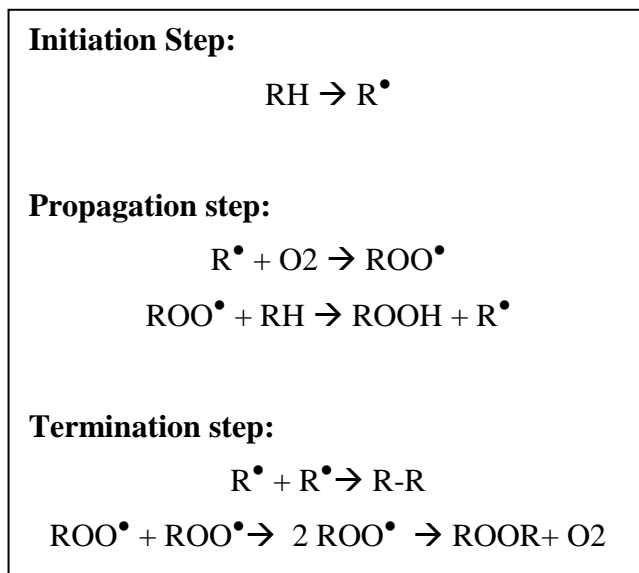
Figure 7.1. General scheme of lipid auto-oxidation.

2.4 Mechanism of action of antioxidant agents

In general, there are 2 major groups of antioxidant compounds, the first group includes direct antioxidants and the second indirect antioxidants.

Direct antioxidants are the compounds that stop the oxidation chain reaction. They are classified in two different subcategories. The first includes preventive antioxidants, which inhibit the initiation step by stopping the formation of radical species. Examples of direct antioxidants are the enzyme catalase, phytic acid and other metal chelators which block the redox process of metal ions like Fe^{+2} and trap it in the oxidized form Fe^{+3}. The second type includes chain-breaking antioxidants. The antioxidant molecule interferes in the propagation step by competing with the substrate to react with peroxyl radicals ($ROO^{\bullet}$).

25
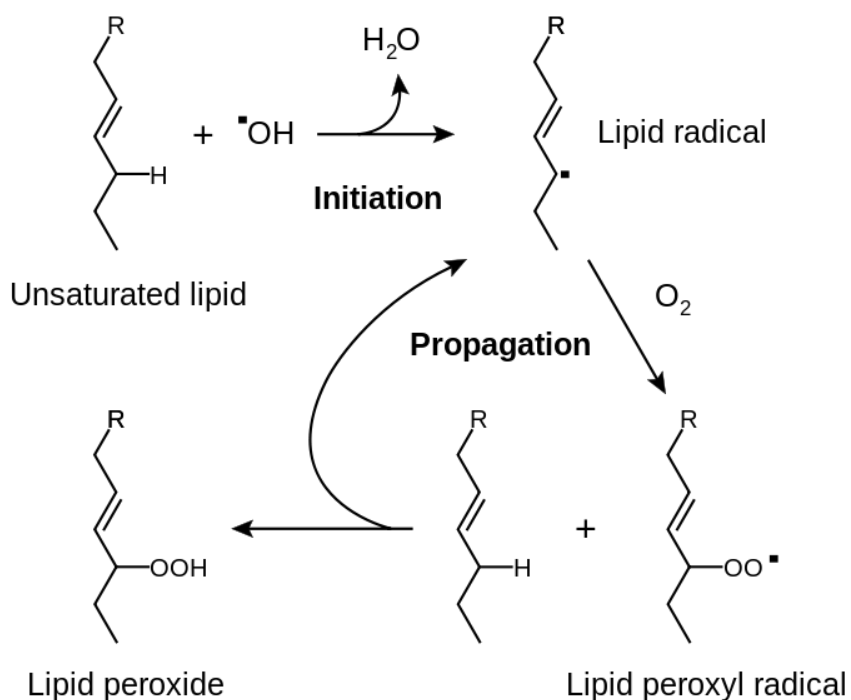
Figure 7.2 Mechanism of unsaturated fatty acids auto-oxidation process (Young and McEneny 2001)

As a result it can inhibit the auto-oxidation process (Amorati et al. 2013). α-tocopherol and essential oils are examples of chain breaking antioxidants (Byun et al. 2010).

Chain-breaking antioxidants are considered the most effective direct antioxidants for two main reasons. The first reason is their interference with the peroxyl radical which is the main motor for the whole chain reaction. The second reason is that preventive antioxidants are not effective if used after the initiation step. Essential oils belong mainly to direct antioxidants. (Amorati et al. 2013)

The second type includes indirect antioxidants, Their mode of action includes intensifying the cellular antioxidant activity via altering gene expression (e.g. glutathione

and superoxide dismutase). If one molecule of direct antioxidants like thymol can quench one free radical, an indirect antioxidant can induce a gene switching system that occurs through the Nrf2/ARE pathway. This action can quench multiple free radicals. Examples of plant extracts containing indirect antioxidants, e.g. Ecklonia cava, Curcumin (Rhim et al. 2006)

An example of indirect anti-oxidation mechanism is the mechanism of action of glutathione in human cells. Glutathione is a peptide containing cysteine amino acid, and it is present in most aerobic living organisms. It is synthesized in the body, hence it is not needed to be ingested through the diet. The thiol group of the cysteine moiety performs its role as a reducing agent that inhibits oxidation. Glutathione is kept in the reduced form by the effect of the enzyme glutathione reductase. It reduces other metabolites and reacts directly with free radicals and oxidants. Glutathione is found in relatively high concentrations in cell to perform it role as a key agent in maintaining the cells redox state. (Fairlamb and Cerami 1992)

2.5   Chemical Composition of essential oils

2.5.1   Thyme essential oil chemical composition

Thyme was used by ancient Egyptians for mummification of the dead (López-de-Dicastillo et al. 2012). It was also used by Greeks and Romans for its desirable odor in their shelters, temples and baths. Thyme was also added in liquors and foods for its appealing aroma. (Grieve 1990) Thyme essential oil has been analyzed by gas chromatography/ mass spectrometry in three different studies. Analyses showed that it is made up of Thymol, p-Cymene, Carvacol, γ-Terpenine, and small amounts of Linalool,

β-Mercene, and Caryophyllene, etc. in a descending order of concentration. The % composition of thyme essential oil is detailed in Table 1.(Lagouri et al. 2011, Jouki et al. 2014)

Several factors contribute to the variation of the chemical composition of essential oils. Examples of contributing factors include the used plant species and genus, the part of the plant where the oil is extracted from (leaves, stem, flowers or roots), the geographical origin of the plant and the harvesting season of the herb, etc. (Ojeda-Sana et al. 2013) chemical composition of thyme essential oil is shown in Table 1.

Table 1. A comparison between thyme essential oils of different origins in terms of chemical composition.(Lagouri et al. 2011, Jouki et al. 2014, Zengin and Baysal 2014)

Component	(Jouki et al. 2014)	(Zengin and Baysal 2014)	(Lagouri et al. 2011)
	% of total wt.	% of total wt.	% of total wt.
Thymol	46.42	4.51	12.76
p-Cymene	22.31	7.84	10.09
Carvacol	12.42	75.27	45.73
γ-Terpenine	7.50	2.96	0.13
Linalool	5.37	0.39	2.23
β-Mercene	2.27	1.10	-
Caryophyllene	0.69	0.85	0.26

2.5.2 Rosemary essential oil chemical composition

The name "Rosemary" originates from the Latin meaning of "sea dew", as "ros" means dew and "marinus" means sea. Rosemary belongs to the mint family like thyme. It is a perennial herb characterized by pointed leaves and a strong fragrance, as well as pink,

violet or blue flowers and a fibrous root system.

Rosmarinus officinalis L. is sometimes called 'anthos', which means flower in Greek. (America 2009)

Rosemary grows as an evergreen shrub and its leaves are usually used in cooking due to their appealing aroma. They are characterized by an astringent flavor inclined towards bitterness, enhancing the meaty note of barbeque grilled meats. Rosemary has also been used for treating certain inflammatory diseases, respiratory problems, etc. Rosemary extracts are also used to release anxiety and to improve concentration and alertness. (Jiang et al. 2011)

Not only has rosemary proven to be an efficient antioxidant and antimicrobial agent, but it is also a strong anti-inflammatory, antiviral and anti-carcinogenic agent.(Ojeda-Sana et al. 2013). Table 2 shows the chemical composition of rosemary essential oil.

2.5.3   Oregano essential oil chemical composition

Oregano also belongs to the same mint *(Lamiaceae)* family as thyme and rosemary. It grows best at warm temperatures in the Mediterranean region. It is a perennial herb that grows up to 80 cm in height, with spade shaped leaves of 1 to 4 cm with violet flowers on spikes. It is also called wild marjoram *(Origanum vulgaris L.). (*Table 3) shows the chemical composition of oregano essential oil.

2.6   Essential Oils and their antioxidant activity

The International Organization for Standardization (ISO) defined essential oils in (SO/DIS 9235.2 1997 P. 2) as *"Product obtained from vegetable raw material, either by distillation with water or steam or from the epicarp of citrus fruits by mechanical process*

Table 2. Chemical composition of Rosemary essential oil analyzed by GC/MS. (Jiang et al. 2011)

Component	% Composition (wt)
a-Thujene	0.27
a-Pinene	20.14
Camphene	11.38
[3-Pinene	6.95
[3-Phellandrene	0.98
a-Terpinene	0.21
p-Cymene	1.59
Limonene	1.32
1,8-Cineole	26.54
-y-Terpinene	1.02
Linalool	0.25
Isopulegol	1.03
Cis-chrysanthenol	1.93
Camphor	12.88
Borneol	3.06
Terpinene 4-ol	0.34
a-Terpineol	1.95
Verbenone	1.36
Bornyl acetate	0.92
Geranyl acetate	0.24
[3-Caryophyllene	2.37
a-Caryophyllene	0.68

Table 3. Chemical composition of oregano essential oil analyzed by GC/MS(Olmedo et al. 2014)

Component	% Composition (wt)
α-Thujene	1.26
α-Pinene	1.09
Sabinene	4.62
β-Pinene	0.44
β-Myrcene	1.78
α-Phellandrene	0.79
a-Terpinene	8.54
r-Cymene	2.65
b-Phellandrene	3.62
c-Terpinene	25.1
Cis sabinene hydrate	0.9
Terpinolene	2.13
Linalool	7.44
Camphor	0.39
Terpinen-4-ol	16.7
a-Terpineol	2.09
Thymol methyl ether	1.87
Carvacrol	16.2
b-Caryophyllene	0.83
Germancrene D	1.03
Total composition	99.5

or by dry distillation" The ISO states also that *"essential oils may undergo physical treatments (distillation, aeration) which do not involve significant change in their composition".* According to Amorati et al. (2013), Essential oils are usually extracted from aromatic plants by steam distillation isolating the essence of the plant in the form of an essential oil.

Reactive Oxygen and Nitrogen species are naturally produced in human cells during metabolism and normal biological activities. Pollution and many other environmental factors influence human cells to produce more free radicals. These species are known to have a potentially negative impact on human health, as they cause direct harm to DNA, RNA, proteins and lipid molecules. Accumulation of reactive oxygen and nitrogen radicals is believed to be the root cause for many diseases like cancer, rheumatoid arthritis etc. The aging process and its associated degenerative processes are believed to be triggered by free radicals.

Biological systems are naturally protected against the damage caused by free radicals through oxidative enzymes like catalase, superoxide dismutase, etc. The body is negatively affected by free radicals when the process of antioxidant activity becomes unbalanced and insufficient to quench the free radicals. In turn, human cells start to suffer degeneration leading to aging and other degenerative diseases. Antioxidant food supplements, especially essential oils are used as one of the ways to increase the body defense against free radicals and associated diseases (Saleh et al. 2010).

The antioxidant activity is usually measured by using colored antioxidant reagents which when they react with free radicals they change color as a result of reduction of the radical. Examples of these chemicals tests are DPPH$^{\bullet}$, ABTS$^{\bullet}$+, ORAC$^{\bullet}$ and Fe^{+3}

radicals/ions.(Amorati et al. 2013)

Phenols and phenolic compounds are believed to be the key compounds contributing to the antioxidant activity of essential oils. They are able to inhibit and delay the oxidation process of organic compounds due to the phenomenon of resonance which stabilizes the phenolic ring (Figure 8). However, the steam distillation process usually used to extract essential oils is not capable of extracting all phenolic compounds present in the plant, due to the fact that some phenolic compounds are not volatile, hence they won't evaporate. On the other hand, some essential oils are phenol free and yet have a clear antioxidant activity. This is a result of the presence of some terpenoids and/or other non-volatile/volatile components.(Valgimigli and Pratt 2012)
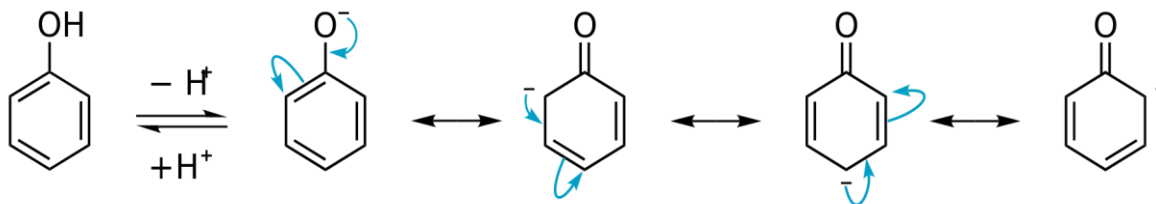
Figure 8. Resonance phenomenon in phenolic type antioxidants.

Some essential oils are free from phenolic compounds yet they show antioxidant activity due to the presence of some terpenic compounds having active methylene group. Examples include γ-terpinene which can contribute to radical scavenging activity of the volatile oil fraction of many essential oils like tea tree (Kim et al., 2004) and citrus essential oils (Takahashi et al., 2003). This activity is due to the presence of two active methylene groups in that molecule in β-position to double bond, which can donate electrons and quench free radicals (Foti and Ingold 2003).

Commercial synthetic preservatives of known antimicrobial and antioxidant activities are generally recognized as safe and allowed to be used in food preservation. However, certain approved synthetic additives present specific health related problems i.e. causing allergies (e.g. sulphites and benzoic acid, Teixeira et al. 2013). The use of Nitrites as preservatives is also questioned in terms of food safety due to the possibility of formation of carcinogenic nitrosamines in humans. (BHT) and (BHA) are also suspected to be carcinogenic in rodents. These risks justify the need for the replacement of synthetic antioxidant and antimicrobial additives by natural ones. (Teixeira et al. 2013) Essential oils are favored as a replacement because they are accepted by consumers and recognized as safe, with no safety tests needed. Figure 9. shows antioxidant activity of some essential oils and plants extracts. They are also acceptable from a sensory perspective as they taste
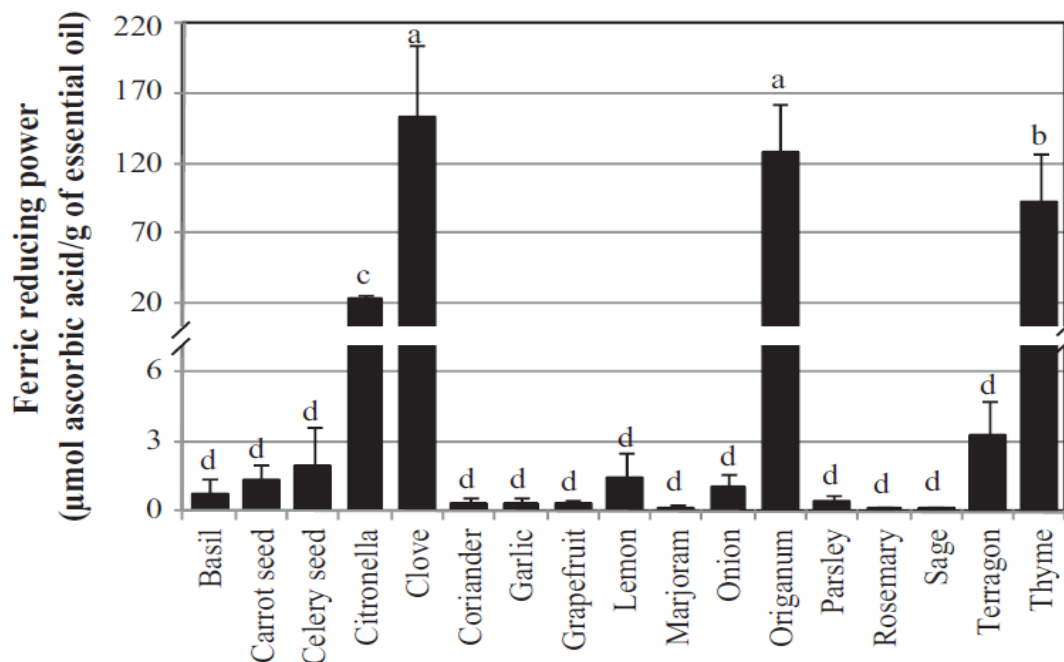
Figure 9. Antioxidant activity of essential oils measured by the ferric reducing power method. Bars express the average, and the error bars represent the standard deviation. Letters show significant differences between essential oils (P < 0.05) (Teixeira, Marques et al. 2013)

and smell good as long as they are used in reasonable concentration.(Olmedo et al. 2014)

Camo et al. (2008) compared the shelf life extension of lamb meat treated with three types of antioxidants by preparing three samples. The first was packaged in a rosemary active film, the second was packaged in an oregano active film while the third meat sample was sprayed with rosemary essential oil on the surface, then packaged in a high oxygen atmosphere. TBARS test was used to assess the antioxidant effect of the three treatments against a control (untreated sample). Color change was measured, and sensory analysis was performed. The results showed that all treatments had a significant effect in terms of antioxidant activity. Direct spraying of rosemary essential oil had a higher antioxidant effect than using rosemary active film. Oregano active film had a similar effect to direct spraying of rosemary essential oil. Sensory testing showed that the treated samples had an extended period of freshness of odor and color from 8 to about 13 days as compared to untreated samples.

Chouliara et al. (2007) evaluated the combined effect of modified atmosphere packaging (MAP) and addition of oregano essential oil 0.1% and 1% w/w to fresh chicken meat. Microbiological, physiochemical and sensory characteristics were monitored through a period of 25 days of storage at 4 $^\square$C. Sensory analysis showed that the usage of oregano essential oil for direct application in a concentration of 1% is not feasible, because it affects the flavor of the stored meat. The TBA test results of all treated samples didn't exceed 1 mg MDA/kg. pH showed a decrease from 6.4 to 5.9 throughout the storage period. Color change throughout the storage period was not affected by the addition of

oregano essential oil or by the MAP. Samples treated with oregano essential oil 0.1% had a shelf life of 3-4 days, samples under MAP had 2-3 days of shelf life, while samples treated with both oregano essential oil and MAP had a longer shelf life of 5-6 days. Karabagias et al. (2011) investigated the effect of thyme and oregano essential oils on shelf life extension of lamb meat with modified atmosphere packaging (MAP). The study included two experiments. In the first thyme and oregano essential oils were used with direct application to meat at concentrations of 0.1 and 0.3% v/w with 2 different types of modified atmosphere packaging, MAP1 had 60% CO_2 and 40% N_2, MAP2 had 80% CO_2 and 20% N_2.

The quality and sensory characteristics as well as microbiological properties of lamb meat were monitored through a period of 20 days. The results showed that the high concentrations of essential oils directly applied caused a noticeable change in flavor of meat. Thyme showed a higher efficiency in terms of antimicrobial activity than oregano and MAP2 showed higher efficiency than MAP1. In a second experiment, MAP2 was used with thyme essential oil 0.1% on fresh lamb meat, for a period of 25 days. Microbial counts decreased significantly and TBA value didn't exceed 4mg MDA/kg. Shelf life of air packaged samples (control) was 7 days at 4 $^{\square}$C, while reaching 10 days for samples with thyme essential oil 0.1%, and 22 days for samples combining MAP and thyme essential oil (0.1%).

Based on the above, it is clear that direct addition of essential oil generates higher oxidative stability of samples than using active packaging films. This may be attributed to the fact that most of the antioxidant agent molecules are trapped in the polymeric matrix, exhibiting only a portion of the original antioxidant activity (Camo et al. 2008). On the

other hand, active packaging should limit the antioxidant effect to the surface of the packaged sample, which is the site where oxidation takes place. Migration process from active film to food supplies longer protection for the food. (Bolumar et al. 2011)

2.7   **Methods of measuring antioxidant activity**

2.7.1   **DPPH antioxidant assay**

1,1-dipheny-2-picrylhydrazyl (DPPH) is a radical with a dark violet color. It has been used first for the purpose of assessing antioxidant activity of compounds by Brand Williams et al. (1995). DPPH violet color turns yellow when reduced with the help of a reducing agent/antioxidant (Figure 10.)

Generally antioxidant activity against DPPH is evaluated either by electron spin resonance (ESR) or by detecting the decrease in absorbance by a spectrophotometer at the wavelength of 517 nm (515 – 528). (Karadag et al. 2009)

Figure 10. DPPH radical color change from violet to yellow color.

EC_{50} is defined as the concentration of the antioxidant compound required to decrease the concentration of DPPH by 50%. The value of EC_{50} indicates the strength of antioxidant

compound. According to Sanchez-Moreno et al., (1997) the time required to reach the steady state with EC_{50} is called TEC_{50}. Based on the value of TEC_{50}, antioxidants are classified into 3 categories. The first includes rapid antioxidants, with a TEC50 of less than 5 minutes. The second includes intermediate antioxidants with a TEC_{50} less than 30 minutes, and the third includes slow antioxidants, with a TEC_{50}, higher than 30 minutes.(Sánchez-Moreno et al. 1998, Karadag et al. 2009)

Using EC_{50} to express antioxidant efficiency is not always accurate because the reaction between the antioxidant and DPPH concentration is not necessarily linear (Karadag et al. 2009)

Sánchez-Moreno et al. (1998) introduced the term of "antiradical efficiency" (AE) to express the strength of the antioxidant molecule in quenching free radicals.

$$AE=(1/EC_{50})$$

The term AE in more descriptive than EC_{50} because it takes into account reaction time besides antioxidant capacity. (Sánchez-Moreno et al. 1998, Karadag et al. 2009)

Another term introduced by De beer et al. (2003) is called "radical scavenging efficiency" (RSE). This term expresses the radical scavenging capacity and the initial scavenging rate. It is calculated as the ratio between the initial rate of radical scavenging and the EC_{50} value.(de Beer et al. 2005, Karadag et al. 2009)

Karadag et al. (2009) demonstrated that the most important advantage of the DPPH assay is the simplicity and speed of application. Instrumentation wise, it only requires a UV-Vis spectrophotometer. On the other hand, Magalhães et al. (2008) have explained that DPPH

assay has its own drawbacks and limitations. DPPH is soluble in organic solvents only, insoluble in aqueous media. This rather limits the application of the DPPH test in assessing antioxidant activity of hydrophilic compounds. Also, the impurities in solvent either acidic or basic can affect the ionization equilibrium of phenols and may result in changing the spectrometric absorbance.(MacDonald-Wicks et al. 2006, Karadag et al. 2009)

Another limitation is the sensitivity of DPPH to light and temperature, as the absorbance changes when subjected to light. This may result in some interference with the obtained results from the assay. Another issue that limits the DPPH assay is its tendency to coagulate, which may decrease the DPPH availability to quench free radicals if not adequately stirred to avoid coagulation, hence affecting the reading of the assay (Magalhães et al. 2008, Karadag et al. 2009). Also, the absorbance of DPPH is affected by pH and solvent type. Such that; a hydrogen ion concentration increase (pH decrease) can inhibit DPPH radical quenching by the antioxidants, which causes acidic samples to show less or no antioxidant activity against DPPH. On the contrary, basic conditions are believed to boost the process of quenching DPPH radicals, and accordingly give a higher antioxidant activity reading than actual.(Onwulata 2014)

Another drawback of DPPH is that certain antioxidants that react rapidly with peroxyl radicals, do not do so with DPPH, while some other antioxidants are inert towards DPPH despite being active towards peroxyl radicals. Furthermore, the tested antioxidant may have a component whose spectrum overlaps with DPPH absorption at 515 to 528 nm like carotenoids. (Karadag et al. 2009). Reaction of DPPH with eugenol was reported to be a reversible reaction, as a result, the obtained readings cannot be reliable if the antioxidant

contains eugenol. It will show lower antioxidant activity than the true value.(Karadag et al. 2009)

2.7.2 TBA test

Monitoring of lipid oxidation is important to food manufacturers in order to assess the oxidative deterioration of food, which is one of the major chemical causes of food spoilage.

It is practically difficult to measure reactive oxygen species because they have short life times, however it is possible to measure the products from damage caused by oxidation like TBA. (Pryor 1991)

Malondialdehyde (MDA) is a byproduct formed during the process of secondary fats degradation through oxidation. TBA assay quantifies the MDA in the target sample, through which the amount of oxidized fat is assessed indirectly.

Thiobarbituric acid (TBA) test is based mainly on spectrophotometric quantification of pink colored compound that is produced by the reaction of MDA with TBA molecules (Figure 11). (Botsoglou et al. 1994)

2.7.3 FRAP test

The ferric reducing antioxidant power (FRAP) antioxidant activity evaluation testing method is mainly based on the reduction of Fe^{+3} complex of tripyridyltriazine $Fe(TPTZ)^{+3}$ into dark blue colored Fe^{+2} complex of $Fe(TPTZ)^{+2}$ by using the tested antioxidant at an acidic pH. The absorbance of the produced solution is measured at 593 nm, and the results are expressed in terms of micromolar Fe^{+2} equivalents. This method is claimed to be a fast and easy method to measure antioxidant activity manually or

automatically. (Antolovich et al. 2002).
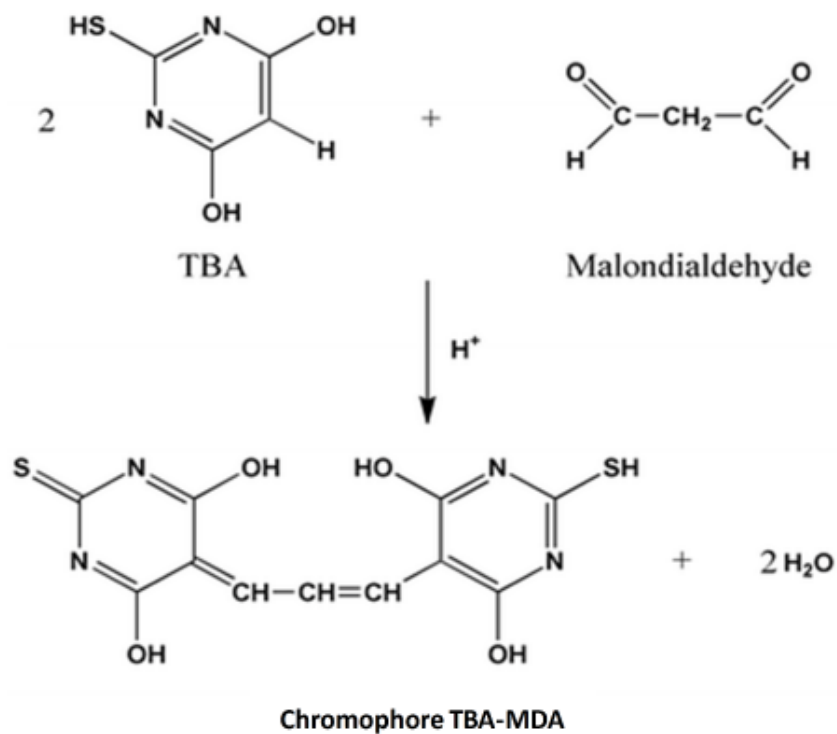
Figure 11. Reaction between TBA & MDA to form the TBA-MDA adduct with pink color.

2.7.4   β-Carotine bleaching test

This method depends mainly on an aqueous emulsion of linoleic acid and β-carotene. The color of β-carotene is discharged by the effect of free radicals generated during the process of fatty acids oxidation at a temperature of 50 ^{o}C. The absorbance is then measured at 470 nm after adding the antioxidant material under test in different concentrations.

This method has some drawbacks that affect the precision of results like the sensitivity of β-carotene towards oxygen and temperature, the complexity of the used reagent, the non-

specific conditions of temperature that can impact the results, the use of a previously set reaction time in different reactions, the uncontrolled conditions that can affect the results like pH value and solvents ratio and presence of metals in the reaction medium. (Prieto et al. 2012)

2.8 **Legislative aspects**

Synthetic antioxidant preservatives are heavily used as antioxidants in the food and plastics industry. In the second, they serve as antioxidants that prevent oxidative degradation of plastics during extrusion process. These compounds are generally recognized as safe for human consumption as long as they don't exceed the allowable limits of ingestion. However, some researchers have suggested that certain chemical antioxidants like BHA and BHT are potential carcinogens after causing cancer to rodents. This has put these chemicals in controversy despite their undeniable functional efficiency as antioxidants (Gonçalves et al. 2013, Teixeira et al. 2013, Samsudin et al. 2014).

The usage of synthetic antioxidant preservatives is currently allowed in some types of foods with a specific limit of the applied quantities. Antioxidant food additives are controlled by many organizations all over the globe, including European Union regulations (Directive 95/2/EC, 1995; EC Regulation No 1333/ 2008, 2008), FDA in USA (FDA, 2001a, 2001b) and Codex Alimentarius (CGSFA, 1995). (André et al. 2010)

The joint FAO/WHO expert committee on food additives (JEFCA) has set limits to these chemicals according to their toxicity. Acceptable Daily Intake (ADI) value was set for synthetic antioxidants. Consumption below these amounts should be safe to human health.

According to the directive (JECFA, 2003), the ADI value of OG, DG, BHA and TBHQ is 0-1.4 mg/kg (mg of additive / kg of body weight). Scientific committee from the (SCF) has set the ADI value to be 0.5 mg/kg. BHT particularly had a lower ADI of 0-0.3 mg/kg. Based on the above, there is a tendency for using natural antioxidants instead of synthetic ones primarily in food stuffs. A typical example of such substances are essential oils which have proven to have antioxidant, antimicrobial, antiviral, antinociceptive and antiphlogistic as well as anticancer properties (André et al. 2010).

According to the FDA Code of Federal Regulations (21CFR182), essential oils are listed among the food additives that are generally recognized as safe (GRAS). Essential oils are safe as long as they are handled as food ingredients applying good manufacturing practices in processing and storage. In particular, thyme, oregano and rosemary are listed as safe food additives. Codex Alimentarius and FAO have also listed essential oils as natural flavoring complexes.

3. Materials and Methods

3.1 Materials

Essential oils used originated from Egypt and Greece. Thyme and Rosemary essential oils extracted from *Thymus vulgaris* and *Rosmarinus officinalis* herbs were donated by "Fridal" and "IFF Egypt" companies, respectively. Oregano essential oil was donated by Eth. Oil company in Greece. It was extracted from *Oreganum vulgare* herb. All oils were extracted by steam distillation of partially air dried herbs.

Polylactic acid (PLA), 2002 D, was purchased in pellet form from Natureworks® Co., Minnetonka (USA).

1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma Aldrich. TBA, BHT, and silicon oil were purchased from Merck-Schuchardet. All used solvents were pro-analysis grade.

Oncorhynchus mykiss fresh fish (rainbow trout) were purchased from Ioannina fish market. Originated from a fish farm on Louros river, having been fished less than 6 hours before purchasing. The fish were transferred to the lab in flaked ice. Fish average weight after removing head and guts was 250 g ± 25 g.

3.2 Film preparation

Ten g of PLA pellets were poured in 100 ml of chloroform in a 250 ml glass beaker, followed by adding 1 ml of tween 80 as an emulsifier and 1 g of essential oil. Concentration of essential oil in PLA was 10% w/v. The beaker was covered by aluminum foil, followed by a layer of para-film to avoid solvent evaporation. The mixture was stirred for 16 hours on a magnetic stirring plate (Arex magnetic stirrer) until

complete dissolution was achieved and the solution had a viscous liquid texture.

As shown in Figure 12, the solution was then poured into glass mold of internal dimensions (40 cm X 20 cm). The glass mold was perfectly leveled on the bench to ensure even distribution of the solution. PLA solution was then left to dry for another 16-20 hours then peeled at room temperature under the fume hood.

Eight films were prepared for every experiment, including 2 control films (straight PLA without adding essential oils), 2 thyme active films, 2 rosemary active films and 2 oregano active films. All the tests were conducted in duplicate on fresh films within 24 hours from film peeling. The experiment was replicated twice (n = 2 X 2). A scheme for the process of film preparation by the casting method is shown in Figure 13.
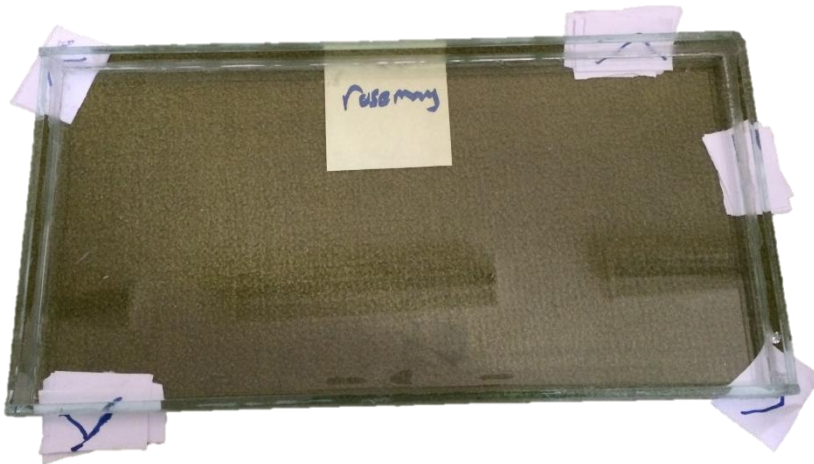
Figure 12. Homogenized solution of PLA, chloroform, tween 80 and rosemary essential oil poured in a leveled glass cast.

1. **Add 10 grams of PLA, 1 g essential oil & 1 ml tween 80 to 100 ml of chloroform**

10 g PLA 1 ml tween 80	10 g PLA 1 g Thyme 1 ml tween 80	10 g PLA 1g Rosemary 1 ml tween 80	10 g PLA 1 g Oregano 1 ml tween 80

2. **Put on magnetic stirrer for 16 h**

No EO   Thyme   Rosemary   Oregano

3. **Pour into glass casts and leave to dry for 16-20 h**

4. **After drying, peel the film off the cast.**

Peeling process of PLA film

Transparent PLA film on white paper

Figure 13. A scheme of PLA film preparation procedures

3.3 Determination of antioxidant activity of pure essential oils and films using the DPPH test

Antioxidant activity was measured by using the method of Brand-Williams et al. (1995) with some modifications. A DPPH solution was prepared by dissolving 0.0040 g of DPPH in 100 ml methanol in a volumetric flask. The flask was wrapped in aluminum foil, to protect DPPH from light effect. The flask was then stirred on a vortex for 10 minutes to ensure DPPH complete dissolution. The obtained solution had a deep violet color. The solution was left in fridge for 2 hours before starting the test in order for its absorbance to stabilize.

All absorbance measurements were performed using a "Perkin Elmer lambda 25" UV-Vis spectrophotometer. A calibration curve for DPPH absorption at 517nm was constructed. DPPH solutions with concentrations ranging from 0 to 40 ppm were used to construct the calibration curve of absorption versus concentration.

The antioxidant activity of pure essential oils was assessed through adding 0.1 ml of essential oil solutions in methanol with different concentrations from 500 ppm to 10000 ppm to 3 ml of DPPH solution in spectrophotometric cuvettes. The cuvettes were placed in a light impermeable box and stored in the dark.

The essential oils quench the free radicals of DPPH causing the dark violet color of DPPH to fade away and change to yellowish color with time, causing change in absorbance of DPPH after adding the essential oils. Every 1 hour, the absorbance was measured using the spectrophotometer. The absorbance decreased until the reading reached a plateau. The obtained absorption values at the plateau are called "A_t".

Antioxidant activity of film extract was assessed by using the same test. Films containing 10% w/w essential oil were cut into small pieces (0.5 cm X 0.5 cm) and immersed in 50 ml methanol and left on a shaker for 48 hours for extraction of essential oil. The resulting solution was filtered through Whatman filter (No. 2) to remove impurities.

5 concentrations of every film extract were prepared in methanol as a solvent (1000 ppm, 2000 ppm, 5000 ppm, 10000 ppm and 20000 ppm). 0.1 ml of every concentration was added to 3 ml of DPPH and went through the previously mentioned DPPH test procedures to obtain "A_t" values.

Results were expressed in terms of % decrease of DPPH absorbance and concentration calculated by the following equations (Brand-Williams et al. 1995), (Anagnostopoulou et al. 2006)

% reduction in DPPH absorbance $= (\dfrac{A_0 - A_t}{A_0})$ X 100 $\boxed{\text{Equation 1.}}$

% reduction in DPPH absorbance is also called in literature "free radical scavenging activity" (FRSA) or "radical scavenging activity" (RSA).

Where A_0 is the absorbance of DPPH before adding the antioxidant agent, while A_t is the absorbance at Plateau.

Concentration of DPPH is calculated from the equation of DPPH calibration curve plotted between absorbance on the (Y)-axis and DPPH concentration on the (X)-axis.

% Reduction in DPPH concentration $= (\dfrac{C_0 - C_t}{C_0})$ X 100 $\boxed{\text{Equation 2.}}$

Where C_0 is the concentration of DPPH before adding antioxidant agent, while C_t is the concentration of DPPH at plateau.

% DPPH Remaining = 100% - % reduction in DPPH concentration

$$\boxed{\text{Equation 3.}}$$

Final results were expressed by the following terms:

EC_{50} is the concentration of sample needed to achieve 50% decrease of DPPH concentration. The value is estimated from the graph constructed by plotting concentration of sample versus % reduction of DPPH concentration.

Antiradical efficiency (AE) = $\dfrac{1}{EC_{50}}$ $\boxed{\text{Equation 4.}}$

Calculation procedures start with obtaining A_t from the absorbance of spectrophotometer after reaching plateau. Radical scavenging activity (RSA) is then calculated by equation 1. Then % reduction in DPPH concentration is calculated by equation 2. Then the DPPH remaining is calculated by equation 3.

A graph is plotted between Essential oil or film extract concentration on X-axis and the % DPPH remaining on Y-axis. EC_{50} is obtained from the equation of the graph by substituting the %DPPH remaining by 50%. Then AE is obtained by reciprocating the EC_{50} value.

3.4 Determination of antioxidant activity using the Thiobarbituric acid (TBA) test.

3.4.1 Film preparation for TBA test

PLA films containing 10% w/w thyme, rosemary or oregano were prepared by the previously mentioned procedures. Every film was cut into small squares with dimensions (10 cm X 10 cm) and used to sandwich the minced fish samples.

3.4.2 **Fish samples preparation**

Fish fillets were prepared from fish after removing head, viscera, skin and bones. Fish fillets were minced by an electric household mincer for 3 successive times to ensure the sample homogeneity. Figure 14 shows the de-headed fish before extracting fish fillets.

The minced meat in the form of burger Figure 15. was sandwiched between 2 films (with dimensions 10 cm X 10 cm) as shown in Figure 16. The packaged fish was stored inside a low density polyethylene (LDPE) sealed bag Figure 17, under refrigeration temperature.

Figure 14. Trout (Oncorhynchus mykiss) fish during sample preparation process (after removing head, and viscera)

3.4.3 **Thiobarbituric acid (TBA) test.**

The test was performed based on the method of (Tarladgis et al., 1960) over a period of 8 days. Sampling was carried out at days 0, 4, 6 and 8. The procedure was as follows:

97.5 ml of distilled water was placed into a 500 ml round bottom flask. 10 grams of minced fish meat were added, followed by 2.5 ml of HCl 4N. 1.5 ml of BHT solution in ethanol (1000 ppm) was added to the mixture to prevent sample auto-oxidation.

Figure 15. Fish meat after mincing

Figure 16. Minced fish sandwiched between 2 layers of PLA
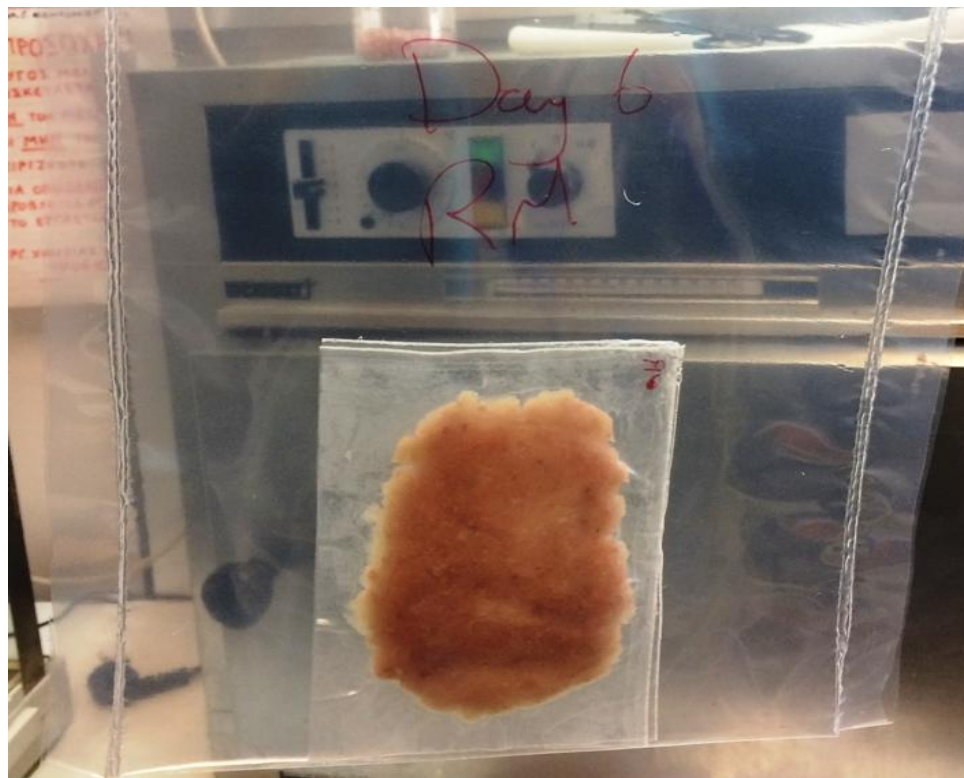
Figure 17. Minced fish meat sample, sandwiched between two PLA layers, and stored in LDPE heat-sealed bag.

Ten drops of silicon oil were added as an antifoaming agent. 3 glass beads were added to the mixture. The mixture was then distilled for about 20 minutes until 50 ml of distillate were collected. Five ml of the distillate were pipetted in a vial containing 5 ml of 0.021 M aqueous solution of 2-thiobarbituric acid (TBA), and 0.6 ml of BHT. The sealed vial was placed in a water bath at 90 $^\circ$C for 40 minutes in order to develop TBA-MDA colored complex. The vial was cooled by water until reaching room temperature. The resulting solution was pinkish in color. The solution was filtered by "Millex syringe driven filters". The absorbance of the filtered solution from every sample was measured using spectrophotometer at 532 nm.

A blank sample was prepared by adding 5 ml distilled water to 0.6 ml BHT, and 5 ml TBA reagents.

Results were expressed in terms of mg MDA/Kg of minced fish meat, and calculated by Equation 5 (Tarladgis et al. 1964)

mg MDA/kg = Absorbance X 7.8 | Equation 5. |

3.4.4 Microbiological spoilage test

This test was conducted to identify the time when the minced fish is microbiologically spoiled so that TBA readings would be discontinued. The test was conducted by the following methodology on day 4, 6, and 8.

A sample of 10 gm of minced fish was placed in a stomacher bag (Seward Medical, UK) and homogenized for 60 sec with 90 ml of sterile buffered peptone water (BPW) solution of concentration 0.1% using a lab blender 400.

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For enumeration, serial dilutions of homogenate were prepared in BPW (0.1 g/ 100 ml) and 0.1 ml aliquots were spread on the surfaces of agar plates. Total viable count (TVC) was then determined on plate count agar (PCA, Merck code 1.05463 Darmstadt, Germany) after incubation for 48 hours at 30°C.

3.5   Mechanical testing

3.5.1   Film thickness

Film thickness was measured by a portable digital micrometer (IS 13109 INSIZE CO., LTD, Japan.). Films were measured at 8 different points of their surface and the average reading was recorded.

3.5.2   Tensile strength, percent elongation at break and Young's modulus

Tensile strength indicates the capability of the film to be stretched. Tensile strength or more accurately, ultimate tensile strength is defined as "The maximum strength that the material can sustain and is taken to be the maximum load exerted on the test specimen during the test divided by the cross sectional area of the specimen".

% Elongation is measured as the percentage of elongation of the film between the machine grips at the point of break. It indicates the ability of the film to elongate before breaking. The higher the value of % elongation, the higher the amount of energy absorbed by the film before it breaks.

Tensile strength tests were conducted using an Instron model 4411 dynamometer (Instron Engineering Corp., Canton, MA) (Figure 18.).

Figure 18. Instron (4411) dynamometer used for mechanical properties measurement (Instron Engineering Corp., Canton, MA)

Tests were carried out according to the American Society for Testing and Material (ASTM) method D882. Samples were prepared from films in the form of rectangles of

dimensions of (1 cm X 10 cm). Tests were conducted at temperature of 25 $^\circ$C, with a crosshead speed of 50 mm/min.

Stress (σ) is defined as the force (F) applied by the machine per unit cross sectional area (A) of the film under test (Equation. 6). The stress is measured in N/m^2 (Figure 19).

$$\text{Stress (Tensile strength) } (\sigma) = \frac{\text{Loading force (N)}}{\text{Cross sectional area (m}^2)}$$

Equation 6.

Strain (Equation 7) is the fractional change in the length of the film under test. It is measured as ($\Delta L/L_0$). Strain is dimensionless.
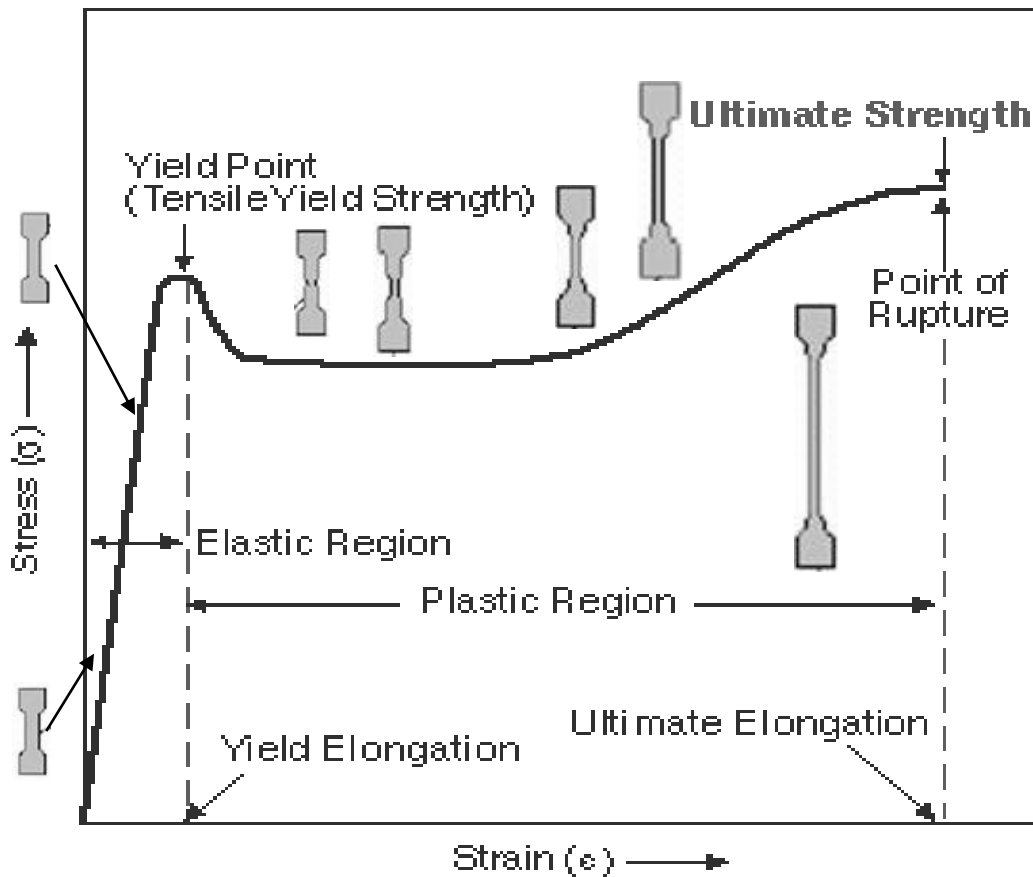
Figure 19. Typical stress strain curve for thermoplastic polymer showing the strain effect on PLA (Onwulata 2014)

$$\text{Strain (\% Elongation) } (\varepsilon) = \frac{L\text{-}L_0}{L_0} \text{ X } 100$$

Equation 7.

L_0 = Original sample length, L= Sample length after extension (by applying stress).

Young's modulus provides an indication of the stiffness of the film material, and it is defined as the ratio of stress to the strain within the elastic region (Equation 8).

$$\text{Young's modulus} = \frac{\text{Stress}}{\text{Strain}}$$

Equation 8.

3.6   Oxygen transmission rate

Oxygen Transmission rate was determined by method ASTM D3985. The test was conducted using the Mocon Oxtran 2/20 permeability tester (Mocon, Inc.). Temperature was set to 23 ^{o}C. The instrument was set to the convergence mode, in which readings are taken until two successive readings have a difference of less than 5%. Test was duplicated twice (n = 2 X 2).

The used test method is called "isostatic method" because pressure and flow rates of gases in both chambers around the film are equal. This method is also called "steady state method" because the concentration gradient of oxygen across the film in both chambers is always constant. The steady state method depends on measuring trace amounts of oxygen in a stream of an oxygen-free carrier gas. This method requires a very sensitive oxygen sensor (Coulometric oxygen sensor) to be able to deliver a clear signal with minimal noise (better signal to noise ratio). The operation principle of the gas permeability apparatus is shown in (Figure 20).

The measurement of oxygen transmission rate by the isostatic method is carried out using

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a cell divided by the film into two chambers. One of the chambers is filled with a flow of pure oxygen while the other chamber is filled with a flow of oxygen free carrier gas (nitrogen).

Oxygen starts to permeate through the film from the oxygen rich gas stream in the first chamber into the oxygen deficient gas stream in the second chamber. The gas flows from the second chamber to a coulometric oxygen sensor, where the oxygen concentration in the carrier gas is measured.
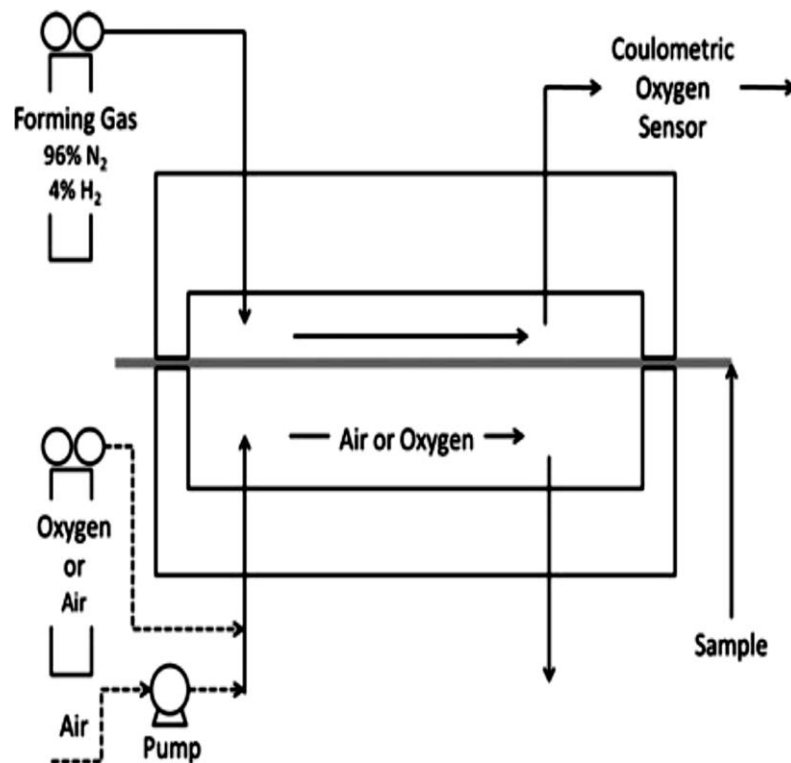
Figure 20. Steady-state, Isostatic method for oxygen transmission rate measurement using the Moccon Oxtran 2/20 permeability tester (Abdellatief and Welt 2013)

3.7   **Migration testing**

Migration testing was carried out with two different food simulants in two different experiments according to Regulation EU No. 10/2011 (Commission Regulation EU 10/2011). Each experiment was duplicated twice (n = 2X2). A film of dimensions (10 cm X 10 cm) was weighed on an analytical balance and then submerged in a sealed glass container containing the food simulant at a ratio of film weight to simulant volume 1:10 (e.g. if the film weight is 1.5 grams, the film is submerged in 15 ml food simulant)

Distilled water was used in the one experiment to simulate aqueous foods. Test conditions were: 10 days at a temperature of 40 ^{o}C. For the other experiment, isooctane was used as a simulant for fatty foods. Test conditions were: 2 days at 20 ^{o}C. After the prescribed t / T contact conditions, the food simulant was transferred to a pre-weighed round bottom flask and the solvent evaporated in a roto-evaporator (Buchi water bath B-480). Then the round-bottom flask was re-weighed to calculate the amount of migrants transferred from the film to food simulant.

4. Results and Discussion

4.1 PLA film properties

The prepared films were colorless, except that with oregano essential oil which had a slightly yellowish color owing to the relatively darker color of oregano essential oil. Films were homogenous in terms of color and thickness with an Average film thickness of 117 μm. PLA films prepared by the solvent casting technique by Rhim et al. (2006) were described to have a similar transparent color.

Each film had the odor of the essential oil used for its preparation, which is an indication of the retention of a large portion of the essential oil within the film matrix after the drying step.

4.2 Determination of antioxidant activity of pure essential oils using the DPPH test

The results of the present study showed that pure essential oils have a relatively high antiradical efficiency (AE), which was similar for all three essential oils tested; indicating the high antioxidant capacity towards the DPPH radical. Oregano essential oil showed the highest radical scavenging activity (% RSA) as well as AE. The spectrophotometric readings of the DPPH test for pure essential oils are shown in Table 4.

Furthermore it was shown that the radical scavenging activity of all essential oils increased proportionally with increasing concentration of essential oil in the tested solution.

TABLE 4

Results obtained in the present study regarding RSA, EC$_{50}$, and AE of the three essential oils tested are in reasonable agreement to those of essential oils from different botanical and geographical origin (Saleh et al. 2010), (Viuda-Martos et al. 2010) (Kulisic et al. 2004) Figures 21, 22 and 23 show the effect of different concentrations of pure essential oils of thyme, rosemary and oregano, respectively on the % RSA as well as the % remaining DPPH (% of unquenched DPPH radicals). It is shown that % DPPH remaining decreases with an increase in essential oil concentration. On the contrary, as essential oil concentration increases, the % RSA increases.
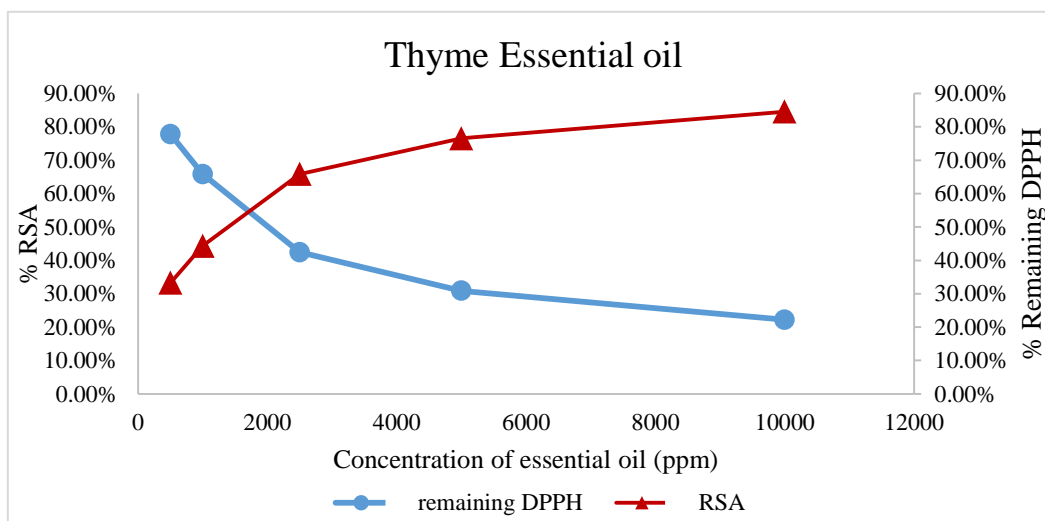
Figure 21. % DPPH remaining & % RSA versus concentration of Thyme Essential Oil (TEO)

Saleh et al. (2010) studied 248 essential oils from different sources, including thyme and oregano from Morocco, Spain, Germany and Turkey. Thyme and oregano were found to have an RSA value higher than 90% at a concentration of 25000 ppm. On the other hand, in the present study, RSA of thyme and oregano were 84.5% and 87.7% respectively at 10,000 ppm. The reduction of the RSA is believed to be the result of the lower concentration of essential oils used in the present study (10000 ppm vs. 25000 ppm).
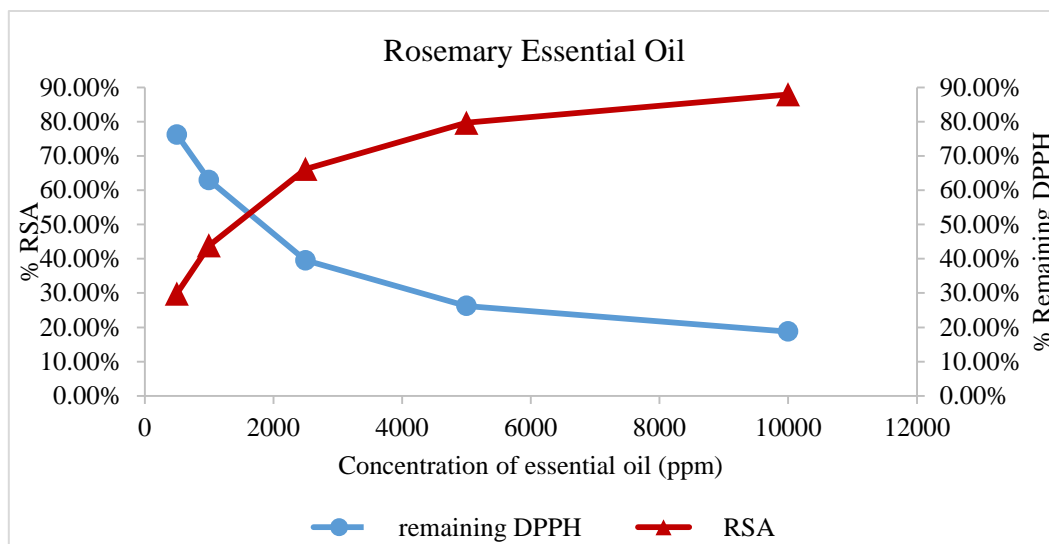
Figure 22. % DPPH remaining & % RSA versus concentration of Rosemary Essential Oil.
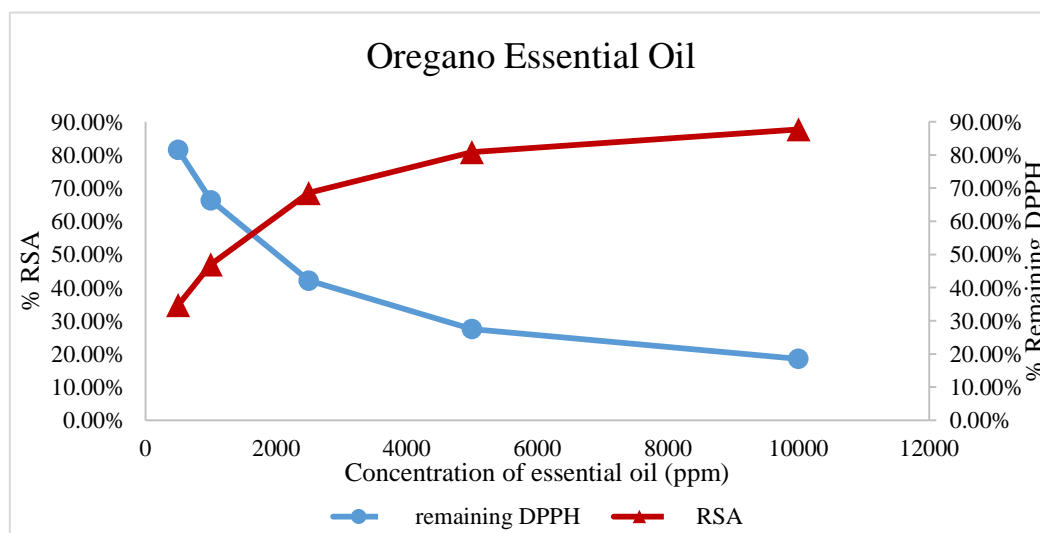
Figure 23. % DPPH remaining & % RSA versus concentration of Oregano Essential Oil

Viuda-Martos et al. (2010) evaluated antioxidant activity of essential oils from several species from Mediterranean countries by using the DPPH method. Thyme, rosemary and oregano were studied. At 10000 ppm concentration, The RSA value were 81.3%,

48.35%, 64.85% respectively. The obtained values are lower than those of the present study by 4.0% for thyme, 44.8% for rosemary and 26.0 % for oregano.

Kulisic et al. (2004) studied the antioxidant properties of oregano essential oil and linked them to its chemical composition. They tested its antioxidant capacity by three methods including the DPPH method. Antioxidant activity of oregano essential oil was found to be lower than ascorbic acid but close to that of α-tocopherol (natural antioxidant) and BHT (chemical antioxidant). It was noted that the antioxidant activity of oregano essential oil is directly proportional to the concentration of essential oil used.

The % RSA obtained by the DPPH method for concentrations 500 and 1000 ppm were 53% and 65% respectively. The obtained results in the present study are relatively lower than those obtained in Kulisic et al. (2004) study by about 34.4% and 27.6% for concentrations 500 and 1000 ppm respectively. Many factors could be responsible for the variation in the RSA% of rosemary and oregano essential oils between the two studies including the part of the plant used for essential oil extraction, the exact geographical origin and soil conditions as well as the harvesting season. All the mentioned factors can actively participate in changing the chemical composition of essential oil and its phenolic content.

4.3 Determination of film extract antioxidant activity using the DPPH test

The DPPH test was performed on the extracts of PLA films containing essential oils. The results are shown in Table 5.

TABLE 5

At a concentration of 10000 ppm, thyme film extract resulted in 79.4% RSA versus 84.6% resulting by direct application of thyme essential oil. The AE of thyme essential oil was 2.8, while that of thyme film extract was 2.0 (28.6% decrease in AE). Regarding rosemary essential oil, the % RSA and AE values were 87.9% and 3 respectively. While rosemary film extract showed a % RSA of 84.3% and AE of 2.4 (20% decrease in AE). Oregano essential oil showed a % RSA of 87.7% and AE of 3.2, while oregano film extract had a % RSA and AE of 83.3% and 2.8 (12.5% decrease in AE) respectively.

These results indicate the loss of a portion of the antioxidant activity of essential oils when incorporated into PLA films by the solvent casting technique (Figures 24 and 25). The results showed that oregano film suffered the lowest loss of antiradical efficiency (i.e. the highest antioxidant activity) among the three types of active films (Figure 25). The loss of antioxidant activity may be attributed to the trapping of a portion of essential oils inside the film matrix. It may be also affected by the evaporation of a portion of the incorporated essential oil during the drying stage of film preparation by the solvent casting method.

Figures 26, 27 and 28 show the antioxidant activity of different dilutions of PLA active film extracts on the % RSA as well as the % remaining DPPH (% of unquenched DPPH radicals). The figures show a similar pattern to that of pure essential oils. As the concentration of film extracts increases, the % remaining DPPH decreases and % RSA increases.
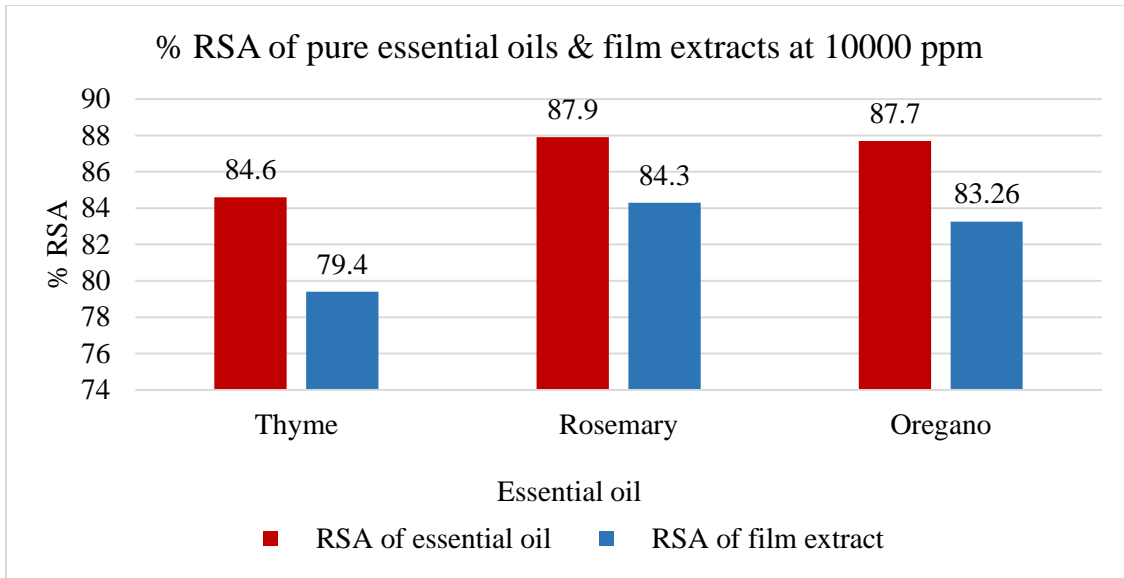
Figure 24. Comparison between the % RSA caused by direct application of tested essential oils versus the % RSA caused by the extracts of active PLA films containing the same essential oils at the same concentration.
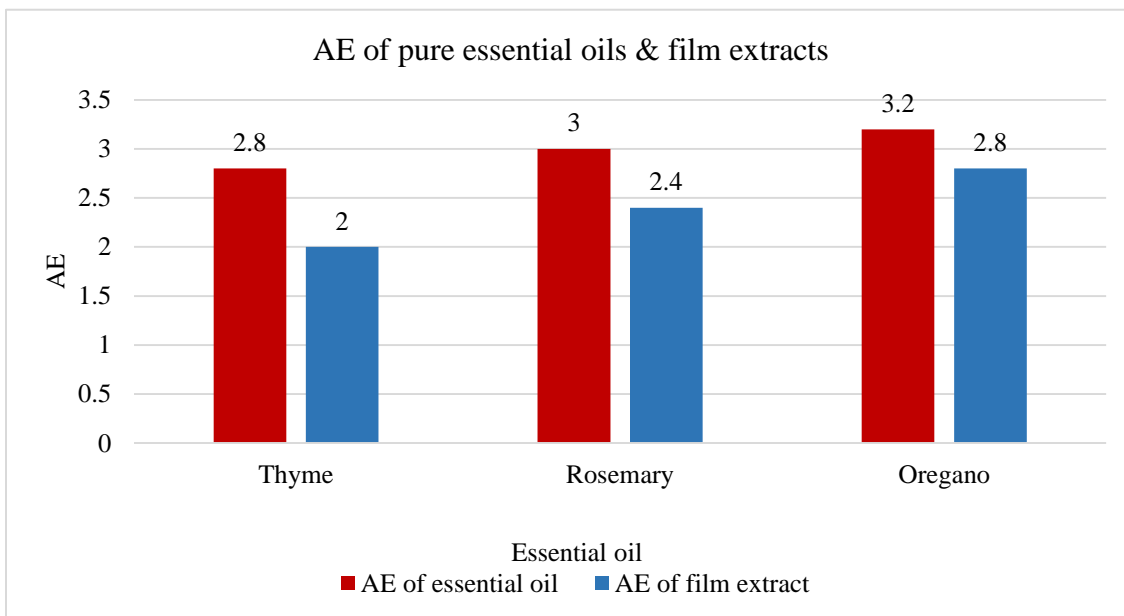
Figure 25. Comparison between the AE caused by direct application of tested essential oils versus the AE caused by the extracts of active PLA films containing the same essential oils
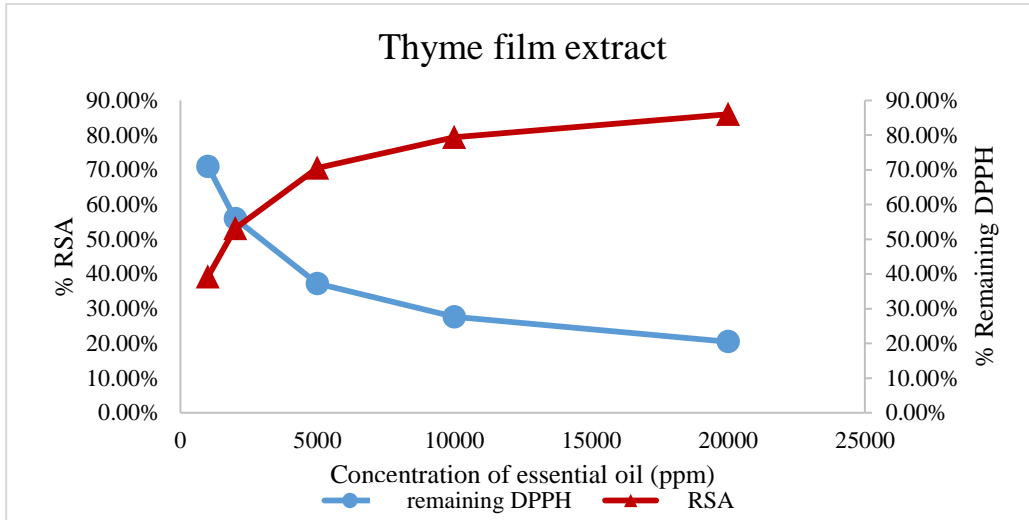
Figure 26. % DPPH remaining versus concentration of Thyme film extract
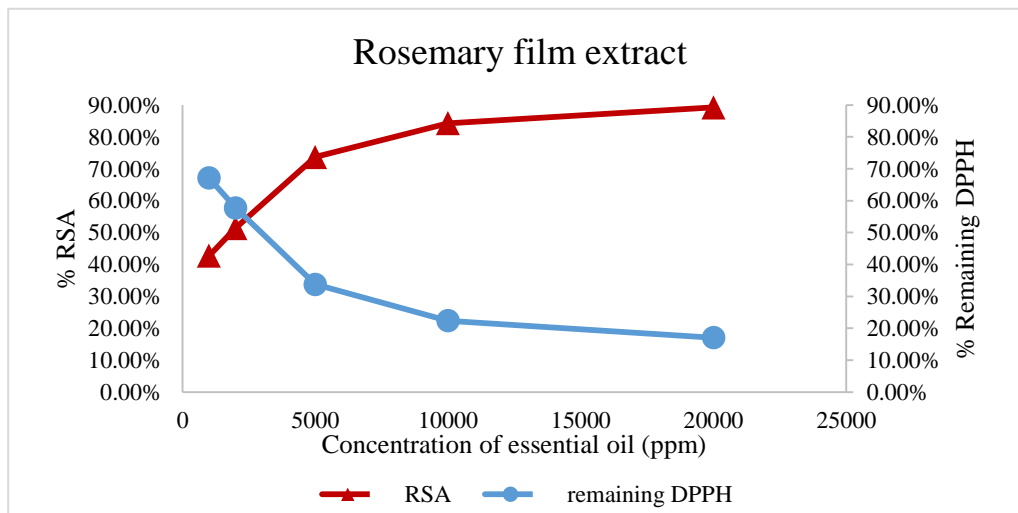
Figure 27. % DPPH remaining versus concentration of rosemary film extract
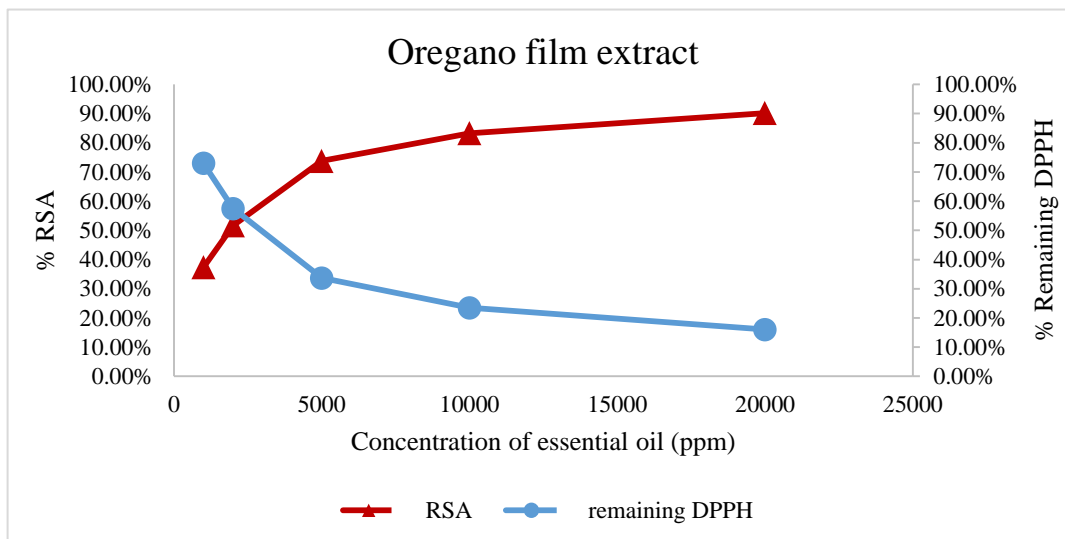
Figure 28. % DPPH remaining versus concentration of oregano film extract.

The obtained results are in line with literature results of similar studies where essential oils were incorporated into biodegradable films. Active packaging films produced by Jouki et al. (2014) incorporating thyme essential oil into quince seed mucilage film. Results showed an increase in antioxidant activity in a direct proportion to the increase of essential oil concentration in the packaging film. The radical scavenging activity of the prepared quince seed mucilage films without thyme addition was 18.39 %. After adding thyme essential oil at concentrations of 1% and 2%, the radical scavenging activity was 30.11%, and 43.14% respectively. In the present study at the concentration of 1000 ppm and 2000 ppm the % RSA induced by thyme essential oil containing film was 39.2% and 53.1%, respectively. This may be attributed to the higher diffusivity of PLA than quince seed mucilage films that allows more essential oil to migrate to the food contacting phase. Also it may be attributed to the nature of the specific essential oils used.

Jamshidian et al. (2013) determined the percentage of reduction in antioxidant activity of a series of antioxidant compounds when incorporated in PLA. This was reported to be less than 10% for α-tocopherol (AT), butylated hydroxyanisol (BHA), Propyl Gallate (PG) and Tertbutylhydroquinone (TBHQ) reaching 13.9% for BHT. The highest antioxidant activity loss was reported for AP is equal to 70.5%. These results show that the lower the volatility of the antioxidant compound, the lower the loss in antioxidant activity.

In the present study, the antioxidant activity loss of essential oil when added to PLA films at 10000 ppm concentration was 6% for thyme EO, 4.1% for rosemary EO, and 5.1% for Oregano essential oil. Obtained results are in line with the values of antioxidant loss of AT, BHA, PG and TBHQ reported in the above study.

Ramos et al. (2014b) worked on developing PLA films containing thymol as an active substance and montmorillonite (D43B) at 2 different concentrations. Antioxidant activity of the generated films was assessed by the DPPH method on adding thymol (8% wt/wt), and D43B at concentrations of 2.5% and 5% wt/wt, respectively.

The thymol containing film had a value of RSA of 71.1%. This value increased significantly on adding D43B at 2.5% concentration reaching 84.3%. The value did not further increase by increasing the concentration of D43B to 5%. The previously mentioned study used pure phenolic compound (thyme) which is one of the active ingredients in thyme essential oil which was used in our work.

Byun et al. (2010) developed a PLA antioxidant film by cast extrusion incorporating α-

tocopherol, BHT and polyethylene glycol 400 (PEG 400) as a plasticizer. Antioxidant activity was quantified by the DPPH method. BHT was added at a concentration of 0.1 g/ kg PLA, while PEG 400 was added at a concentration of 100 g/kg PLA. The generated film achieved a 90% RSA. Similar results were achieved in the present study by adding 20000 ppm of oregano essential oil. Adding thyme and rosemary essential oil at the same concentration resulted in an antioxidant activity of 86% and 89.2% respectively.

4.4   **Thiobarbituric acid test (TBA)**

The values of MDA in minced fish (Table 6) showed a measurable antioxidant activity for the PLA films containing essential oils as compared to the control film. More specifically, thyme showed a 5% decrease in degree of oxidation of minced fish muscle on day 4. On day 6 the respective decrease in the degree of oxidation was 10.8%. Finally, on day 8, the respective decrease in oxidation was 31.3 %. It should be noticed that the shelf life of minced fish meat doesn't normally exceed 5 to 6 days, thus a more realistic value for reduction in degree of oxidation would be between 10% and 11%.

Regarding Rosemary there was a 20.3% decrease in degree of oxidation on day 4. Respective values for days 6 and 8 were 24.2% and 11.5% respectively.

Finally regarding Oregano, there was a 47.9% decrease in degree of oxidation on day 4, while respective values for day 6 and 8 were 19.3% and 24.6%

For all the three above mentioned essential oils/films tested, there was a decrease between 5.1% and 47.9 % in degree of oxidation on day 4 of storage. Overall results show that films containing essential oils are able to extend the shelf life of minced fish with regard to the degree of oxidation.

Camo et al. (2008) packaged fresh lamb steaks in three different active films. The first sample was packaged in a rosemary active film (4% wt/wt), the second was packaged in an oregano active film (4% wt/wt) and the third one was sprayed with rosemary extract. TBA test was conducted to assess the antioxidant activity of each treatment.

On day 8, the increase in the value of MDA of meat in the control film was 2.7 (mg MDA/ kg). Rosemary active film, Oregano active film and direct application of rosemary essential oil showed a lower increase in amount of MDA/ kg with values of 2, 0.7 and 0.6 respectively. Direct application of rosemary essential oil gave the lowest mg MDA/ kg value. The percentage of inhibition of MDA formation was 25.9%, 74% and 77.7% for rosemary, oregano and direct application of rosemary extract respectively. In the present study, the percent reduction in amount of MDA on day 8 was 31.3%, 11.6% and 24.7% for thyme, rosemary and oregano active films respectively.

Table 6. Average absorbance values of distillates of packaged fish with 4 different films, and the corresponding average content of MDA in (mg MDA/kg fish).

(A) Control film			(B) Thyme active film		
Day	Av. Abs ± SD	mg MDA /kg fish	Day	Av. Abs	mg MDA /kg
0	0.063 ± 0.003	0.49 ± 0.02	0	0.063 ± 0.003	0.49 ± 0.02
4	1.51 ± 0.07	11.75 ± 0.5	4	1.43 ± 0.06	11.15 ± 0.5
6	1.66 ± 0.06	12.92 ± 0.5	6	1.48 ± 0.06	11.52 ± 0.5
8	2.14 ± 0.09	16.67 ± 0.7	8	1.47 ± 0.06	11.45 ± 0.5
(C) Rosemary active film			(D) Oregano active film		
Day	Av. Abs	mg MDA /kg fish	Day	Av. Abs	mg MDA /kg
0	0.063 ± 0.003	0.49 ± 0.02	0	0.063 ± 0.003	0.49 ± 0.02
4	1.20 ± 0.05	9.37 ± 0.4	4	0.79 ± 0.03	6.12 ± 0.2
6	1.26 ± 0.05	9.79 ± 0.4	6	1.34 ± 0.05	10.42 ± 0.4
8	1.89 ± 0.06	14.74 ± 0.5	8	1.61 ± 0.06	12.56 ± 0.5

Bolumar et al. (2011) developed antioxidant active packaging films by applying rosemary essential oil (10% solution in methanol) on the surface of household low density polyethylene (LDPE) used for food wrapping. Minced chicken breast and thighs were used to make patties that were packaged under vacuum in the produced films. The level of lipid oxidation was determined on the surface as well as in the inner parts of packaged patties. The results showed that lipid oxidation in the inner parts were lower than that on the surface. It was also shown that active packaging was able to delay oxidation throughout the whole period of 25 days. The TBA test showed that on day 3, the oxidation level on the surface of the control was higher than that on the surface of samples wrapped in active films, while the internal parts had equal amounts of MDA/kg of meat. Throughout the storage period, the surface of control samples experienced the highest values of oxidation. On the other hand, the inner parts of samples packaged with active film showed the lowest oxidation values among all samples. Figure 29 shows the TBA values obtained in both control and active packaging samples on the surface and internal parts of tested chicken patties.

In the present study, results on day 4 showed a 20% decrease in the amount of MDA in rosemary treated fish sample compared to the control sample. On the other hand, Bolumar et al. (2011) study shows a decrease of 25% in the amount of MDA/kg of chicken patties on the surface while internal parts had almost no oxidation until day 3.

Differences between the results of the two studies may be attributed to the time variation (3 vs. 4 days in the present study), the variation in the type of meat used, fat content and variation in sampling techniques (using the entire sample in the present study versus using surface samples in Boulmar et al. study).

Contini et al. (2012) conducted TBA analysis in cooked turkey meat wrapped in PET trays coated with citrus extract for a period of 4 days.

From day 0 to day 4, the value of MDA in turkey meat in control samples (wrapped with PET only) increased by 4.6 (mg MDA/ kg), while in PET films coated with citrus extract the value increased by only 2.2 mg MDA/ kg of turkey meet. There was a 47.8% decrease in MDA amount in the presence of citrus extract.

Thyme and rosemary in the present study exhibited a lower antioxidant activity than citrus extract. This may be attributed to the fact that citrus extract was coated in direct contact with the food, unlike thyme and rosemary which were incorporated into the PLA film matrix. On the other hand, oregano achieved a similar decrease in MDA amount as citrus extract. This results from the strong antioxidant activity of oregano essential oil that compensated for the fact of being less exposed to the packaged food surface.

4.5 Microbiological spoilage test

Total viable count (TVC) was measured for samples of minced fish in control film and treated films to monitor the spoilage time of the fish, after which, there would be no need to continue monitoring oxidation of fish meat by TBA (Table 7).

Results showed that on day 8, values of total viable count (TVC) showed complete spoilage of control sample (>7 cfu/g), while thyme rosemary and oregano were very close to the limit of spoilage.

Results in Table 7. shows that antioxidant activity should not be evaluated beyond day 8 of storage.

Film	Bacterial count (cfu/g)
Control film (PLA)	7.68 ± 0.27
Thyme film (PLA + Thyme)	7.25 ± 0.36
Rosemary film (PLA + Rosemary)	6.76 ± 0.28
Oregano film (PLA + Oregano_	6.78 ± 0.31

Table 7. Total Viable Count (TVC) of minced trout fish samples at day 8.

4.6 **Sensory Assessment**

Minced fish samples were tested for off-odor on day 4, 6 and 8 by 5 untrained panelists. Fish began to have unpleasant/pungent odor by day 4 in the control film samples, while

the odor was clearly less pungent in active films samples, most probably, as a result of 1) the masking effect caused by the odor of essential oils transferred to fish, 2) the lower values of oxidation induced by the presence of essential oils, and 3) the antimicrobial effect of the essential oils Oregano had the strongest protective effect and highest masking effect on unpleasant odors.

On days 6 and 8, the unpleasant odor increased and was clearly detectable in all samples, while being slightly masked by essential oil odor. Samples were judged as acceptable on day 4 and unacceptable beginning with day 6.

4.7 Mechanical Properties testing:

The obtained results (Table 8) show that the tensile strength at break was reduced in thyme, rosemary and oregano films by 26.1%, 21% and 28.2% compared to control film This is believed to result from the modification of the polymer matrix after the incorporation of essential oils. The % elongation at break slightly decreased in rosemary and oregano films by 2.8% and 4.2% respectively, while thyme film showed a higher reduction in % elongation by 13.7%. The decrease in Young's modulus showed a decrease in material stiffness after adding essential oils. Young's modulus decreased by 62%, 68.1% and 70.8% in thyme, rosemary and oregano films respectively.

Rhim et al. (2006) worked on developing and comparing PLA films produced by thermocompression and solvent casting. Mechanical properties (e.g. tensile strength at break, % elongation at break) were tested and compared for both production methods. Based on the conducted gravimetric analysis by Rhim et al. (2006), the solvent cast films had a dry matter of 86.3% and residual solvent content of 13.7% that act as a plasticizer, while thermocompressed films were made up of more than 99% dry matter. Dry matter represents PLA content of the film. The thermocompressed films are similar in composition to solvent cast PLA films after being dried to remove retained solvent residues in the film matrix.

PLA films prepared by thermocompression were stiff and brittle with values of tensile strength and elongation at break of 44 MPa and 3% respectively; while solvent cast films had a tensile strength of 16.6 MPa and elongation at break of 204%. The tensile strength

of solvent cast film was about 63% lower than that of thermo-compressed film. On the other hand, the elongation at break of solvent cast films was more than 70 times higher than that of thermocompressed films.

Table 8. Mechanical properties of control film (PLA), thyme antioxidant film, rosemary antioxidant film and oregano antioxidant film.

Film type	Mean ± SD			Young's Modulus (MPa)
	Thickness µm	% Elongation at break	Tensile strength at break (MPa)	
Control film	112 ± 5	368.4 ± 17	19.5 ± 0.9	125.9
Thyme antioxidant film	122 ± 5.6	317.7 ± 16.0	14.4 ± 0.6	48.77
Rosemary antioxidant film	118 ± 5.5	358.2 ± 16.1	15.4 ± 0.5	40.1
Oregano antioxidant film	126 ± 5.8	353.1 ± 16.0	14.0 ± 0.7	36.7

The tensile strength and elongation at break obtained for pure PLA film in the present study were 19.5 MPa and 368% respectively, which are closer to those obtained by Rhim et al. (2006) with partially dried solvent cast films than they are to results obtained in other studies on dried solvent cast PLA films (Jamshidian et al. 2012), (Ortiz-Vazquez et

al. 2011), (Rhim et al. 2009).

Comparison of the results of Rhim et al. (2006) to those of the present study, shows variation in mechanical properties between solvent cast films (containing solvent residues) and dried solvent cast films or thermocompressed films (free of solvent residues). Such variations result from the presence of solvent residues in the film matrix that act as a plasticizer. The effect of plasticizers includes decreasing brittleness through reducing the high inter-molecular attraction forces, accordingly, increasing the mobility of polymer chains and increasing the flexibility of films. Eventually, the result is a decrease in tensile strength, and an increase in Young's modulus and elongation at break values.

Jamshidian et al. (2012) incorporated Ascorbyl palmitate (AP) and a-tocopherol (AT) into PLA films of average thickness 100 μm by extrusion. Tensile strength, Young's modulus and % elongation at break of the produced films were measured. The results of pure PLA sample were about 58 MPa, 2240 MPa and 3.6% respectively. The obtained results of mechanical properties are different than those obtained in the present study due to the effect of extrusion which differs considerably than solvent casting, especially regarding thermal treatment of the polymer resin.

Jamshidian et al. (2012) incorporated synthetic phenolic antioxidant compounds BHA, BHT, PG and TBHQ at a concentration of 1% wt/wt in PLA films using solvent casting method. Mechanical properties were assessed including tensile strength, Young's modulus and percent elongation at break at a temperature of 20 ^{o}C, a relative humidity of

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52% and a stretching rate of 25 mm/min. Pure PLA had a tensile strength value of 58 MPa. Tensile strength decreased on adding BHA, BHT, PG and TBHQ by 14.5%, 5.7%, 19.1% and 21% respectively. The value of Young's modulus of pure PLA film was 2240 MPa. Adding BHA, BHT, PG and TBHQ reduced Young's modulus by 12.5%, 3.1%, 17.4% and 17.4% respectively. % Elongation at break was also measured to be 3.6 % for Pure PLA film. On adding BHA, BHT, PG and TBHQ it decreased to 3.4, 3.0, 3.2 and 3.3 % respectively.

Ramos et al. (2014b) developed nano-biocomposite PLA based films of thickness 210 µm. Thymol was incorporated into the films as active agent in addition to modified montmorillonite (D43B) at 2 different concentrations. Thermal and mechanical testing was performed on the produced films. The results showed that thermal stability of PLA was not significantly changed by incorporating thymol into films. On the contrary, mechanical properties of films were improved by the addition of D43B and thymol.

Samples containing PLA-thymol showed a decrease in tensile strength and Young's modulus of approximately 15% versus the control film (PLA). This change may be attributed to the plasticizing effect of thymol. Samples incorporating D43B and thymol showed an increase in Young's modulus and a decrease in % elongation at break. This indicates an increase in film brittleness caused by the addition of D43B. Films containing both thyme and D43B showed lower Young's modulus versus pure PLA films closer to the values obtained in the PLA-Thyme films. These results indicate that the plasticizing effect of thymol in PLA films is dominant over the reinforcement effect

caused by nanoclay (D43B).

In the present study, adding thyme to PLA caused a decrease in tensile strength of 26.2%, and a decrease of Young's modulus of 38.7%. The higher reduction in tensile strength and Young's modulus may be attributed to the higher concentration of thyme used in the present study.

Ortiz-Vazquez et al. (2011) studied the changes in mechanical properties of PLA films produced in a pilot plant scale extruder (extrusion blow molding) after the addition of BHT (1.5% wt/wt). Film thickness was 51 µm. They measured the tensile strength at break found to be 48.2 MPa in pure PLA film, decreasing by 7.3% on adding BHT to the films. % Elongation at break of pure PLA film was 5.8%, decreasing by 29.3% after adding BHT.

Rhim et al. (2009) developed PLA based composite films using several types of nanoclays by the solvent casting technique. Mechanical properties were evaluated including tensile strength and % elongation at break. Tensile strength was 50.5 MPa and % elongation at break was 3% respectively at a film thickness of 71 µm.

4.8   Oxygen transmission rate testing

The produced films in the present study were tested for oxygen permeability. Results are shown in Table 9. Thyme and rosemary films showed a similar oxygen permeability value to control. On the other hand, oregano films showed a small increase in oxygen permeability equal to 8.8%.

According to Abdellatief and Welt (2013) PLA films showed to be of rather low barrier because the oxygen transmission rate (OTR) values were higher than 200 ml/m^2.24h.atm.

Usually, antioxidant additives perform a plasticizing role in the films, causing changes in film crystallinity and internal structure that in turn increase the free spaces between molecules and allow for higher gas permeability rates (Samsudin et al. 2014). This justifies the increase in the oxygen transmission rate values for films containing essential oils that act as plasticizers with the exception of films containing thyme and rosemary essential oil.

According to Onwulata (2014) PLA films prepared by solvent casting have an average OTR value of 800 ml/m^2.24h.atm, which is in line with the obtained results of the present study. PLA film has an oxygen permeability value substantially lower than that of LDPE (approx. 5000 ml/m2.24h.atm/50 µm). On the other hand EVOH and PVDC have a much lower oxygen permeability of about 1 to 10 ml/m^2.24h.atm/10 µm. Such polymers are considered to be high barriers to oxygen.

Table 9. Oxygen permeability of generated PLA films

Film type	Oxygen transmission rate (ml/m^2.24h.atm)
Control film (PLA)	865 ± 38.9
Thyme film (PLA + Thyme)	870 ± 27.8
Rosemary film (PLA + Rosemary)	911± 36.1
Oregano film (PLA + Oregano)	941± 38.6

Yuniarto et al. (2014) developed PLA films with different thickness and determined their barrier properties with respect to film thickness. The results showed that the higher the film thickness the lower the oxygen transmission rate value.

They also studied the effect of increasing the plasticizers concentration on the barrier properties of PLA films by using PEG as a plasticizer. The increase in plasticizer concentration decreased the oxygen transmission rate value up to the concentration reached 5%. At concentrations above 5%, the oxygen transmission rate increased proportionally. This may be attributed to the induced increase in polymer crystallinity caused by the plasticizer according to the author. The oxygen transmission rate of pure PLA film with 10% PEG was approx. 800 ml/m^2.24h.atm that is 8.1% lower than the value obtained in the present study. The lower oxygen transmission rate as compared to that in the present study may be attributed to the plasticizing effect caused by the solvent residing in the film as suggested by Rhim et al. (2006)

Thymol and nanoclay (D43B) were incorporated into PLA films using compression molding technique by Ramos et al. (2014b). The produced films with a thickness of 210 μm were tested for oxygen permeability and the results showed that thymol caused a slight increase in oxygen transmission rate of about 4%. The increase in transmission rate of oxygen is caused by the ability of thymol to increase the mobility of polymer molecules and chains which in turn reduces the polymer orientation within the film, therefore, increasing oxygen transmission rate. In the present study, thyme essential oil incorporation into PLA film caused no increase in OTR.

According to Jamshidian et al. (2012) the, the normal oxygen transmission rate value for

extruded PLA is between 1.9×10^{-18} and 6.0×10^{-18} m^3 m m^{-2} s^{-1} Pa^{-1}. Adding BHT to

PLA increased the oxygen transmission rate by 30%. BHA, PG and TBHQ resulted in almost no change in the oxygen transmission rate of PLA. In the present study, the value of oxygen transmission rate of PLA was 1.1×10^{-17} m^3 m m^{-2} s^{-1} Pa^{-1}. The value obtained in the present study is higher than that in literature by a factor of approximately 5 but within the same order of magnitude. This may be due to the difference in the technique of film preparation, since the present study was performed on solvent cast films rather than extruded films.

Samsudin et al. (2014) incorporated marigold flower extract into PLA films and showed that the addition of the antioxidant agent had an insignificant effect on the permeability of PLA. Pure PLA had an oxygen transmission rate of 55.6×10^{-17} kg m/m^2 s Pa, while films containing marigold flower extract had a similar oxygen transmission rate of by 17.4% (45.9×10^{-17} kg m/m^2 s Pa). The study suggested that changes in permeability may be due to the specific nature of the antioxidant used in relation to the nature of the polymer matrix.

4.9 Migration Testing

Migration is the property of the packaging material that indicates releasing of a substance or substances from the packaging film into the packaged food as a result of contact or interaction between food and package. Overall Migration testing is used to assess the total amount of migrants transferred from the packaging material into food or food simulant under specific test conditions.

The results (Table 10 and 11) show that the migration occurring from PLA films in aqueous and fatty food simulants did not substantially increase after the addition of essential oils. The total migration level is below the limits set by European directive 2002/72/EC of 6 August 2002 relating to plastic materials and articles intended to come into contact with foodstuffs in both control film and test films (10 mg/dm^2 or 60 mg/kg).

Data shows that the overall migration in water was substantially higher than that in isooctane. This can be justified by the partially hydrophilic nature of PLA which is rather incompatible to isooctane.

Auras et al. (2004) tested PLA migration in contact with ethanol 95%. The total amount of migrants in simulants were lower than the average daily intake quantity of lactic acid from all proposed uses as an indirect food additive (22 mg/day). The value of overall migration from PLA was 12.9 mg/kg of food contacting medium. This is a smaller amount of lactic acid than that found in a cup of yogurt (10 g/kg). Compared to the present study, the value of overall migration reported in the above study is substantially higher. This may be primarily due to the different food simulant used.

Table 10. Migration testing in isooctane (fatty food simulant)

Film type	Overall Migration (mg/dm^2)
Control film (PLA)	0.15 ± 0.01
Thyme film (PLA + Thyme)	0.16 ± 0.01
Rosemary film (PLA + Rosemary)	0.16 ± 0.01
Oregano film (PLA + Oregano)	0.17 ± 0.02

Table 11. Migration testing in water (aqueous food simulant)

Film type	Overall Migration (mg/dm^2)
Control film (PLA)	0.99 ± 0.05
Thyme film (PLA + Thyme)	1.08 ± 0.06
Rosemary film (PLA + Rosemary)	1.05 ± 0.07
Oregano film (PLA + Oregano)	1.11 ± 0.06

Fortunati et al. (2013) prepared PLA films by the solvent casting method to be used as active food packaging materials incorporating high performance nano-materials. They investigated the overall migration in isooctane. The results showed that pure PLA had overall migration values that were below the limits set by the EU of 10 mg/dm^2 or 60 mg/kg. Pure PLA had an overall migration value of 0.015 mg/kg of isooctane. In the present study, the migration value of PLA film into isooctane was 0.15 mg/dm^2 of film. The variation in the obtained migration results may be due to the use of a different food simulant of different concentration.

5. **Conclusion**

Based on results of the present study, PLA films prepared by the solvent casting method containing thyme, rosemary or oregano natural essential oils have the potential to protect fish such as trout from oxidation.

6. **Suggestion for future work**

A promising future research opportunity would be to investigate the antioxidant and/or antimicrobial activity of PLA films coated with the antioxidant/antimicrobial active compound(s) in order to increase its/their concentration on the surface of the food thus increasing their efficiency. Coating efficiency may be enhanced using plasma or corona treatment of the film surface.

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