UNIVERSITA' DEGLI STUDI DI MILANO

FACOLTA' DI SCIENZE E TECNOLOGIE

Dipartimento di Chimica

XXIX Cycle of Ph.D. Industrial Chemistry

Bio-based materials to deliver and to supply natural antioxidants: intrinsic active materials for food packaging and biomedical applications

Tutor: Dr. Luisella VEROTTA

Co-Tutor: Prof. Giuseppe DISILVESTRO

Dr. Stefano ANTENUCCI

Matricola: R10796

Anno Accademico 2014 - 2016

Introduction

Abstract

The main focus of my Ph.D. research is to study a new way to supply natural antioxidant molecules within a specific substrate using biocompatible polymers, the latter being either synthetic or natural.

Collins Dictionary defines the word “antioxidant” as:

1. any substance that inhibits oxidation, as a substance that inhibits oxidative deterioration of gasoline, rubbers, plastics, soaps, etc.

2. an enzyme or other organic substance, as vitamin E or beta carotene, that is capable of counteracting the damaging effects of oxidation in animal tissues and food.

The first definition is closer to the field of polymers used for the production of goods that are commonly used. If the antioxidant protects the material during its processing (that usually occurs at high temperatures), it is identified as secondary antioxidant: on the other side, an antioxidant used to prevent goods degradation (i.e. weathering) is defined as primary antioxidant. The common practice consists in a proper combination of primary and secondary antioxidant in order to obtain a final material that is able to be processed and to resist for the entire life of the products. The choice of the proper antioxidant depends on the polymer and therefore on the technological field of use and must be done according to three important parameters:

1. The temperature required for the process (i.e. melt extrusion, mold injection etc.): the higher the processing temperature, the higher must be the degradation temperature of the antioxidant.
2. The time required for the transformation: the kinetic of decomposition must be considered as a crucial parameter because, even if the antioxidants are stable in a range of temperatures and can prevent degradation, a prolonged thermal stress, occurring at temperature close to the decomposition temperature, can promote the reaction and consume all the antioxidant leaving the polymeric material without protection against oxidation.
3. The external stress exposure during goods life: UV light (indoor or outdoor applications), thermal stress (oxidation kinetic is dramatically dependent to temperature), moisture (antioxidants can be soluble in water therefore continuous washings can promote migration of antioxidant leaving polymer without protection).

Radical attack is the most common route of polymeric material degradation; active radicals can be generated by thermal stress and ultraviolet irradiation and in both cases an active radical can attack

the polymeric chain modifying the structure and changing material properties. A general scheme of the reaction is reported in figure 1. The role of antioxidant is to interrupt the cycle preventing the degradation of the material.

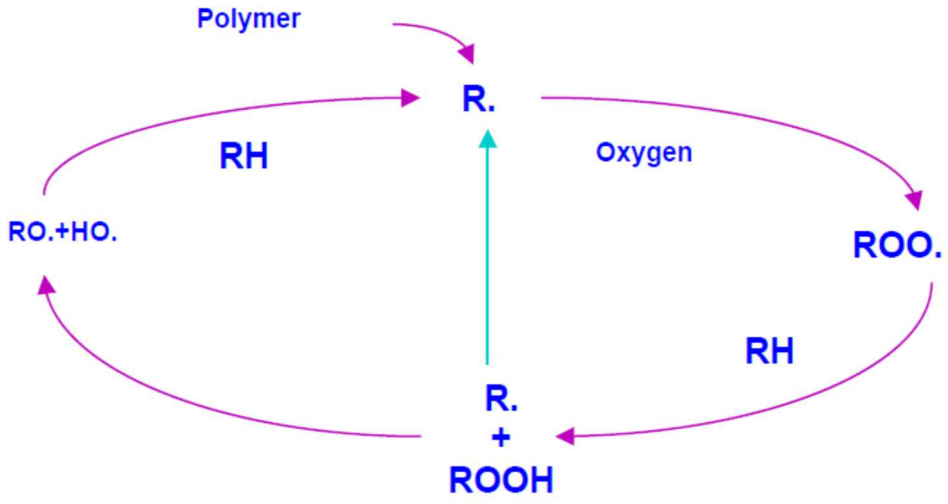


Figure 1: general scheme of radical attack on organic substrate

The second definition, more familiar with biochemical field, defines another kind of antioxidant, or better, another kind of role that antioxidant should have in a process. Oxygen radicals can attack, with the same mechanism shown in figure 1, a lot of biological substrate leading to degradation. If the attack occurs in non-living animal substrate (i.e. food) the degradation leads a low-quality or even a non-comestible product. If the degradation occurs at a cellular level, an “oxidative stress” is present: this can promote a lot of diseases, and even lead to cancer in living tissues.

The use of active substances, i.e. of substances that can have active functions beyond the inert passive containment and protection of the product to preserve packaged food is a novel approach used in packaging that lies beneath the field of the so-called “active packaging”. Food companies, and also the academic world, have a great interest in this field. Economists have estimated that active packaging is a business worth 2.8$ billion 2014 that will reach 4.0$ billion in 2019 only in the US. In the academic field, the interest around active packaging is steadily growing: indeed, the key words “active packaging” give almost 15000 results related to papers and patents (SciFinder® - 2016) with a trend that dramatically increases in the latest 10 years. Up to 1996 the total amount of publications in the field was 1886 whether in 2016 year only, there are more than 1000 publications.

The framework here presented is the background of the present research, active packaging is defined as the field related to “packaging having active functions beyond the inert passive containment and protection of the product”: it is one of the most promising novel strategies in the

field of food packaging. Some active packaging solutions are already present in the market, and examples are: devices for moisture control, oxygen scavengers, CO_2 emitter, antibacterial coating and also radical scavengers. Active packaging solutions rely on two main techniques, one involving the use of external devices put in the package (i.e. silica small bags to absorb moisture or iron sachets as oxygen scavengers) – this solution is not appealing for consumers in food packaging – the other involving the use of additives compounded with the polymers used for packaging. One of the most relevant issue of the latter is related to the migration of active substances into food over time that causes food contamination and alteration. The PhD project is dedicated to the development of a new approach for active packaging, capable of potentially solving (or at least dramatically limit) the problems evidenced: such approach relies on the "in situ" synthesis of intrinsically active polymers – antioxidant being the preferred ones – with the potential of becoming materials used to produce films for packaging having intrinsic active features. Another issue related to packaging (and food packaging in particular) is the huge environmental impact (waste food packed with standard PE, PP or PET is disposed as general waste, thus raising two ethical issues, one related to food waste and the other related to health concerns related to traditional plastics end-life in the last years the use of biopolymers as packaging materials has rapidly grown, even if they are still a niche market. The PhD project was therefore focused on the synthesis of intrinsically active biopolymers, to combine both the environmental sustainability of such biopolymers and the new approach related to active packaging solutions: some natural antioxidant molecules (or their simple derivatives) were chosen according to their potential reaction in "in situ" polymerization of lactide to give poly lactic acid (PLA). Lactide reacts giving PLA via Ring Opening Polymerization (ROP) that can be initiated by an aliphatic alcoholic moiety whereas aromatic ones do not react with lactide. Benzyl alcohols bearing a phenolic moiety can act as polymer initiator leaving unreacted the phenol groups, responsible for antioxidant features.

The first step was to synthesize intrinsic antioxidant polymers using bulk polymerization of lactide. Different antioxidant compounds were found as possible active molecule, namely:

Tyrosol (Tyr), Vanillyl Alcohol (VA), Methyl Ascorbic Acid (AA), Pyridoxine (Pyr) and dihydroxy benzyl alcohol (DBA).

The criterion of choice was based on the presence of at least an aliphatic alcoholic moiety allowing potential reaction with lactide.

The second criterion used was the resistance at high temperatures, since PLA bulk polymerization involves the use of high temperatures of about 190°C. The study of thermal properties (performed via TGA) revealed that only VA and Tyr have the required long-term thermal stability at 190°C.

Thanks to these two criteria, VA and Tyr were chosen as the molecules that could be added in the feed used for the bulk polymerization of lactide. Given the presence of a phenolic and of a $–CH_2$-OH moiety in both molecules, they could act as bi-functional comonomer in the polymerization. Nevertheless, the phenolic moiety is too acid to form a stable bond with lactide: this means that VA and Tyr act as mono-functional initiators in the ring opening polymerization (ROP) of lactide. They were therefore used in low concentration in the polymerization feed (0,1% mol/mol on lactide) to allow having polymers with high molecular weight, necessary to obtain films via solution casting.

To evaluate "in vitro" antioxidant features of such polymers, DPPH assay were used to assess the antioxidant power of the new polymers.

Despite VA and Tyr were used in low concentration, PLA bearing VA resulted having 8.3% of radical scavenging (whereas pure PLA has no radical scavenging activity, as expected), where the scavenging ability of pure VA is 94%. Besides, the relatively high molecular weight (comparable to the one of Natureworks PLA Ingeo® 4043D, commonly used in food packaging) of this polymer allowed the production of films via solution casting: this indicates that the polymer, in a future, could be used also for film production via melt extrusion. The research project was then devoted to the research of further improvements to the antioxidant power of PLA+VA and to find a way to widen possible application fields of this new class of intrinsically antioxidant polymers. Polymerizations were performed with increasing VA content (up to 0.2%); this strategy revealed some problems:

1) The antioxidant molecule acts as chain initiator therefore as its concentration increase, the average degree of polymerization (DPn) decreases, jeopardizing the possibility to process the polymers (i.e. to obtain homogeneous films via solution casting).

2) The Vanillyl alcohol has an intrinsic high reactivity due to its antioxidant moiety that leads to unexpected side reactions.

Regarding point 1, the theoretical approach relies on the studies about the degree of polymerization (DPn). DPn at full conversion (i.e. 100%) can be determined with the following equation:

$$DPn = \frac{n_{monomer}}{(n_{initiator} \times f)} \quad 1)$$

$n_{monomer}$ are the moles of monomer, $n_{initiatior}$ are the moles of initiator and f is the number of functionalities of active molecules able to react with the monomer. Equation 1 shows that the increase of chain initiator concentration decreases DPn value. For instance, a 0,1% mol/mol concentration leads to a polymer with maximum DPn = 1.000 (real DPn is lower) while 0.5%

mol/mol decreases maximum DPn to 200. Since melt viscosity strongly depends upon molecular weight, and therefore DPn, low DPn leads to low melt viscosity, that doesn't allow for melt extrusion of films: DPn of industrial PLA used for film production is higher than 600. This consideration led us to choose a maximum concentration of VA lower than 0.2% mol/mol.

Regarding point 2, VA has an intrinsic high reactivity. If its concentration is higher than 0,5 mol%, at 190°C and in the presence of a catalyst, side reactions occur.

Due to what described about the above points 1 and 2, the experiments were conducted on PLA containing 0,1% mol/mol of VA.

The very promising DPPH results on this sample allowed to perform shelf life test with a real industrial food matrix: an industrial salami was chosen due to its high fat content that makes it a critical food in terms of shelf life requirements. The test required a long preparation time since, to perform reliable tests, a minimum of 160 salami slices must be tested: PLA films were used as interlayer between two salami slices. For each packaging of two salami slices, a film of about 100cm^2 is required. It means that for a reliable shelf life test at least 8000cm^2 of active PLA (the surface of 12 A4 sheets) was required; solvent casting deposition permits to produce only one A4 sheet of PLA film every 15h, therefore the production of all polymer sheets for the test took more than 180h.

The results obtained were very good:

1. PLA+VA enhances the stability of food matrix decreasing the oxidation kinetic: the degradation begins after 15d whereas, in non-active packaged salami, the degradation starts after few days.
2. DPPH value does not decrease during the test (i.e. no migration of VA occurs over time)

Therefore, at the end of the work, a new class of polymeric materials with intrinsic antioxidant properties was developed, tested with "in vitro" assay and also with "in vivo" shelf life test.

CONCLUSION

During the Ph.D project, several issues related to intrinsically antioxidant polymers were addressed:

- The possibility to obtain intrinsic antioxidant environmentally friendly polymers via ROP of lactide was verified: antioxidant molecule such as Tyr and VA can react with lactide using the same approach used for industrial process of PLA synthesis.

- The possibility to increase the quantity of VA in lactide bulk ROP was tested: the maximum quantity of VA should be below 0,2% mol/mol and therefore no real change in antioxidant feature could probably be achieved in comparison to the polymers with 0,1% of VA.
- The polymers obtained were tested in-vitro confirming that the antioxidant moiety (phenolic moiety) still remain active after the polymerization leading to an "active" material. Afterwards, the PLA containing VA was tested in-vivo using a standard procedure for shelf-life studies: the in-vitro results were confirmed by these assays, since the material reduces the oxidation rate of packaged food enhancing the shelf-life of the products.

The second part of the Ph.D. project was devoted to the use of natural antioxidants for biomedical application. Oxidative stress, as was previously mentioned, is one of the main causes of a series of degenerative diseases (like cancer, heart failure, infections), including those pathologies affecting the Central Nervous System like Parkinson's disease, Alzheimer's disease, and also depression. The presence of free radical, as reactive oxygen species (ROS) in mitochondrial ambient, can disturb the normal redox cellular equilibrium leading to damage the cellular ambient, including lipids, protein and also DNA; however, reactive oxygen species can be beneficial as they are used by the immune system as a way to attack and kill pathogens. Short-term oxidative stress may also be important in prevention of aging by induction of a process named mitohormesis, an adaption of human body of stress caused by the presence of endogenous ROS. Not only the endogenous ROS uncontrolled production can cause diseases but also an external trauma, for instance a fracture of bone or muscle sprain, can modify the cellular redox equilibrium leading to an exceeded production of radical. The administration of antioxidant in the damaged area can restore the normal redox equilibrium enhancing the healing process.

A possible way to prevent, or at list to reduce, the effect of oxidative stress is the consumption of antioxidants, that are abundant in vegetable and fruits; the most famous are Vitamin C, Vitamin E, natural flavonoids, carotenoids. Even if a well-balanced diet can provide many nutrients, sometimes it could not be enough for many causes (for instance exceeded stress due to the life style, high intensity training, unhealthy habits, smoke etc.): to complete the daily supply of antioxidants with supplements can be a good solution. Antioxidant supplements represent a 23$ billion/year industry (n.d.r. 2015) that continue to spread its market.

One of the relevant issue of antioxidant supplement, and also for antioxidant molecules present in foods, is related to the "real" availability of the active molecule inside the human body. In other terms, the effects of antioxidant are not only dependent on their concentration in food or

supplements but especially on the so-called “bioavailability” and “bioaccessibility”. Bioavailability is defined as the proportion of an antioxidant that is digested, absorbed, and utilized in normal metabolism; measurement of bioavailability heavily relies upon estimates of amounts of antioxidant absorbed. On the other hand, bioaccessibility is a commonly used term defined as the amount of an ingested nutrient that is available for absorption in the gut after digestion. In these terms, the bioavailability strictly depends on the bioaccessibility. Bioaccessibility rapidly decreases over time: in fact, the concentration of antioxidant dramatically decreases 2h after digestion: therefore, in the human body the concentration of antioxidants rapidly increases after the assumption of food and supplements and, with the same velocity, decreases.

A system that is able to protect and to release an antioxidant under specific conditions could be a solution to overcome these problems. Scientists have developed a lot of systems for controlled release of substances: dendrimers, nano and micro particles, brush polymer, hierarchical scaffolds etc., Even if the structure and therefore the production methods are different, the key concept is almost the same: the active principle molecule is entrapped in a system which can create weak reversible interactions and, with a specific variation of pH or solvent solubility (the active principle should be more soluble in water than in the macromolecular chains of the scaffolds), the release occurs. A controlled release has great advantages in comparison to “standard” method:

- The release occurs in the target substrate reducing dramatically the loss of activity and preventing the eventually side effects.
- The active principle concentration can be kept constant obtaining better results in terms of performance and efficacy.
- The concentration of active substances in supplements could be decreased reducing the costs of production.

Two different release systems were investigated during this Ph.D. thesis:

- High porous scaffolds
- Release system for ingestion

Biomedical scaffolds are defined as a “solid framework able to hold cells or tissue together” therefore they required specific features: thermal stability (T_g > human body’s Temperature), resistance to standard sterilization procedure (i.e. thermal treatment, UV irradiation, cryo-treatment etc.), biocompatibility (reduced inflammatory response due to chemical structure of material in contact with tissues), and maybe bioresorbable properties tuned for specific scopes (decomposition time, due to enzymatic and chemical attack has to be tailored on the time required for medical

treatment). A lot of materials can be used for scaffolds production, for instance metals and ceramic materials present a lot of advantages in terms of resistance to sterilization treatment and resistance to human body ambient; however, they promote inflammatory process and cannot be absorbed by human body requiring a surgery at the end of treatment. Polymer-based scaffolds can overcome these problems; polymers can be tailored for specific purpose avoiding problem related to inflammatory process and bioresorbable property that can be finely tuned (for instance the ambient in gastrointestinal tract is widely different than ambient presents in broken bone). Differently, polymers present a less stable structure (i.e. low thermal stability in comparison to metals or ceramics) that leads to a low resistance to sterilization treatments.

A lot of polymers with high biocompatibility for this purpose were developed: polyacrilates (PA), polyurethanes (PU), polyvinylpyrrolidone (PVP), polyglicolide (PLGA),poly (lactic acid) (PLA) etc., Another possibility is the use of natural polymers, extracted from natural sources, for the formation of scaffolds, the most common being collagen and chitosan (from animal sources) and pectin and alginate (from vegetable sources). Synthetic polymers have the great advantage that they can be synthetized with the same features and controlling impurities: the reproducibility of the release in terms of kinetic and released quantities is higher than in other systems. On the other hand, natural polymers are completely biocompatible with human body, in particular with the digestive system, even if they have an intrinsically variability in their structure (ex. the quantity of methyl ester in pectin or the molecular weight or the percentage of acetylation in chitosan).

During the Ph.D. project, different polymeric systems were studied for the delivery of antioxidants in human body. Two different approaches were pursued:

- The first approach consists in the use of a standard industrial poly (lactic acid) for the delivery of a modified natural antioxidant: oligotyrosol (oligoTyr).
- The second approach considers the use of natural polymers (i.e. pectin and alginate) for the delivery of Ellagic Acid.

Oligotyrosol was obtained by horseradish peroxidase coupling of Tyrosol, a natural phenol obtained from olive and also green tea. Tyrosol does not present exceptional properties, having a radical scavenging power of 24% whether oligoTyr has a radical scavenging power of 48% (these values were obtained via DPPH analysis in standard conditions). The increased antioxidant power of oligoTyr leads to obtain new properties, since oligoTyr promote the ossification process in human osteoblast cells. The healing process of broken bone takes at least 8 weeks and a continuous administering of oligoTyr could promote the healing process. To have a continuous administering of oligoTyr, two approaches can be used:

- periodical targeted injection in the damaged zone.
- Insertion of a scaffold loaded with oligoTyr that is able to release the active principle and that, at the end of the process, can spontaneously degrade.

The second option presents two great advantages: first of all, a patient can avoid continuous annoying injections and moreover a constant rate of active substance release avoids toxicity issues due to high concentrations. From this regard, PLA seems a good candidate for this purpose: it is biocompatible and can be digested via enzymatic attack of proteinase K.

The first step of the work was dedicated to the study of a reliable procedure to obtain a hierarchical scaffold (high porous scaffolds promote the adhesion of bone cells and, increasing the surface area, can promote the release). Two different methods were used:

•**Method A**. PLA was dissolved in THF in a 100 mL glass flask. Methanol was added at room temperature under mechanical stirring up to 95/5 v/v THF/methanol ratio. The solution was frozen by immersion of the flask into liquid nitrogen and was then poured in warm water. The solid PLA scaffolds that separated were recovered after removal of the solvent by filtration.

•**Method B**. PLA was dispersed in 1,4-dioxane in a 100 mL glass flask at room temperature and taken under mechanical stirring overnight. The resulting homogeneous solution was frozen by immersion of the flask into liquid nitrogen and the solvent was removed by sublimation at room temperature under vacuum (2.5×10^{-3} Bar).

Both methods were used for the preparation of scaffolds loaded with Gallic Acid (GA), used as a model molecule, Tyrosol (Tyr) and oligoTyrosol (oligoTyr).

SEM images of samples prepared with the two methods showed that different structures can be obtained, revealing differences in superficial area and in pores dimensions. The first method leads to obtain scaffolds with very small pores (minimum measured dimension = 300nm) whereas scaffolds prepared with the method B have larger pores (minimum measured dimension = 2µm); method B also allows to obtain scaffolds with a smooth surface. The first important result achieved is that a method that allows to control the morphology of the scaffold that can be tailored for each application was developed. Once a method to control the morphology was obtained, another important parameter was to verify how the active molecules affect the formation of crystalline domains and of the amorphous phase. Gallic Acid was chosen for this purpose. It was also verified if the concentration of GA decreases during the scaffold preparation: UV quantitative analyses revealed a decrease of GA concentration due to migration in water promoted by melted THF during the solvent elimination phase. On the other hand, method B intrinsically prevents the leakage of

substances, therefore this method was used for the preparation of scaffolds loaded with Tyr and oligoTyr.

The following step was the preparation of scaffolds loaded with oligoTyr and Tyr: given their antioxidant features, Tyr and OligoTyr are able to reduce oxidative stress and promote osteoblastic cell growth. The aim was to obtain a scaffold able to constantly release oligoTyr in a period of about 2 months. In collaboration with the group of Prof. A. Napolitano from University of Naples Federico II, the release kinetics of oligoTyr and Tyr in phosphate buffer were investigated, in order to simulate human body condition. The group of Professor B. Burlando of University of Piemonte Orientale studied the biological effect of pure oligoTyr, Tyr and PLA and also verified the effect of osteoblastic cells growth, expressed as alkaline phosphatase (in particular ALP) activities. It was verified that PLA, Tyr and oligoTyr are not toxic for human osteosarcoma cells and Tyr and also oligoTyr promote cells growth.

Besides the use of a synthetic polymer as bulk material for the production of scaffolds, natural polymers were also tested for the vehiculation of antioxidant molecules. Pectin and Alginate were chosen due to their easy availability and processability. Both polymers are water soluble polysaccharides that can be extracted from plants; in particular, alginates are widely extracted from brown seaweeds whereas pectin is refined from citrus peel but also from apple, apricot and carrots. Both polymers present carboxylic groups along the polysaccharides chain which can be used to coordinate metals; the coordination of bivalent ion leads to obtain a gel-like structure. Such structure permits to use the alginate and pectin as a material for the encapsulation of an active principle. Even if both materials are able to form gels, the different macromolecular structure between pectin and alginate (for each repeating unit alginate has a carboxylic moiety while the concentration of carboxylic moiety in pectin depends on the amount of galacturonic acid and on the percentage of its deacetylation) leads to different mechanical properties of the gel. Pectin-Ca(II) gel is reversible and stiffer than the gel obtained with Ca(II) coordinated to Alginate. These differences could be used to obtain different materials able to use different substrates.

The first part of the work was devoted to the study of the material that can be obtained through calcium complexation with pectin: the materials obtained were characterized in terms of rheological properties, analyzing the gel obtained with different concentration of pectin in water. Also the rheological properties of the alginate gels were assessed; these analyses were performed in order to assess which are the best materials (in terms of rheological properties and gel stability) that should be used for the encapsulation of active molecules.

The further step was to individuate an active molecule with the potential to be exploited in the future also in scale-up processes; one of the most appealing substances for that purpose was Ellagic Acid, an aromatic polyphenol present in many fruits especially berries and pomegranate. One of the great advantages of using such polymers is the possibility to work using water as a solvent, therefore the antioxidants that will be encapsulated should be soluble in water. Ellagic acid is almost insoluble in water (10 µg/ml) therefore a method to enhance such solubility was studied: the easiest way is to obtain a salt. Strong bases, such as NaOH or ammonia lead to deprotonation of ellagic acid and promote the decomposition via quinon formation. Weak and medium bases, such as substituted amine, are able to deprotonate the ellagic acid avoiding the degradation. The weak bases chosen for this purpose is the L-lysine: an essential amino acid, bearing an extra amine group, is the best possible solution due to its intrinsic biocompatibility and low cost. The use of L-lysine allowed to increase the solubility of ellagic acid up to 400.000 times obtaining a solubility of 40mg/ml.

The last step was to encapsulate the ellagic acid-lysine salt in pectin and alginate gels and to study the kinetic of the release. The kinetics were studied in different conditions modifying the environment (water and phosphate buffer at pH 7,4). The results were very good: both pectin and alginate can control the release of ellagic acid-lysine salt. Moreover, modifying the production process, the release can be tailored: the release can be accelerated or decelerated reaching well-defined concentrations of ellagic acid. It is possible to obtain very low release rate (5% of loaded feed in 72h) or, modifying the environment and the structure, to reach very high release rate (25% of loaded feed in 2h).

CONCLUSION

During the Ph.D. project, several issues were addressed:

- High porous scaffolds based on synthetic industrial polymer (PLA) able to release active substances were produced; the structure of scaffolds can be tailored modifying the method of preparation.
- Two natural polymers, pectin and alginate, were used for the production of scaffolds with elevated biocompatibility able to control the release of an active principle. The release can be modulated modifying the composition of scaffolds.

Acknowledgments

This work has been carried on thanks to the financial support of Fondazione Cariplo and Regione Lombardia for the project ‘‘BIOPLANT’’ given according the three guidelines: Action 1: ‘‘Bando per interventi di ricerca industriale e sviluppo sperimentale finalizzati al potenziamento di centri di

competenza di rilievo regionale’’, Action 2: ‘‘Avviso a presentare programmi di valorizzazione del capitale umano nell’ambito della ricerca e di sostegno alla mobilità dei ricercatori attraverso progetti ad alto valore innovativo anche internazionali’’, Action 3: ‘‘Promuovere progetti internazionali finalizzati ad incrementare l’attrattività del sistema ricerca regionale’’.

1. Introduction

The most common processes of degradation that occur in nature involve oxygen. The formation of oxygen radical can be promoted by ultraviolet radiation (1), thermal stress (2), metals (3), pollution (4) or by a combination of these factors. Many examples of the effect of oxidation are provided in literature and concern very different fields, from the oxidative stress in life organisms to the oxidation of organic products during synthesis (5) (6) (7) (8). In all of these cases, the aim of research is to reduce the impact of oxidation using specific substances called antioxidants. An antioxidant is a molecule that inhibits the oxidation of other molecules allowing the degradation of specific moieties on its structure and avoiding the propagation of radical reactions; in this way, radical reactions are terminated preventing the damage of a high value substrate. Most common antioxidants are phenolic compounds, secondary arylamines, organo-phosphites, and thioesters (9).

Two different branches of “antioxidant chemistry” could be defined:

- Antioxidants used for preventing material and goods degradation
- Antioxidant used for preventing degradation of animal and vegetable substrate both in processed products and life organisms.

1.1 Antioxidants in Polymers

The first definition is most related to plastics world: plastics are organic substances which can be degraded both during the processing, that normally require high temperature, and during the life of goods. The protection of materials against oxidation, during the processing or during the life of products, requires different kind of substances with different reactivity; it is a common practice to divide antioxidants in two families: primary antioxidant and secondary antioxidant. A primary antioxidant has the important role to prevent the degradation of plastics during the life of goods: atmospheric agents, such as the light irradiation and high temperature, can promote the oxidation of polymers jeopardizing the properties of the material: molecular weights decrease and therefore the loss of mechanical properties (10) and the variation of optical properties can occur (11). Secondary antioxidants are used to prevent polymer degradation during the processing of the materials: melt extrusion (12), injection molding (13), blown processing (14). All of these techniques require high temperature (the temperature should be higher than melting temperature or glass transition temperature) that promotes the oxidation of polymers.

The structure of the antioxidant needs to be varied in relation of its application: time and temperature required for the processing. A proper combination of primary and secondary

antioxidant leads to obtain a material that can be processed avoiding degradation and can resist for the entire life of goods that is used for. A generic scheme of antioxidants structures related to their possible applications is shown in figure 1.

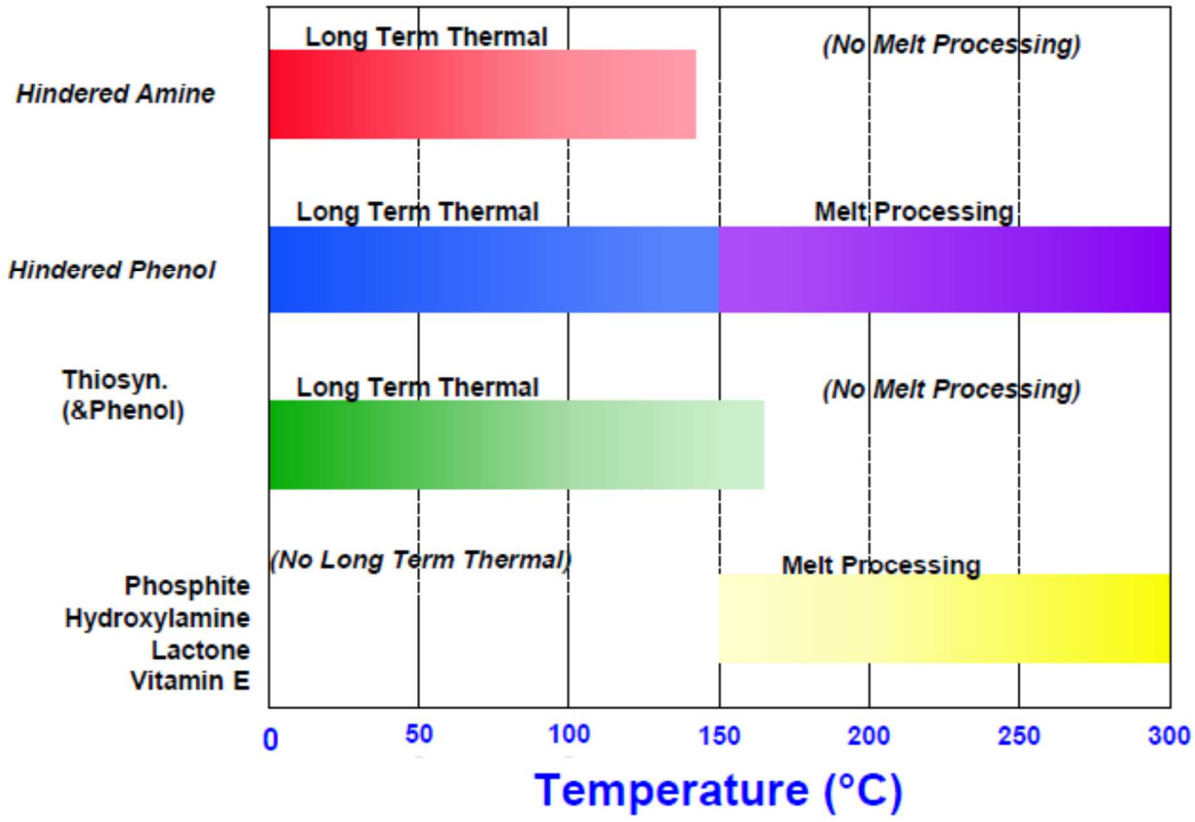


Figure 1.1: scheme antioxidant molecules and their application related to processing temperature

1.2 Antioxidants in food

The most serious cause of food degradation is related to the attack of free radical species: in particular researchers have individuated a group of oxygen radicals defined “reactive oxygen species” (R.O.S.). The mechanism of food degradation is practically the same described above: a radical attack the food leading to the development of off-flavors, color and flavor changes, besides nutritional losses (15). The presence of an antioxidant can block, or at list decelerate, the kinetic of degradation increasing the shelf-life of a product; with the term *shelf-life* it is intended the period in which a food, conserved in proper conditions, maintains its nutrition characteristics in term of taste, flavor and nutritional facts after packaging. The shelf-life enhancement of a product has a great economic impact: the product can be exposed in shelves for a longer time reducing the production of wastes. Antioxidant can be provided to food in two ways: mixing with food during the transformation process or adding to the packaging.

The first approach is the most consolidated and older: in fact, the use of spices for the curing of meet is a practice that was normally used since the 20th century B.C., for instance the use of cinnamon and black pepper is widely reported in historical documents (16). Even though since 20th century B.C. the food conservation industry has been evolved, adding preservative to food normally occurs; citric acid, phosphoric acid, vitamin C are very few examples of a long list which includes the additives used as food preservative. On the other hand, customers' concern towards additives in food has dramatically increased, in particular during the last years when a lot of researches have been published demonstrating the negative effects of some additives on human health. The awareness of customers' concern pulls up the industrial and academic research to discover a new way to prevent food degradation leading to the developing of "active packaging". Even though the first concept of active packaging was originally used up to late 60s with the work of ethylene scavenging insert in banana packs (17) the widening of the study, and therefore the interest of academic world in this field, began during the 90s.

Active packaging has an active role in food preservation whereas the normal concept of packaging is related to a passive physical barrier against external environment. Packaging industries have developed a lot of devices able to enhance the shelf-life of foods: oxygen scavenger, moisture control, ethanol releaser, radical scavenger, antimicrobial scavenger etc., The role of antioxidant, in the active packaging field, is circumscribed to the radical scavenger devices which present the ability to inhibit the reaction of R.O.S. The common approach consists in mixing the polymeric matrix with active substances that will gradually migrate into food controlling the activity of food. This approach requires edible substances that minimally affect the food in terms of taste and aspect; most diffused additives (according to the European Union legislation, Directive 2006/52/EC) are synthetic antioxidants whose use is authorized only in processed food; few examples are: Butylated hydroxytoluene (BHT), Butylated hydroxyanisole (BHA), tertbutylhydroquinone and propyl gallate.

Besides the use of synthetic antioxidants, the possibility to use natural antioxidant were investigated during the last years: a lot of active packaging prepared by adding α-tocopherol, caffeic acid, catechin, quercetin or carvacrol (natural substances extracted from cinnamon, oregano and in general from plants) in polymers are reported in literature. The use of these substances has many advantages, first of all their intrinsic biocompatibility and also the appeal on customers' feeling: it is a common idea that "natural" is synonymous of "healthy" and "non-toxic" hence the choice of customers will be oriented on these products.

The concept of food packaging and active packaging will be fully explained in the chapter dedicated to the packaging.

1.3 Antioxidants for human diet

During the 50s, Denham Harman conceived the free radical theory of aging (FRTA) assuming that the free radical of oxygen (ROS), produced during cellular respiration, can cause cumulative damage leading to loss of functionality and actually death (18). This concept was further spread by Harman himself, including not only aging as a cause of ROS exposure but also a lot of diseases such as cancer, arthritis, atherosclerosis, Alzheimer's disease, and diabetes. At the beginning of 70s, the theory was modified again: the exposure to radicals remains the cause of aging and disease but Harman changed the site where the reaction occurs. In fact, he supposed that the reaction of cellular breathing, that normally occur in mitochondria, promotes the production of ROS which can react with protein and lipids and most important with mitochondrial DNA (mDNA). (19)

Free radicals can react with many substrates in order to pair their electron shells, the most relevant issue is related to the radical transfer to biological molecules which loss, or worst change, their activity. For instance, the reaction ROS with DNA can induce DNA cross-linking that could promote cancer growth; another reaction of ROS can promote reaction of coupling of protein with lipid that is the cause of wrinkles (20).

The first approach, in order to overcome these problems, was to administrate high quantity of natural antioxidant, such as vitamins A, C, D, beta-carotene and superoxide dismustase (an enzyme able to regulate the dismutation of superoxide radical $O_2^{-\cdot}$) in order to tackle and reduce the effect of ROS reaction. Many evidences reported how this approach did not have effects, rather, in many cases the use of antioxidants promoted the aging effect. Even if theoretically the assumption of antioxidant should reduce the effects of ROS, the results reported shown opposite results: scientists discovered that a high quantity of exogenous antioxidant could reduce the capability of cells to auto-regulate ROS. This process, normally called hormesis, is a spontaneous adaption to external stress induced in human body: it auto-reacts in order to restore the normal condition “training” the organism to tackle future stress. In the case of disequilibrium of redox ambient in mitochondria, a spontaneous reduction of ROS can occur with enzyme dismutase but, if the antioxidant species are introduced with supplements, the temporary ROS excess were overcome but the spontaneous adaption is inhibited. Hence, the organism will not be able to manage future radical attacks and the effect of aging are, indirectly, promoted. On the other hand, a low concentration of antioxidants can avoid the inhibition of hormesis helping the organism to restore the perfect condition. Mechanism

of antioxidant action can include suppressing reactive oxygen species (ROS) formation, either by inhibition of enzymes or by chelating trace elements involved in free-radical generation, scavenging reactive species, and up regulating or protecting antioxidant defenses. (21) (22)

Many antioxidants are presents in fruits and vegetable such as vitamin E (tocopherol), vitamin C (ascorbic acid), β – carotenoids, polyphenols etc., but in many cases, the diet cannot supply the need of antioxidant required for correct redox equilibrium. Elevate stress due to the life style (i.e. high intensity training, smoke, unhealthy habits etc.) can modify the standard request of antioxidant and the use of supplements filled with extract from natural sources could be a great solution.

The academic world has a great interest in this field: in fact, the key words “antioxidant supplements” give almost 50000 results related to publications on scientific journals and patents; the great interest the antioxidant supplements receive from the academic research, is related to the high economic impact that the supplements industry have: economists have estimated that the industry of antioxidant supplements is a business of 23$ billion (n.d.r. 2015) (23).

2. Food Packaging

Food conservation has always been one of the most important goals for humanity; since the prehistoric era, men always tried to find a way to enhance the time duration of a food avoiding problems related to the food taste and to health safety. The first approach to food conservation concerned the use of preservatives: salt and spices were the first preservative for meat, fish and vegetables. Also the curing process, that is already used for the production of hams and cured meats in general, required the use of salt and spices:

- salt, reducing the amount of water, permits a prolonged “shelf-life” avoiding the bacterial growth (24)
- spices, which contain a lot of antioxidant substances, give new flavors changing the taste and sometimes covering the off-flavors of rotten food, in particular of meat (25).

During the centuries, the technology of food preservative has evolved a lot: in particular, the food conservation was revolutionized during the ‘40s when the diffusion of fridges allowed a great part of population to conserve, for a longer period, foods in their houses. Besides the development of this technology, another need was spreading among people: since with fridge it is possible to conserve foods for many days, customers needed devices for goods transport. The modern concept of packaging was born.

Although the developing of food packaging started in the ancient era with use of paper as wrapping for spices dates back to 1035, it was only in the 18th century, when tinplate cans were firstly used to package snuff, that the modern concept of food packaging was developed. The most used materials were metals, paper and glass. During the 19th century, corrugated paper was invented and patented and the first pre-cut paper box was produced. The great improvement in packaging technologies occurred during the 20th century, when plastic was invented: plastic has great advantages in comparison to the other materials, for instance plastic is water resistant, it has low weight and it can be coupled with other materials (paper, metals, wood, pottery etc.) enhancing their properties.

After this brief historical overlook on packaging world, it is time to define the packaging concept: it can be described as a coordinated system of preparing goods for transport, warehousing, logistics, sale, and end use. The meaning of this definition leads to define at least three different levels of packaging:

1. Primary packaging: it is the material that first wraps and holds the product and it is normally the smallest unit.

2. Secondary packaging: it is the material that holds and groups the primary packaging. It normally helps the customer to transport goods unit to home.
3. Tertiary packaging: it is the latest packaging unit and its purpose is to facilitate the transport for the big distribution.

An example of these three units is: a can is the primary packaging, the polyethylene film that wrap cans is the secondary packaging and the pallets used for the big distribution are considered as the tertiary packaging. According to the scopes, materials features must be changed; in particular primary packaging material should be tailored in order to obtain a material able to be in contact with the goods: in the specific case of food packaging, the material cannot contain substances that could modify nutritional facts, taste, or worse, to jeopardize consumers' health in general.

Packaging industry has an estimated business worth up to 797$ billion in 2013 and economists have forecasted that it will reach 975$ billion by 2018. This huge amount of money leads to a great interest in this field; in particular, companies and academic have great interest in developing new materials able to improve the preservation of goods. If at the beginning the aim of primary packaging was to passively protect goods against external ambient effects, the current trend is a packaging system able to interact with external environment (radical, oxygen, lights etc.) preventing degradation: this is the latest step of the evolution of packaging technique. This new packaging approach, developed during the latest 30 years, is called "intelligent packaging" or "smart packaging".

2.1 Smart Packaging

The smart packaging is the newest approach in packaging field; in particular, it was developed for food and pharmaceutical products that have an elevated instability and, in many cases, requires specific condition for conservation. The active packaging acts not only as an inert barrier, as all packaging devices do, but it also has an active role in food and drug preservation (26). This is one of the most promising strategies for food preservation and there are many devices that are already used: oxygen scavenger bags, ethanol emitter, antibacterial coating, moisture control, radical scavenging etc.

2.1.1 Oxygen scavenger

Oxygen scavenger devices are commonly used in food packaging; they are able to reduce the amount of oxygen in packed food preventing the oxidation of products. The first example of oxygen scavenger devices, patented in 1869, was an alkaline solution of pyrogallic acid in an air-tight

vessel (27); even if this device could be a good solution it cannot be used in modern food vessel therefore, scientists have studied and developed new solutions. Modern oxygen scavengers are metal powder-filled sachets able to react with atmospheric oxygen. The trigger of the reaction is moisture: in anhydrous condition the oxidation of metal powder does not occur whereas in wet ambient the reaction begins. The most used metal is iron, due to its low toxicity, that is able to reduce the concentration of oxygen below to 0.01% (the complete oxidation of 1g of iron powder can remove up to 300cm^3 of oxygen). Other metal powder can be used for this purpose, for instance Cobalt, and other organic substances like ascorbic acid derivate. (28)

2.1.2 Moisture Control

The presence of water is the fundamental requisite for bacterial and fungal growth, therefore a control on moisture level can enhance the shelf life of many products. Moisture control devices are probably the most diffused and famous active packaging devices. In fact, it is possible to see this kind of device in a lot of packed products not only in food packaging, electronic devices require low quantity of humidity therefore the need of a system able to reduce, or at least to control, the humidity. The most diffused substance, used for this purpose, is silica gel [formula $(SiO_2)n$], an inorganic polymer obtained by acid dehydration of sodium silicate.

2.1.3 Ethanol Emitter

The bactericide power of ethanol is well known and, for many years, it was used as a disinfectant in a lot of processes; ethanol is normally used as preservative in many foods in particular it is widely used for sliced bread (the high level of moisture permits the microbial growth, moreover sliced bread cannot be packed using modified atmosphere due to its intrinsic high porosity that keeps inside the slices a lot of oxygen). Ethanol can be directly sprayed on products obtaining a concentration of 0.5-1.5% but the use of devices, able to release ethanol, could be a great solution. Ethanol is normally encapsulated in polymeric film; for instance, the most produced ethanol emitter film (Ethicap®) is a composed material made of food grade alcohol (55%) and water (10%) adsorbed onto silicon dioxide powder (35%) and contained in a sachet made of a paper and ethyl vinylacetate (EVA) copolymer laminate.

2.1.4 Antimicrobial coating

The coating with edible polymer containing antimicrobial agents is one of the most promising techniques in this field: for instance, nisin (polycyclic antibacterial peptide) has a great impact on the growth of Staphylococcus aureus and Listeria Monocytogenes (29) and, when it is encapsulated in methylcellulose (MC) and hydroxypropyl methylcellulose (HPMC), nisin can be released

reducing bacterial growth. Nisin is only an example, many substances (also natural extract of cinnamon, oregano etc.) can be encapsulated reducing the possibility of microbial food attack.

2.1.5 Antiradical packaging

Natural based (ascorbic acid, ferulic acid, quercetin, and green tea extract, tocopherol etc.) or synthetic based (BHA, BHT etc.) antioxidants can be incorporated in polymeric film. Using the same mechanism explained for antimicrobial coating, antioxidant can be released during the shelf-life time avoiding the oxidation of foods (30) (31) (32) (33) (34) (35).

2.2 Active packaging Limits

Even if active packaging seems to be a great solution for the enhancement of food shelf life, it has its own limits. In all the cases previously mentioned, the active part of the packaging is a component that is added during the production of the material and therefore can migrate into food. Although the migration is the trigger that allows active packaging to work, the leakage of active substances has tremendous impact on treatment duration: the release has its kinetic and, when the active substances are completely released, the activity decreases leaving the products free of protection. The other problem related to the migration of active substances is related to the variation of mechanical properties of the material: the active substances, generally low molecular weights species, act as plasticizer and their migration can modify the mechanical properties of the material (31) (36). Even though all technical problems are fixed, the most relevant limit of active packaging is the perception of customers about the “invasion” of chemistry in their products; in other words, the common idea is that the additives can modify and alter packaged products leading to a low quality product or worse to non-safe products. In order to limit these phenomena, two different strategies have been pursued:

- use of natural substances, that are easily well accepted by customers.
- development of materials that are intrinsically active, bonding to the polymer backbone the active moiety.

These two points are the focus of this Ph.D. work that aims to obtain new materials intrinsically active bonding to polymer chains the active substances deriving from natural sources.

2.2 Biodegradable packaging

Market researches have estimated that every year industry produces 78 million metric tons of plastic for all packaging: 40% of that are landfilled and 32% are leaked into the environment. Another

interesting datum is related to the amount of plastic recovered for further application: the recycled plastic is only the 28% (37). These dramatic data promote the development of a new environmental-friendly conscience among people, clarifying the need of low environmental impact materials. The idea, firstly developed at the end of 80s, was to find new materials based on removable sources able to replace the conventional oil-based materials (PET, PE, PP, PVC etc.). During these years, several scientific publications have been produced and many materials have been also industrially produced; one the most successful biodegradable polymers is poly(lactic acid) but there are other examples of biodegradable industrial plastics: MaterBi®, Ecoflex®, Ecovio® etc., Although the results were impressive – all of these plastics are completely or partially bio-based and can be rapidly decomposed – all of such materials have a lot of disadvantages that limit their applications. The mechanical properties, in comparison with other industrial material like PE, PP, PET, PAs, do not allow biopolymer to substitute the common oil-based materials; Young's modulus of PLA is approximately 3500MPa whereas the same modulus, registered for PET is in the range of (200 to 4100) MPa (38); also gas permeability is higher in bio-polymers. For instance, PLA has an oxygen permeability, measured as $cm^3mm/(m^2$ day atm), in the range of 3.5–15 whereas PET value is in the range of 1–5 and polyamide value is 0.1–1 (39). Nevertheless, environmental-friendly conscience pushes on towards the biopolymer research leading to the development of materials able to compete with oil-based polymer in some fields. It is possible to find biodegradable material used for the production of shoppers, disposables and primary food packaging. Every day a high quantity of food is wasted and it should be disposed separately from packaging material or disposables; the separation process requires a lot of energy and therefore costs. Biodegradable packaging can help to overcome the problem: packaging material and disposable could be disposed together with food. This approach is already used for biodegradable shoppers used for food-waste collection: they are made with MaterBi®, a bio-based and biodegradable plastic produced by Novamont S.P.A., The shoppers are directly composted with wasted food avoiding all the problem concerned to the separation of polyethylene shoppers and increasing the yield of compost plants.

2.3 Shelf Life Study

Shelf life is defined as the time that a food can be stored without becoming unfit for use, consumption, or sale. The shelf-life of a food begins from the time the food is produced and/or packed (40). The main objective of food packaging is to protect food products, particularly from oxidative and microbial spoilage and to extend its shelf-life; the standard concept of food packaging involves a passive barrier whereas the latest development in this field aims to produce packaging with an active role on shelf-life (31).

The shelf-life is affected by multiple factors: exposure to light, heat, moisture, transmission of gases, mechanical stresses, and contamination by micro-organisms. The study of a combination of these factors permits to determine the best preservative conditions, in terms of health safety and food and beverage taste and properties. The main result of a shelf-life study is the “expiry date” but it is not the only one. Shelf-life is a fundamental tool for customers and sellers to obtain an overarching knowledge of food characteristics and how processing, storing and transport can affect them.

Shelf-life study strictly depends on food characteristic (quantity of fats, moisture content, food processing etc.) therefore it is not possible to develop a unique general protocol applicable for all kind of foods. Nevertheless, a general systematic procedure can be created to approach to shelf-life study; an example of a flow chart is reported in figure 2.1: this flow chart was written by “Food Safety Authority of Ireland”.

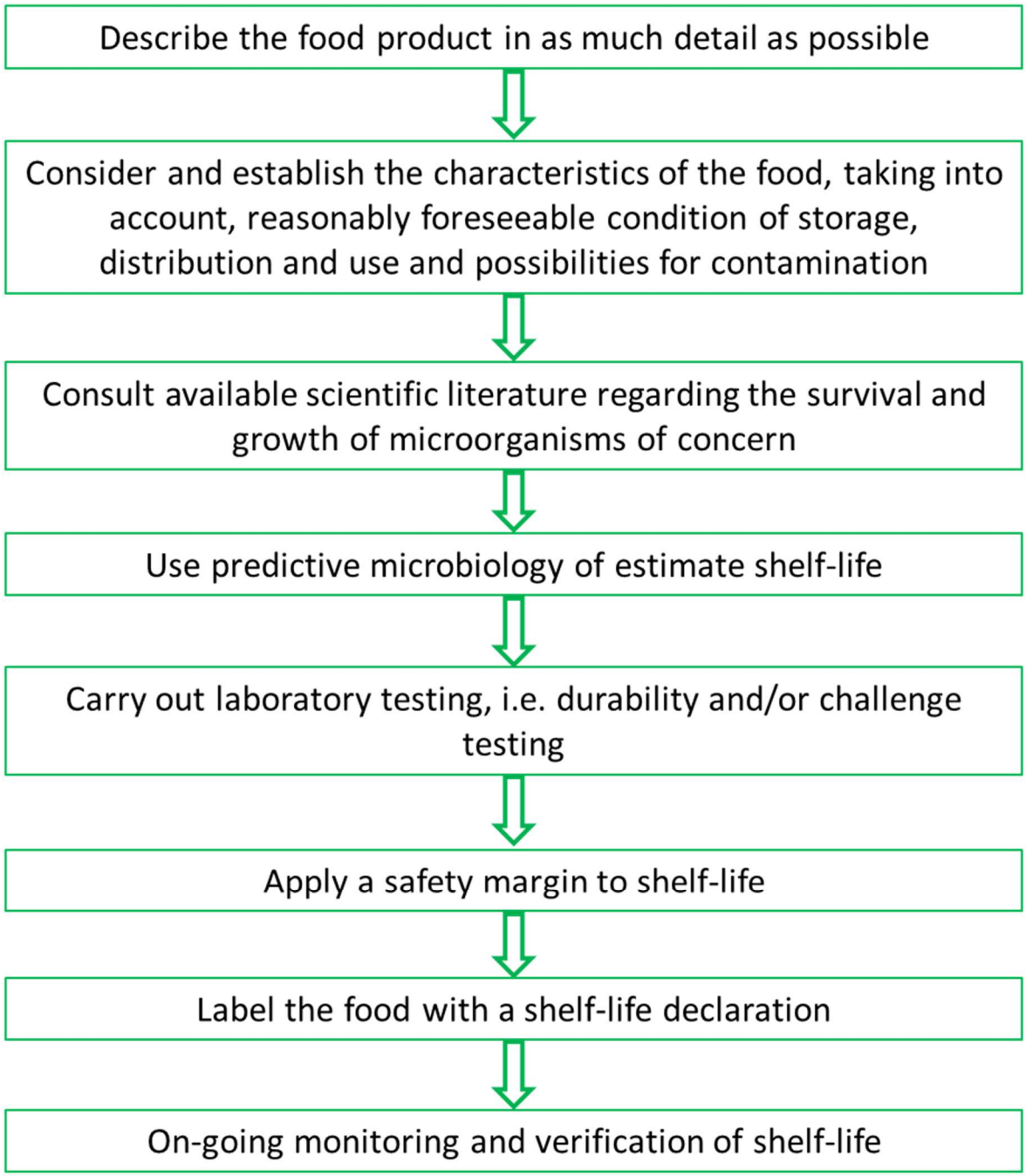


Figure 2.1: flow chart of shelf-life assay procedure

Active packaging, as was previously explained, aims to enhance the shelf-life of a product therefore shelf-life study can be performed in order to evaluate how a new packaging method can modify it. Active material or devices are targeted for specific features therefore (antioxidant, oxygen scavenger, antimicrobial etc.), choosing an appropriate food as a standard, is possible to determine how the active packaging works. The previously protocol include several aspects that, for preliminary studies on new packaging methods, can be neglected. For instance, the shelf-life study, for the development of new antioxidant materials, can neglect the microbiological assays focusing on the study of the oxidation level; in this case the choice of “standard food” will be targeted on

high fatty matrix since fat is the easy oxidizable substratum in food; on the other hand, the shelf-life study for the developing antimicrobial devices, can ignore the oxidation aspects focusing on the aspects which affect the bacteria and mildew formation.

3. Antioxidants

As explained in chapter 1, the antioxidants have a multipurpose role: they can act as radical scavenger for packaging application and, with the same mechanism, they can act as radical scavenger in human body in order to restore the normal redox equilibrium.

The radical scavenging mechanism of phenols (the mechanism is shown in figure 1) is widely studied and there are many literature examples. The aromatic hydroxyl moiety is able to react with radicals due to its relatively high acidity, this reaction leads to the formation of a new radical stabilized by the aromatic ring resonance. The new radicals can react with water obtaining new stable species. (41) (42) (43) (44) The scheme proposed in figure 3.1 is specific for phenols but it can be assumed as a general radical reaction mechanism also valid for substituted phenols. The substitution of phenols can seriously modify the activity against radicals; for instance, the presence of an electro attractive moiety like RO- can increase the radical scavenging of a phenol a lot. This is the case of vanillyl alcohol (VA) and tyrosol (Tyr): the radical scavenging, measured with DPPH assay, is 24% (45) for Tyr, which not presents electro attractive moiety, whereas the radical scavenging obtained for VA is significantly higher reaching 96%.

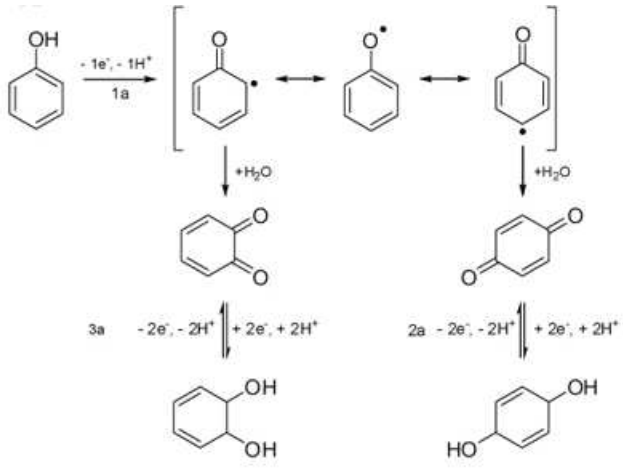


Figure 3.1: general scheme of phenols oxidation via radical attack

The phenols used in this work are: tyrsol (Tyr), vanillyl alcohol, oligo-tyrosol (oligo-Tyr) and Ellagic Acid (EA). The structure and the properties will be plenary explained further in the paragraph.

3.1 Tyrosol

Tyrosol, or 4-(2-Hydroxyethyl) phenol according to IUPAC, is a low molecular weight phenol present in a lot of natural sources, mainly in olive and olive oil: its structure is shown in figure 3.2. In many cases, tyrosol, or its derivates such as hydroxy tyrosol, is extracted as acetylated form: the increased lipophilicity, due to the acetyl moieties, enhances the activity thanks to the higher permeability through cell barrier. (46)

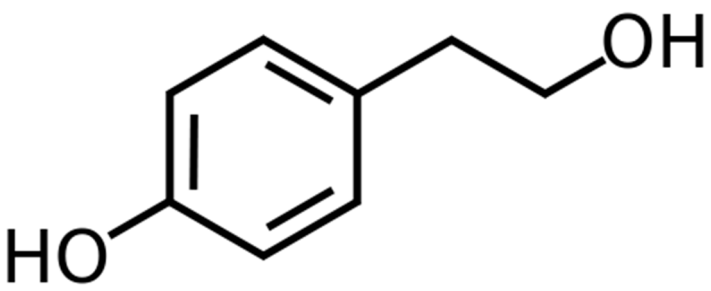


Figure 3.2: molecular structure of Tyrosol

Antioxidant properties of tyrosol are well-known (47) and a lot of studies were conducted in order to investigate how these properties can enhance its biological activity. Tyrosol was investigated as histamine suppressor in allergenic disorder treatments (48), also tyrosol anticancer properties were investigated in particular as olive oil extract (49). Tyrosol antimicrobial properties were also reported: it can act as biofilm suppressor in human oral cavity. (50)

Oxidative enzyme mediated reaction of phenols permits to obtain a more or less homogenous mixture of oligomeric species with enhanced properties regard to the original molecule. Phenols coupling can be achieved with other oxidant reactives (for instance ferricyanide and persulfate) but the enzymatic catalysis works with high efficiency both with phenols and substituted phenols under biomimetic condition []; moreover, the enzymatic process obtained the attention of researchers due to its high biocompatibility and environmental friendly process.

Oligomer of Tyrosol (OligoTyr) were firstly obtained in 2015 by the group of Prof. Napolitano using horseradish peroxidase and H_2O_2. The structure has been determined via CP - MS NMR and via size exclusion chromatography: the oligomer, whose structure is shown in figure 3.3, has a polydispersity index (D) of 1.28 and a weight average molecular weight of 2861 Da; the narrow D indicates that the molecular weights of oligomers are homogenous.

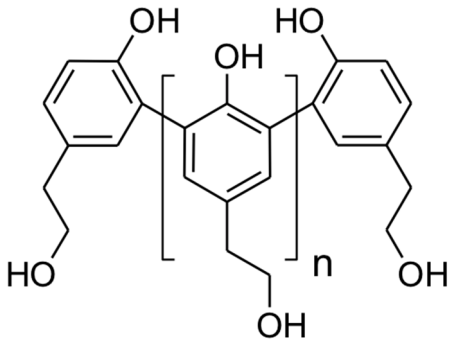


Figure 3.3: molecular structure of oligo-Tyrsol determined via CP-MS-NMR

The antioxidant properties of oligomers of Tyrosol (OligoTyr) were assessed using different tests: 2-Diphenyl-1-picrylhydrazyl (DPPH) assay, Ferric reducing/antioxidant power (FRAP) assay and Hydroxyl radical scavenging assay. Results reveal how the oligomerization can increase the antioxidant properties of the new synthetic polyphenol: DPPH and Hydroxyl radical scavenging increase to 100% whereas FRAP index practically does not change. As was previously mentioned in this paragraph, Tyrosol has shown many properties related to its antioxidant skills: for instance, tyrosol can promote the human ossification process reducing the free radicals that are normally produced in a damaged part. Due to their similar structures, it has been supposed that oligoTyr could have at least the same properties of Tyrosol: the results shown the alkaline phosphatase is mainly promoted by the oligomeric species than the Tyrosol.

3.2 Vanillyl Alcohol

Vanillyl Alcohol, or 4-(Hydroxymethyl)-2-methoxyphenol according to IUPAC.

Lignin is one of the most abundant waste products and it represents the 20% of total biomass nevertheless almost the 98% is burned. One of the most abundant building blocks of lignin is identified as the β-O-4 linkage block (51), the structure of the block is shown in figure 3.4. The oxidative extraction, this technique represents one of the goal of the bio-refinery, permits to obtain vanillin and many others chemicals. Vanillin is the aroma of vanilla that is widely used in food industries as flavor for sweets (ice cream, chocolate, bakery products etc.) and represents a great substrate for pharmaceutical synthesis (52).

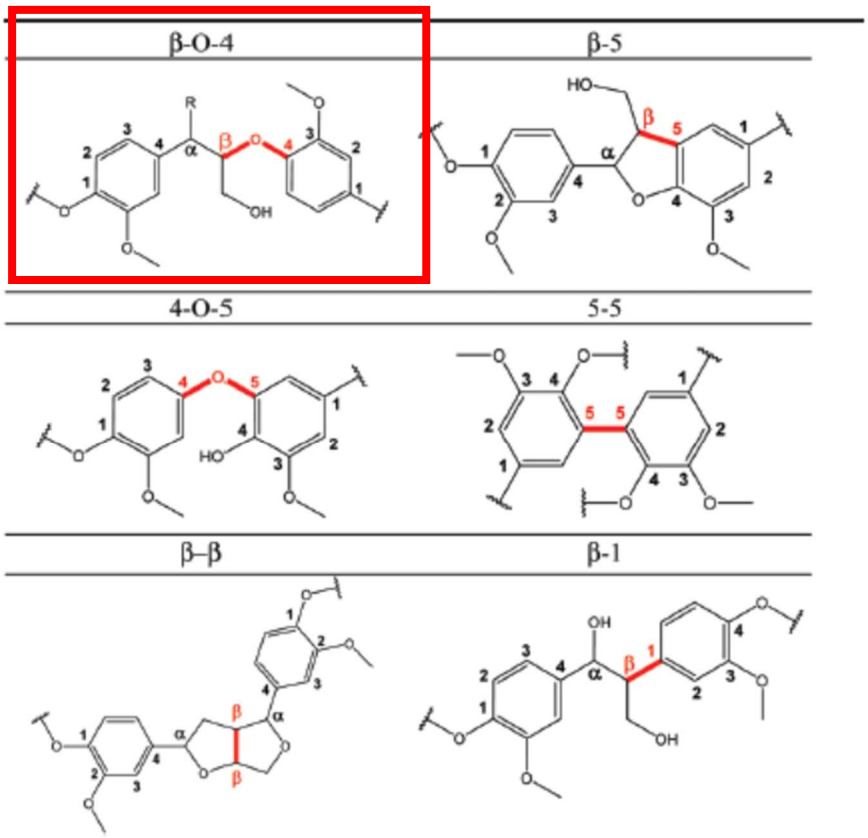


Figure 3.4: structure of lignin repeating units (51)

Nowadays the 85% of vanillin supply is obtained via chemical oxidation of petroleum-derived guaicol transformation and, the other 15%, is obtained by biotechnological extraction from lignin. The synthesis from guaicol was firstly proposed by Karl Reimer in 1876 using the Reimer–Tiemann reaction that permits to obtain ortho-formyl derivate of phenols. The scheme proposed in figure 3.5 explains the Reimer-Tiemann reaction applied to guaicol that gives 2 different products: vanillin and the o-vanillin (53).

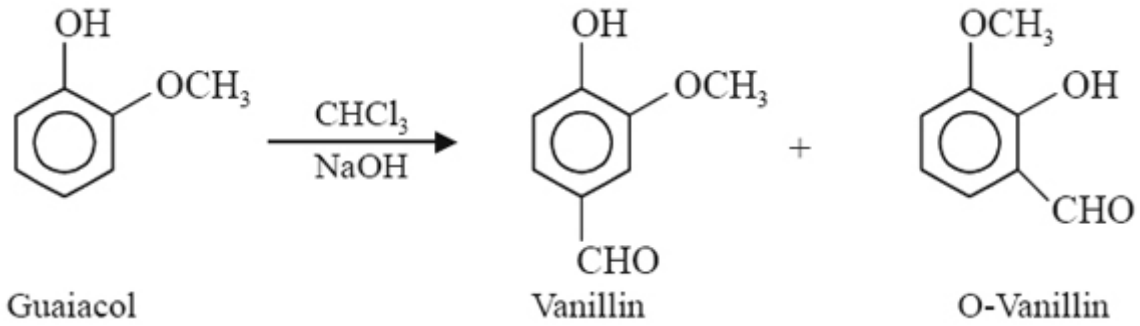


Figure 3.5: Reimer-Tiemann synthesis of vanillin via guaiacol oxydation

Besides this technique, the biotechnological synthesis of vanillin from lignin was developed in order to valorize the great amount of biomass. Lignin has a high concentration of aromatic compounds and its conversion into fine chemicals is one of the most important goals of the bio-

refineries. (54) (55) Many techniques have been developed but one of the most studied biotransformation is from ferulic acid, the structure is shown if figure 3.6.

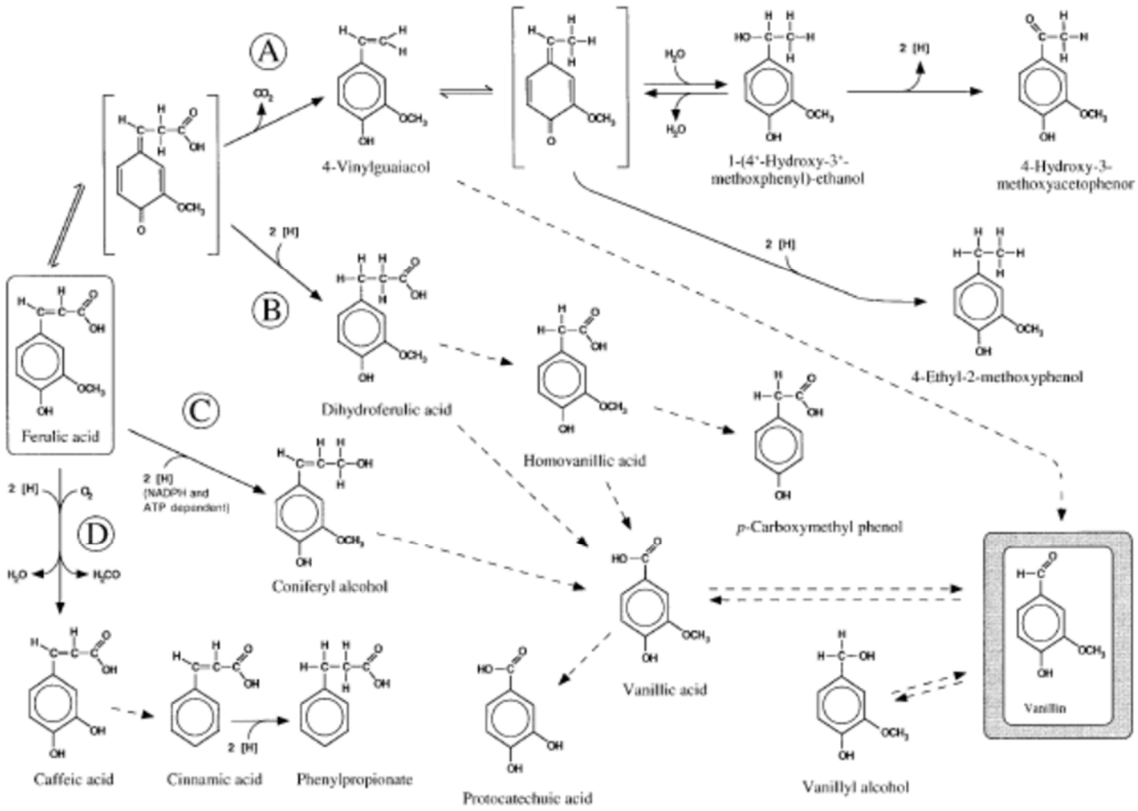


Figure 3.6: biotransformation of ferulic acid to vanillin (51)

In this complex scheme four different reaction pathways are explained:

- Path A: Decarboxylation: the phenolic acid decarboxylases enzyme begins the reaction removing the carboxylic moiety from the ferulic acid.
- Path B, C, D: reduction of ferulic acid mediated by microorganism.

Although the reaction pathways are biotransformation, the extraction of ferulic acid from lignin can be achieved only by alkaline hydrolysis therefore this ferulic acid cannot be considered “natural”. There are many studies concerning the enzyme-catalyzed extraction of ferulic acid from lignin avoiding the problem related to the use of strong alkali (potassium hydroxide or sodium hydroxide) and obtaining a green process. (51)

Another substrate used for the Vanillin synthesis is Eugenol (scheme is proposed in figure 3.6) that is the main constituent of Syzygium aromaticum essential oil and with a market price about US$5 kg^{-1}. It is a cheap, commercially available raw material for biotransformation processes. The first process was proposed in 1977 by Tasada using Corynebacterium for biotransformation of Eguenol passing through the ferulic acid and then vanillic acid. A fed-batch bioconversion process from

eugenol to coniferyl alcohol using resting cells of the fungus Byssochlamys fulva V107 was reported to yield 123 mM (21.9 g l–1) coniferyl alcohol within 36 h, with a molar yield of 94.6%. (56)

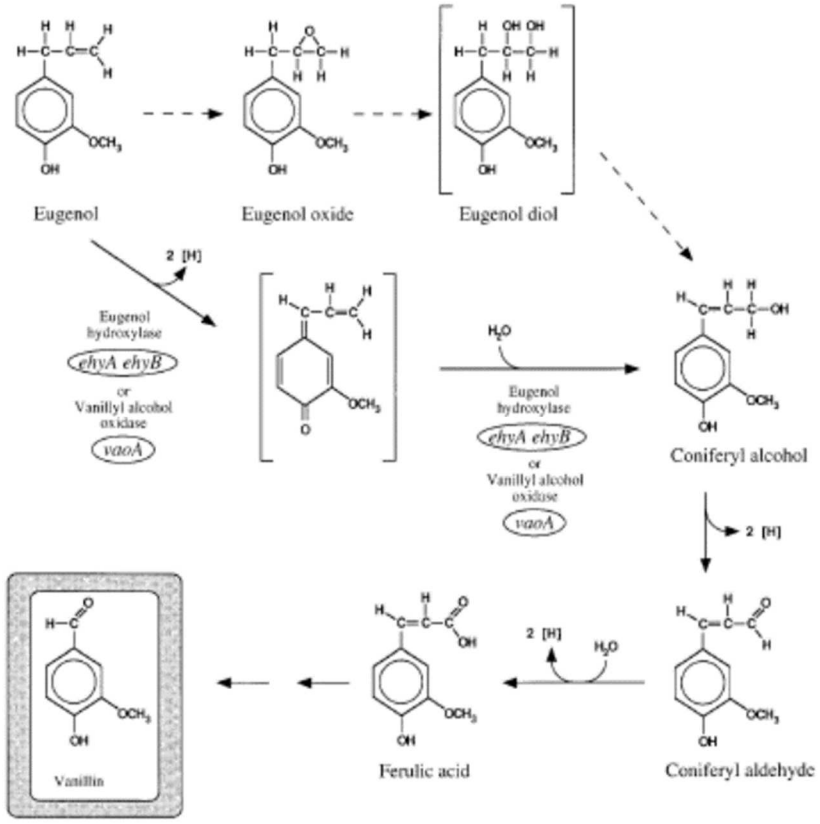


Figure 3.6: Eugenol biotransformation reactions scheme to vanillin (51)

Vanillin can be reduced in order to obtain Vanillyl Alcohol. Many literature works reports a rapid easy reduction using $NaBH_4$ or using $Zn(BH_4)_2$. (57) (58) (59)

Vanillyl alcohol is widely use as model molecule for lignin extraction model development: as was previously described, VA can be easily synthetized from vanillin reducing its cost, therefore it represents a good solution for the studies related to the development of lignin digestion model. Moreover, VA represents a valid phenolic building block for a lot of chemicals and many examples have been reported in literature: VA can be modified in order to obtain substituted bisphenol used for the synthesis of epoxy resins (60). Antioxidant properties of Vanillyl Alcohol have been already reported in literature: there are many examples about the use of VA as antioxidant for biomedical application. (61) (62) (63)

3.3 Ellagic acid

Ellagic acid, or 2,3,7,8-Tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione according to IUPAC, is a metabolite of natural phenols that are present in a large variety of fruits and vegetables, the structure is reported in figure 3.7. It was discovered by Henri Braconnot in 1831. Pomegranate juice has the highest concentration of ellagitannins, which are bioactive polyphenols, and contains the unique ellagitannin: punicalagin. (64) Pomegranate ellagitannins, and punicalagin in particular, are not absorbed intact into the blood stream but are hydrolyzed to EA over several hours in the intestine. Ellagitannins are also metabolized into urolithins by GUT flora, which are conjugated in the liver and excreted in the urine (65) (see scheme reported in figure 3.8).

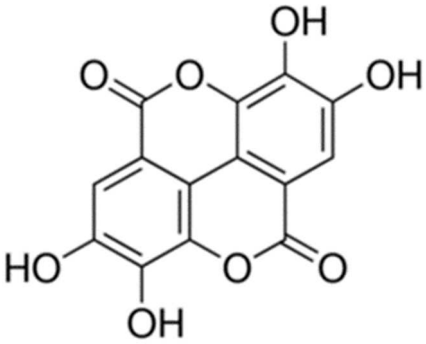
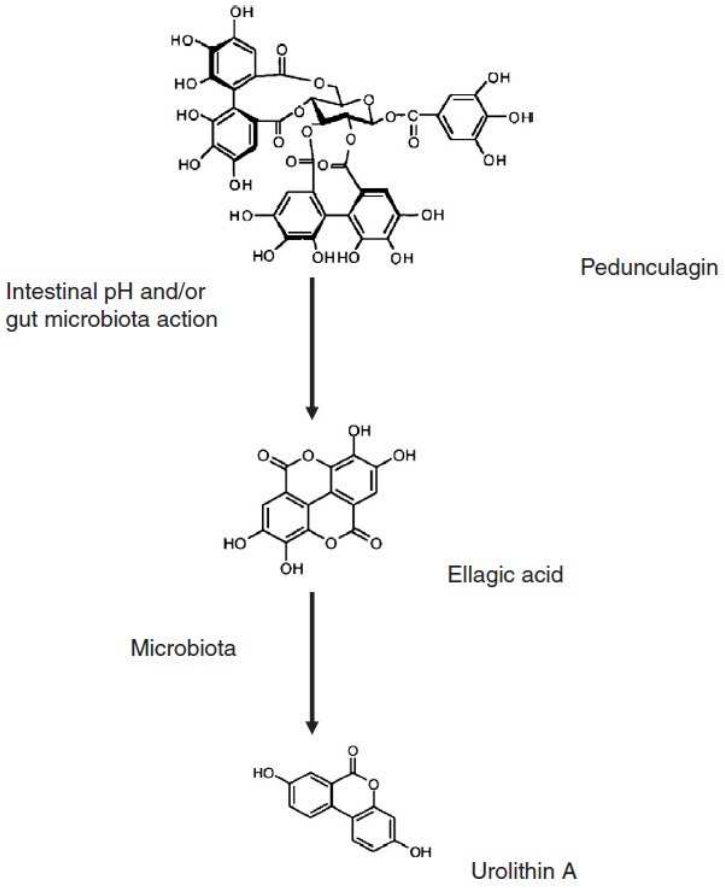


Fig 3.7: Ellagic Acid Structure

The highest levels of EA precursors polyphenols are found in blackberries, cranberries, pecans, pomegranates, raspberries, strawberries, walnuts, wolfberries, grapes, peaches and EA is also found in oaks species and in the medicinal mushroom Phellinus linteus (65). Commercial EA is obtained by chemical extraction using acid-methanol mixtures as solvents to hydrolyze the rich-ellagitannin plant materials. However, during the last years, fermentation technology has been the most used technique for the production of EA (66).

EA is one of the most active compounds derived from pomegranate and it exhibits both antioxidant effects and apoptosis-inducing activity against certain types of cancer cells in “in-vitro” tests (65) (67). In addition, epidemiological studies indicate that intake of EA-rich foods may be protective against certain chronic diseases (65). EA shows antiproliferative and antioxidant properties that have prompted several researches both in the academic and pharmaceutical world. The antiproliferative properties of EA may be due to its ability to directly inhibit the DNA binding of certain carcinogens, such as nitrosamines and polycyclic aromatic hydrocarbons (68). In addition, EA also shows antifungal properties and can be used in agriculture as agrochemical. (69) Thanks to

these anticancer and preventing-cancer effects, EA has been marketed as dietary supplement to prevent medical problems. In addition, intestinal metabolites of EA, like urolithins, are under study as anti-cancer agents and are known to effectively inhibit cancer cell proliferation like HepG2 hepatic carcinomas cells. Some studies suggest that consuming a diet rich in urolithins-precursors foods could benefit individuals undergoing liver cancer chemotherapy. (70)



Scheme 1- Methabolic pathways of EA

In addition, In vitro studies have shown that pomegranate extracts inhibit the growth of prostate, breast, colon and lung cancer cells. (71) (72)

To sum up EA has not been proven to treat or prevent cancer in humans, but it has no side effects and several researches about EA and its metabolites health benefits have been published during the recent years (more than 400 during 2016 and more than 550 during 2015, source SciFinder). Epidemiological evidences indicate that EA and its metabolites may be protective against certain chronic diseases, like heart diseases and cancer, but discrepancies are observed between *in vivo* and *in vitro* experiments. This could be explained by their low bioavailability. In fact, *in vitro* studies operate in ideal conditions and absorption, metabolism and detoxification pathways are not considered in this type of experiments. One of the most relevant issues related to food intake of EA

is its low absorption in gastrointestinal tract. In addition, some studies reported the EA detection in human plasma post-ingestion of pomegranate juice but it is rapidly eliminated in few hours (71). Moreover, EA is metabolized to urolithins, which have been reported as a less potent antioxidant if compared to EA.

Antioxidant and cancer-preventing effects of EA are strongly inhibited by the above mentioned absorption and excretion issues. Consequently, EA-rich supplements are needed to obtain its potential health benefits, but a high concentration can dangerously involve the detoxifying systems, obtaining a harmful result.

4 Polymers

The general trend of the latest years has moved on developing bio-based material, or at least biocompatible material, in order to substitute the “conventional” oil-based material: the first dreamlike approach was to obtain natural biodegradable materials overcoming the use of oil-deriving polymers. Nevertheless, it was clear that the bio-based materials cannot completely substitute the “conventional” polymers, therefore the research was also focused on the developing of new methods to obtain either monomers from natural sources or to obtain biodegradable polymers from oil-based monomers. One of the most impressive case is the development in very few years of MaterBi® (polyester based on starch derivatives) produced by Novamont S.p.a.: the production started at the beginning of 90s with only 4000tons/year in 1997 reaching the production of 120000 tons/year in 2015 becoming, in Italy, the most diffused biodegradable material. (73) The Novamont’s case is only an example cited in order to explain how the interest in bioplastic and biomaterial is a very topical issue. Many big companies, all around the world (BASF, Cargill, Dow Chemicals, DuPont etc.) have made huge efforts in this direction investing many economical resources in research and development of new bio-materials. Economists have estimated that the worth of bioplastic market in 2015 was approximately 4.5$ billion and a growth is expected to reach the worth of 5.1$ billion in 2021 (74). The bar chart in figure 4.1 shows the amount in metric tons of the global production of bio plastic.

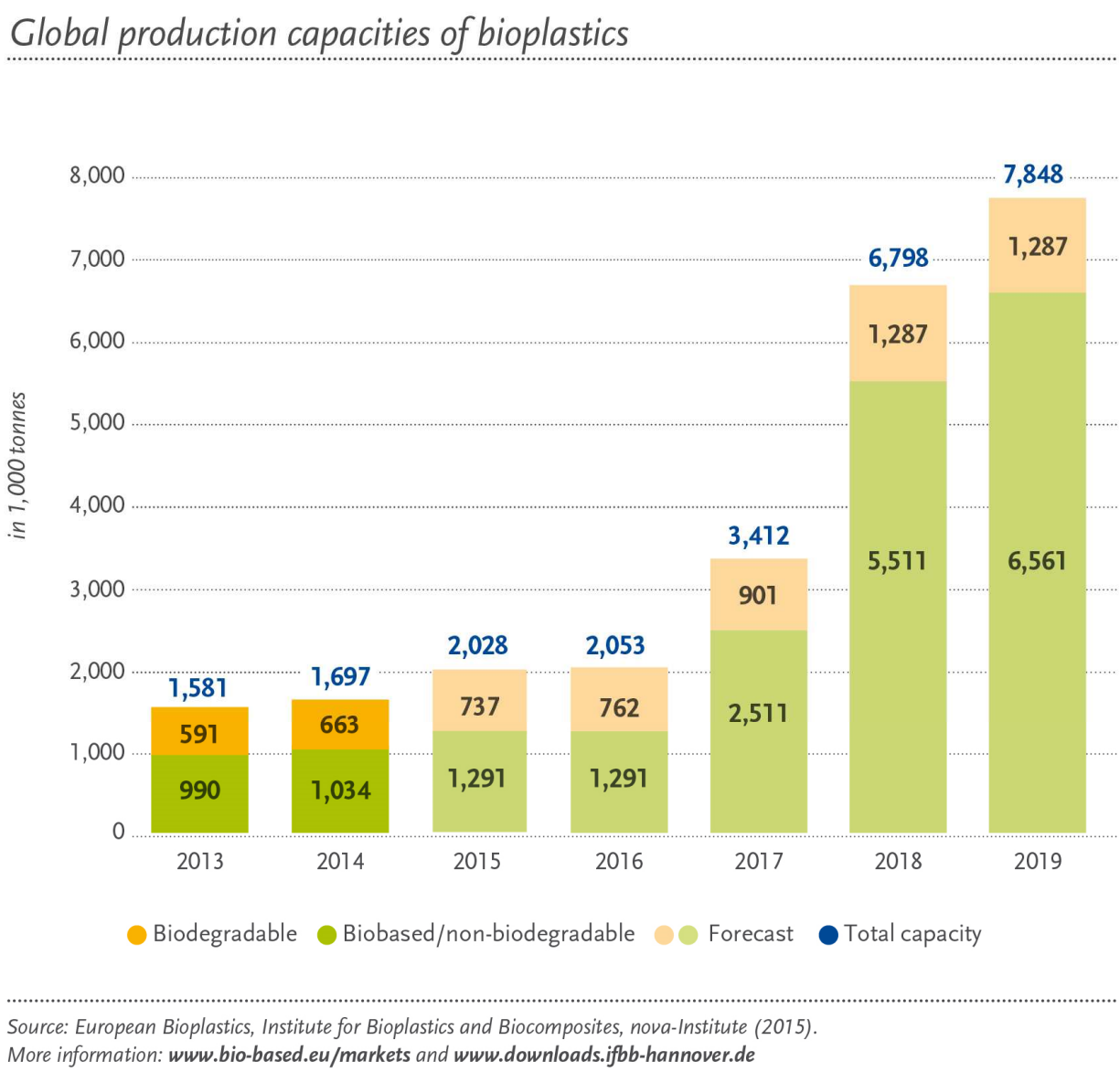


Figure 4.1: Global production of bioplastics since 2008 to 2015 and forecast from 2016 to 2019 production (75)

Besides the efforts made by private companies, also the academic world has focused its attention on bioplastic development and applications: the key-words "biodegradable polymer" generate an impressive long list of 40055 references with at least 14000 patents (the aim of these researches still be high industrially oriented).

The data provided in pie chart in figure 4.2 show that, without considering bio-PE and bio-PET that are not biodegradable polymers but materials obtained via polymerization of bio-based monomers, poly(lactic acid) (PLA) is the most produced and diffused biodegradable polymer.

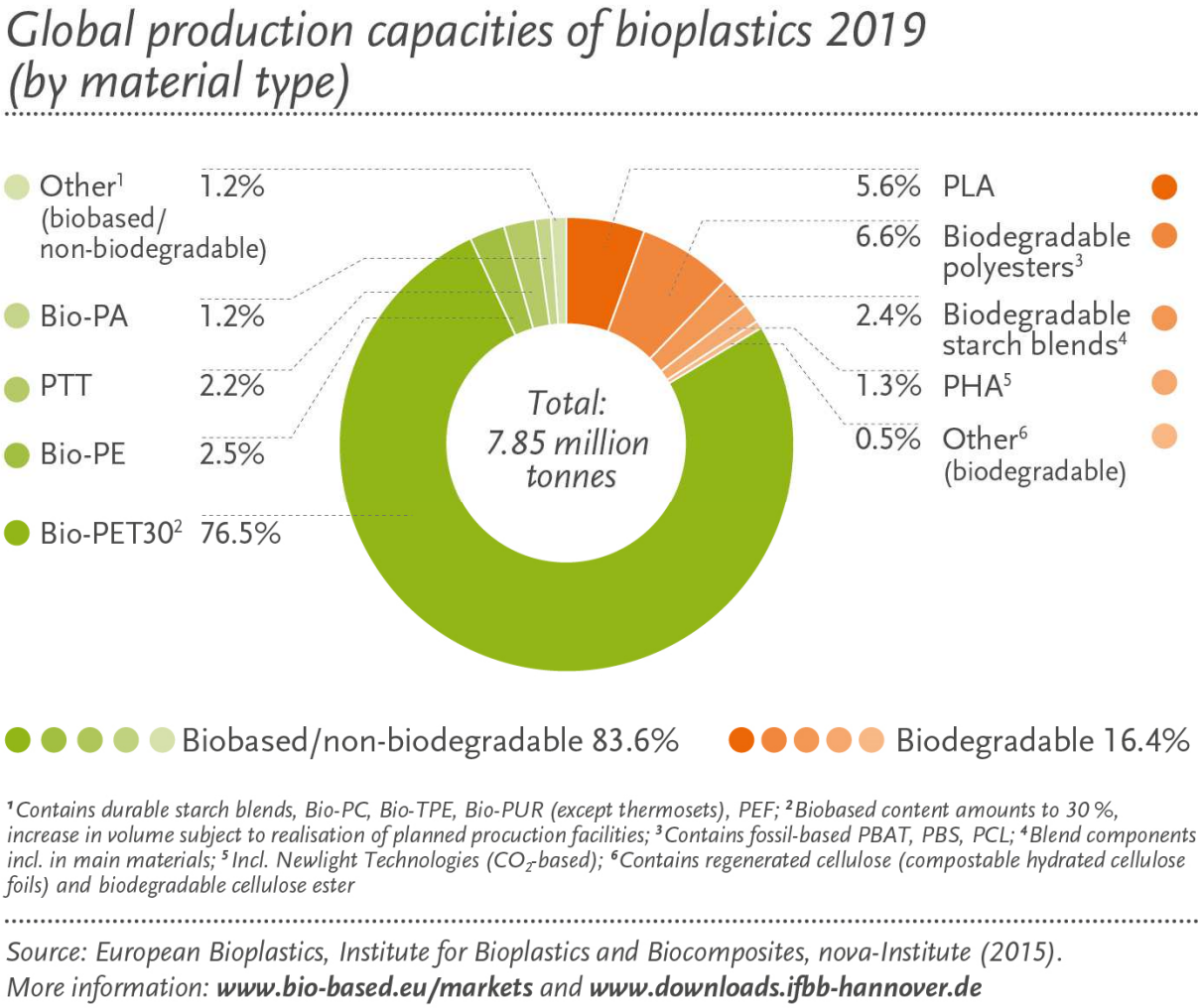


Figure 4.2: pie chart of global quantities (metric tons per year) of bio-based plastics (75)

The high interest in PLA, due to its biodegradable and biocompatible properties, has generated a market of 4.3$ billion with multidisciplinary interests in many fields: packaging, agriculture, commodities, disposable and medicine. PLA can be considered the forefather of industrial bio plastics.

The following paragraphs explain the synthesis and features of polymers used in the present work, highlighting the aspects related to this research.

4.1 Poly (lactic Acid)

Poly(lactic acid) or poly(lactide) (PLA) is one of the most produced biocompatible and biodegradable polymers. Since it was synthetized for the first time, it has been considered one of the most promising biomaterials and several researches have been made to enhance its properties in order to obtain an efficient alternative for oil-based polymers. (76) (77) (78) (79) (80) (81) (82) (83) The general structure of PLA is shown in figure 4.3.

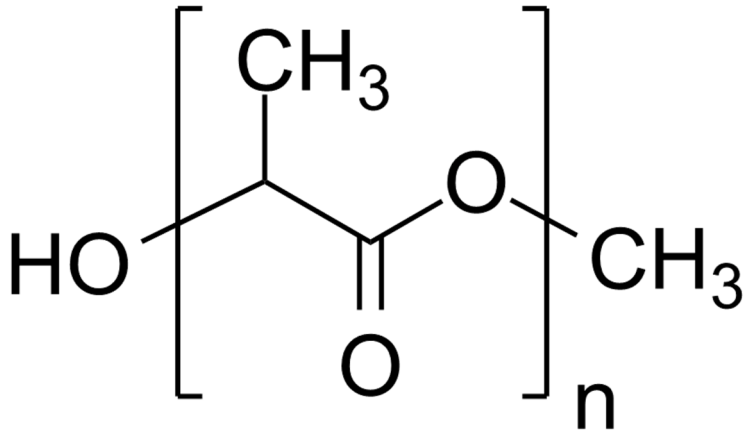
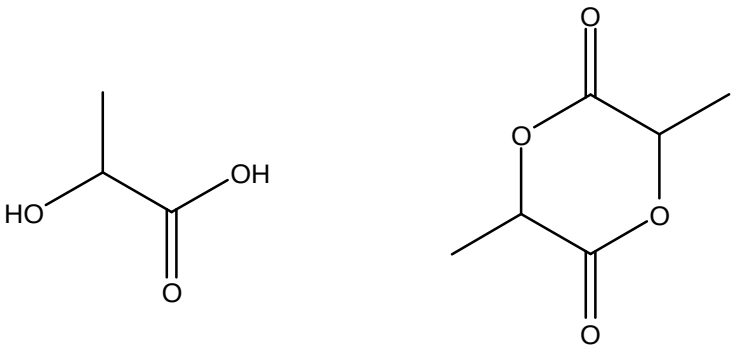


Figure 4.3: poly(lactic acid) molecular structure

PLA is a thermoplastic biodegradable semi-crystalline polymer obtained via polycondesation of lactic acid or via ring opening polymerization (ROP) of lactide, the cyclic dimer of lactic acid (the structures of lactic acid and lactide are shown in figure 4.4).



Lactic Acid Lactide

Figure 4.4: structure of lactic acid and lactide

PLA thermal and mechanical behavior strictly depends on the tacticity of polymer chains: lactic acid has a stereogenic carbon therefore it is possible to obtain atactic, syndiotactic and isotactic chains; since the polycondesnsation does not affect the stereocenter (side reaction promoted by

temperature and acidity promotes the racemization) the tacticity of polymer only depends on monomer stereochemistry. Lactic acid exists in L and D form, therefore, it is possible to obtain the LL, DD and DL lactide; the reaction of DL lactide permits to obtain syndiotactic polymer whereas the reaction of LL and DD lactide leads to isotactic polymers. Lactic acid, obtained via bacteria fermentation, is in the L form therefore the bio-based lactide has the LL configuration. Poly(L-lactic acid) (PLLA) is the standard commercial PLA.

Commercial PLA presents a glass transition temperature (T_g) between 55°C and 60°C and a melting temperature (T_m) between 160°C and 180°C. (84) The crystal structure consists of spherulitess composed of lamellae separated by amorphous regions. (85) Stereo defects dramatically affect the thermal properties lowering the Tm even to 130°C and, above certain concentration of atactic portion, the crystalline structure disappears leading to an amorphous polymer. (83)

4.1.1 PLA synthesis

The mechanical properties are affected by crystallinity, induced by tacticity of polymer: crystalline PLLA has higher elasticity values, strength, impact, low gas permeability and thermal stability in comparison to an atactic amorphous PLA.

The first reported synthesis of PLA dates back to 1932: poly(lactic acid), obtained heating under vacuum lactic acid, were synthesized in DuPont laboratories by Carothers (86). The synthesis developed by Carothers permitted to obtain only low molecular weight polymers: lactic acid, heated under vacuum, can simultaneously react in two ways:

- Polymerization
- Cyclization

The first way leads to obtain polymer chains and can be interpreted with the model developed by Flory and Carothers for the polycondensation of monomer AB, whereas the second way leads to the formation of lactide, the cyclic dimer of lactic acid. (87) (88)

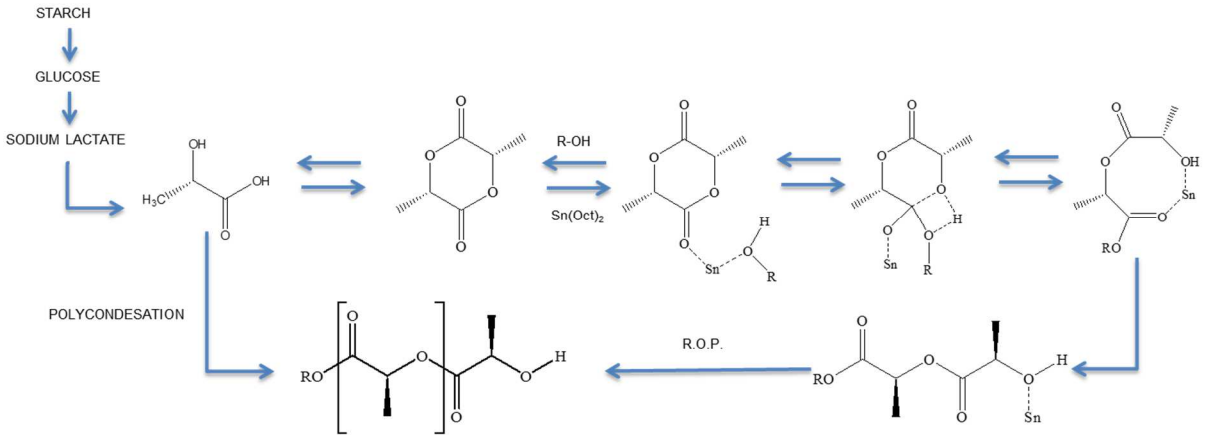


Figure 4.4: PLA polymerization process

Lactide, as well as low molecular species, is volatile in the reaction condition (i.e. heat and reduced pressure) therefore its production, and consequently sublimation, leads to promote the reaction of cyclization instead of polymerization. (89) To overcome this problem, it is necessary to provide the reactor with a reflux system in order to keep the lactide and low molecular weight species inside the reactor shifting the equilibrium by the side of polymerization. Another way to prevent lactide formation, promoting polymerization reaction, is to work using high boiling point organic solvent performing the reaction with a Dean-Stark apparatus. (90) This approach has two relevant issues:

- The use of organic solvents has a high environmental impact although they can be recovered at the end of the reaction; moreover, the use of organic solvents intrinsically increases the costs for the production decreasing the commercial appeal for the polymer.
- High boiling temperature solvents, together with acidity of reaction ambient, promote the racemization of monomer compromising the properties of the polymer: racemization leads to obtain atactic chains that are unable to crystalize.

These issues have promoted research to focus its attention on the development of another way for PLA synthesis based on the ring opening polymerization (ROP). ROP is widely used for the synthesis of aliphatic polyesters allowing a high control of molecular weights and macromolecular structures, the reaction steps are shown in figure 4.4. The ROP of lactide can be performed using metal catalysts (Sn(II), Zn(II), Ti(IV) etc.) both by organic solvent solution polymerization (THF, CH_3Cl, Toluene etc.) and by bulk polymerization. (91) Solution polymerization is normally performed for academic purpose: the proper choice of solvents, and therefore reaction temperature, permit to finely control the molecular weight and the macromolecular architecture; in this way it is possible to predict how a co-monomer can react with lactide in order to develop a model for further bulk polymerization. (92) (93) On the other hand, lactide bulk polymerization (industrial plant

conditions: inert atmosphere, 130°C<T<200°C; 90min<t<240min), even if it proceeds with the same steps of ROP in organic solvent, presents some differences:

- The higher temperature promotes backbiting reactions which affect the polydispersity of the polymer; lactide R.O.P. proceed as a multi-steps reaction that can be interpreted using the polyaddition model: polydispersity, without chain regulator, should be equal to 1. Backbiting reactions (transesterification of growing chains promoted by catalyst and T) increase the polydispersity obtaining values ≈ 1.6 or higher. (94)
- The thermal properties (boiling temperature and decomposition) of co-monomers are a crucial parameter: the evaporation of monomer modifies the feed of reaction changing the material properties; moreover, side reactions, promoted by catalyst, affect the properties of co-monomer leading to a loss of properties. For instance, anti-UV monomers have high reactivity and can degrade during the reaction, preventing the transfer of anti-UV properties to polymeric chains.

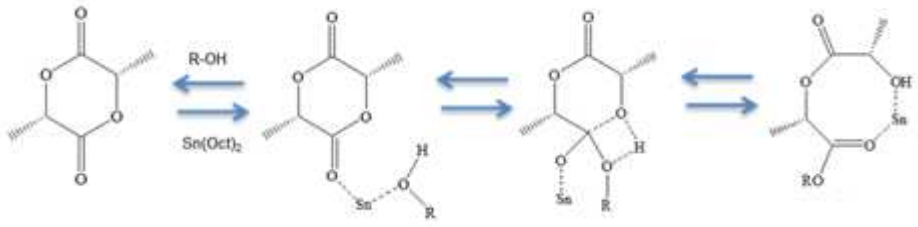


Figure 4.5: ROP reaction mechanism: coordination with Tin and further nucleophilic attack by alcohol

Lactide ROP requires a nucleophilic chain initiator and a metal catalyst to begin the reaction; figure 4.5 shows the first three steps of lactide reaction (95). Among catalysts used in ROP, compounds based on Tin(II) and Tin(IV) oxides and carboxylate, especially tin 2-ethylhexanoate [$Sn(Oct)_2$], are those that are more frequently used in the polymerization of lactide due to their versatility, ease of use and excellent solubility in melted lactide; furthermore, FDA agency approved the use of Sn(Oct)2 as food additive, that can be used in substances for food contact. The catalyst required to be activated using a species with mobile hydrogen: a large amount of compounds with hydroxyl moieties have been already investigated in literature (96) (97) (98).

The molecular weight of PLA can be easily controlled varying the amount of hydroxyl moiety in the reaction feed. Several works were made to explain the complex mechanism of lactide polymerization; the most accredited mechanism involves the activation of monomer through the coordination with Sn(II), then the polymerization can proceed through the nucleophilic attack of hydroxyl moiety of the initiator. The coordination of Sn(II) with the hydroxyl functionality of open lactide keeps the catalyst active for the propagation of the reaction. The reaction ends after the

cleavage of tin-polymer bond forming a hydroxyl end group. The use of tin as catalyst permits to obtain high molecular weight polymer with conversion higher than 90% and racemization lower than 1%. (99)

The reaction proceeds with a first order kinetics with respect to the lactide and the catalyst; the nucleophilic attack of the complex tin-alcohol to the carbonyl carbon of lactide is the rate-determining step of the ROP reaction. The kinetic can be also affected by the presence of octanoic acid which is obtained from the hydrolysis of Sn(Oct)2 that occurs at higher temperature: the presence of an acid promote the cleavage of metal-growing chain bonds slowing down the kinetic, or worst, interrupting the chain-growth process.

Two possible paths can be pursued in order to accelerate the kinetics:

- Increase the amount of initiator; increasing the concentration of hydroxyl reactive moieties the kinetic speeds up, however, the molecular weight of polymer depends on the ratio [monomer]/[-OH] therefore high concentration of alcohol leads to a steeply decrease of molecular weight.
- Increase the amount of catalyst; two relevant issues coming from this approach: first, the high concentration of catalyst enhances side reaction as backbiting and coloring. The latter, is that FDA imposes 300ppm of tin as the highest concentration admitted for food contact material therefore high concentration of catalyst dramatically reduce application fields.

PLA, obtained via ROP of lactide, can be further polymerized using the solid state polymerization (SSP). (100) (101) (102)This approach aims to increase the molecular weight of polymers and to eliminate the byproducts that may be present; SSP is industrially used for other polyesters in particular for PET or for polyamides. PLA SSP is normally used not only for the synthesis of PLA from oligomers but also for the increase of molecular weights and the removal of residual monomers and low molecular weight species (monomers and low molecular weight species act as plasticizer altering the rheological and mechanical properties of materials).

SSP is a very simple process that involves three key parameters: mobility of chains in amorphous phase, kinetic of the reaction and removal of volatile compounds to shift the equilibrium of the process. The polymer is heated at temperature above the Tg but below Tm in order to increase the mobility of the chains and promote the reaction among the end-groups of chains in the amorphous phase. Moreover, the removal of volatile products (monomer and other reaction products) is enhanced by vacuum or high inert gas flow. Since the SSP reaction is performed at temperature lower than bulk polymerization and considering the reduced mobility of polymeric chains, the time

required to obtain high molecular weight is very long. This technique is widely used to increase the molecular weight of polymers obtained via polycondesation of lactic acid whereas, in the case of polymer synthesized via ROP, SSP is normally used to remove the residual lactide. (102)

4.1.2 PLA application

The high cost of lactide production has limited for several years PLA use only to biomedical applications, in particular, PLA was used for the production of wound suture yarns and stent applications, drug delivery system-based PLA, orthopedic and fixation devices, tissue engineering and regenerative medicine. (103) (104) (105) (106) (107) The reducing costs of lactide production, together with the technological improvements of synthesis and processing, has opened the possibility to employ PLA in other fields: disposable, food and beverage packaging, textiles, "durables" such as engineering components for automotive and electronics, and so on. (108)

PLA has attracted the attention of food packaging world due to its biodegradability and biocompatibility; every day tons of packaged food are wasted and the common waste processing requires a separation of food and plastics which have a different disposal process (food is addressed to compost plants whereas packaging to recycling plants). The use of PLA, substituting non-biodegradable plastics, can avoid this step, dramatically reducing costs of waste disposal. Tenova (Sweden) was the first company introducing PLA for packaging application: they delivered biodegradable shops made in PLA/Ecolex®. Tenova was then followed by a lot of companies spread all around the world: Biota (USA), which introduced biodegradable water bottle; Wal-Mart (USA), which introduced the PLA clamshells for strawberries and brussel sprouts; Hypermarket chain Auchan (France), which introduced PLA packaged salads; Sant'Anna (Italy), introduced the "bio-bottle" made in PLA; Shiseido-Urara (China), which developed a shampoo bottle made with composite material 50 wt.% PLA and 50 wt.% HDPE; Polenghi LAS (Italy), which developed a PLA bottle for lemon juice (acid liquid); Ceramis® (Switzerland), which developed high oxygen barrier PLA film for snacks, bread and fruits packaging and so on. One of the most hilarious case happened when Frito Lay introduced a compostable PLA bag for their Sunchips® brand in 2010: the production was ended in 2014 because this bag underwent major public scrutiny over the loud crinkling sounds during bag handling. (109)

PLA has also found application in agriculture field: PLA is used for the production of mulching films, substituting the widely used PE: films degrade and can be absorbed by the ground after the use. On the other hand, the relatively high Tg of PLA (≈60°C) together with less amorphous region (polymeric phase edible by microorganism) dramatically reduces the low-temperature degradation

rate of PLA. In order to overcome this issue, PLA is blended with other materials as poly(hydroxy alcanoate), starch or poly(butylene adipate-co-terephthalate). (110)

PLA has found many applications also in different fields, moreover, research continues to work in order to improve PLA properties and to identify new possible applications. It could be interesting to know the trend (the bar chart provided in figure 4.5 shows the number of scientific publications per year) of research reports since 1990 based on the Web of Science search using keywords “PLA”, “PLLA”, “PDLA”, “polylactic acid”, “polylactide”, and “poly(lactic acid)”: scientific interest in PLA has always had a positive trend meaning that the interest to find and to develop alternative materials is one of the most relevant issue of these years. (109)

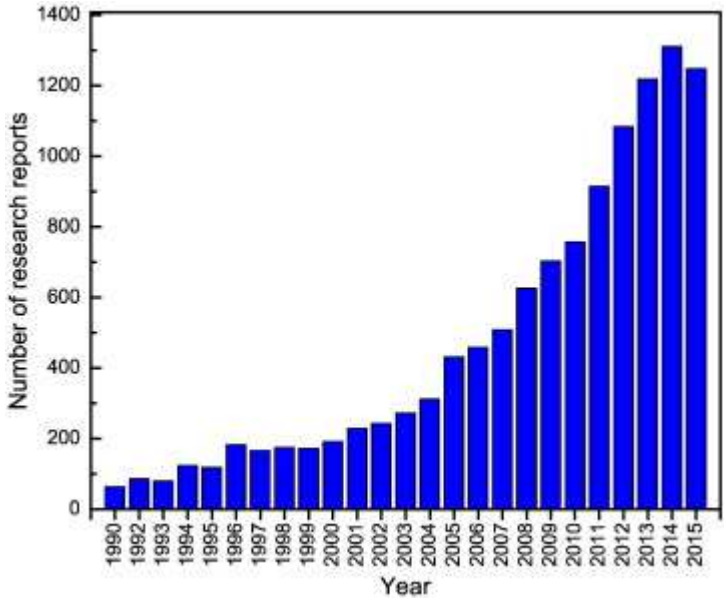


Figure 4.5: number of scientific publications per year concerning the key-words “PLA”, “PLLA”, “PDLA”, “polylactic acid”, “polylactide”, and “poly(lactic acid)” (109)

4.2 Pectin

Pectin is a hetero-polysaccharide, isolated and described for the first time in 1825 by Henri Braconnot, contained in the cell walls of plants especially in the non-woody parts. Its amount, structure and chemical composition differs among plant species; during the ripening process of fruit pectin is broken down by specific enzymes, called pectinase and pectinesterase and the fruit becomes softer.

Pectin structure includes several types of saccharides, as D-Xylose, D-apiose and D-galactose, and galacturonic acid that is the most abundant. In other terms, pectin could be identified as a multi-block copolymer where monosaccharide (units of D-Xylose or D-Apiose) units bond together blocks of homogalacturonan, i.e. near chains of α-(1–4)-linked D-galacturonic acid; the structure is shown in figure 4.6.

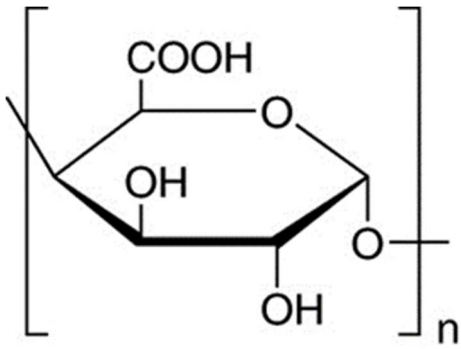


Figure 4.6: α-(1–4)-linked poly (D-galacturonic acid)

Although the pectin structure previously described is the most abundant, other categories of pectin can be extracted from fruits,: Rhamnogalacturonan I pectins (RG-I), which contain a backbone made of disaccharide 4-α-D-galacturonic acid-(1,2)-α-L-rhamnose that is branched by various neutral sugars, like D-galactose, L-arabinose and D-xylose. The average molecular weight of pectin is usually in the range between 50000 and 150000 g/mol (111); these data were obtained through GPC analysis made after GPC calibration with monodisperse pectin samples. The broad range depends on pectin source and extraction conditions: for example, a study regarding the extraction conditions used to obtain pectin from peach pomace have shown that harsh conditions of temperature and pH increase the overall yield whereas milder conditions can lead to samples with better gelling characteristics, since the molecular weight of obtained pectin is higher. The structural properties of pectin are related to their structure:

- Level of branching
- Molecular weight of homogalacturonan chains

- Amount of free carboxylic moieties along the chain

A part of -COOH groups of galacturonic acid is naturally esterified forming the low polar and hydrophilic methyl-galacturonan derivative. The ratio of esterified units on the total amount of galacturonic acid units defined the degree of esterification (D.E.); the solubility of pectin and its gelling property seriously depend on D.E.; for instance high D.E. leads to shorter setting time of the gel whereas low D.E. leads to a stronger gel, since a higher number of crosslinking interactions are formed between carboxyl groups and divalent cations (see “Gel formation” paragraph below). In conclusion, the D.E. depends on the origin of the plant source, harvesting and processing conditions, such as storage, extraction, isolation, and purification (112).

According to D.E. pectin are classified as:

- High Methoxylated (HM), in which the D.E. is higher than 50%
- Low Methoxylated (LM), in which the D.E. is lower than 50%

Also pH has a serious influence on pectin behaviour: the carboxylic moiety of galacturonic acid has a pKa ≈ 4; by neutralizing pectin with sodium hydroxide or potassium hydroxide, it is possible to obtain the sodium or potassium salt, stable at pH higher than 6. On the other hand, pH higher than 8 leads to the hydrolysis of esterified galacturonic acid and the α bond between the saccharide units modifying the structure, and therefore, the properties of the polymer.

4.2.1 Extraction Process

Pears, apples, guavas, quince, plums, gooseberries, oranges and other citrus fruits contain large amounts of pectin. The most used raw materials for production are dried citrus peel or apple pomace, both by-products of juices production. In 2009 the worldwide production of pectin was about 42000 tons and considering that the extraction yield of pectin from citrus peel is equal to 3%, 1,4 million tons of waste materials have to be processed to supply the world demand of this polymer.

Pectins are extracted with a multi-step process:

hot dilute acid solution (usually HCl or H_2SO_4) at pH from 1.5 to 3.5 is added to raw materials in order to promote the hydrolysis of cross-linked structure permitting its solubilisation; this step takes several hours (minimum 2h).

The acid solution is then filtered in order to remove insoluble part of raw material that cannot be hydrolyzed. The solution is finally concentrated and, in order to promote the precipitation of pectin, ethanol or isopropanol is added to the acid solution.

The result is a white, or slightly light brown, solid that after fine grounding process is dried and sell.

4.2.2 Applications

Pectin is classified as soluble dietary fiber and is a natural part of the human diet; its daily intake from fruits and vegetables can be estimated in 5 g (assuming consumption of approximately 500 g fruits and vegetables per day). In the gastrointestinal tract, pectin binds to cholesterol and traps carbohydrates inducing a slower absorption of glucose. Its consumption has been shown to reduce blood cholesterol levels by increasing the viscosity of the gastro intestinal fluids. This leads to a reduced absorption of cholesterol from bile and food since the fluid mobility is reduced, by the viscosity increment, and consequently the contact time between intestinal content and villi is lowered (113).

One of the most relevant issues related to the use of natural polymer, is connected to their intrinsic variability that can limit the reproducibility of the results of the extraction batches. On the other hand, their non-toxicity - FAO (Food and Agriculture Organization of the United Nations) and WHO (World Health Organization) have not set any acceptable daily intake (ADI) - and low cost (pectins are mainly produced from food industry waste material) made pectin a widely used natural polymer for many applications.

Pectin is widely used in food industries (food additive E440 according to International Numbering System for Food Additives “INS”) as gelling agent, thickening agent, in sweets production and as stabilizer in fruit juices and milk drinks; one of the most diffused use is as gelling agent for the production of marmalades. As food additive, pectin is typically used in the range between 0.5 and 1.0% on weight, which is about the same amount present in fresh fruit. In addition, pharmaceutical industry has a great interest in pectin use; it is used as bulking agent in drugs production, it is sold as supplement of dietary fiber, it is used in wound healing preparations and medical adhesives, such as colostomy devices and it could be used for oral drug delivery formulations (e.g., controlled release systems, gastro-retentive systems, colon-specific delivery systems and mucoadhesive delivery systems) (114).

4.2.3 Gel formation

Pectin can form a stable reversible gel that normally is created at temperatures depending on the chemical composition of pectin chains. On the other hand, gelation can be also achieved via

complexation of bivalent cations (in particular alkaline earth metal cations). The next two paragraphs will explain how temperature and complexation affect the toughness of pectin's gel.

Thermal gelation

Temperature is one of the key parameter of pectin gel: it is possible to identify a specific temperature, called Gelation Temperature, that activates the formation, or the demolition, of gel. Gelation temperature is the switcher from the liquid viscous form of pectin solution to the frozen solid structure. Thermal gelation normally occurs during the process of marmalade production: pectin is added to the boiling fruits mixture and, when the mixture is cooled below gelation temperature, the gel structure starts to form.

The beginning of gel formation depends on the temperature which is influenced by the degree of esterification: gelation is a complex process that involves the formation of aggregates due to hydrogen bonds formation and non-polar interactions. The amount of polar and non-polar interactions dramatically depends on the degree of esterification: the higher the D.E. the higher the gelling rate. On the other hand, the toughness of the gel (a crucial parameter that influence the texture of foods) is affected by different parameters:

- Concentration of pectin in water solution
- Concentration of sugars as sucrose, glucose and fructose
- pH

High concentration of pectin leads to a tough gel; nevertheless, the toughness of gel is also influenced by the concentration of a co-solute (usually sugars like sucrose) that reduce water activity promoting hydrophobic interactions between methoxy groups; literature data report that a concentration of sucrose higher than 55% w/w_{gel} is required for gel obtaining and a changing of sugars (glucose or fructose) widely affects the mechanical properties of the gel.

Also the pH affects the formation of the gel: the lower is the pH the lower is the dissociation of carboxylic moieties along the polymer backbone, therefore, the non-polar interactions are promoted. A pH lower than 3,5 enhance the formation of HM pectin gels whereas pH higher than this value promotes the formation of LM pectin gels.

The combination of these factors influence the texture of the final product: in particular, when pectin is used as gelling agent (for instance in marmalade production), tough gel can lead to a final syneresis (the expulsion of a liquid from the gel) or a granular texture whereas a weak gel leads to excessively soft material (115).

Bivalent Cations Complexation

Besides temperature, that still remains a trigger parameter for the formation of pectin gels, gelling can be achieved also through complexation of divalent cations (usually earth alkaline metals) by non-esterified carboxylic moieties along the polymer backbone. Linear blocks of homo-galacturonan modify their spatial disposition, orienting the galacturonic acid ring in order to bind a bivalent cation: the self-assembly idealized structure, called “egg-box model”, is shown in figure 4.7.

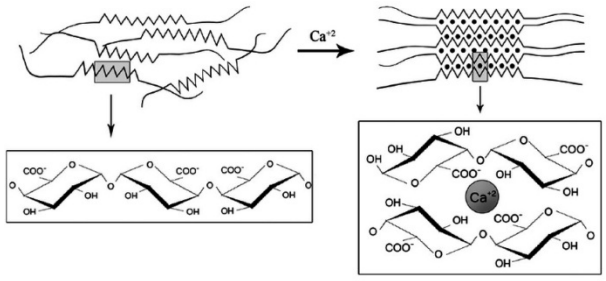


Figure 4.7: “Egg-box” complexation model of Ca2+ by pectin

Calcium is the most used divalent ion to form gels due to its high biocompatibility and non-toxicity; normally a pH range between 2.6 and 7.0 is used to increase the quantity of deprotonated carboxylic groups to form a higher amount of ionic bridges. The lower is the D.E. of pectin the higher is the number of crosslinking points increasing the toughness of gel. (116)

4.2.4 Pectin methoxylated: degree of esterification

The binding of Ca(II) by carboxylic moieties along pectin chains promotes the formation of “ionic bridges” through the polymeric chains leading to gel structure.

The determination of D.E. is fundamental in order to determine the stoichiometry quantity of calcium required for the gel formation: low quantity of calcium cannot promote ion bridges from pectin chains reducing toughness of the gel whereas a high concentration of calcium promotes the complete gelation of polymeric, but the non-bonded calcium affects the rheological properties of the material.

In order to understand how the chemical properties of pectin can affect the release, two types of pectin with different D.E. were purchased from Sigma-Aldrich:

- pectin from apple (Poly-D-galacturonic acid methyl ester), having a D.E. between 50 and 75%;

- pectin from citrus peel, having a degree of esterification higher than 6,7 percent;

The data about the degree of esterification provided by the supplier are not sufficient to properly calculate the amount of $CaCl_2$ needed to obtain stoichiometric gels, i.e. gels with the highest amount possible of ionic bridges between the polymer chains or, in other words, the strongest possible gels.

An IR based methodology (117) was used to determine the degree of esterification (DE) of pectins. The bands used for DE determination are those at ≈1610 and ≈1740cm^{-1} as reported in litterature. The first one corresponds to the symmetrical stretching vibration of carboxylate moiety whereas the second is assigned to stretching of carbonyl groups both from carboxylic acid moiety and methyl-ester derivate. Considering that in partially methoxylated pectin at pH 6, the carboxylic groups are completely deprotonated, the 1740 cm^{-1} band can be attributed exclusively to the carboxymethyl groups. The ratio between the absorbance of 1740 cm^{-1} band divided by the sum of those at 1610 and 1740 cm^{-1} is proportional to DE. The equation found by Manrique et al. that correlates DE to absorbance parameters was used for DE determination of the purchased pectins and it is reported in figure 4.8.

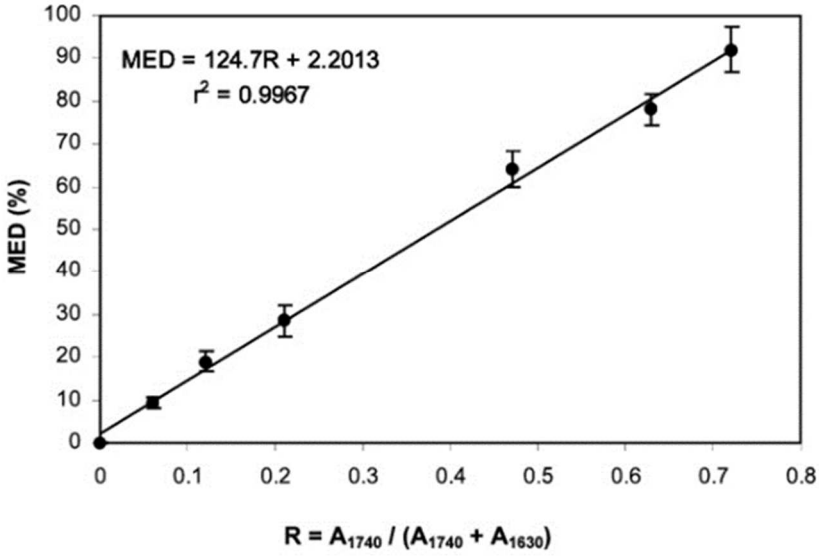


Figure 4.8: Equations used for DE determination reported by Manrique et al. (117)

4.3 Alginate

Alginic acid, also called alginate, is an anionic natural polysaccharide and is one of the major components of cell walls of brown algae. It is sold in filamentous, granular or powdered forms and its colour ranges from white to yellowish-brown. It is a linear copolymer constituted by homopolymeric blocks of (1-4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues, covalently linked together in different sequences (118). Alginic acid structure is reported in figure 4.9.

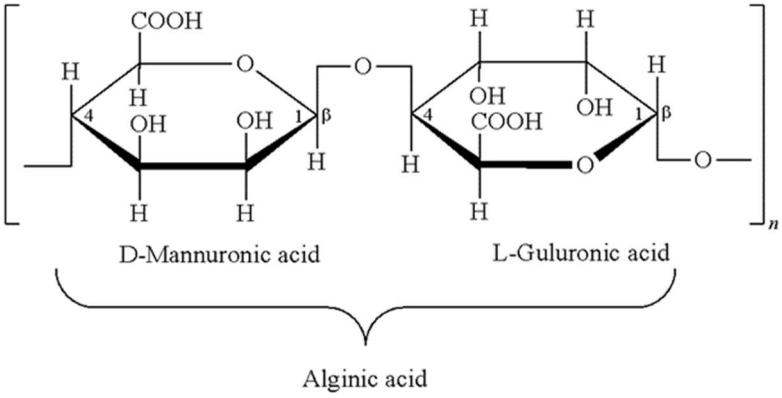


Figure 4.9: Alginate chemical structure

The monomers can appear in homopolymeric blocks of consecutive G-residues (G-blocks), consecutive M-residues (M-blocks) or alternating M and G-residues (MG-blocks) (119). As mentioned, it is an important component of algae and is also an exopolysaccharide constituent of bacteria including Pseudomonas aeruginosa (119).

Commercially available alginate is typically extracted from brown algae by treatment with aqueous alkali solutions, usually with NaOH (118). Sodium alginate has a wide use in food, textile printing and pharmaceutical industries. An example of widely diffused application is dental impression that utilizes alginate. (120).

4.3.1 Commercial sources

Industrial processes used to make sodium alginate from brown seaweeds are relatively simple and they fall into two categories. In the first one, the principal intermediates are calcium alginate and alginic acid, while in the second no calcium alginate is formed but only alginic acid. The two processes are illustrated in figure 4.10.

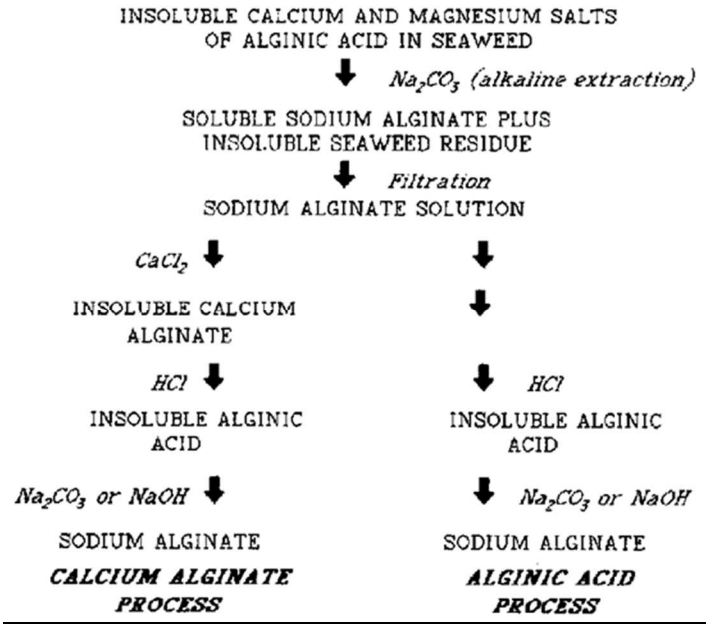


Figure 4.10: Industrial processes for alginate production (121)

The advantage of the first process, called “calcium alginate” process, is that calcium alginate can be precipitated in a fibrous form which is easy to separate; subsequently it can be converted into alginic acid by using an acidic treatment. An advantage of this process is that some calcium alginate can be allowed to remain in the final product. The presence of low concentrated calcium ions in an alginate solution increases its viscosity while larger amounts will cause the formation of a gel. The addition of Ca^{2+} is therefore a way of increasing the viscosity of an alginate solution without increasing the amount of alginate dissolved or its molecular weight.

The second process, called “alginic acid process”, does not need the step of the formation of calcium alginate, nevertheless it presents some disadvantages. In fact, when alginic acid is precipitated, it forms a colloidal material that is very difficult to separate and this implies a high loss of alginic acid. The removal of liquid from within the gel structure, after the treatment with Na2CO3 or NaOH to form the soluble alginic acid sodium salt, the so called “dewatering” step, also presents difficulties. In fact, the water content in alginic acid sodium salt is very high, close to 98%, and squeezing or centrifuging step is needed to remove the greatest part of the water. However, it is not removed completely and a mixing with alcohol step is used, usually methanol or ethanol, for the conversion to sodium alginate in solid form. This last treatment makes the process more expensive, since an alcoholic solvent is used, if compared to the calcium alginate process.

One of the most relevant issue of alginate production is related to the physical separations: the filtration of residues from viscous solutions or the separation of gelatinous precipitates, which hold large amounts of liquid within their structure, are crucial steps of the extraction of alginate from algae; these steps acts an important role in the economy of the process (121).

4.3.2 Gel formation

The mechanism of pectin gelation has been described in paragraph 4.2.3 and pectin gels are defined as reversible gel: temperature, also in presence of Ca^{2+}, promotes the breaking process of intermolecular bonds destroying the gel structure. Alginate acts in a very similar way: they are soluble natural polymers which can react with divalent cations (Alginate can create stable structure also with trivalent cations) forming stable gels at room temperature. The Alginate-Ca(II) gel, contrary to what happen with pectin, is stable and neither the temperature leads to its disaggregation. Alginate solutions can also form gels if they are carefully acidified; these gels are generally softer than gels obtained by calcium complexation giving a different texture profile and melt with temperature arise (approximately T>30°C) making such gels interesting for food application.

4.3.3 Bivalent Cations Complexation

Alginate carboxylic moieties can bind bivalent cations leading to a non-reversible gel. One of the most used bivalent cations is Ca(II) due to its high biocompatibility and low cost; on the other hand, the complexation of bivalent cations is not limited to Ca(II) but it is reported how alginates can form stable gels, even though with different mechanical and texture properties, with different metals: alkaline earth metals, Iron, Copper etc., Ca(II) concentration has a key role in gel toughness: high concentration of Ca(II) leads to obtain very tough gel in very short time, whereas low concentration of Ca(II) – obtained using low soluble salt such as calcium citrate – leads to obtain soft in long time (118).

The standard procedure for Alginate-Ca(II) gel preparation consists in dropping a high viscosity water solution of $CaCl^2$, at 1.3% w/w, and a water soluble thickening agent (the most diffused are carboxymethyl cellulose and dextran) in a water solution of alginic acid sodium salt, with a concentration 0.5-4% w/w. When a drop of Ca(II) solution sinks into the alginate solution, the gel structure sets immediately obtaining an Alginate sphere. Retarding agents can also be used, such as sequestrants (e.g. EDTA), to complex calcium ions and make them unavailable and slowly released.

The gel strength depends on the source of the alginate, i.e. the algal species, its concentration, its degree of polymerization and the calcium concentration. Alginates from different seaweeds can have differing ratios of mannuronic acid, guluronic acid and different proportions of M, G and MG blocks. This ratio, and the way in which the acids are distributed in the polymer chains, have a marked effect on gel formation and gel strength. Alginates with a high proportion of G blocks form rigid gels while alginates with mainly M blocks form gradually, softer and more elastic gels. Gel

formation occurs since calcium ions substitute hydrogen ions, H+, on the carboxylic acid groups and form ionic bridges between chains. This three-dimensional model is called "egg-box model". While calcium holds the molecules together via ionic bridges, their polymeric nature and their aggregation bind the calcium more firmly forming chain entanglements; this phenomenon has been named "cooperative binding" (121).

4.3.4 Acid Gels

The variation of pH affects the properties of alginate modifying the ratio between protonated and non-protonated carboxylic moieties: increasing the number of protonated carboxyl groups the electrostatic repulsion between chains is increased promoting hydrogen bond formation and increasing viscosity. For equivalent alginate concentrations, acid gels have approximately half strength of calcium gels. Soft behaviour is useful in some food applications since acid gels can mimic the effect of gelatine; on the other hand, acid gels are less stable than Alginate-Ca(II) gels: when they are heated they melt and, in acid condition, depolymerizes (121).

4.3.5 Applications

The uses of alginates are based on three main properties: first their ability, when dissolved in water, to thicken the solution, second their ability to form gels when a calcium salt is added to a water solution of sodium alginate, and third the ability to form films of sodium or calcium alginate and fibres of calcium alginates.

In textile printing, alginates are used as thickeners for the paste containing the dye. Alginates do not react with the dyes and they are easily washed out of the finished products. Textile printing accounts for about 50% of the global alginate market.

The thickening property of alginate is used in food industries for the preparation of sauces, syrups and toppings. In addition, it is used to stabilize water-in-oil emulsions, such as mayonnaise and salad dressings, and to improve the texture, body and sheen of yoghurt and fruit drinks. It is used as a binder and thickening agent for pet-food. Another application for alginates is not related to their viscosity or gel properties, but to their stabilizer behaviour. In facts, they act as stabilizers in ice cream, since addition of alginate reduces the formation of ice crystals during freezing, giving to the final product a smooth texture. As food additive it is labelled as E400 on food packaging as disposed by the International Numbering System for Food Additives (INS) (122).

4.3.5.1 Pharmaceutical and medical uses

Thanks to total biocompatibility alginate has been widely used for pharmaceutical and medical purposes. For example, stable fibres have been produced from mixed salts of sodium and calcium alginate and used in wound dressings. They have very good wound healing and haemostatic properties and can be absorbed by body fluids since the calcium in the fibre is exchanged with sodium from the body to give a soluble sodium alginate (123). Thanks to swelling behaviour of alginate, it has been used as a tablet disintegrant. In addition, it finds applications in products such as Gaviscon® tablets, which are designed to relieve heartburn and acid indigestion; since the swollen, alginic acid helps to keep the gastric contents in place and so reduce the likelihood of reflux irritating the lining of the oesophagus. Alginate is used in the controlled release of drugs and other bioactive chemicals. The active ingredient is generally placed in a calcium alginate bead and slowly released as the bead is exposed to the appropriate environment. In addition, oral controlled-release systems involving alginate microspheres, sometimes coated with chitosan to improve the mechanical properties, have been tested as a way of delivering various drugs (124).

4.5 Polyvinylpyrrolidone

Polyvinylpyrrolidone, or Polyvidone or povidone, (PVP) is a synthetic polymer obtained by free radical polymerization of 1-vinyl-2-pyrrolidone (NVP). The polymerization does not modify the structure of lactone rings that maintain their chemical-physical properties in terms of reactivity, solubility in solvents etc., PVP chemical formula is shown in figure 4.11.

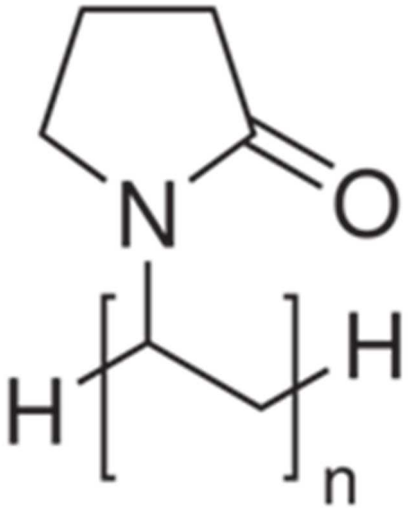


Figure 4.11: PVP chemical structure

4.5.1 PVP synthesis

PVP was firstly obtained in 1938 by Walter Reppe in BASF's laboratories (125). The radical polymerization of NVP can be performed either in bulk or in solution or in suspension, and the resulting polymer has a degree of polymerization between 10 and 105. Changing the reaction conditions, a wide range of molecular weights can be achieved, extending from low values of a few thousand Daltons to approximately 2.2 million Daltons. In particular, higher molecular weight PVPs are obtained in aqueous solutions, whereas lower molecular weight polymers are obtained doing the polymerization reaction in organic solvents, since these may act as chain transfer agents (126).

PVP molecular weight dramatically affects the thermal properties of the material: the glass transition temperature (Tg) can range from 100°C (for Mw=2500Da) to 175°C (for Mw≈1000000Da). On the other hand, the Tg values is seriously affected by the amounts of water absorbed into the polymer: small quantities of water even lead to a steeply decrease of Tg values.

PVP is a high hygroscopic polymer therefore moisture can dramatically affect its thermal properties. (127) (128) (129)

Industrially NVP polymerization is usually performed in water between 50°C and 80°C using hydrogen peroxide as radical initiator. The reaction is performed in water at 20-60% of NVP concentration depending upon the desired viscosity range. The polymer may be spray dried in order to obtain a solid product or directly used as water solution. Besides hydrogen peroxide also azobisisobutyronitrile (AIBN) can be used as radical initiator in the temperature range 50-60°C. (130) The global PVP market size was 161.8 kilo tons in 2015 (131).

4.5.2 Applications

PVP has both hydrophilic and hydrophobic moieties therefore it can interact with a lot of solvents; in fact, it is soluble in cold water and in many organic solvents too, such as alcohols, chloroform, methylene chloride, ethylene dichloride, nitroparaffins and amines. (132)

Its physical and chemical properties, such as biocompatibility, non-toxicity, chemical stability, good solubility, affinity to both hydrophobic and hydrophilic substances, made it suitable as a biomaterial in a lot of applications, in pharmaceutical industry and medicine, optical and electrical applications, membranes, adhesives, ceramics, paper, coatings and inks, lithography and photography, household, fibers and textiles and environmental applications. (132)

Pharmaceutical industry is the first field that has benefited from the use of PVP, due to its excellent biocompatibility and ability to form stable association complexes with many active substances. The global PVP demand for pharmaceutical application accounted for over 58.0% of its volume shared in 2015. Growing demand for PVP in pharmaceutical application as drug solubilizer, co-solvent, sterilization disinfectant and dispersion stabilizer is expected to boost the market growth in the next years. Starting as blood plasma expander, now PVP is a common component of drug manufacture, presents into all kinds of formulations such as tablets, granules, pellets, capsules, gels, films, coatings, injectable solutions and contact lenses. (133) For example, iodine added to PVP forms a complex called povidone-iodine that has disinfectant properties, which is used in various products like solutions, ointment, liquid soaps and surgical scrubs. It is known under the commercial names Pyodine® and Betadine® (133). PVP, thanks to its amphiphilic behavior, is used to solubilize in water either lipophilic or low water soluble substances; several examples of solubilization of drugs and polyphenol have been reported in literature. Increasing the water solubility is an easy way to enhance the bioavailability of an active substance.

Besides the pharmaceutical field, PVP displays good electrical properties and it is used in various electrical and optical applications, such as screens, printed circuit boards, cathode ray tubes and energy storage devices. PVP is an additive and a pore-former agent in membrane fabrication for water purification, wastewater treatment, desalination, food processing (e.g. beer and wine filtration) and gas separation. Adhesive properties of PVP have been exploited for many years for different uses as skin adhesives, hot-melt adhesives and glue sticks. A wide range of paper products (copying paper, printing paper, electric insulating paper, thermal paper,etc.) and office supplies have been reported to use this polymer. PVP has been applied in coatings for photo-quality ink-jet papers and in inks for printers thanks to its high polar behavior that allows PVP to bind exceptionally well to polar molecules. PVP is also used in personal care products, such as shampoos, toothpastes and in formulas for hair sprays and hair gels (132). It has also been used in contact lens solutions and in steel-quenching solutions. PVP finds applications in paints and adhesives that must be moistened, such as old-style postage stamps and envelopes.

Food industry is another important field in which PVP finds applications. In particular, the market of beverage manufacturing is increasing the use of PVP especially as clarifying agent in wine production. It has wide application in manufacturing non-alcoholic beverages such as juices, tea drinks, soy sauce and vinegar and for improving solid food texture and flavour. Food and beverage field of application is expected to growing at over 8.0 percent rate over the period from 2016 to 2024 (131). As food additive, PVP act as stabilizer and it is labelled as E1201 on food packaging as disposed by the International Numbering System for Food Additives (INS).

5. Biomedical Delivery System

The aim of biomedical delivery system is to delivery for a certain time in a specific target tissue an active substance. A controlled delivery permits, in comparison to the standard drugs assumption, to reduce premature degradation, to improve drug uptake, to sustain drug concentrations within the therapeutic window, and reduces side effects (134).

In figure 5.1 the curves related to the release of active molecules during the time are shown:

- The yellow line represents the pulsatile release that normally occurs with traditional drugs intake: the concentration of active principle burst arise after the intake and then decrease.
- The red line (first order release) represents the release rate that normally occurs in drug release device: the concentration of active principle rapidly increases and gradually decreases after the intake.
- The blue line (zero order release) represents the release rate of advanced drug release device: the concentration of active principle is kept for a long time in the therapeutic concentration range.

The zero order release is the goal of the modern controlled release devices: in this way it is possible avoid the problems related to the toxicity of active molecules in high concentrations and, maintaining the concentration in the therapeutic window, improving the efficiency of the therapy. (135)

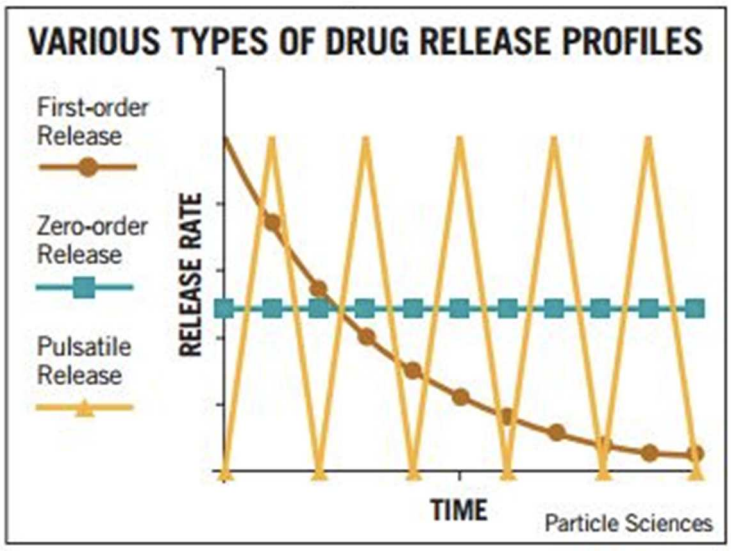


Figure 5.1: active principle release rate during the time of therapy (136)

Many drug delivery systems are commonly used every day: therapeutic patch for local inflammatory treatment, vaginal ring for female hormones delivery for contraceptive therapies, insulin deliverer for type 1 diabetes etc.

Even though the drug delivery is a young discipline, it is considered that this field is only 40 years old; many works have been done. The continuous attention on the development of new materials and technology allow the development of different devices shifting the scale from macro- to nano-. The release of small drugs molecules is only one of the aim of release devices, many examples are reported of release of other therapeutic factors as proteins (137) and genes (138).

The need of extremely versatile and tailor-made material makes polymers as the main used materials for the production of drug delivery devices but also inorganic devices as ceramic scaffolds (139) or metal-based material (140) are used as drug carrier. On the other hand, this work is focused on the use of polymers as release matrix therefore this chapter aims to explain the main characteristics, issues and advantages of the use of polymer-based drug delivery systems.

5.1 Mechanism of Release

The mechanisms involved in the control of the release through a polymer matrix are 4: diffusion, swellings systems, erosion or by an external stimulus. (141) (142) (143) (144)

The first mechanism studied was the diffusion that leads to obtain a concentration gradient of drug during the time: the Fick's low of diffusion regulates the release, resulting in nonlinear Fickian release profiles.

The release in swelling-controlled systems is controlled by the water swelling in the delivery system: the swelling improves the polymer flexibility and therefore pores dimensions, enhancing the drug molecules mobility. The profile of drug release kinetic is linear for first period but, in a second time, the release will have a Fickian release profile (a combination of release due to polymer disentanglement for the linear part and Fickian-shape kinetic for the non-linear part).

The erosion-controlled devices are produced using bio-erodible polymers; the mechanism of release is complicated and involves mass transport and chemical reactions: drug dissolution, polymer degradation, porosity creation, micro-environmental changes in pH, diffusion in polymer matrix, and autocatalytic effects. The kinetics of the release is a multi-step process depending on the polymer and its interaction with the body.

Stimulus-controlled release systems control the release of drug upon an external stimulus able to modify the structure of the polymer that permits a release of the active molecules. External stimuli

can be pH variation, temperature arising, ionic strength or water composition (i.e. glucose concentration). (143) (144)

The four release mechanisms are graphically summarized in figure 5.2.

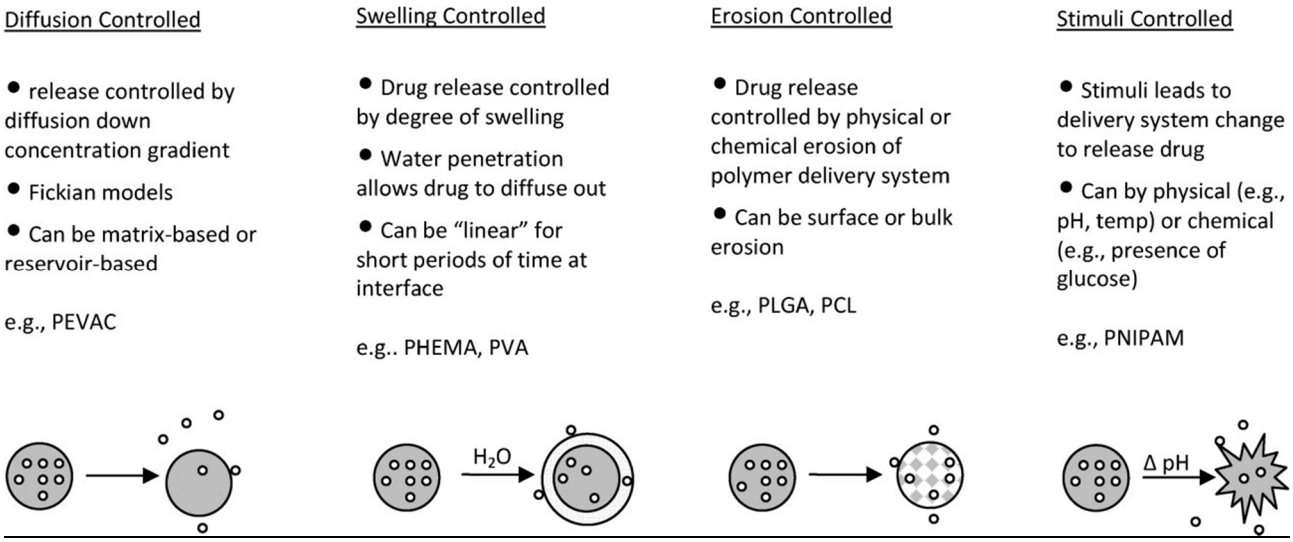


Figure 5.2: mechanisms involved in drug released from polymer-based devices. (145)

The released mechanisms are analyzed excluding the interactions (physical and chemical) among polymeric matrix (moieties born along the chain) and the moieties of the active molecules delivered. Wang and Von Recum introduced in their review the concept of affinity-based drug delivery: *affinity* can be defined as the tendency of a molecule to associate with another molecule.

The interactions among active molecules and polymeric matrix (i.e. ionic interaction, van der Waals forces, hydrogen bond, hydrophobic interaction and their combinations) are able to affect the release modifying the shape of the kinetics release curves. Three examples, of how the interaction among active molecules and release device affects the release, are shown in figure 5.3: it is important to notice how the release can be tuned modifying tailoring the molecular properties of the release device. (145)

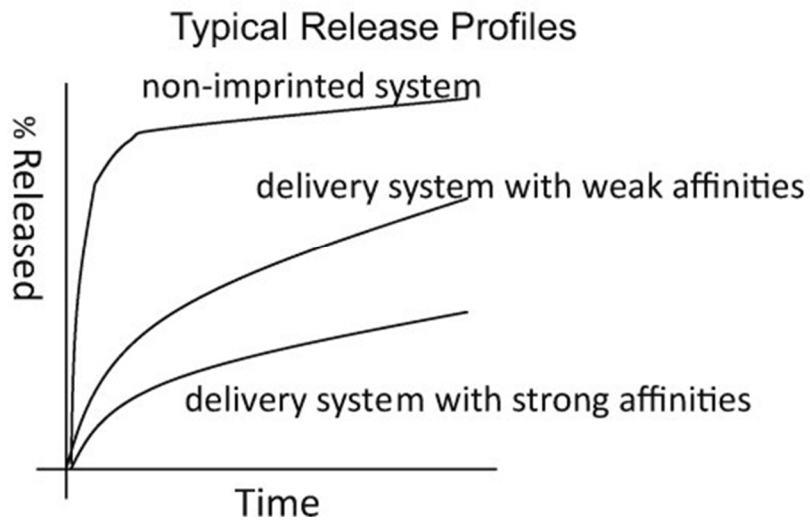


Figure 5.3: kinetic release curves with different interaction among active molecules and polymer

5.2 Molecular Imprinting

Besides conventional methods for the release previously explained, the *molecular imprinting* play an important role: it is a very high specific method able to fine control the release of an active molecule that is called *imprinted molecule*. (145)

The creation of an active site, compatible only with a specific active molecule that is defined imprinted molecule, is called *molecular imprinting*. Polymer is synthetized using functional monomers able to modify the features of the macromolecular network improving interactions with active molecules. Both covalent and non-covalent interactions can be achieved but the latter is preferred due to many reasons: slow kinetic covalent bond cleavage, readily adaptable and rapid synthesis, close resemblance to molecular recognition and availability of functional monomer libraries. The non-covalent interactions include hydrogen bonding, hydrophobic interactions, ionic, and van der Waals forces and obviously their combinations. (146)

The synthesis of polymer is performed with the active molecules already bond to the active monomer: after the polymerization the active molecules are removed leaving cavities, compatible only with their, along the polymer network. This technique mimes the activity of enzymes that are able to interact only with specific substrates; this mechanism can be explained with the analogy of *the lock and the key* where the key is the substrate and the lock the enzyme: the lock can be opened only by its key. The figure 5.4 shows an example of this mechanism. (147) (148)

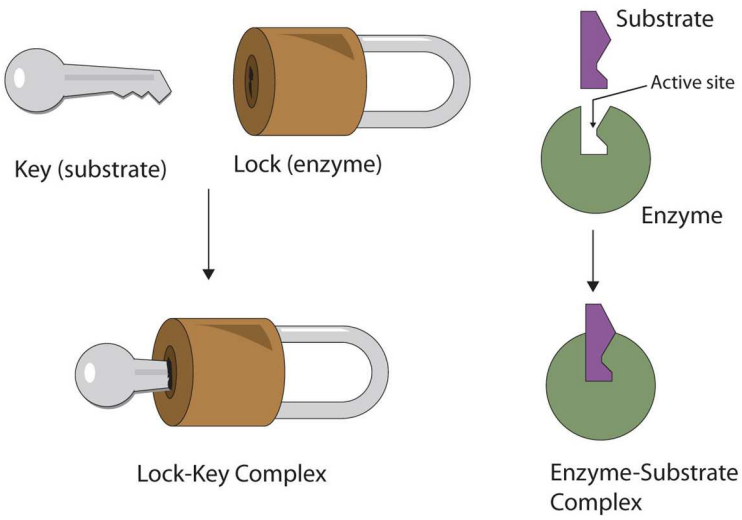


Figure 5.4: lock and key activity mechanism (149)

The first example of this kind of release system was obtained by Vlatkis e all.: they synthesized a cross linked polymer using as functional monomer the methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) as cross-linker. The methacrylic acid is able to perform ionic interaction with amines and hydrogen bonds with polar group of active molecule. Even in the presence of molecular similar molecule, the selectivity of the polymers for the imprinted molecules is exceptionally high. (150)

The ratio of cross-linker, monomer and active molecules dramatically affects the kinetics release. Increasing the quantity of active molecule, even though the drug load cavities are formed, the release occur immediately due to low amount of monomers that consist the cavities: too weak interactions occurs and the release is rapidly achieved. On the other hand, increasing the amount of monomer multiple cavities are formed in polymer network leading to a decreased release kinetic. (151)

Results and Discussion

6. **Lactide polymerization**

Vanillyl alcohol (VA) and Tyrosol (Tyr) were identified as potential antioxidants able to react with lactide acting as chain initiator for the ROP reaction: both molecules have an aliphatic alcoholic moiety (fundamental for polymerization with lactide) and a phenolic moiety (this moiety acts as radical scavenger). The focus of the work is produce antioxidant material using the industrial condition in order to rapidly scale up the process; Tyrosol was chosen a *model molecule* in fact, the non-substituted phenols do not show interesting antioxidant activity. On contrary, VA is a substituted phenol activated by the -OCH3 that slightly increase the acidity of the phenol (VA pka 9.75 and Tyr pka 10.17) increasing the activity against radicals. Other molecules could be used, for instance 3,5-Dihydroxybenzyl alcohol or dihydroxy Tyrosol, but they are too instable at polymerization temperature. Moreover, the increased acidity due to aromatic ring substitution deactivates the complex Lactide-Tin-initiator blocking the chain growth process. (152)

As was already explained in paragraph 4.1.1, the polymerization of lactide via ROP requires a nucleophilic chain initiator; even though primary amines have a better nucleophilic feature and give more stable reaction products, aliphatic alcohols are the most widely used chain initiators for lactide polymerization and their reactivity with lactide is extensively reported in literature. (153) (154)

The structure of vanillyl alcohol and tyrosol is reported in figure 7.1; both molecules present both an aliphatic primary alcohol and a phenolic moiety. According to the scheme proposed in paragraph 4.1.1, both moieties can react forming the complex Tin-Lactide-Alcohol for the beginning of ROP reaction. On the other hand, the phenolic moiety does not react with lactide whereas the aliphatic alcoholic moiety does; previously literature examples reported the bulk polymerization of Lactide with Tyr, showing that the authors obtain only linear mono functionalized polymeric chains (155). Furthermore, the higher steric hindrance (in particular in VA), together with the high stability of aliphatic ester bonds, disadvantages the reaction of lactide with phenolic moiety promoting the reaction with primary aliphatic alcohol.

The relatively high acidity of phenolic moiety can promote the hydrolysis of the metal complex Tin-Polymer-Alcohol required for chain growth. In order to overcome this problem, $Sn(Oct)_2$ was used in higher concentration (3000ppm) in comparison to the limits indicated for material for food contact (300ppm).

6.1 Solution Polymerization

Solution polymerization of lactide were performed as *model synthesis* in order to assess the reactivity of VA with lactide for PLA synthesis. The reactions were performed in anhydrous tetrahydrofuran as was previously reported by Basilissi in his Ph.D. thesis. (156) The reactions were conducted at 67°C for 165h: the kinetics of the reaction at such temperature is very low therefore very long reaction time is required in order to obtain high monomer conversion.

Three samples were synthesized; feed, degree of polymerization (DPn), monomer conversion (p) and percentage of VA bonded to polymer are provided in table 6.1. DPn was calculated via ^{1}H-NMR (spectra are shown in figure 6.1 and 6.1) using equation 1 whereas p is calculated using equation 2.

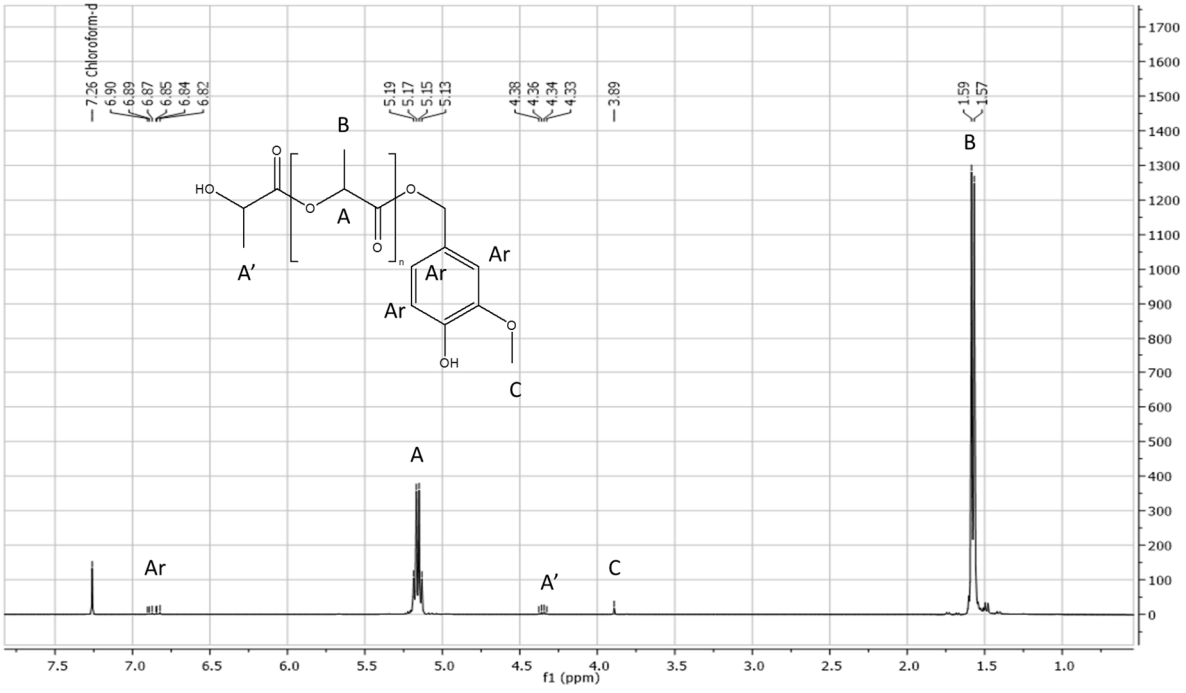


Figure 6.1 :1H-NMR of PLA with 0.5% of VA

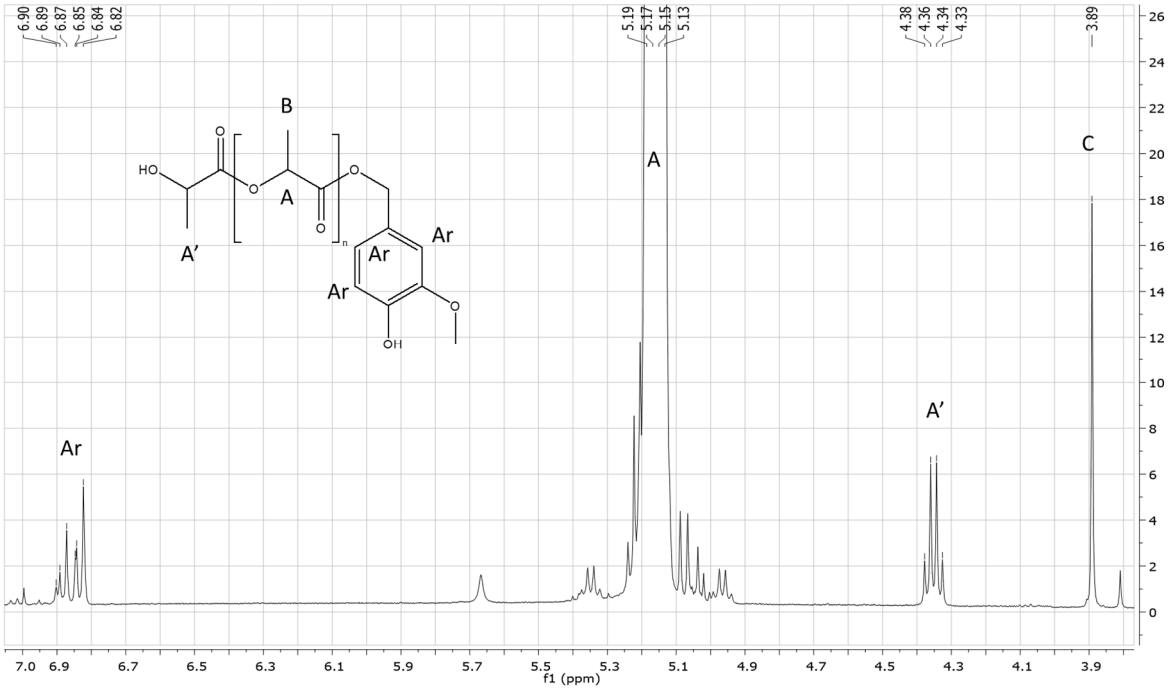


Figure 6.1: zoom in the region between 3.7-6.5 ppm of 1H-NMR of PLA with 0.5% of VA

$$1)\ DPn = \frac{\int CH}{\int CH_{end}}$$

$$2)\ p = 1 - \frac{1}{DPn}$$

Where ∫CH is the value of the NMR integral of multiplet at (5.19, 5.17, 5.15, 5.13), $\int CH_{end}$ is the value of the NMR integral of multiplet at (4.38, 4.36, 4.34,4.33)

Sample	PLA+0.1%$_{mol/mol}$AV		PLA+0.5%$_{mol/mol}$AV		PLA+10%$_{mol/mol}$AV
Feed (%$_{mol/mol}$)	0.1%*	0.1%	0.5%*	0.5%	10%
DPn	68	55	52	64	6
p (%)	98.5	98.2	98.1	98.4	83.3
Molar percentage AV (%$_{mol/mol}$)	0.096	0.102	0.456	0.470	5.228

*Table 6.1: data of PLA solution synthesis; * sample was analyzed before solution pouring in methanol.*

The VA reaction proceed with high conversion (p > 98%) for concentration lower than 0.5%$_{mol/mol}$ whereas high concentration of VA (10%) dramatically affects the reaction lowering the conversion to 83%. As previously stated, the acidity of phenolic moiety deactivates the complex Tin-Lactide-Initiator inhibiting the polymerization. Moreover, VA acts as chain initiator since the quantity of VA, measured via ^{1}H-NMR, slightly increase after the pouring process in methanol: VA, lactide and low molecular weight PLA chains have high solubility in methanol whereas high molecular PLA fraction has not; therefore, the non-bonded VA should be solubilized by methanol while the bonded VA should not.

Number average molecular weight (Mn), Weight average molecular weight (Mw) and poly dispersity index (D) are provided in table 6.2 (the curves are reported in figure 6.2). It is possible to observe how the molecular weights, and therefore the hydrodynamic volume, decrease increasing the amount of VA in the feed. Moreover, the polydispersity index, obtained via GPC, is slightly higher than 1 for samples *PLA+0.1%$_{mol/mol}$AV* and *PLA+0.5%$_{mol/mol}$AV* whereas it seriously increases for sample *PLA+10%$_{mol/mol}$AV*: the high amount of VA, which deactivates the complex among lactide, catalyst and initiator, does not allow the formation of polymeric chains but only oligomeric species.

SAMPLE	Mn (Da)	Mw (Da)	D
PLA+0.1%mol/mol AV	10872	13632	1,25
PLA+0,5%mol/mol AV	7781	9236	1,19
PLA+10%mol/mol AV	632	1014	1,61

Table 6.2: GPC results

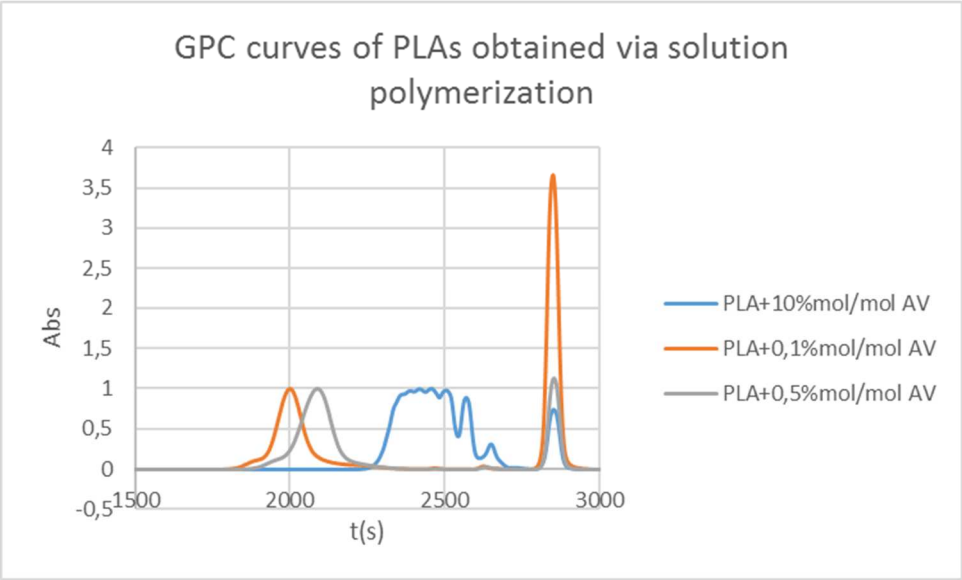


Figure 6.2: GPC curves of PLAs obtained via solution polymerization of lactide with VA as chain initiator.

The preliminary studies permit to conclude:

- VA is able to react with lactide acting as chain initiator; moreover, VA does not negatively affect lactide polymerization for concentration lower than 0,5%
- VA conversion is higher than 90% for concentration lower than 0,5%mol/mol

6.2 Bulk polymerization

The aim of the project is to obtain intrinsic antioxidant polymers using the standard industrial condition used for PLA synthesis. Therefore, the further step was the setup of the reaction of lactide with VA via bulk polymerization conditions. The reaction was performed under N_2 atmosphere at 190°C: at this temperature the lactide is melted and the kinetics of reaction steeply increase leading to a polymerization time of about 1.5h. On the other hand, the high temperature, together with the presence of tin as catalyst, can promote side reactions of VA. Tin is used for degradation of phenolic compounds in water treatment and enzyme catalyzed oxidative coupling reaction of VA was already reported in literature. (157) (158) Nevertheless, the low concentration of tin in the reaction feed together with the absence of oxygen should prevent degradation of VA.

Besides the VA, which has high antioxidant power, Tyrosol (Tyr) was identified as potential model molecule for bulk polymerization: tyrosol has higher thermal stability in comparison to VA therefore the temperature should not lead to degradation during the polymerization, permitting to determine how the bulk polymerization proceeds in presence of phenolic compounds.

The molecular weight of the resulting polymer can be controlled changing the feed of reaction, the DPn of the polymer depends on the ratio [M]/([Initiator] x *f*) where the [M] is the molar concentration of monomer, [Initiator] is the molar concentration of initiator and f is the number of active moieties (in this case *f*=1). As for any other thermoplastic polymers, the mechanical properties of a PLA depend on its DPn: the entanglements molecular weight was reported as 9000Da (159), that means a DPn value of approximately 125 (standard industrial PLAs have DPn higher than 500). Using a concentration of initiator equals to 0,5%$_{mol/mol}$, limit DPn is 200 therefore the polymer cannot have the same performance of industrial materials. The maximum concentration of chain initiators allowed is 0,2%$_{mol/mol}$ (DPn =500 for conversion equals to 1).

Sample	M_n (Da)	M_w (Da)	D
PLA+0.1%Tyr	73456	124742	1,71
PLA+0.25%Tyr	16977	43304	**2,55**
PLA+0.5%Tyr	13055	34166	**2,62**

Table 6.3: GPC results of bulk polymerization reactions with Tyr as chain initiators

Bulk polymerization of PLA was performed using Tyr concentration of 0,1%$_{mol/mol}$, 0.25%$_{mol/mol}$ and 0.50%$_{mol/mol}$ (maximum theoretical DPn are respectively: DPn=1000; DPn=400; DPn=200): experimental results confirm that Tyr reacts with lactide as chain initiators since increasing the concentration of Try, the values of M_n and M_w decrease; results are provided in table 6.3. It is important to notice that, for concentration of 0.25%$_{mol/mol}$ and 0.5%$_{mol/mol}$, an unexpected decrease of molecular weight is registered and also the D values distance themselves from the ideal value of 1.6: the presence of relatively high concentration of phenolic moiety affects the polymerization process.

Nevertheless, the molecular weights of *PLA+0.25%Tyr* and *PLA+0.50%Tyr* are unexpectedly similar: for concentration of Tyr higher than 0.1%$_{mol/mol}$, Tyr seems to negatively affect the polymerization reaction blocking the propagation step. Probably the acidity of tyrosol inhibits the formation of complex among lactide-Tin-chain initiator blocking the propagation of the reaction; moreover, the acid ambient, together with the high temperature, catalyzes the backbiting reactions that increase the polydispersity (160). Thus results permit to understand how a phenolic compounds affects the polymerization of lactide and to proceed with the reaction of VA with lactide.

Using the same Tyr concentration, polymerization reaction was then repeated for the synthesis of VA modified PLA. GPC results are provided in table 6.4

Sample	M_n (Da)	M_w (Da)	D
PLA+0.1%VA	70781	114003	1,61
PLA+0.25%VA	25809	118632	4,61
PLA+0.5%VA	10799	31842	2,95
Nature Works 4043D	90702	171573	1,89

Table 6.4: GPC results of lactide bulk polymerization reaction with VA at different molar concentration

As in the case of Tyr, only 0.1%$_{mol/mol}$ of VA leads to high molecular weight polymer whereas concentrations of 0.25%$_{mol/mol}$ and 0.50%$_{mol/mol}$ affects the polymerization reactions. The presence of a phenol compounds, in concentration higher than 0.1%mol/mol, inhibits the Lactide polymerization reaction affecting not only the molecular weights and polydisperisity index but also the aspect of polymers: PLA with 0.25%$_{mol/mol}$ and 0.50%$_{mol/mol}$ of VA have different colors related to the degradation phenomena: the bulk polymerization conditions promotes side reaction of VA, which are disadvantaged in high diluted sample, leading to light brown polymers.

Coupling reaction of phenols compounds, in presence of enzyme and oxidative ambient (O_2 or H_2O_2) are widely reported in literature. Similar reaction was described for the the preparation of oligoTyr (161) (162) (163) (164). Even though the polymerization reaction was performed in inert atmosphere (N_2 flux) the presence of metal catalyst can promote degradation reactions of phenolic compounds. Tin is reported to be used as catalyst for phenolic compounds degradation in polluted water: very low concentration of phenolic compounds (concentration lower than 0.2%$_{mol/mol}$) disadvantage the side reaction of VA and Tyr whereas for concentration higher than 0.2%$_{mol/mol}$ the side reactions start to occur.

The attention was therefore focused on 0,1%$_{mol/mol}$ VA and Tyr modified PLAs samples. GPC results of these polymers are provided in table 6.5. NatureWorks 4043D is an industrial PLA used as a standard industrial material: it is produced and sold for flexible pakcaging.

Sample	M_n (Da)	M_w (Da)	D
PLA+0.1%Tyr	73456	124742	1,71
PLA+0.1%VA	70781	114003	1,61
Nature Works 4043D	90702	171573	1,89

Table 6.5: GPC results of lactide bulk polymerization reactions with VA and Tyr

The effects of VA and Tyr on molecular weights are practically the same: the molecular weight of *PLA+0.1%VA* and *PLA+0,1%Tyr* is very similar as the polydispersity of the material. The presence of low concentration of phenols does not affect the polymerization process even in bulk polymerization.

Polymers having higher VA concentration were synthetized in order to check the highest level of phenols that is possible to load in the feed without compromising the polymer properties. Samples with $0.25\%_{mol/mol}$ and $0.50\%_{mol/mol}$ were synthetized and M_n, M_w and D data, obtained via GPC analysis, are provided in table 6.4.

6.2.1 Thermal properties

Thermal properties of polymers were assessed using differential scanning calorimetric (DSC), Glass transition temperature (T_g), cold crystallization temperature (T_{cc}), cold crystallization heat (ΔH_{cc}), melting temperature (T_m), melting heat (ΔH_m) and crystallinity (X_c) data are provided in table 6.6; all the data reported are referred to the second heating cycle.

Sample	T_g	T_{cc}(°C)	ΔH_{cc}(J K^{-1} g^{-1})	T_m(°C)	ΔH_m(J K^{-1} g^{-1})	Xc
PLA+0,1%Tyr	53,00	107,25	69,12	168,34	72,07	3,17
PLA+0,1%VA	52,74	108,34	55,34	167,39	58,34	3,23
Nature Works 4043D	59,08	124,29	14,23	155,26	15,28	1,13

Table 6.6: thermal properties of synthetized polymers in comparison to Ingeo NatureWorks 4043D

All samples shown a clear glass transition temperature, an exothermic peak related to the cold crystallization and melting peak. DSC show that all samples have a marked tendency to have a cold crystallization but *PLA+0.1%VA*and *PLA+0,1%Tyr*, that have very similar behavior, show a lower

crystallization temperature (about 15°C lower than the temperature registered for PLA 4043D) but a higher cold crystallization enthalpy. On contrary, the Tm measured for *PLA+0.1%VA*and *PLA+0,1%Tyr* is higher than the Tm obtained for PLA 4043D (about 11°C) also the melting enthalpy of *PLA+0.1%VA*and *PLA+0,1%Tyr* are higher than PLA 4043D. The Ingeo 4043D behaviour indicates a lower crystallinity content in comparison to *PLA+0.1%VA*and *PLA+0,1%Tyr*; Xc values show that *PLA+0.1%VA* and *PLA+0,1%Tyr* have a crystallinity content three time higher than the one of Ingeo 4043D. The lower crystallinity of PLA Ingeo 4043D is due to its D stereisomer content: the producer has declared that the total amount of D isomer is approximately 4,3% whereas in the lactide, used for the synthesis of *PLA+0.1%VA*and *PLA+0,1%Tyr*, the D isomer content is lower than 1% as declared by producers.

Sample	$T_{1\%}$ (°C)	$T_{5\%}$ (°C)	$T_{50\%}$ (°C)	$T_{95\%}$ (°C)	$\Delta T_{1\%-95\%}$ (°C)
PLA+0,1%Tyr	270	301	346	370	100
PLA+0,1%VA	274	302	353	381	107
Nature Works 4043D	351	351	380	395	44

Table 6.7: degradation temperature determined via thermo gravimetrical analysis (TGA)

Thermal stability of *PLA+0.1%VA*and *PLA+0,1%Tyr* were also assessed in comparison to PLA Ingeo 4043D via TGA analysis checking the temperature of 1%, 5%, 50% and 95% of weight loss ($T_{1\%}$, $T_{5\%}$, $T_{50\%}$, and $T_{95\%}$). Data, provided in table 6.7, show that PLA+0.1%VA and PLA+0,1%Tyr have a lower thermal stability compared with PLA Ingeo 4043D: the later has a temperature of degradation $T_{1\%}$ of approximately 351°C, almost 80°C higher than *PLA+0.1%VA* and *PLA0,1%Tyr*. Comparable degradation temperatures were previously reported by Basilissi et al.: they synthetized in bulk, with the same method reported in this work, PLA nanocomposites and measuring a $T_{1\%}$ for a non-modified PLA 248°C, a temperature 26°C lower than the one measured in this work for *PLA+0.1%VA* and 22°C for *PLA+0,1%Tyr*. The presence of antioxidant linked to the chain enhance the thermal stability of the new polymers, in addition it is important to notice that the producer supplies processing stabilizer into PLA Ingeo 4043D is (165). Anyway, although the degradation of *PLA+0.1%VA* and *PLA+0,1%Tyr* starts at low temperature, the processability of the material is not affected since PLA is commonly processed at temperature about 200°C that is widely lower than the temperature of degradation beginning. TGA curves are shown in figure 6.3.

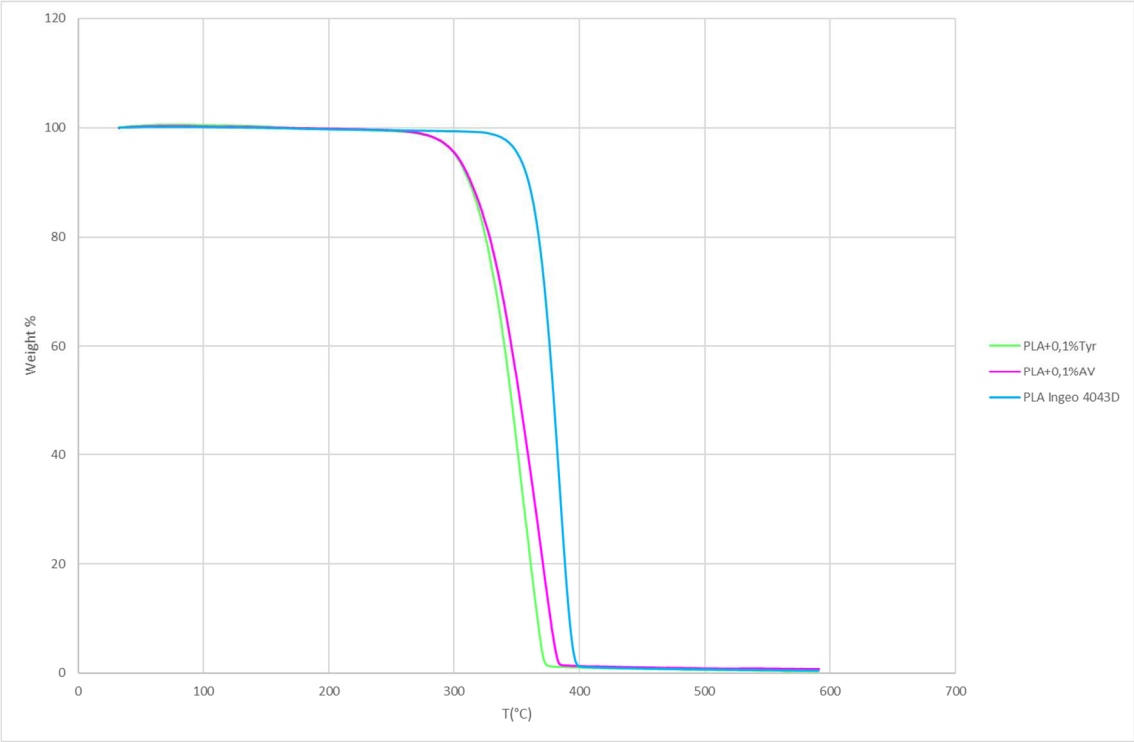


Figure 6.3: TGA curves of PLA+0,1%VA, PLA+0,1%Tyr and NaturWorks 4043D

6.2.2 Antioxidant features

The evaluation of antioxidant features of the polymers is one of the aim of the project, therefore it was necessary to find a way to determine if the polymerization reaction could affect the phenolic moiety responsible for such property. The antioxidant power was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. DPPH is a stable organic radical with high solubility in organic solvents such as methanol, THF, dichloro methane etc.; the UV-vis spectra of the unpaired molecule presents two absorbance maxima one at 350nm (yellow solution) and one at 515nm (dark purple solution) whereas the reduced form presents only one absorbance maximum at 350nm (yellow solution); the UV-Vis spectra of both forms and their structure are reported in figure 6.4. An antioxidant, such as VA or Tyr, is able to reduce the DPPH molecule leading to solution color change: the dark purple solution turns into a pale yellow solution and the absorbance decrease, measured at 515nm, is used to determine the antioxidant power.

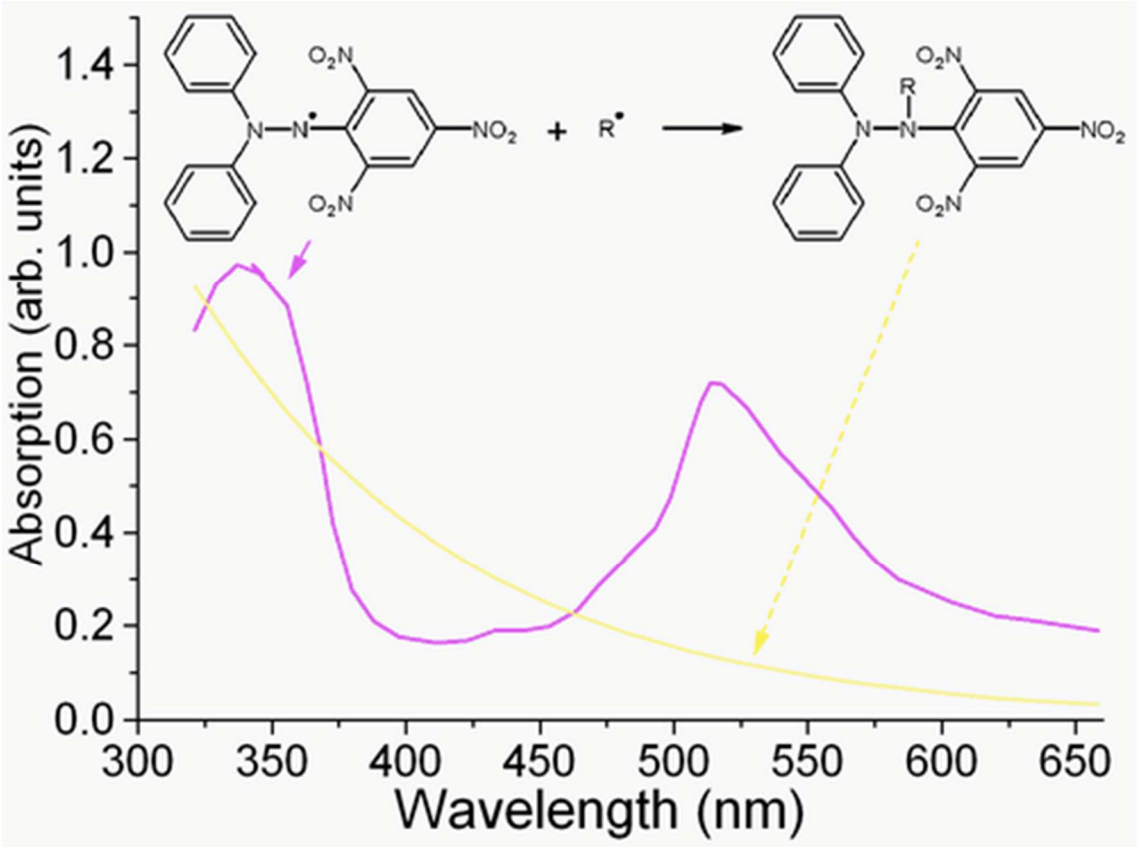


Figure 6.4: DPPH and DPPH reduced form UV-vis spectra

The assay is normally performed using methanolic antioxidant solution; nevertheless, in this case the antioxidant molecule is bond to a polymeric chain that makes it insoluble in methanol. The standard methodology was modified for this purpose: a polymeric film was cut in small portions that were left in contact for 30 minutes with DPPH solution; then the absorbance was measured obtaining the antioxidant power of polymeric films. DPPH assay results are provided in table 6.7.

Sample	Antioxidant Power (%)
VA	**91 ± 1,4**
Tyr	7.6 ± 0.2
PLA+0.1%Tyr	4.3 ± 4,5
PLA+0.1%VA	***12.4 ± 1.5***
Nature Works 4043D	1.0 ± 0,2

Table 6.7: antioxidant power of pure substances and polymers

The PLA 4043D having no additives, and *PLA+0.1%Tyr*, does not show any antioxidant features whereas the *PLA+0.1% VA* shows an interesting activity. The antioxidant power of VA has been therefore successfully transferred to polymeric chains.

6.3 Conclusion

With one-pot synthesis is possible to obtain intrinsic antioxidant material with properties, in terms of thermal stability and molecular weight, comparable to standard PLAs. Moreover, interesting antioxidant properties have been achieved even using very low concentration of VA (0.1%mol/mol), which is covalently bond to the polymeric chains: the VA cannot migrate into food therefore the antioxidant properties of polymer cannot decrease during the time.

7. Shelf-life Study

Even though in literature are reported higher value radical scavenging activity for antioxidant films (31), the very promising DPPH results on *PLA+0.1%VA* sample allowed to perform shelf life test with a real industrial food matrix. The test was performed in I.R.T.A. laboratories (Monells, Catalunya, Spain) in collaboration with Dr. Marcos B.M.,

The shelf life test aims to discover how a packaging method affects food or beverage properties. In this case, the aim of shelf life test is to verify the antioxidant properties of the VA modified PLA, therefore it is important to individuate an easily oxidizable food in order to rapidly asses how the VA modified PLA affects the oxidation kinetics. Cured meat products are ideal candidates due to their high level of fat and due to the low pH value of meat:

- Fats are easily oxidizable by radical attack: ROS react with lipids promoting the formation of byproducts (*i.e.* malondialdehyde) that rapidly change the aspect, taste and flavor of food
- Low pH obtained in cured meet products is fundamental to avoid bacterial growth: pH lower than 5 disadvantage microbial activity therefore the degradation of food is only due to radical exposure.

A 1m long industrial salami was chosen as standard food, this kind of products are normally used for industrial preparations. Even though preservatives are widely used in industrial foods, the use of handcrafted products will be avoided the effects of preservatives highlighting the effects of VA modified PLA but, at the same time, it does not allow to obtain reliable results due to the intrinsic variability in food production.

Salami was sliced and the VA modified PLA was used as interlayer between two slices; then the slices were packed into a low density polyethylene bags. The air inside the PE bags was removed and then the packaged salami was thermo-sealed. The package was exposed to standard shelf-conditions of light exposure (12h of lights and 12h of darkness) and temperature (4°C ±2°C).

The test required a long preparation time since, to perform reliable tests, a minimum of 160 salami slices must be tested: PLA films were used as interlayer between two salami slices. For an each packaging of two salami slices, a film of about 100cm^2 is required. It means that for a reliable shelf life test at least 8000cm^2 of active PLA (the surface of 12 A4 sheets) was required; solvent casting deposition permits to produce only one A4 sheet of PLA film every 15h, therefore the production of all polymer sheets for the test took more than 180h.

During the shelf life assay, properties of food and material were checked: water activity (a_w), *pH*, color, thiobarbituric acid reactive substances (TBARS) and DPPH of *PLA+0.1%AV*. a_w and *pH* are standard parameters correlated to the microbial growth; they are normally controlled during a shelf life test whereas color is a crucial parameter used to assess how the light exposition affects the aspect of packaged food.

7.1 pH and Water Activity

Water activity (a_w) and pH were assessed during the shelf-life process. These parameters are normally used to control the microbial growth.

Low pH value, obtained during curing process of meat, dramatically disadvantage the microbial growth even in products with high quantity of water. Normally pH lower than 5 inhibits microbial growth, for instance: the growth of E. Coli occurs at 5.6<pH<6.8 at 15°C as was reported by the study of Gibson and Roberts (166). During this test the pH was constant between the range 4.63 to 4.75 therefore the E. Coli growth is inhibited. Figure 7.1 shows the trend of pH during the shelf life test.

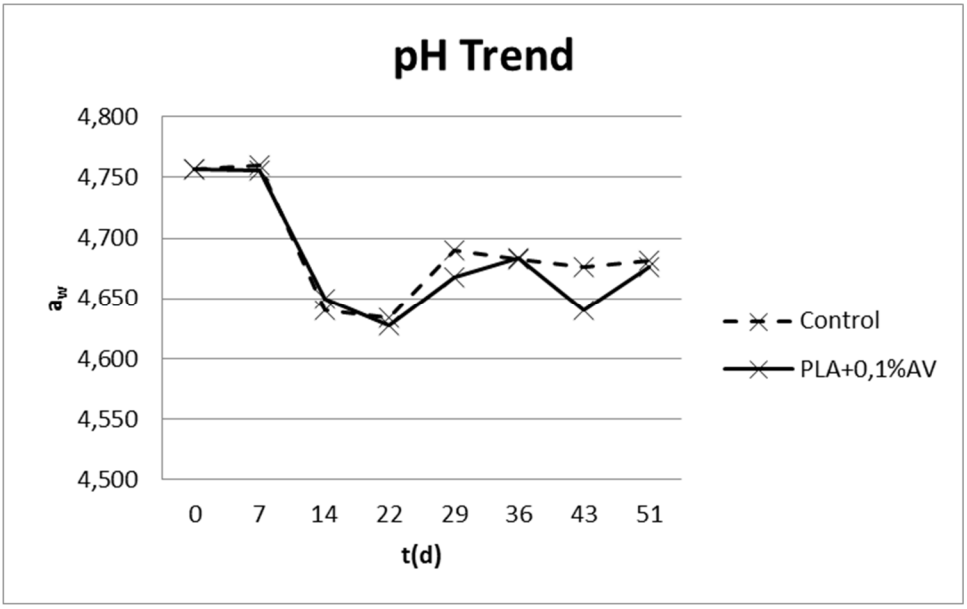


Figure 7.1 pH trend measured during the shelf life test

Also a_w is checked in order to verify the microbial growth, W. J. Scott correlated in 1953 the a_w to the bacterial growth. (167) (168). Sperber reported in his study that E. Coli does not survive for a_w lower than 0,95 and also Penicillium survive for aw higher than 0,92 (169) (170). The trend of a_w during the shelf life test is reported in figure 7.2.

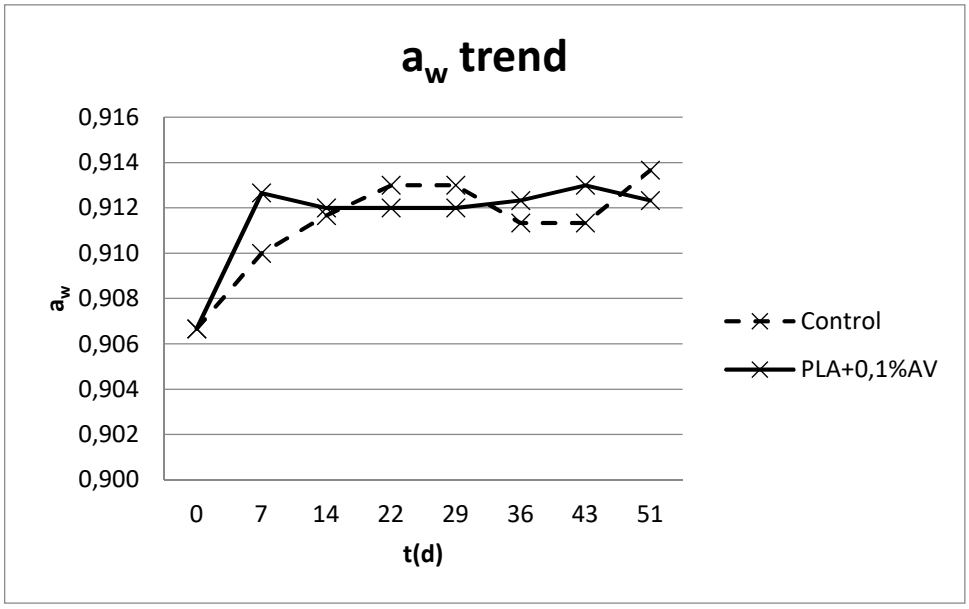


Figure 7.2: a_w trend during the shelf life study

7.2 Color

The PLA films were used as interlayer between two salami slices whereas the external wrapping was made with PE/PA. The light exposure normally affects the aspect of food therefore the color was measured only on the surface of slice exposed to the cabinet light. Even though the *PLA+0.1%VA* does not interact with the light it is important to control this parameter. The parameters a*(yellowness), b* (redness) and L* (lightness) were registered; in figure 7.3 is the spectrum of visible light related to the value a* and b* is given.

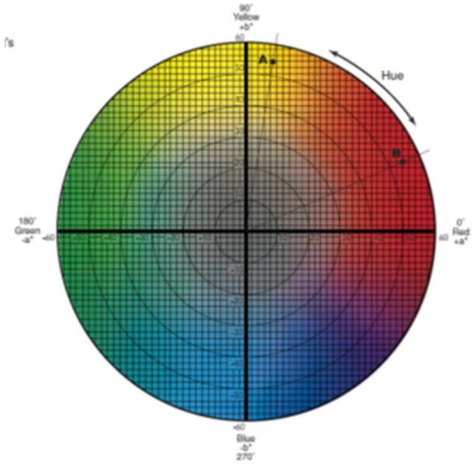


Figure 7.3: colors spectrum of visible light

As it possible to see from the graph reported in figure 7.4 and 7.5, L* trend and a*, during the assay slightly change. On the other hand, most interesting results were obtained analyzing the b* parameters: during the assay, the value of b* decrease moving from the pale grey to grey. At the

beginning of the test the b* value was approximately 9 whereas at the end was almost 4. This decrease in b* value indicates a surface color modification of salami slice that changed their color during the test. The surface exposed to the cabinet light appeared grey whereas the side, which is not exposed, does not present color modification. Only slight difference can be observed between *control* and *PLA+0.1%VA*

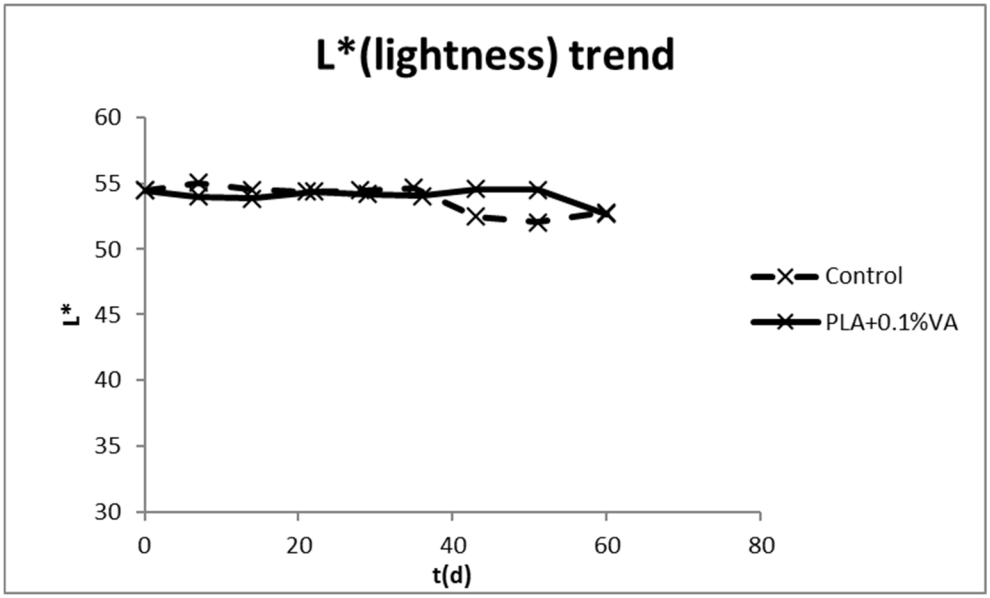


Figure 7.4: L trend during shelf life study*

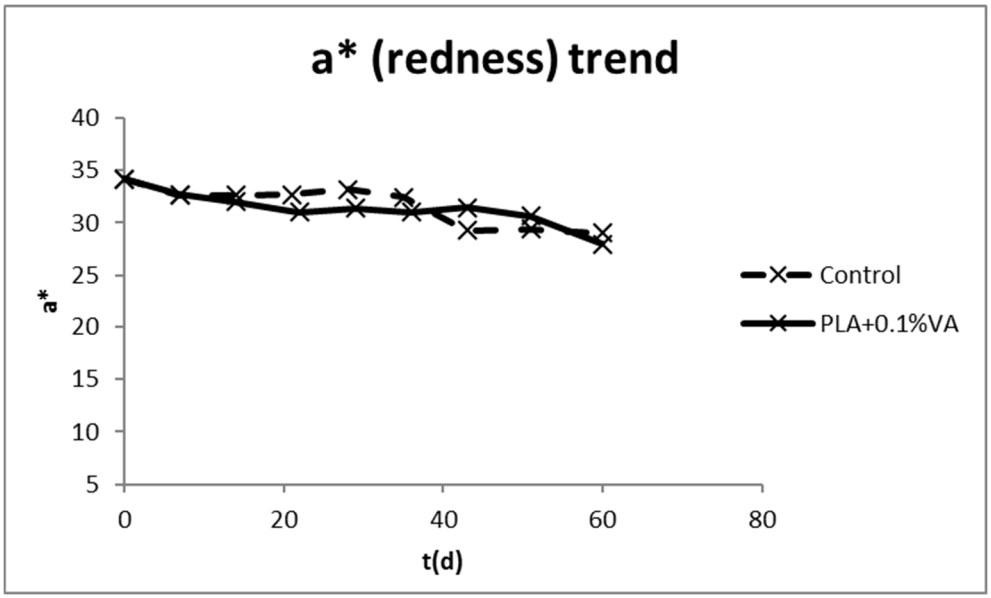


Figure 7.5: a trend during shelf life study*

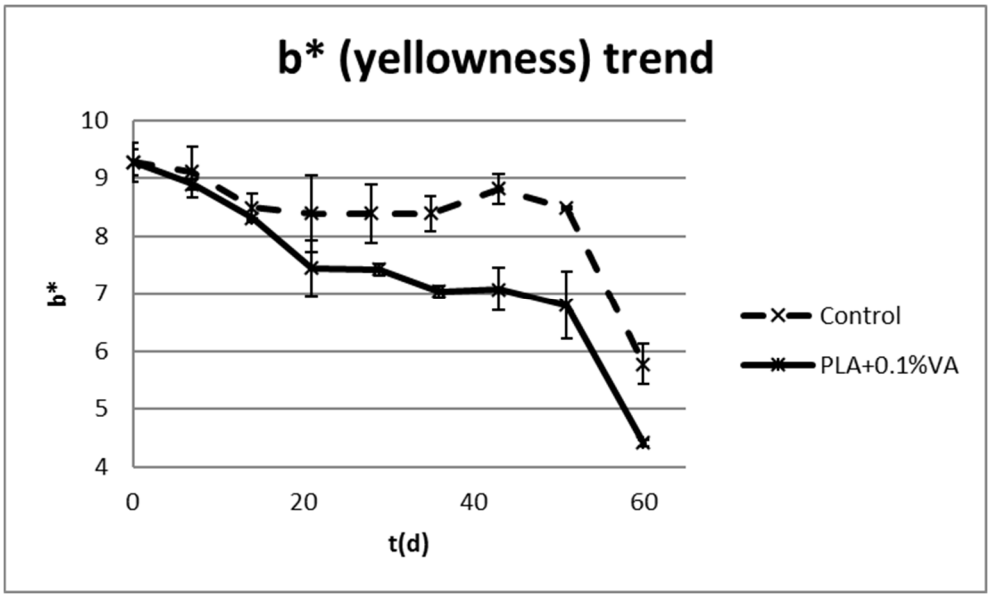


Figure 7.6: b trend during shelf life test*

7.3 TBARS

Thiobarbituric acid(TBA) reactive substances (TBARS) is a typical assay used to determine the oxidation level in meat products. It is a colorimetric system and measures the concentration of malondialdehyde (MDA), which is one of the most important by-products of meat oxidation (171).

The reaction between MDA and TBA is shown in figure 7.7: the reaction leads to the formation of a product detectable via UV-Vis analysis. In this study, TBARS determination was performed since this parameter is one of the most widely used tests for evaluating the extension of secondary lipid oxidation that can limit quality and acceptability of meats products.

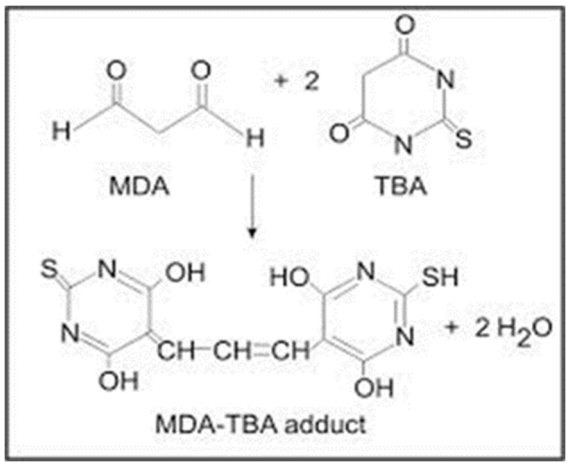


Figure 7.7: reaction of TBA with MDA used for the determination of TBARS

Measuring the TBARS it is possible to determine the threshold of oxidation and how the *PLA+0.1%VA* affects the kinetic of oxidation of food. The TBARS trend (see figure 7.8) shows that the oxidation of salami immediately starts for "control" products whereas for samples prepared using *PLA+0.1%VA* as interlayer the oxidation begins later, i.e. after 20 days.

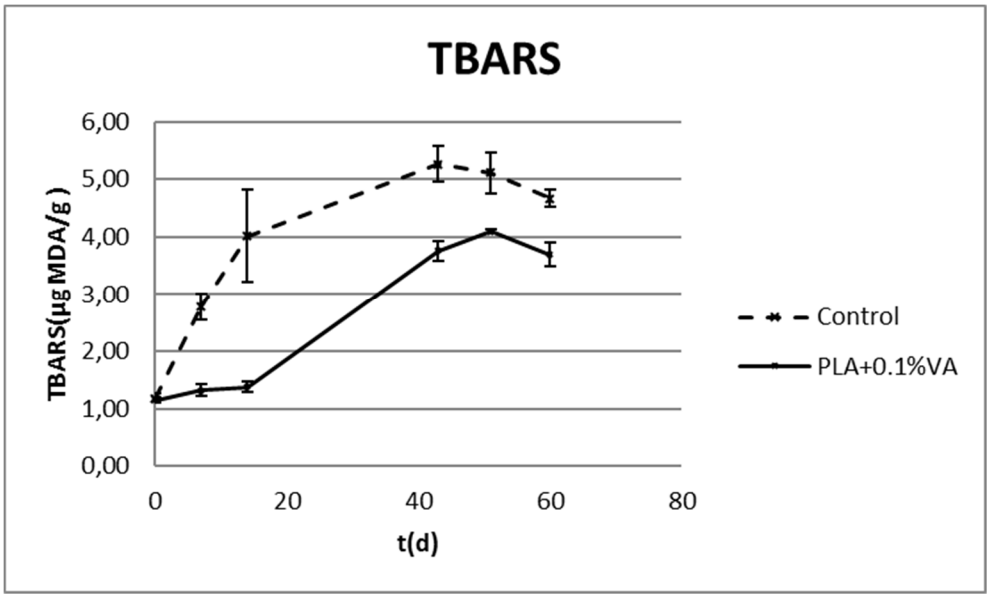


Figure 7.8: TBARS trend during shelf life test

The presence of VA, bond to PLA, freezes the oxidation of lipids for the first 20 days but, after this time, the kinetics increase. In any case, when the oxidation begins in samples with *PLA+0.1%VA*, the concentration of MDA never reaches the concentration of MDA registered for control samples.

7.4 DPPH

Besides the test performed on food, also the PLA used as interlayer was analyzed. Radical scavenging power of the films was assessed during the shelf life test: *PLA+0.1%VA* films, taken from the salami slice, were washed with n-hexane in order to remove the residual lipids form the surface and then they were analyzed following the same procedure used for original films.

Normally the DPPH values decrease during the time; the active molecules, used as additives migrate into food (31). In this case, as it is possible to see from the DPPH trend shown in figure 7.9, the radical scavenging power remains constant during the all test.

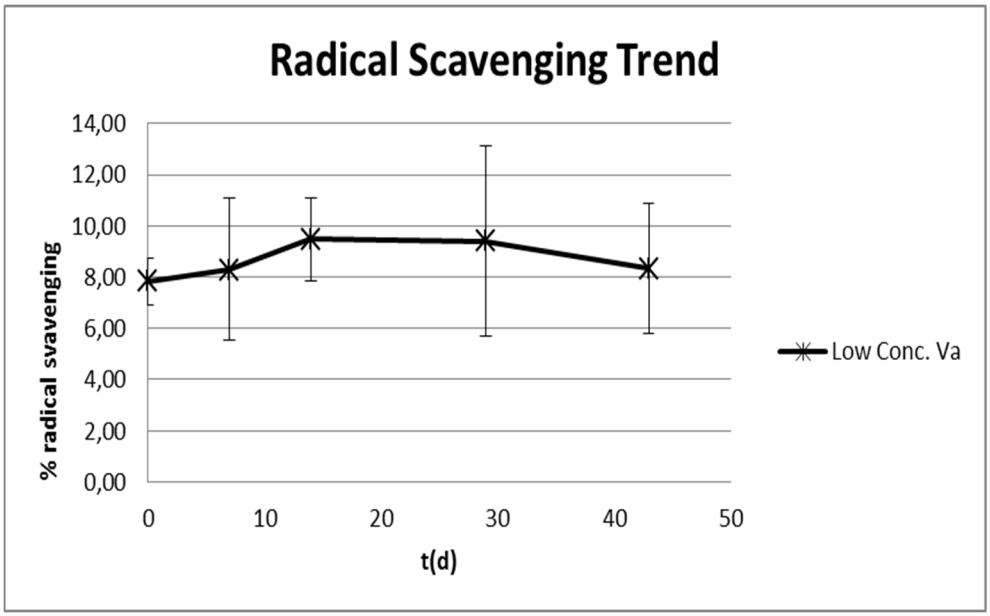


Figure 7.9: radical scavenging activity trend measured via DPPH

Constant values of antioxidant power could be addressed both to the non-migration of of VA to food matrix or to a negligible consumption of VA for food stabilization. On the other hand, the covalent bond of the antioxidant molecule was previously assessed (GPC analysis do not show low molecular weight peaks addressing to unreacted VA of Tyr) therefore, a constant value of radical scavenging power indicates a high performing material. The contact with radicals, since the degradation of salami indicates the presence of radicals species, does not affect the activity of the *PLA+0.1%VA*: it means that with very low quantity of antioxidant bonded to the polymer [i.e. 0.1%, in other works the quantity are approximately 5% (31)] it is possible to obtain a material with very high activity able to prevent oxidation and maintaining constant its radical scavenging power.

7.5 Conclusion

Intrinsically antioxidant biobased materials were obtained. The study conducted allows to understand how aromatic hydroxyl moieties, in VA and Tyr, affect the bulk polymerization of lactide: concentration lower than 0,2%mol/mol of phenolic compounds permits the polymerization whereas high concentration dramatically affects the chain growth process; the acidity of phenolic compounds promotes backbiting reaction and deactivates the complex metal-lactide-chain initiator.

Vanillyl Alcohol and Tyrosol have an aliphatic alcoholic moiety that can react with lactide acting as chain initiator transferring their radical scavenging activity to the polymeric chains. The radical scavenging powers of polymers were assessed: *PLA+0,1%Tyr* and pure PLA do not show antioxidant effect whereas *PLA+0.1%VA* shows interesting antioxidant features.

The shelf life assay demonstrated how the VA modified PLA is able to counteract the oxidation in salami samples packaged using *PLA+0.1%VA*as interlayer between two salami slices. The oxidation begins after 20 days in the samples in contact with *PLA+0.1%VA* whereas in *control* samples the oxidation starts since the first day.

This second part of the project is to develop biomaterials to be used for the delivery of bioactive antioxidants. The idea is to investigate and to tailor the proper morphology of delivery systems using commercial materials already approved for human body contact.
Focus directly on a future industrial development systems here studied.

The antioxidants identified for our scopes are the oligomers of the Tyrosol (oligoTyr), which is obtained via enzymatic coupling of Tyrosol, and Ellagic Acid (EA) that is one of the most abundant and active compounds in pomegranate. Even though these antioxidants have different activity, the method used for the development of the delivery system was the same:

1. Identify a polymer compatible both with active molecules and with target tissue.
2. Identify the best morphology for the release systems in order to enhance the interaction with target tissue.
3. Set up a method for preparation of a release system.
4. Verify the release properties and understand the parameters that affect the release.

8. OligoTyr Release System

8.1 OligoTyr properties

OligoTyr is the oligomer of Tyrosol and it can be obtained by horseradish peroxidase, in presence of H_2O_2, catalyzed coupling reaction (45). This is a new product therefore it was necessary to assess its properties in terms of antioxidant features and biological activity. Prof. Burlando's group performed the biological assays in order to determine the toxicity and the activity for ossification.

8.1.1 OligoTyr Structure

OligoTyr structure was determined via CP MAS NMR, ESI (-) MS analysis and GPC analysis. The combination of the data obtained permits to determine that the oligoTyr is mainly linear oligomeric molecules with some branching point, the hydroxyethyl chain is not involved in the polymerization process (the signals are visible in CP-MS ^{13}C-NMR). The structure proposed is shown in figure 8.1, the narrow molecular weight distribution, obtained via GPC analysis, confirms the non-branched structure.

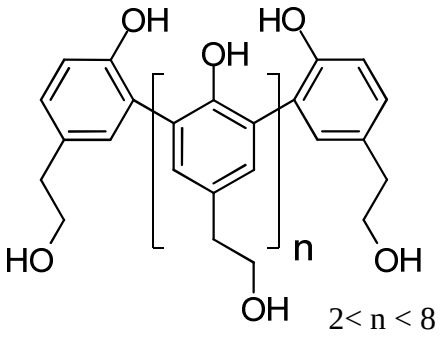


Figure 8.1: oligoTyr structure proposed

8.1.2 OligoTyr Properties

The lowering of pk_a of oligoTyr, in comparison of Tyr, [pk_a(Tyr) = 10.3; pk_a(oligoTyr) = 7.3] suggests that the aromatic rings (coupled each other) resonance promotes the phenolic activity. The antioxidant features were determined as DPPH radical scavenging, Trolox and hydroxy radical scavenging results are provided in table 8.1

Sample	DPPH reduction (%) *0.06mg ml^{-1}*	Trolox eqs (FRAP assay)	OH radical scavenging assay (IC50, mg ml^{-1})
Tyrosol	24 ± 1	0.054 ± 0.002	0.73 ± 0.01
OligoTyr	48 ± 2	0.072 ± 0.002	1.16 ± 0.02

Table 8.1: antioxidant assay results of Tyrosol and oligoTyr

The increased activity of oligoTyr as radical scavenger was assessed. In the DDPH assay, OligoTyr was found to exert a modest but significant antioxidant effect as compared to trolox (97 ± 1% reduction), superior to that of the parent compound tyrosol. The hydroxy radical scavenging, measured as salicylate reduction, the oligoTyr shows a reduction capacity 60% higher than tyrosol and even higher than ascorbic acid [IC50 (0.81 ± 0.02) mg ml^{-1}].

Antioxidant compounds have recently been exploited in tissue engineering as promoters of osteoblast differentiation, since they can counteract the inhibitory effects of reactive oxygen species (ROS) on the process of bone formation by osteoblastic cells. The oligoTyr shows enhanced antioxidant features due to the conjunction of the aromatic rings in ortho- position; therefore, it was studied as ossification process promoter.

Firstly, the biocompatibility of oligoTyr was studied in comparison with Tyr by determining the calcein-AM cell. (172) The value obtained (IC50 > 500 mg ml^{-1}) indicated a very low cytotoxicity of OligoTyr, as well as of tyrosol.

In a further step the ability to improve the ossification in human osteosarcoma cells (SaOS-2) was assessed: the activity of oligoTyr and tyrosol were determined in a range of 7 days as *Alkaline Phosphatase* (ALP) (173); Data are expressed as mean SD (n ¼ 8–16) of p-nitrophenol optical density (OD) at 405 nm, standardized as percent of control. *p < 0.01 with respect to control, according to multiple t test with Bonferroni correction. The results are shown in figure 8.2.

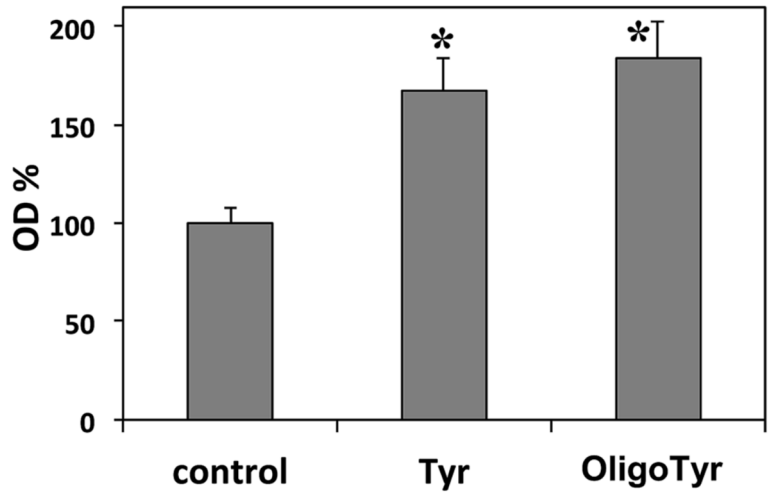


Figure 8.2: ALP activity of oligoTyr, tyrosol and control in 7 days on human osteosarcoma cells

Tyrosol and OligoTyr have a significant effect on ALP activity in respect of untreated cells (control). These results, together with the low cytotoxicity, demonstrated that the oligoTyr and Tyr are able to promote the ossification of human cells. The activity of oligoTyr (enhanced antioxidant features, ALP activity, low cytotoxicity) permits to focus the attention on its delivery.

8.2 Scaffolds

PLA was chosen for the production of scaffolds: it is already used for production of scaffolds and, in human body environment, its *biodegradation time* is higher than 24 months (174). Industrial PLA NatureWorks Ingeo 4043D (≈4.3%of D-lactic content) was used. Primary and secondary antioxidants, which are added by the producer to the polymer during its synthesis, have to be removed in order to avoid any influence on oligoTyr and Tyr activity in biomedical assays.

The active molecule (i.e. Tyr and OligoTyr) has to be soluble both in extractive media (in this case phosphate buffer solution) and in polymer amorphous phase. PLA4043D was therefore chosen due to its very low fraction of crystalline phase: according to DSC, only 0,4%w/w of polymer is crystalline. Moreover, PLA is relatively hydrophobic: its static water contact angle is 67°C, between hydrophobic of 90° and hydrophilic of 0°. These characteristics of PLA (high biocompatibility, low crystalline phase, lipophilic behavior) make it a good candidate as material for the delivery of oligoTyr.

The poor water solubility of oligoTyr requires a high water contact to allow its extraction from the polymeric matrix, therefore the scaffolds must have the highest possible surface in order to promote the release. A sponge-like morphology was chosen for this purpose: other systems, such as nanoparticles can satisfy this request but, although a lot of work regarding the use of nanoparticles as release system have been done in these years, many doubts about their safety have to be clarified

and, due to the aim to focus the attention on the immediately scale-up of these devices for practical application, the use of nanoparticles was avoided. Scaffolds with sponge-like morphology are properly called *Hierarchical Scaffolds*: the structure of the scaffolds presents very high surface area and they are widely used for bone regeneration: since the porous structure is *bone-like* structure and supports the cells growth. (175) (176) (177) (178)

8.2.1 Scaffold Preparation

In paragraph 6.11, two methodologies for scaffolds preparation are reported. PLA scaffolds were first prepared solubilizing both PLA and oligoTyr and Tyrosol (oligoTyr in concentration of 5% $_{w/w}$; 4.5% $_{w/w}$ and 3% $_{w/w}$, Tyrosol in concentration of 5%$_{w/w}$) in a 95:5 THF : Methanol solution (the latter permits the completely solubilization of oligoTyr). The solution was then frozen and the porous material was recovered after pouring in warm water in order to remove solvents. The dried materials were analyzed with scanning electron microscopy (SEM).

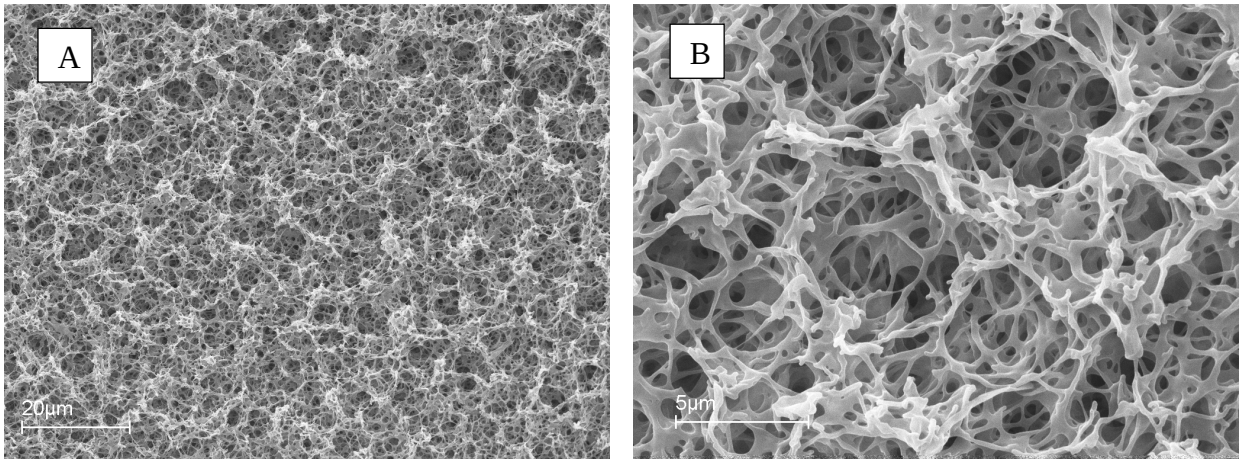


Figure 8.3 A and B: SEM photographs of PLA scaffolds prepared without active molecules loaded

Figure 8.3 A and B shows, with different magnification, the micro-morphology of the scaffolds: high surface area was obtained with dimension of pores are approximately of 600nm. The morphology of scaffolds prepared loading oligoTyr were also studied: figure 8.4 A and B shows the morphology of PLA scaffolds loaded with 5%$_{w/w}$ of oligoTyr. The structure is more compact and the dimensions of pore smaller: moreover, highlighted by the red circle in figure 8.4B, crystal of oligoTyr are identified.

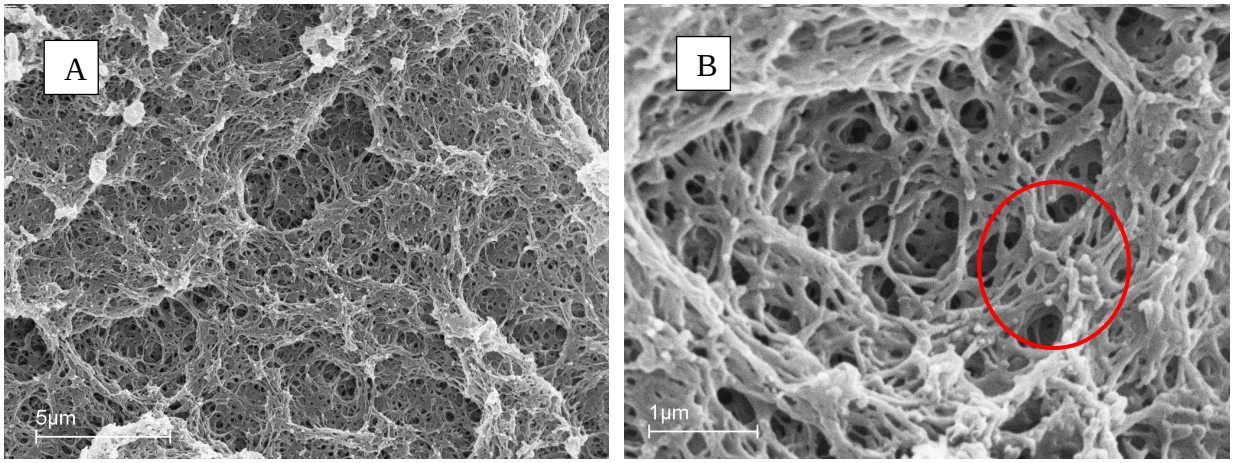


Figure 8.4 A and B: SEM photographs of PLA scaffolds prepared loaded with 5% of OigoTyr

The amount of OligoTyr actually loaded in the scaffold was evaluated by UV/vis spectrophotometry. OligoTyr concentration was found to vary significantly within the scaffold depending on the sampling site, and in all cases was lower (4–4.5%) than the theoretical one based on a 5% w/w loading. This indicated that the dispersion of OligoTyr in PLA was non-homogenous and that some leakage of OligoTyr occurred during scaffolds preparation: melted solvents promote the extraction of OligoTyr in water reducing the load inside the scaffolds.

The loss of oligoTyr, together with the variability of the morphology and non-homogenous dispersion of oligoTyr, does not permit to use this methodology for preparation of scaffolds due to the poor reproducibility of the results.

In order to avoid these problems, another methodology was set up: method B (see paragraph 6.11). PLA and active molecules were solubilized in 1,4-dioxane and then the solution was frozen. The solvent was removed under vacuum avoiding the contact with water.

As a first advantage 1,4-dioxane shows a sublimation temperature (284.1 K) far higher than that of THF (164.8 K), allowing for a convenient removal of the solvent from the bulk material under reduced pressure (179) (180). In addition, 1,4-dioxane has already been reported to afford PLA hierarchical scaffolds (181) (182) (183).

This method, in comparison with method A, presents two advantages:

1. Avoiding the contact with other solvents, the concentration of Tyrosol and oligoTyr fed remains constant
2. The morphology of the scaffolds is determined by the concentration of PLA in solvents and no significant variability between different batches were highlighted.

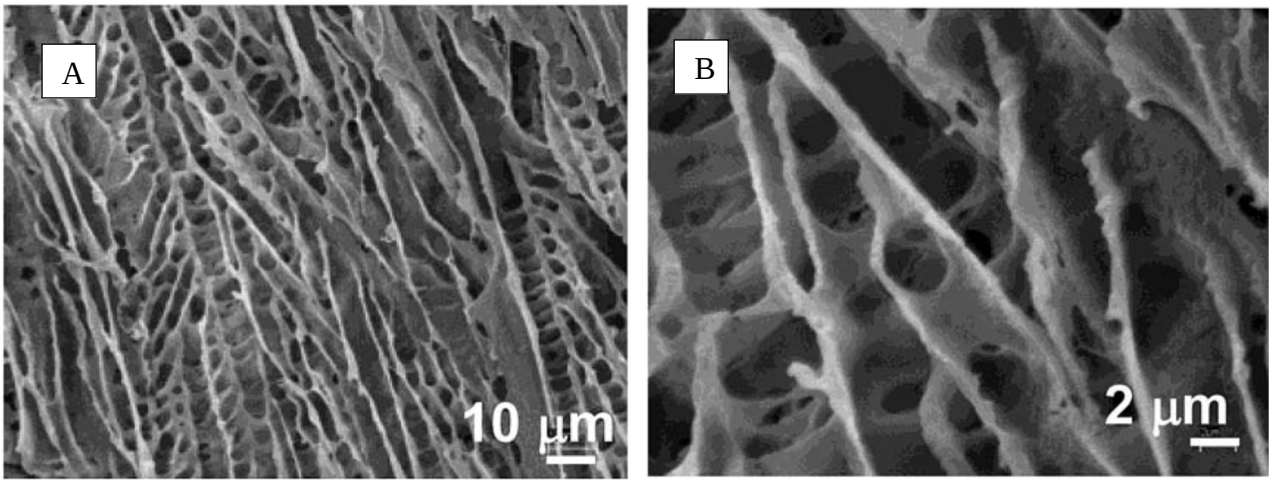


Figure 8.5 A and B: SEM micrographs of PLA scaffolds obtained with method B without oligoTyr

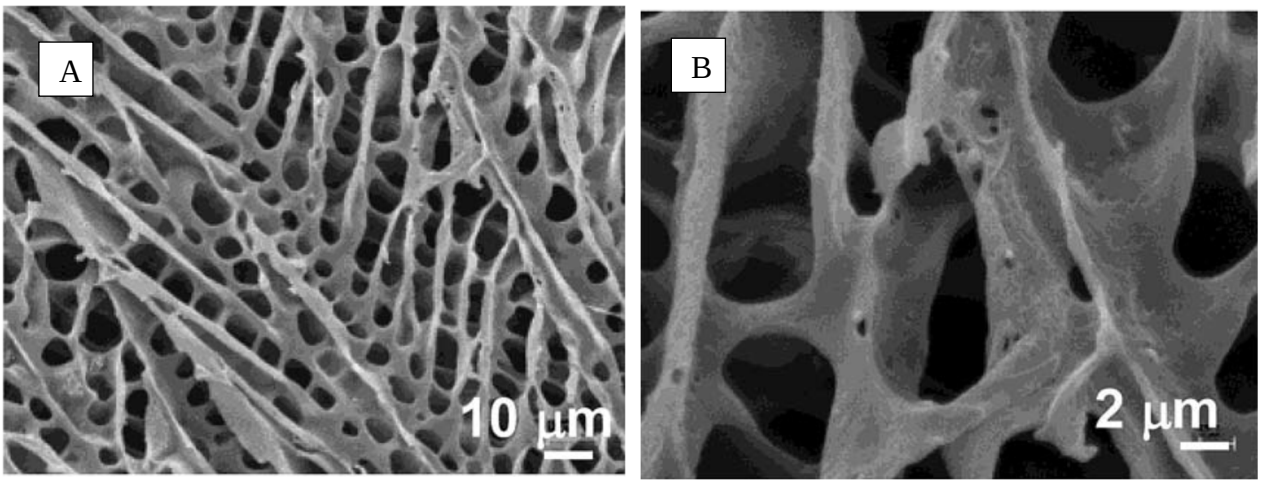


Figure 8.6 A and B: SEM micrographs of PLA scaffolds obtained with method B loaded with 5% of oligoTyr

Figure 8.5 A and B and figure 8.6 A and B show that the morphology of scaffolds does not change between two different batches. Furthermore, no crystals of oligoTyr are identified on the surface of the polymer surface.

Method B permits a fine control and reproducibility of the scaffolds therefore this method was chosen for the production of scaffold utilized for delivery test and for biological assays.

8.2.3 Porosity and Wettability

The porosity of the PLA scaffolds prepared according to method B was determined using two different gravimetric methods. In the first one, relative porosity was calculated by eqn (1) using the density of the dry scaffold (ρs) and the density of raw PLA (ρPLA), determined as the ratio between the dry mass and the volume of the scaffolds:

$$\text{Porosity (\%)} = [1 - (\rho s/\rho PLA)] \times 100 \quad (1)$$

In the second method, the porosity was evaluated using eqn (2):

$$\text{Porosity (\%)} = \{(m_w - m_D)/[(m_D/\rho PLA) + m_w]\} \times 100 \quad (2)$$

where m_w is the wet weight of the scaffold after 96 h contact with water and m_D is the dry mass. In this case the porosity is determined as the total amount of water absorbed by the scaffold.

The most important difference between the two methods is that with equation 1, which determines the apparent porosity, the connection among pores are not considered. In fact, porosity is determined as a ratio between two densities. On the other hand, equation 2, which determines the porosity using the absorbed water, considers the connection among pores. The results are very different: the porosity obtained with equation 1 is 86.7% whereas the porosity measured with equation 2 is 54.5%.

The other parameter, tested to determine how the water solution interacts with scaffold during the release of oligoTyr, is the swelling that was evaluated according equation 3.

$$\text{swelling (\%)} = [(m_w - m_D)/m_D] \times 100 \quad (3)$$

Where m_w is the wet weight of scaffold and m_D is the dry weight of the scaffold. The swelling of PLA, measured after 96h is 120.1% and it does not change even after 120h. The lower value obtained with eqn 2 indicates that, even though there are high quantity of pores, most of them are not linked together. The reduced accessibility to the most internal pores counteracts the extraction a portion of oligoTyr that is homogenously solubilized in polymer. The kinetics of swelling and porosity (obtained with eqn. 2) are reported in figure 8.7.

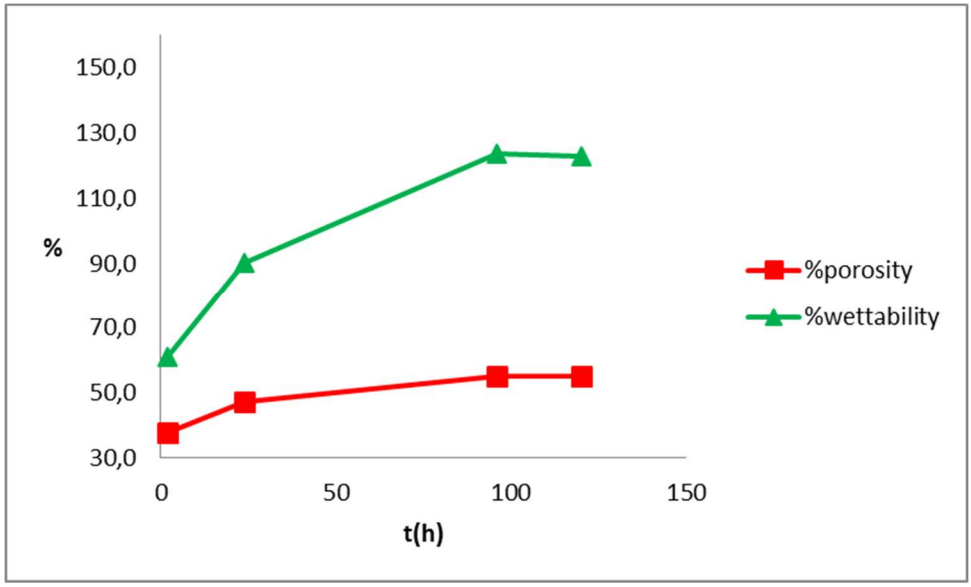


Figure 8.7: swelling and porosity measured in time interval of 120h

Porosity and swelling where measured only on PLA scaffold without oligoTyr; it is reasonable to assume that the low quantity of oligoTyr loaded should not affect swelling and porosity. Moreover,

SEM micrographs shows that the morphology (pores number and dimension) is not influenced by the presence of oligoTyr.

8.3 Release Test

The aim of the project is to develop a method for the release of oligoTyr to be used in order to promote ossification in human bone tissue. The release was assessed using samples prepared loading 5% of tyrosol (reference), 5%, 4.5% and 3% of oligoTyr and 5% of oligoTyr and β-tricalcium phosphate (β-TCP). It was chosen to prepare the composite since this biomaterial has been widely used in view of its osteoconductive properties and cellular activities for regenerating bone tissues (184) (185).

The punctual release kinetic, obtained in PBS solution at pH 7.4, is shown in figure 8.8 whereas the cumulative release kinetic, also obtained in the same conditions, is shown in figure 8.9.

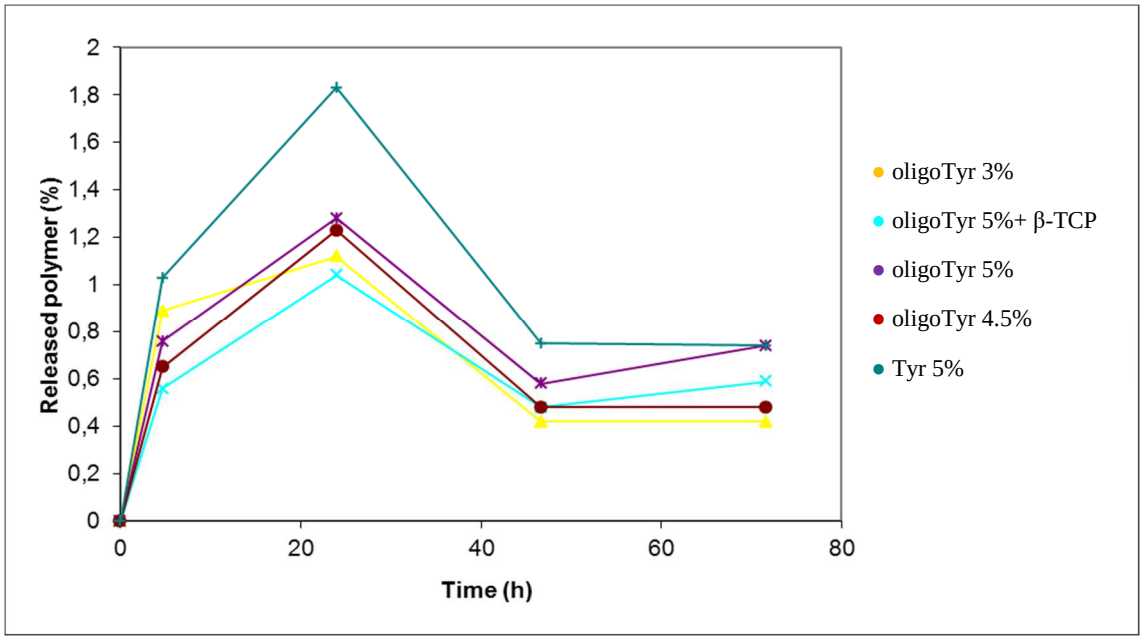


Figure 8.8: punctual release kinetic of PLA scaffolds loaded with Tyr, OligoTyr and Calcium

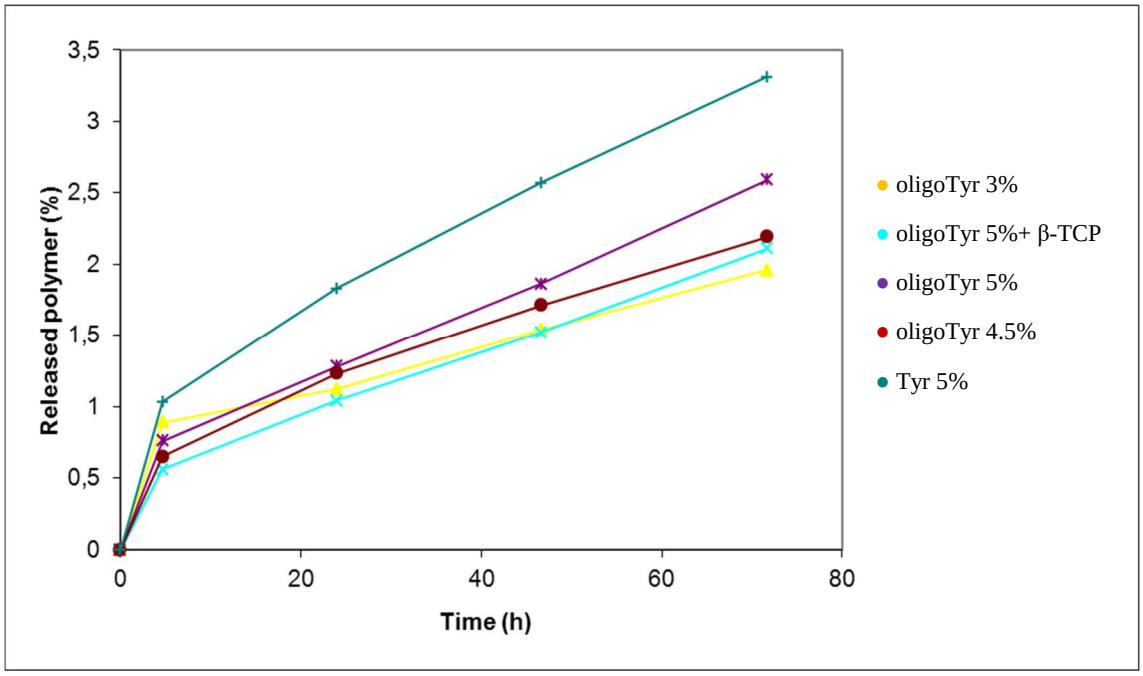


Figure 8.9: cumulative release kinetic of PLA scaffolds loaded with Tyr, OligoTyr and Calcium

The amount of oligoTyr loaded in the scaffold does not affect the release; the poor solubility of oligoTyr determines the concentration available in PBS solution and the concentration achieved during the release study is always the higher concentration obtainable for oligoTyr in water. Neither the calcium phosphate affects the release since the quantity of oligoTyr released is the same measured in the sample without calcium. On the other hand, the quantity of tyrosol released is significantly higher in comparison with oligoTyr: the higher molecular weight of oligoTyr decrease its water solubility reducing the release speed. In all cases the scaffolds release very low quantity of tyrosol and oligoTyr.

The last step was to assess the release of calcium; the kinetics of the release was performed in a prolonged time (28 days) in comparison with oligoTyr release. The aim of this assay is to determine how the presence of oligoTyr affects the release of β-TCP.

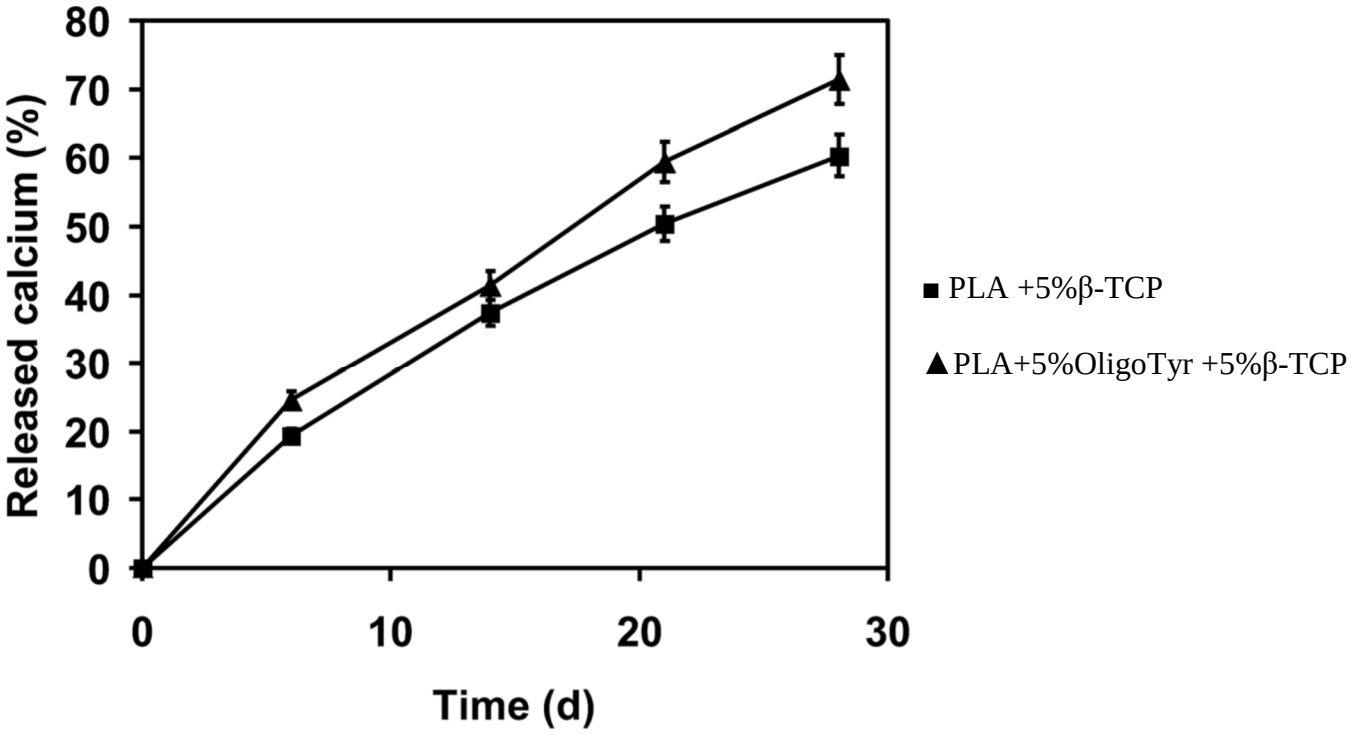


Figure 8.10: β-TCP release in PBS from scaffold loaded with oligoTyr and not

PLA scaffolds loaded with 5% of oligoTyr reaches almost 70% of calcium release in 28 days, whereas the sample prepared only loading β-TCP in PLA achieved the release of 60%. Interestingly, oligoTyr not only does not interfere with calcium release but also can actually promote its release: this can be explained considering a lack of chelating action, which is critical to permit a continuous flux of cationic nutrients throughout the damaged area.

8.4 Biological Assay

Understood the activity of oligoTyr and developed a reliable methodology for scaffolds preparation, the composite containing 5% oligoTyr prepared with method B was tested on human osteosarcoma cells SaOS-2. The aim of this assay was to investigate if the PLA scaffolds affects cells growth and how the release of oligoTyr can promote the ossification process measured as ALP.

Three scaffolds were tested: PLA, PLA loaded with 5% of Tyrosol and PLA loaded with 5% of oligoTyr. Results are provided in figure 8.11.

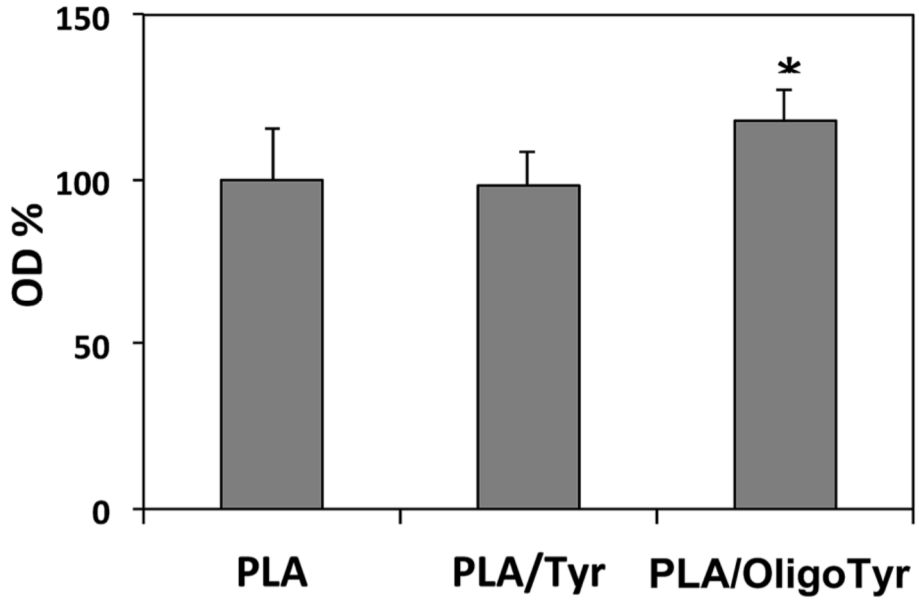


Figure 8.11: ALP activity measured on pure PLA scaffold, PLA with 5% of Tyrosol and PLA with 5% of oligoTyr

Cells were left growing for 7 days on finely ground composites. Data reported in figure 8.11 show that PLA and PLA with tyrosol do not affect the ossification whereas, even the difference is small, the PLA with OligoTyr significantly enhances the ALP for human osteosarcoma cells. Moreover, it was confirmed that PLA has not toxic effect. Also, the Tyrosol loaded scaffolds does not show activity even if, in previous analysis, tyrosol displayed interesting activity.

This assay was conducted for only 7 days; according to the results obtained with release assay, very low quantity of oligoTyr was provided to the osteosarcoma cells. This consideration allows to predict better results for prolonged exposure time.

8.5 Conclusion

A method for the production of PLA scaffolds, which permits a fine control of morphology and of the loading of active molecules (tyrosol and oligoTyr) was developed: the method permits a control on pore dimensions and, moreover, fair dispersion of active molecules avoiding problems related to the presence of high concentration in some areas of the scaffolds.

OligoTyr was synthetized with a relatively simple green enzymatic reaction and its properties and structure were assessed (biocompatibility due to low cytotoxicity). OligoTyr displays interesting antioxidant properties higher than tyrosol and even better than ascorbic acid. Moreover, the oligoTyr behaves as ossification promoter significantly increasing the ALP activity in human osteosarcoma cells SaOS-2.

The scaffolds display very low release kinetics: ossification process requires several days therefore a very slow release of ossification enhancer is required in order to supply a continuous dosage of

oligoTyr: moreover, PLA does not affect the activity of oligoTyr even though it does not display any kind of activity. The tyrosol released, although it has interesting effects on ALP activity if used as pure molecule, is not able to improve the ossification process.

9. Ellagic Acid Release System

Diet and nutrition are fundamentals factors in the promotion and maintenance of good health conditions. They occupy a prominent role in the prevention of chronic diseases like obesity, diabetes, cardiovascular diseases, cancer and osteoporosis (186). In the Western world, cardiovascular diseases and many types of cancer are a major problem leading to high mortality. Nevertheless, medical research has uncovered the causes of these chronic diseases: virtually, the underlying mechanisms of all of these diseases depend on oxidative processes which lead to products that display high reactivity and are able to affect specific molecular targets in the body, like cellular DNA (187). FAO and WHO established the Codex Alimentarius or "Food Code" in 1963 to develop harmonised international food standards to protect consumer health. In addition, in 2003 they published the report “Diet, nutrition and the prevention of chronic diseases” after an international meeting in Geneva, focused on the prevention of chronic diseases through diet, in which they suggest diet changes (186). Epidemiological studies have revealed a lower incidence of many chronic diseases in areas and populations that regularly consume vegetables, fruits and anti-oxidant rich foods, like tea and spices. Many of these foods are sources of antioxidants, like polyphenols, carotenoids, and vitamins. Among them, ellagic acid (EA), a natural polyphenol mainly presents in pomegranates, berries and grapes, is one of the most studied bioactive compounds in both academic and pharmaceutical worlds, since it displays apoptosis-inducing activity against certain types of cancer cells and antioxidant effects.

9.1 Ellagic Acid solubility enhancing

One of the most relevant issues related to food intake of EA is its low absorption in the gastrointestinal tract. In fact, EA is poorly soluble either in water (about 10 μg/mL) or in hydrophobic solvents and it is difficult to be incorporated in a formulation. In order to overcome the problems related to intake of EA; in the present work, a water soluble form was obtained simplifying the incorporation process and modifying the bioavailability of EA.

9.1.1 Water Solubility

As reported in the experimental part (see *paragraph 14.3*), different ratios of polyphenol/base were tested and a soluble salt (EALYS) of EA was obtained using 4 equivalents of L-lysine on EA, displaying considerable water solubility (approximately 13 mg ml^{-1}). According to literature, the water solubility of EA is 8.7 μg ml^{-1} (188)], therefore considering that EA is 34%$_{w/w}$ of EALYS, the water solubility of EA has been increased more than 400 times.

Solubility studies were also conducted using a $CaCl_2$ water solution, since the polysaccharide gels production procedures, used for the incorporation of EALYS, require the use of $CaCl_2$ as gelling agent both for pectin and alginate gels. The solubility in $CaCl_2$ solution was assessed via UV-Vis analysis fixing the wavelength at 280 nm: the solubility of EALYS dramatically decreases when the calcium is added to the water solution, reaching values lower than 5% (results are provided in table 5.1).

Sample	EALYS weight (mg)	CaCl2 weight (mg)	Water (ml)	EALYS quantity in solution (%)
W1	18.5	0.0	30	100.83
W2	18.2	0.0	30	98.48
W3	18.7	0.0	30	99.39
$CaCl_2$1	11.2	8.0	50	2.31
$CaCl_2$2	11.7	8.7	50	2.51
$CaCl_2$3	11.6	8.9	50	2.44

Table 9.1: Results of solubilization tests in water and in $CaCl_2$ solution

Examples of limited solubility of calcium salts of phenols were already studied (189) therefore the formation of a very low water soluble Calcium-EA salt explains the reduction of EALYS in water solution.

Nevertheless, Ca^{2+} is fundamental to obtain pectin and alginate gels; rheological studies were performed in order to quantify how the presence of EALYS affects gel structures in terms of mechanical stability and toughness.

9.1.2 Thermal Analysis

Differential scanning calorimetry (DCS) analyses were performed on ellagic acid L-lysine salts with different ration between EA and L-lysine: EA: L-Lys 1:4 (water soluble salt), 1:3, 1:2 and 1:1. DSC thermographs and results are reported in figure 10.1 and table 10.2.

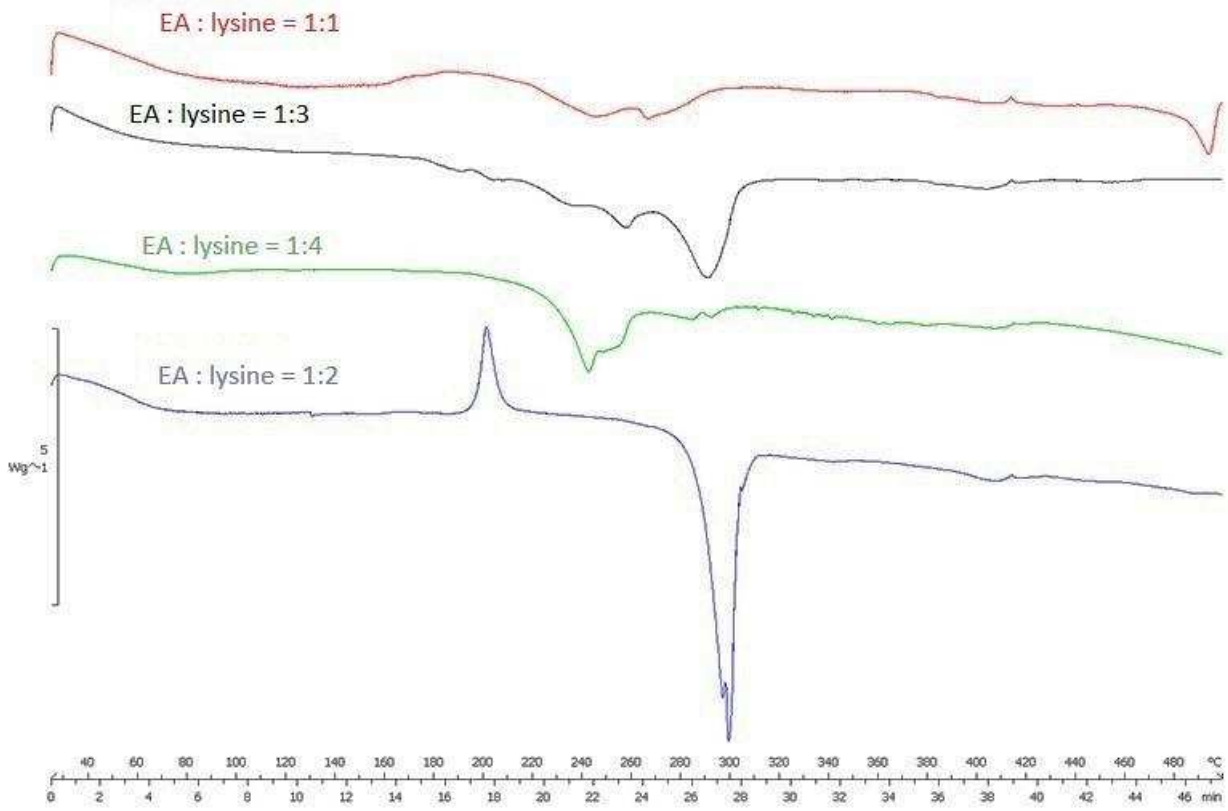


Figure 9.1: DSC curves of EA-lysine salts

Salt EA-lys molar ratio	Tcc (°C)	Tm(°C)
1:1	---	266
1:2	202	296; 298
1:3	---	291
1:4 (EALYS)	---	242

Table 9.2: DSC signals of EA-lysine salts

According to literature, EA melts and immediately degrades at temperature higher than 360°C (190) (191).The crystal structure gives great stability and prevents degradation but, when the crystal melts, the shield effect due to the crystal disappears and degradation phenomena start to occur. The formation of salts modifies the crystalline structure, dramatically affecting the thermal behaviour: in the range of 160°C-320°C, all samples present endothermic signals related to melting processes. Sample prepared with 1:1 molar ratio presents weak signals that cannot be ascribed whereas the sample with 1:2 molar ration present clear and well-defined signals:

- Exothermic signal at 210°C
- Endothermic signal at 298°C

The first signal is ascribed to a cold crystallization phenomenon whereas the second signal is ascribed to a melting process. The double melting peak indicates the presence of two crystalline phases: one is formed during evaporation process whereas the second is formed during the heating process. In addition, the sample does not exhibit degradation signals after melting point. The other two samples, 1:3 and 1:4, present an interesting behavior:

- The sample 1:3 presents a broad melting phenomenon with a peak approximately at 291°C ascribable to melting process of Ellagic Acid L-lysine salt (1:2); on the other hand, endothermic signals were registered approximately at 257°C attributable to L-lysine melting and degradation [Tm(L-Lysine) =215°C].
- The sample 1:4 presents only a broad melting phenomenon with a peak approximately at 242°C ascribable to melting process of L-Lysine.

Electrochemical studies conducted by Verbić et al. (192) together with these data lead to conclude that the EA acts as a diprotic acid; in fact the salt obtained with 1:2 molar ratio shows a clear melting point and high thermal stability. The sample obtained with 1:3 molar ratio, even if the melting signal of 1:2 salt is recorded, presents the signals of non-bonded L-lysine degradation which is also present in sample 1:4. In this case the excess of lysine does not permit to register the melting point of 1:2 salt.

9.1.3 ^{1}H-NMR

EALYS was characterized via ^{1}H NMR (400 MHz, D_2O): δ 7.13(1H, s), 3.58 (2H, t, J= 6Hz), 2.90 (4H, t, J= 8Hz), 1.75 (4H, m), 1.60 (4H, q, J= 8 Hz), 1.35 (4H, m). Integration values are consistent with weighted amounts of EA and lysine used to perform the salification.

9.1.4 Conclusion

In conclusion, the excess of L-lysine permits the solubilization of EALYS acting as a compatibilizer between the phase water and the phase EA-lysine diprotic salt. Lysine is an essential amino acid and therefore its excess does not present toxic issues. EALYS can be used therefore as water soluble form of EA.

10. Pectin

Pectin was chosen as biocompatible polymer for the incorporation of EALYS; but it needs to undergo gelification process to be able to release the active principle in a controlled way. As reported in the materials and methods part, pectin gel can be classified as weak gel, since $CaCl_2$ can diffuse out of the pectin matrix leading the solubilization of pectin in water. Experiments were made in order to determine the Degree of Esterification (D.E.) and the concentration of pectin and $CaCl_2$ needed to obtain suitable materials for EALYS incorporation.

10.1 Pectin *Degree of Esterification* evaluation

D.E. of pectin was determined using the method developed by Manrique et al. (117): the concentration of esterified carboxylic moieties and carboxylate moieties were determined using IR absorbances, as reported in paragraph “Pectin methoxylated degree” in the Materials and Methods part. The IR spectra of LM and HM pectin sample are reported in figures 5.2 and 5.3 and DE results are provided in table 5.3.

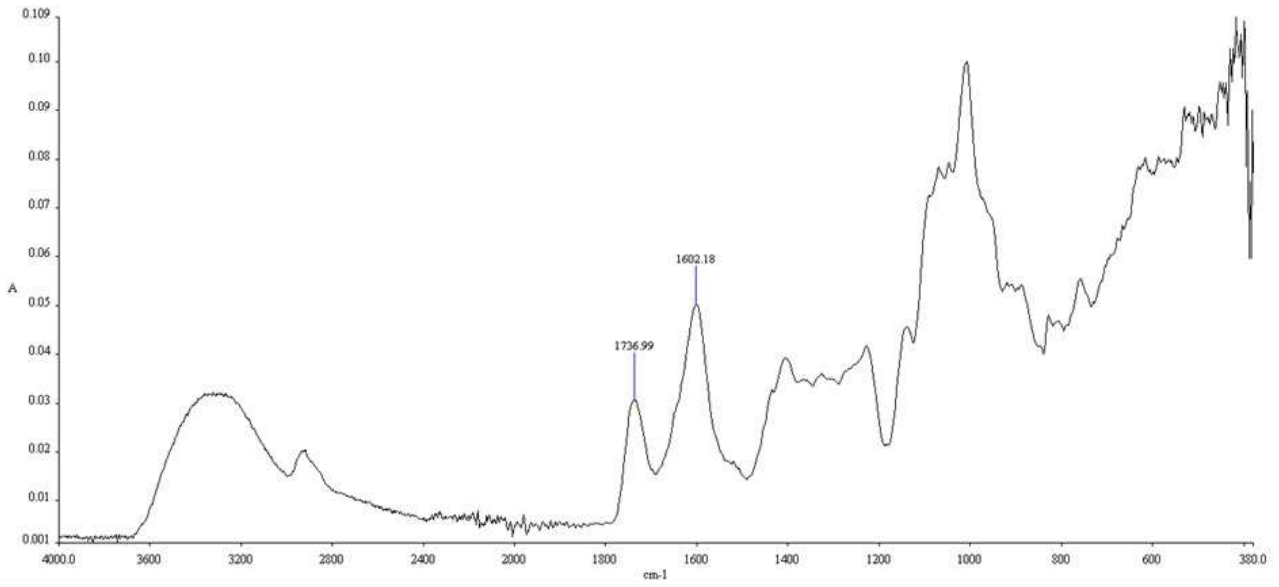


Figure 10.1: IR spectrum of pectin from citrus peel (LM)

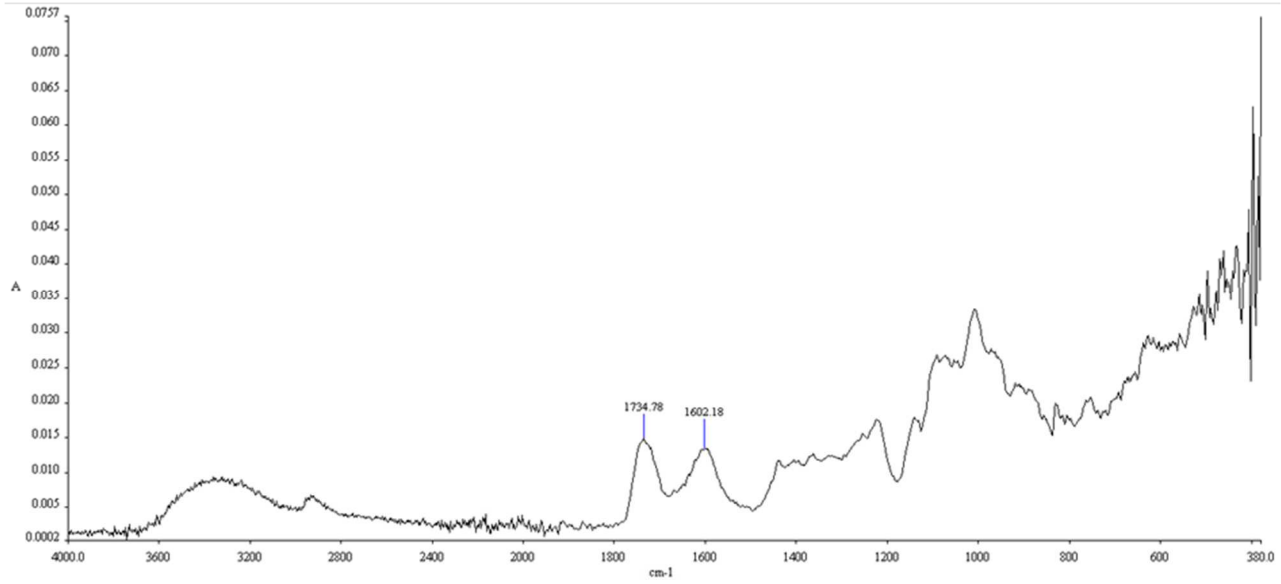


Figure 10.2: IR spectrum of pectin from apple (HM)

Type of pectin	**Degree of Esterification**
LM pectin from citrus peel	49.53%
HM pectin from apple	70.87%

Table 10.1 – D.E. of purchased pectin

10.2 Rheological studies

4 pectin gels were prepared in NaCl solution using LM pectin with a concentration of 3 g/l, 5 g/l, 7.5 g/l and 10 g/l. Rheological studies were performed in order to determine the lowest concentration required to obtain a gel. The amount of $CaCl_2$, which was used for gel preparation, was calculated using equation 1. R is a constant parameter (R=0.58), $[Ca^{2+}]$ is the molar concentration of calcium and $[COO^-]$ is the concentration of non-esterified carboxylic moieties determined by D.E.,

$$R = \frac{2[Ca^{2+}]}{[COO^-]} \quad (1)$$

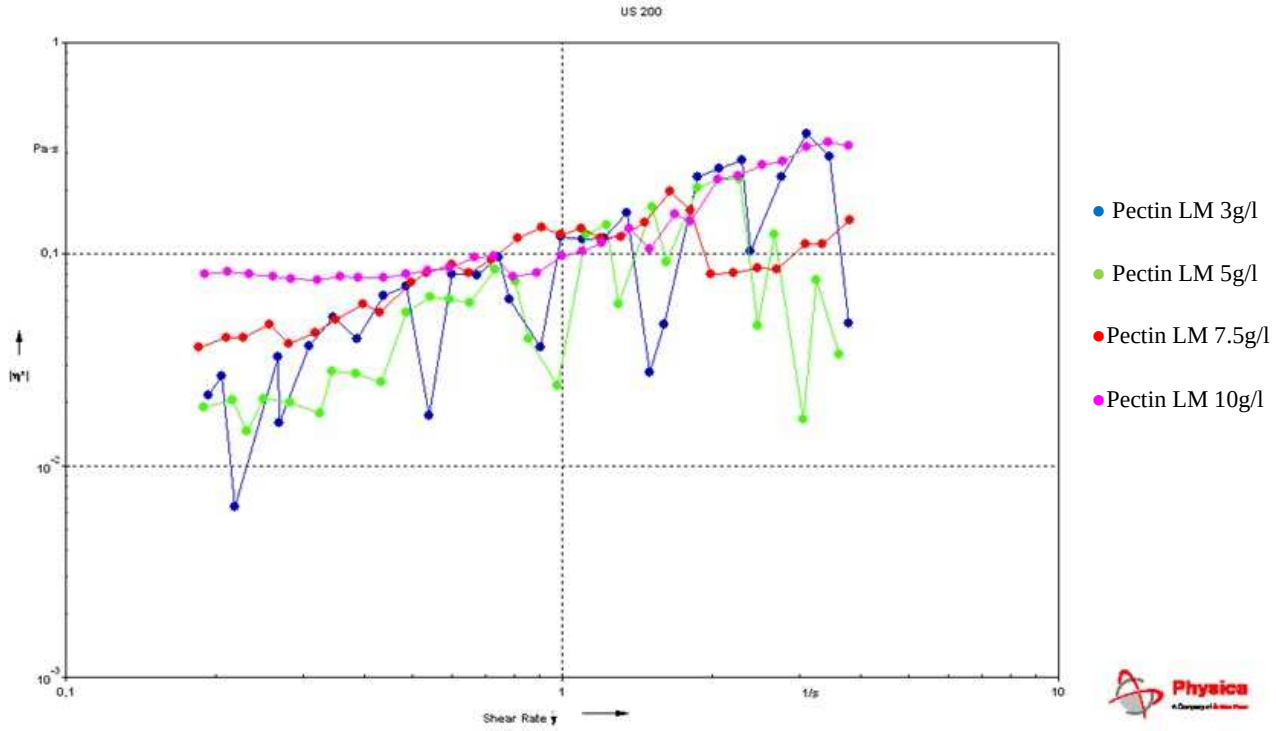


Figure 10.3: Rheological measurement of 3 (blue lines), 5 (green lines), 7.5 (solid red lines) and 10 (hollow red lines) g/l LM pectin samples

Figure 10.3 shows the rheological behavior of LM pectin gels obtained using a concentration of 3, 5, 7.5 and 10 g/l: the very low concentration does not permit to obtain a compact gel structure, and this results in a very low viscosity, even lower than the detection limit of the instrument. A correct interpretation of the analysis is not possible. Although the values obtained cannot be used due to their intrinsic inaccuracy, it is clear how higher concentrations are required in order to obtain tough gels: three more samples were prepared using pectin concentration of 20 g/l, 30 g/l and 40 g/l.

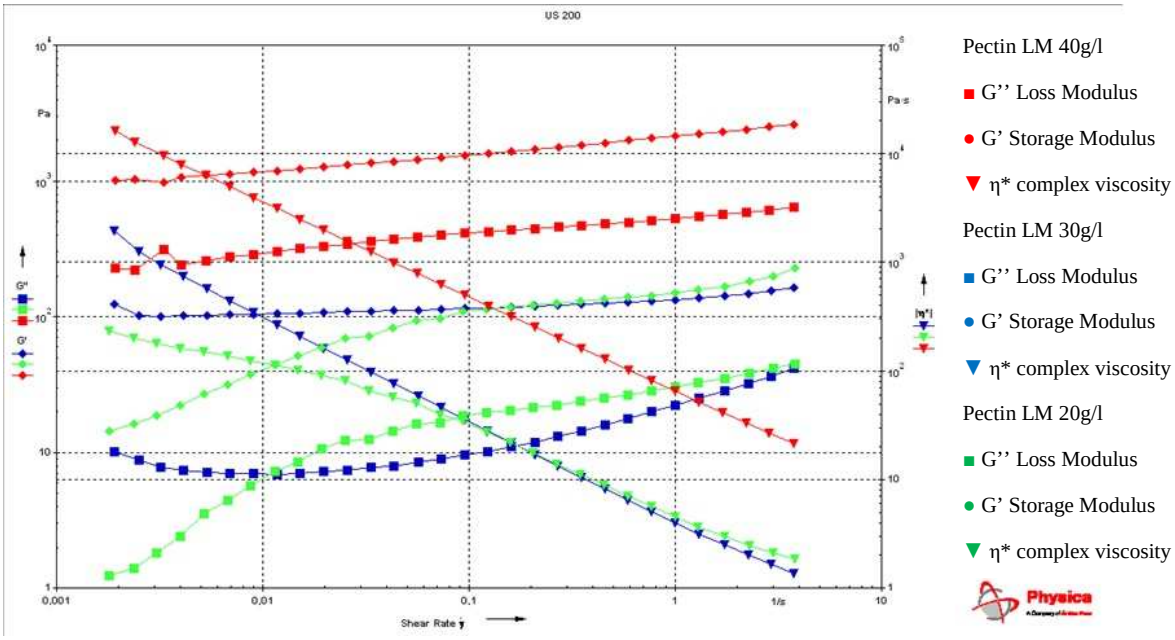


Figure 10.4: Rheological measurement of 20 (green lines), 30 (blue lines) and 40 (red lines) g/l LM pectin samples

The trends of storage modulus (*G'*), elastic modulus (*G''*) and complex viscosity ($|\eta^*|$) are provided in figure 10.4. If *G'>G''* the solid component dominates and the material acts predominately as a solid whereas if *G'<G''* the liquid component dominates and the material acts as a liquid. It can be noticed how the storage modulus (*G'*) of pectin gels analyzed is always higher than the loss modulus (*G''*): this is the experimental evidence of gels formation in all samples.

The samples with 20 g/l and 30 g/l do not show great difference in terms of viscosity and moduli , in particular at shear rate higher than 0.1Hz, whereas the sample 40 g/l shows a significant arise in viscosity and moduli values. In figure 10.5, a general trend of viscosity, related to concentration, is reported; this is obtained using a thickening agent. It is possible to identify a specific concentration, called "entanglement concentration" (EC), which determines a dramatically change in rheology behavior: at concentration lower than EC the fluid has a viscosity that linearly increases with concentration, otherwise, at concentration higher than EC, the shear thickening effects takes place leading to a pronounced arising in viscosity with a variation of slope due to the increasing number of entanglements among polymeric chains. The 20 g/l samples have not a sufficient concentration to arise the critical amount of entanglements, while 30 g/l and 40 g/l sample have a concentration high enough to obtain interactions among polymeric chains, explaining the great difference in rheological behavior among these three samples. Moreover, 20 g/l sample shows a non-linear decrease in viscosity for shear rate lower than 0.1Hz, which is a typical behavior of pseudo plastic fluids and not of gels: these observation leads to conclude that this concentration is not enough to form a gel whereas 30 g/l sample shows a typical behavior of a lattice. 30 g/l was assumed as the lower concentration required for the formation of a stabile gel with LM pectin in water solution.

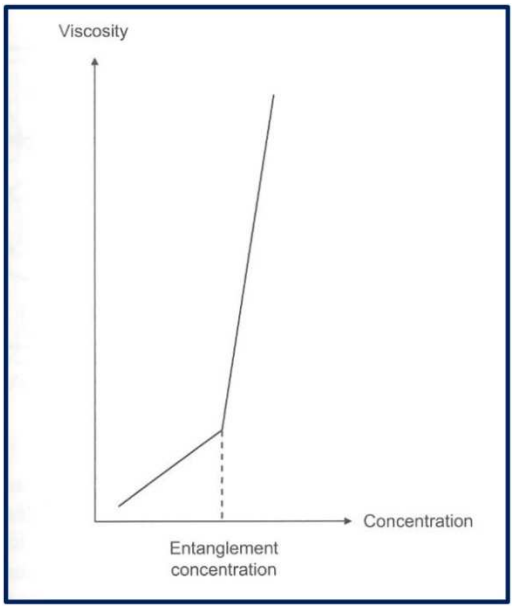


Figure 10.5: Viscosity vs. Concentration trend for thickeners

The mechanical stability of the gel was assed undergoing the 30 g/l gel to an opposite frequency sweep test: the sample, previously analyzed in the frequency interval 0.01 to 20 Hz, was analyzed in the interval 20 to 0.01 Hz. Using these analysis parameters, it is possible to evaluate how fast the polymer chains are able to form entanglements and therefore the reversibility of the phenomenon.

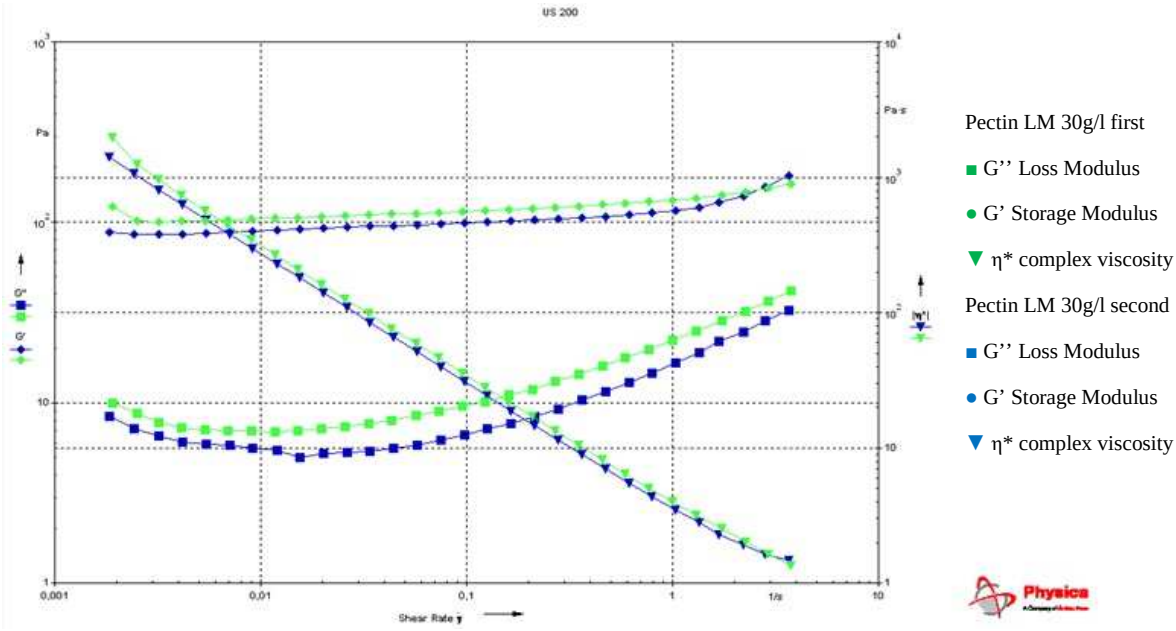


Figure 10.6: Rheological measurement of 30 g/l LM pectin samples from 0.01 to 20 Hz (green lines) and vice versa (blue lines)

The curves shown in figure 10.6 show that the viscosity does not change significantly by reversing the frequency. This behavior can be observed in materials having a high amount of entanglements and it is possible to conclude that a concentration of 30 g/l of LM pectin is enough to obtain a

material with sufficient interactions between polymer chains or, in other terms, a gel with good strength and stability.

As reported in literature, pectin gels display degradation phenomena over time due to depolymerization of pectin chains (193). The rheological analysis was performed on the same sample of LM pectin at concentration of 30 g/l also after a week to evaluate if some rheological changes had occurred.

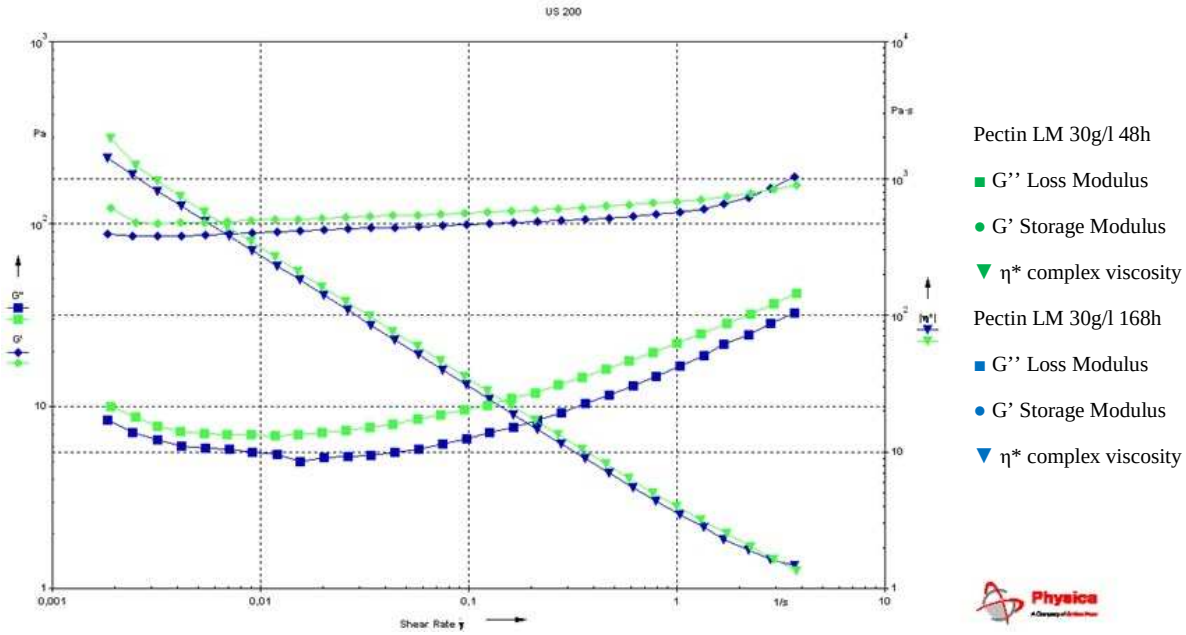


Figure 10.7: Rheological measurement of 30 g/l LM pectin samples after 48 h (green lines) and after a week (blue lines)

Figure 10.7 shows that there are no evidences about viscosity changes in the sample when stored at room temperature for one week. This result permits to conclude that no changes in the material behavior had occurred during the time required to perform the controlled release tests.

The data collected on pectin gels are necessary to set up the material for the incorporation of EALYS. According to the results previously shown, 30g/l is the best condition for the formation of a pectin gel stable in water and stable over the time, required for the release assays.

The same rheological analysis was performed also on sample LM_10%EALYS_30g/l in which EALYS salt was added at 10% on pectin weight.

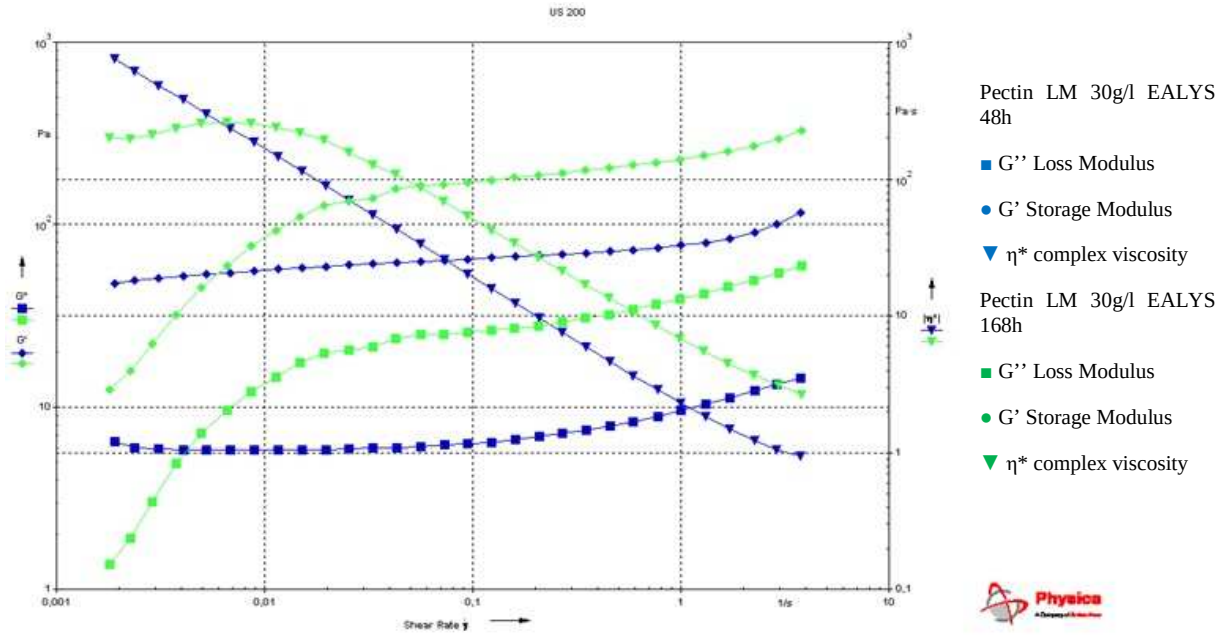


Figure 10.8: Rheological measurement of 30 g/l LM pectin with EALYS samples after 48h (blue lines) and after a week (green lines)

It is possible to notice from figure 10.8, that after 48h from the end of the preparation, the viscosity linearly decreases by increasing the shear rate. After a week, at low shear rate the viscosity displays an anomalous decreasing trend. EALYS is a water soluble salt, but in the presence of Ca(II) ions it shows a decreased solubility leading to a formation of EALYS-Ca(II) salt. Probably, the reaction of EA with calcium ions subtracts the Ca(II) from the pectin gel reducing the ionic bridges among polymeric chains and leading to a material with lower gel features.

Pectin gels are known to be used as culture medium for incubation of molds and bacteria (194). In order to prevent their formation, the materials were freeze-dried to remove the water present avoiding the possibility of microbial growth. The dehydrated gel is able to swell water and return to the hydrated gel form. However, the right amount of water has to be used to re-obtain the hydrated gel form with the same mechanical properties. Rheological tests were performed in order to verify if the rheological properties of the rehydrated material do not change after the freeze-drying process. Curves have been reported in figure 10.9.

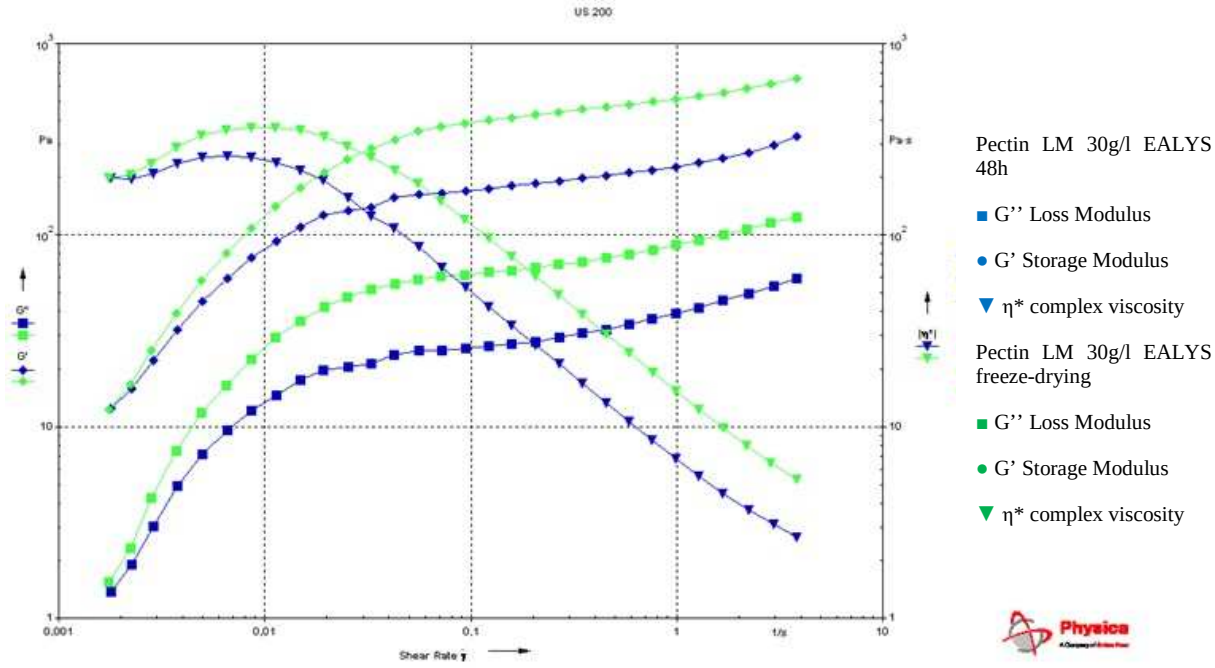


Figure 10.9 - Rheological measurement of 30g/l LM pectin with EALYS samples before (blue lines) and after submitted to freeze-drying and rehydration process (green lines)

The complex viscosity is higher in re-hydrated sample as both moduli *G*' and *G''*. The differences can be attributed to the amount of water used to rehydrate the material: even small differences of water amount inside the gel can largely affects the rheological properties.

Rheological behavior of HM pectin was assessed using the same approach described above for LM pectin. The structural differences between HM and LM pectins (hydrogen interactions and non-polar interactions) affect the rheological behavior.

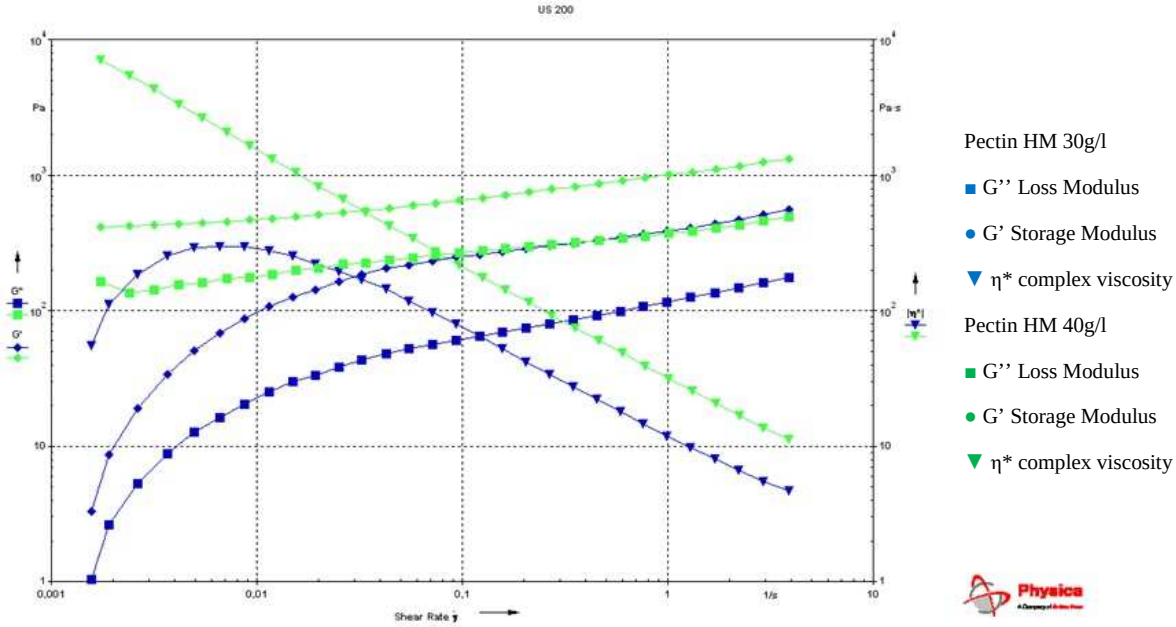


Figure 10.10: Rheological measurement of 30 (blue lines) and 40 (green lines) g/l HM pectin samples

The EC of HM pectin was assessed and rheological curves of 30 g/l and 40 g/l are provided in figure 10.10: it is possible to observe that the 30 g/l sample presents a decreasing in viscosity due to the non-complete formation of pectin lattice whereas 40 g/l sample presents the behavior of a cross-linked material. This can be attributed to the fact that HM pectin has lower physical crosslinking points due to the lower concentration of carboxylate groups, which can interact with Ca^{2+}. Higher concentration of HM pectin is required to obtain the chain entanglements concentration if compared to the LM pectin.

Rheological changes due to the introduction of EALYS were also investigated for HM pectin gel, on the sample HM_10%EALYS_40g/l, and the results are reported in the next paragraph.

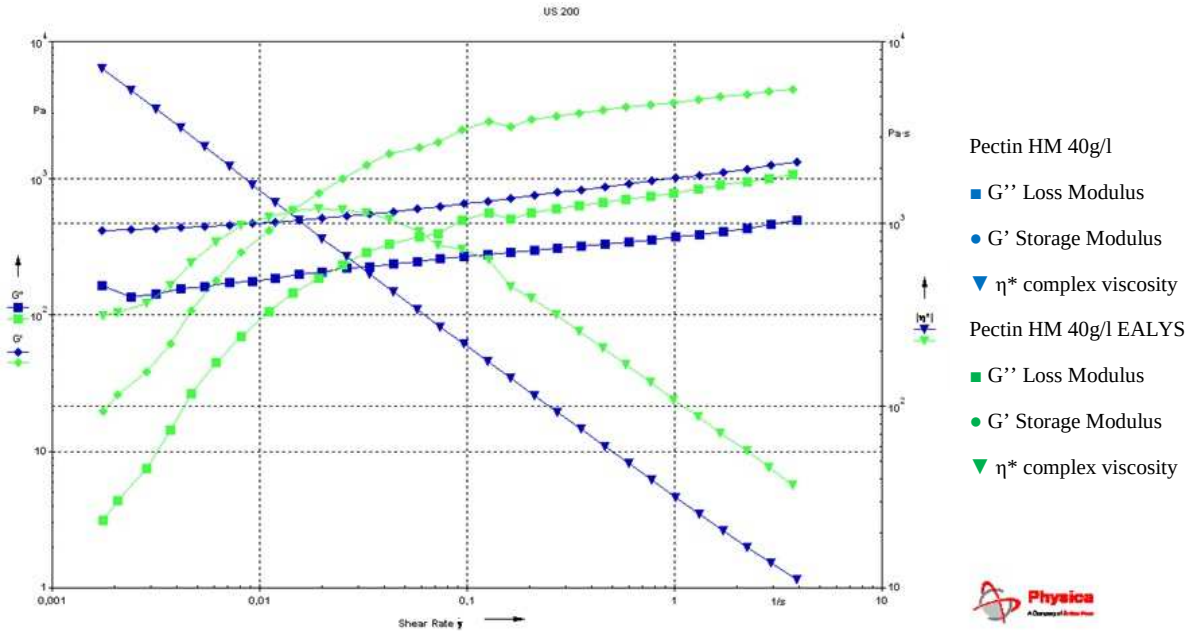


Figure 10.11: Rheological measurement of 40 g/l HM pectin with EALYS (green lines) and without it (blue lines)

The presence of EALYS in HM pectin affects the stability of the gel: EALYS reacts with Ca(II) reducing the ionic bridges among pectin chains compromising the stability of lattice. Rheological curves are reported in figure 10.11. Although a decreasing in viscosity for shear rate lower than 0.01Hz was registered, *G'* modulus is still higher than *G''* modulus therefore the solid structure of pectin gel is not compromised yet.

In conclusion, of this rheological study, the best concentrations of pectin were identified in order to develop a formulation for the incorporation of EALYS. The concentration of 30 g/l in water was selected to compare HM and LM pectin, although a higher concentration is needed for HM pectin to obtain a strong gel. Since the goal is to compare the release properties of the two formulations, the use of the same concentration for both pectin can allow us to understand how the interaction among pectin chains can affect the release.

The rheological properties, and therefore the mechanical behavior of pectin gels, can be related to the release properties of the materials. A complete knowledge of pectin gels behaviour is necessary in order to tailor the materials for the purposes.

10.2 Pectin release studies

Once pectin matrices were selected, EALYS was incorporated. In order to evaluate their release abilities, materials were submitted to release tests, as described in the “Formulations release studies” paragraph of the experimental part.

Since the media in which the tests were conducted was a phosphate buffer at pH 7,4 (PBS) preliminary solubility studies of EALYS in PBS were made and the percentages of EALYS present in solution were determined via UV-Vis analysis. Results are provided in table 10.2.

Percentage of EALYS in water (%)	Average (%)	σ (%)
100.83	99.56	1.19
98.48		
99.39		
Percentage of 10% EALYS in PBS (%)	Average (%)	σ (%)
66.18	68.88	4.13
73.64		
66.82		
Percentage of 2% EALYS in PBS (%)	Average (%)	σ (%)
60.46	61.42	1.13
62.66		
61.14		

Table 10.2: UV-Vis solubility tests results

As it is possible to observe, PBS reduces the solubility of EALYS if compared to water, in which EALYS is 100% soluble. This can be explained considering that phosphate ions interfere with lysine and leads to EA partial precipitation. However, PBS was used for further release tests to mimic the intestinal environment.

In order to evaluate the release properties of pectin gels, three parameters were modified:

- the degree of esterification using two different types of pectin, LM and HM
- the quantity of EALYS present
- the amount of $CaCl_2$ used to obtain the gel

Each pectin sample, used in the release studies, was produced in film formulation, using the procedure described in the experimental part.

First HM and LM pectin samples containing 10%$_{w/w}$ of EALYS were compared in order to identify if there are any differences, due to the D.E., on the release properties. Results are reported in figure 10.12 and table 10.3. The reported percentages were calculated based on the detected amounts of EALYS in PBS used to perform the release tests. The total amount of EALYS present in the materials was taken as 100% reference value.

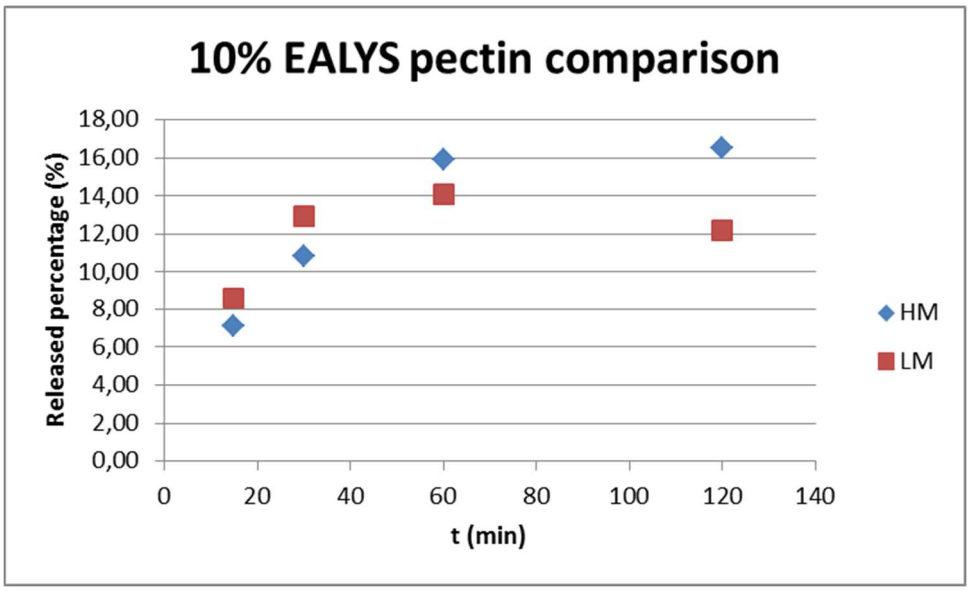


Figure 10.12: Comparison of LM_10%_100 and HM_10%_100

Sample	15’	σ	30’	σ	60’	σ	120’	σ
LM_10%_100	8.6%	2.3	13.0%	0.6	14.1%	0.5	12.2%	0.8
HM_10%_100	7.2%	2.3	10.8%	2.7	15.9%	1.4	16.5%	1.3

Table 10.3: Release data of LM_10%_100 and HM_10%_100

An increase trend of released EALYS over time can be observed for both pectin formulations. In principle, HM pectin should release higher amounts of EALYS, since a lower amount of $CaCl_2$ is required to produce HM pectin gels if compared to LM pectin gels. $CaCl_2$ seems to reduce the solubility of EALYS, as stated in the “synthesis of soluble formulation” paragraph above, the higher the amount of $CaCl_2$ in the formulation, the less EALYS should be present in solution. However, from the release experiments, the two types of pectin seem to behave in the same way and no great differences in the release profile were founded.

EA presents biological activity in concentrations of 1–100 μM (195), therefore formulation with lower concentration of EALYS were prepared (2%$_{w/w}$ of EALSY loaded in HM and LM pectin). A

high concentration of EALYS can dangerously involve the detoxifying systems, obtaining a harmful result. The rheological studies were not performed on these samples because it was previously demonstrated how the gels still maintain good mechanical stability with higher concentration of EALYS, therefore it is reasonable to assume that lower concentrations do not affects their mechanical properties. The aim is to obtain a delivery system able to better control the release efficiency (measured as quantity of EALYS released) and time of release (low concentration of EALYS has reduced interactions with gel structure avoiding its properties variation). The release studies were performed on these materials and the results are reported in figure 10.13 and table 10.4.

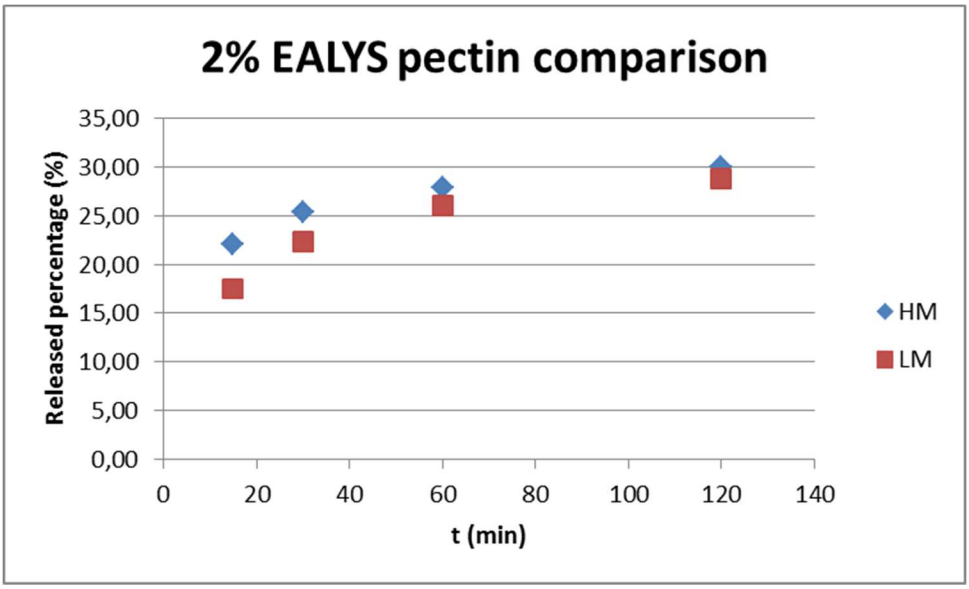


Figure 10.13 - Comparison of LM_2%_100 and HM_2%_100

Sample	15’	σ	30’	σ	60’	σ	120’	σ
LM_2%_100	17.6%	1.6	22.5%	3.0	26.1%	2.3	28.8%	1.5
HM_2%_100	22.1%	4.8	25.1%	2.8	27.9%	8.5	30.0%	3.0

Table 10.4: Release data of LM_2%_100 and HM_2%_100

Again, these formulations were able to control the release of EALYS during time; also in this case the two pectins (HM and LM) do not show strong differences on kinetic release: however, some differences between the 2% EALYS loaded pectin and 10% EALYS loaded pectin gels can be observed:

- 2% EALYS loaded gels are able to release faster in comparison to 10% EALYS loaded gels; in only 15 minutes the formulation can release 22% of EALYS while the 10% EALYS loaded gels require 120 minutes to obtain lower releases.
- The efficiency of release is higher in formulation obtained with 2% of EALYS loaded in pectin; almost 30% of loaded EALYS was released while the 10% EALYS formulation can release only the 15%. Nevertheless, the total amounts of EALYS released from 10% EALYS materials are higher in absolute values. In fact, the 2% EALYS loaded formulation releases 0.6 g in 120 minutes whereas the same quantity of pectin gel loaded with 10% of EALYS is able to release 1.5 g in 120 minutes.

Sungthongjeen et al. reported that the calcium amount in the pectin based materials could modify the drug release behaviour from the formulations (196). In order to verify how the concentration of calcium affects the release, 4 samples were produced modifying the R parameter of equation 5.1: HM and LM films were obtained setting R=0.29 and R=0. EALYS was incorporated in all these films using a concentration of 10% $_{w/w}$ on pectin weight. Results of release tests of these materials are reported in figure 10.14; 10.15 and table 10.5; 10.6.

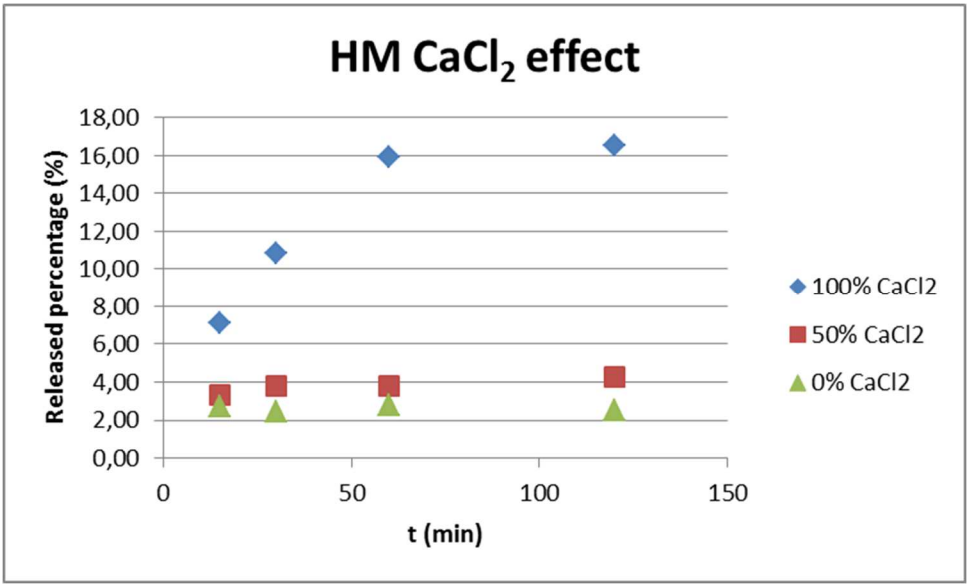


Figure 10.14: Comparison of HM_10%_100, HM_10%_50 and HM_10%_0

Sample	15’	σ	30’	σ	60’	σ	120’	σ
HM_10%_100	7.2%	2.3	10.8%	2.7	15.9%	1.4	16.5%	1.3
HM_10%_50	3.3%	0.38	3.8%	0.1	3.8%	0.1	4.3%	0.6
HM_10%_0	2.7%	0.16	2.5%	0.1	2.8%	0.2	2.5%	0.3

Table 10.5: Release data of HM_10%_100, HM_10%_50 and HM_10%_0

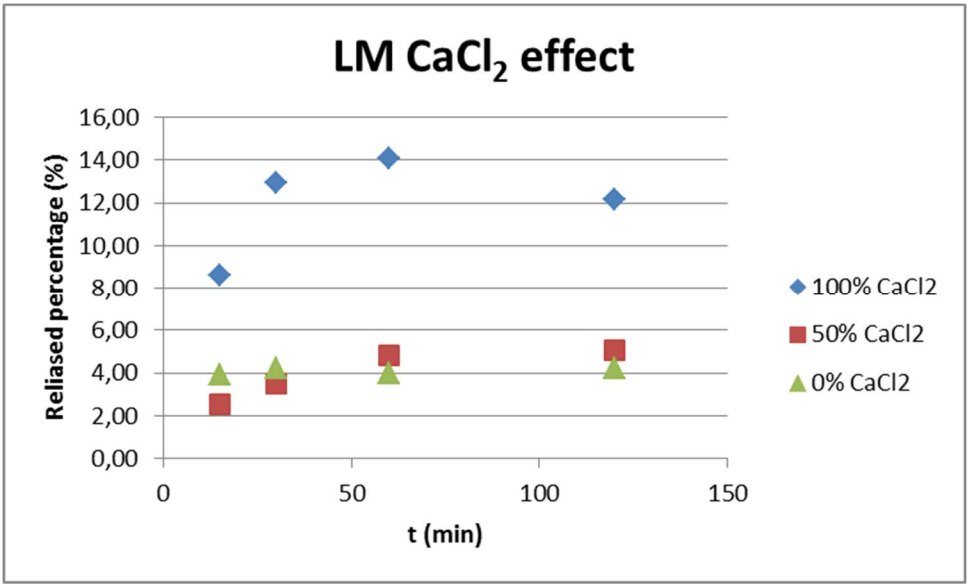


Figure 10.16: Comparison of LM_10%_100, LM_10%_50 and LM_10%_0

Sample	15’	σ	30’	σ	60’	σ	120’	σ
LM_10%_100	8.63%	2.3	12.97%	0.6	14.11%	0.5	12.17%	0.8
LM_10%_50	2.55%	0.3	3.48%	0.5	4.82%	0.2	5.04%	0.1
LM_10%_0	3.90%	0.3	4.23%	0.2	3.97%	0.3	4.23%	0.1

Table 10.6: Release data of LM_10%_100, LM_10%_50 and LM_10%_0

A lower calcium chloride concentration dramatically affects the release of EALYS in PBS solution obtaining values lower than 5% in both cases. Furthermore, the pectin films obtained with R=0.29 and R=0 have very similar behaviour leading to conclude that the correct ratio between carboxylic moieties and Ca(II) is fundamental to obtain release. The presence of free carboxylic moieties steeply increases the local ionic strength reducing the availability of EALYS (it was previously demonstrated that PBS at pH 7.4 leads to precipitation of EA from EALYS).

Conclusions

In conclusion it is possible to assess that:

- Since no strong differences in the release profile of EALYS were found by changing the pectin type, i.e. LM or HM, the choice of the pectin matrix should be based on the market availability and on costs especially with a view on future industrial scale up.
- Pectin release properties are closely related to $CaCl_2$ concentration which also leads to obtain a very tough gel.

To summarize, pectin gels are able to control the release of EALYS during time of two hours (average time required for digestion). However, since the release of EALYS should occur at colon level in the GUT, pectin formulations must be protected against strong acidic conditions due to stomach ambient, which can lead to EALYS protonation and therefore precipitation of EA. In fact, pectin is reported to be able to surpass the upper gastrointestinal tract without being degraded, since it is stable in acidic environment, while it is attacked and degraded by the colonic microflora [49]. However, the use of coating materials is suggested to protect pectin-based formulations in the stomach and allows the release of EALYS in the colon only.

11. Alginate

Alginate gels are widely used as biocompatible polysaccharides for the incorporation of active molecules (118). As reported in the materials and methods part, alginate gels are classified as strong gel, since $CaCl_2$ cannot diffuse out of the matrix in water. Although alginate has a structure similar to pectin (polysaccharides with carboxylic moieties along polymeric chains) it presents different gelling process, therefore it was chosen in order to evaluate how the gel strength influences the release features of materials.

11.1 Beads swelling degree

Swelling is the consequence of interactions between a solvent and a matrix and it is the first step before its total solvation, if it is possible. However, a crosslinked polymer does not dissolve when kept in contact with a solvent, but it will absorb a portion of it and swell. Two forces are competing during the swelling process: the free energy of mixing causes the solvent to penetrate and try to dissolve the polymer; polymer chains start to elongate under the swelling action of the solvent, but, since in a cross-linked polymer they are chemically bounded each other, elastic retroactive forces are generated in opposition to this stretching.

A steady state of swelling is reached when the two forces balance each other; the balance point is a direct function of the cross-linking degree; consequently, swelling experiments are a simple and low-cost technique to characterize polymer networks, since they can serve as an indexing tool for polymer systems with different levels of crosslinking.

Swelling experiments were conducted on White Alginate Beads (WAB) as described in the experimental part. The swelling degree (SD) was calculated using the equation 1 (197).

$$SD\% = \frac{Final_{weight} - Initial_{weight}}{Initial_{weight}} \times 100 \quad (1)$$

Figure 11.1 and table 11.1 report the weight percentage changes of WAB that were submitted to swelling test in water.

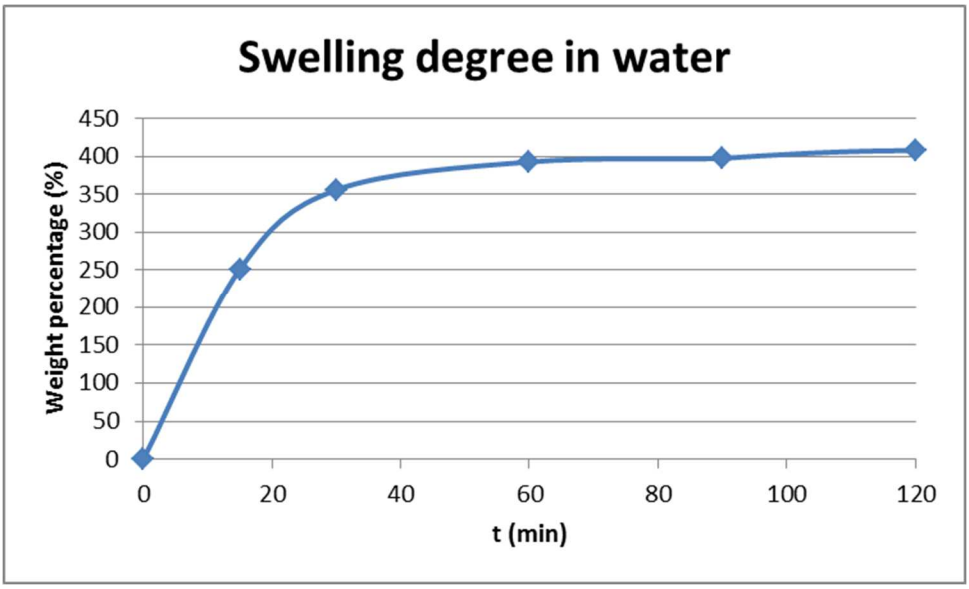


Figure 11.1: Swelling of WAB in water

Time	0 m	15 m	30 m	60 m	90 m	120 m	24 h
Swelling percentage	0%	251%	355%	392%	397%	407%	412%

Table 11.1: Data of WAB swelling in water

After 60 minutes, a swelling plateau was reached; WAB can swell water up to four times their weight. Datum after 24 hours has not been reported on the graph since no significant differences occurred.

The same experiment was repeated using EALYS water solution at 5 mg/ml concentration, in order to assess if EALYS can affect the swelling behaviour of alginate. The results of the assay were provided in table 5.10 whereas the curve is reported in figure 11.2.

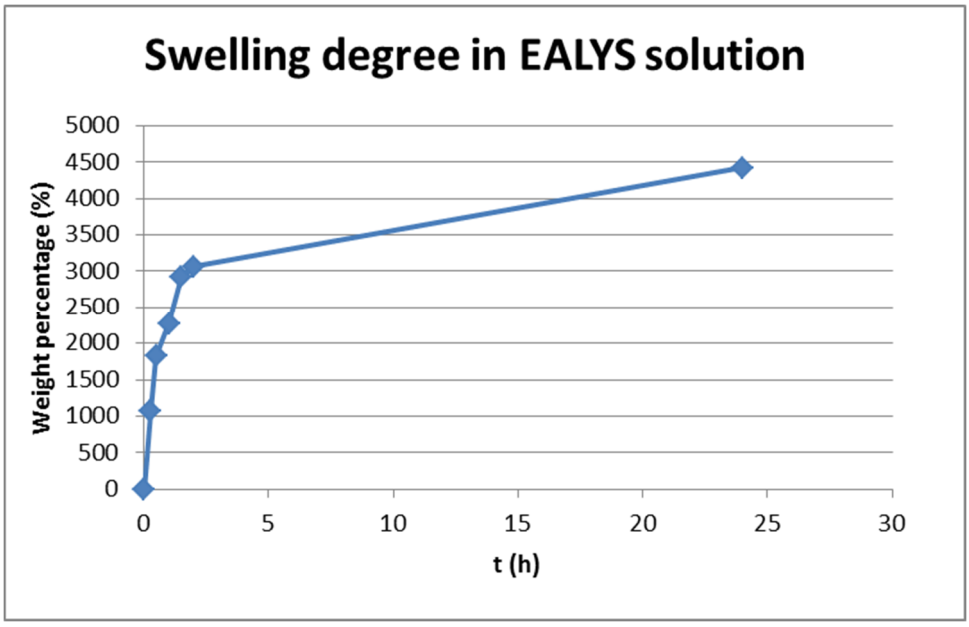


Figure 11.2: Swelling of WAB in EALYS water solution

Time	0 m	15 m	30 m	60 m	90 m	120 m	24 h
Increase of weight percentage	0%	1076%	1824%	2271%	2924%	3067%	4419%

Table 11.2: Data of WAB swelling in EALYS water solution

It can be observed that the beads swelled in EALYS water solution, even after only 2 hours, are able to swell up to 45 times their weight, 10 times more than when beads were made swelling in pure water. However, after 24 hours in the EALYS solution alginate beads started to disrupt: EALYS-Ca(II) salt forms in the solution subtracting Ca^{2+} ions from the alginate beads reducing the ionic bridges and compromising the stability of the gel network. It is possible to conclude that alginate network can be damaged with an excess of EALYS; nevertheless, due to the very high SD, it is possible to do beads swell in lower amount of EALYS solution avoiding problem related to network degradation.

11.2 Rheological study

The rheological properties of alginate films were assessed using the same method previously used for pectin.

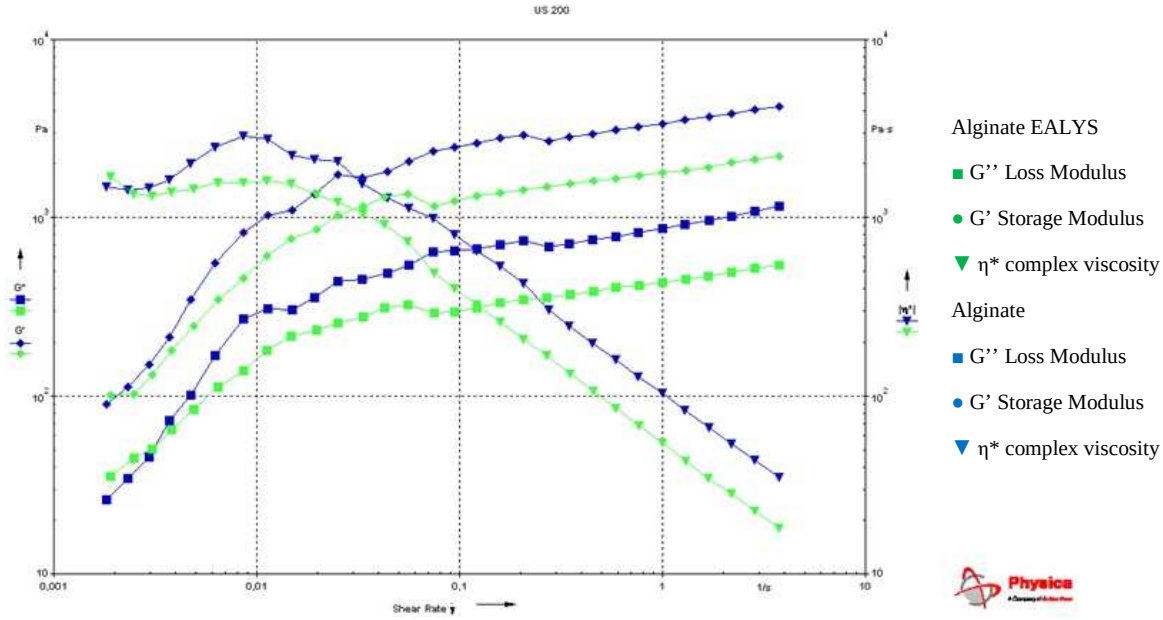


Figure 11.3: Rheological measurement of AF_EALYS (green lines) and WAF (blue lines)

It can be observed that the storage moduli (*G'*), both for film of alginate (WAF) and film of alginate with EALYS (AF_EALYS), are always higher than the loss moduli (*G''*), as shown in figure 11.3: the gels show high stability without mechanical properties loss even under high frequency stress. In addition, the presence of EALYS does not compromise the gelling process. Bonds between calcium and alginate carboxylic moieties, although during swelling test EALYS compromise the alginate lattice, have a higher stability in comparison to bonds present in pectin; in fact, calcium reaction with EALYS required prolonged exposure time and high quantity of water in order to jeopardize the lattice structure.

11.3 Alginate controlled release

Once alginate matrices were selected, EALYS was incorporated. In order to evaluate the release, assays were performed as described in the "Formulations release studies" paragraph of the experimental part.

In order to evaluate the release properties of alginate and to identify the best in class formulations some parameters were modulated, in particular:

- The physical form of the alginate material: beads and films
- concentration of carboxymethyl cellulose (CMC) in film formulations
- concentration of alginic acid sodium salt used in film preparation

PBS is able to dissolve alginate gel, as reported in literature (198), because Na^+ cations, present in the solution, undergo ion-exchange process with Ca^{2+} ions, which are bound to COO^- groups; as a result, the electrostatic repulsion among negatively charged COO^- groups increases, which ultimately causes the chain dissolution and the release of EALYS.

11.3.1 Release results

Alginate production procedures and release experiments are described in the experimental part. If compared to pectin release experiments, extra data were taken after 24 hours of release, since the majority of alginate materials submitted to the tests did not stop releasing EALYS after two hours. 24 hours time was selected in order to mimic the release at infinite time.

First, “Produced beads” (PAB), in which EALYS was incorporated in the solutions used to produce the beads, and “Swelled beads” (SAB), in which EALYS was incorporated by making WAB swelling in an EALYS water solution, were compared to identify differences in their release behaviours; release trends are shown in figure 11.4, whereas results are provided in table 11.3.

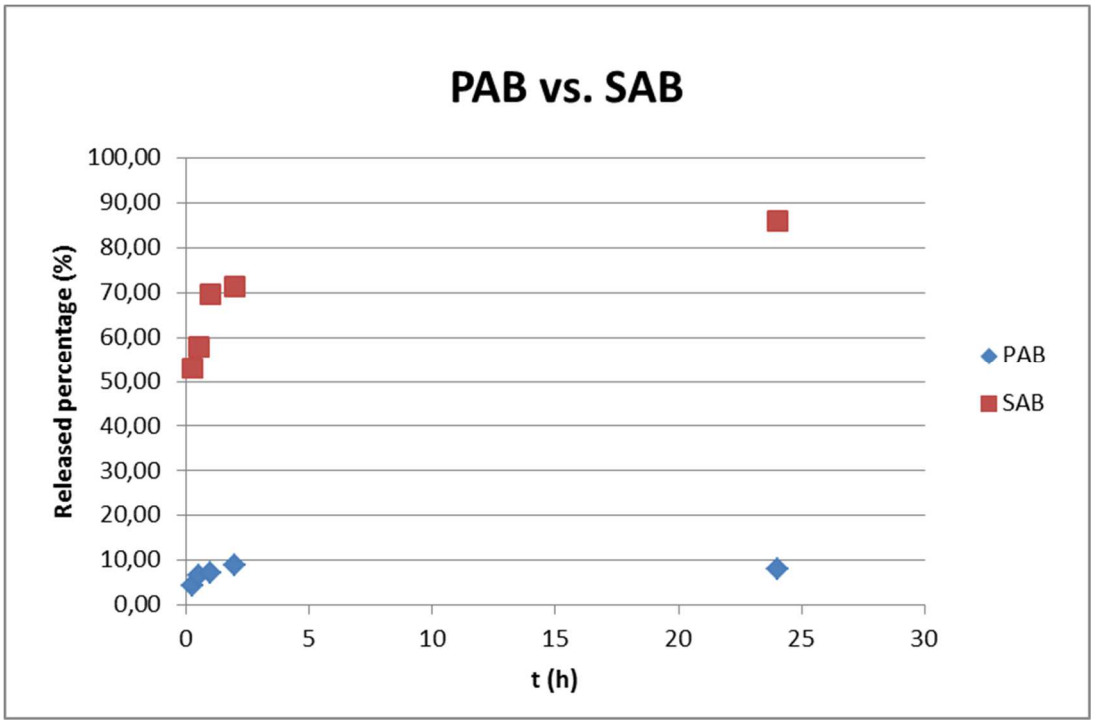


Figure 11.4: Comparison of SAB and PAB alginate beads

Sample	15’	σ	30’	σ	60’	σ	120’	σ	24 h	σ
SAB	53.0%	2.2	58.0%	2.0	69.7%	21.6	71.5%	15.2	86.0%	25.7
PAB	4.2%	1.0	6.5%	0.7	7.1%	0.6	8.9%	0.1	8.0%	0.3

Table 11.4: Release data of SAB and PAB alginate beads

Both materials displayed a release trend over time, but PAB reaches the plateau of release curve after 2h EALYS, whereas, SAB could release EALYS even after this time. After 15 minutes SAB releases 53% of EALYS present in the formulation and during the following 2 hours 33% more EALYS is released by SAB. The preparation method used for SAB leads to obtain an incorporation of EALYS both inside the beads and on the surface of the beads: the EALYS present on the surface of beads is easily dissolved leading to a rapid rise of EALYS concentration, while the incorporated EALYS is less accessible to PBS solution leading to a slow rate release during the assay time. Therefore, almost 60% of loaded EALYS is lost during the first 15 minutes while 40% is held inside alginate beads which are able to control its release.

Since the literature procedure reported the use of CMC in order to increase the viscosity of the solution used to produce alginate gel (199), two samples were prepared to investigate the effect of CMC on the release behaviour; in one sample CMC was used to produce a film, while in the other sample no CMC was used. Results are reported in figure 11.5 and table 11.4.

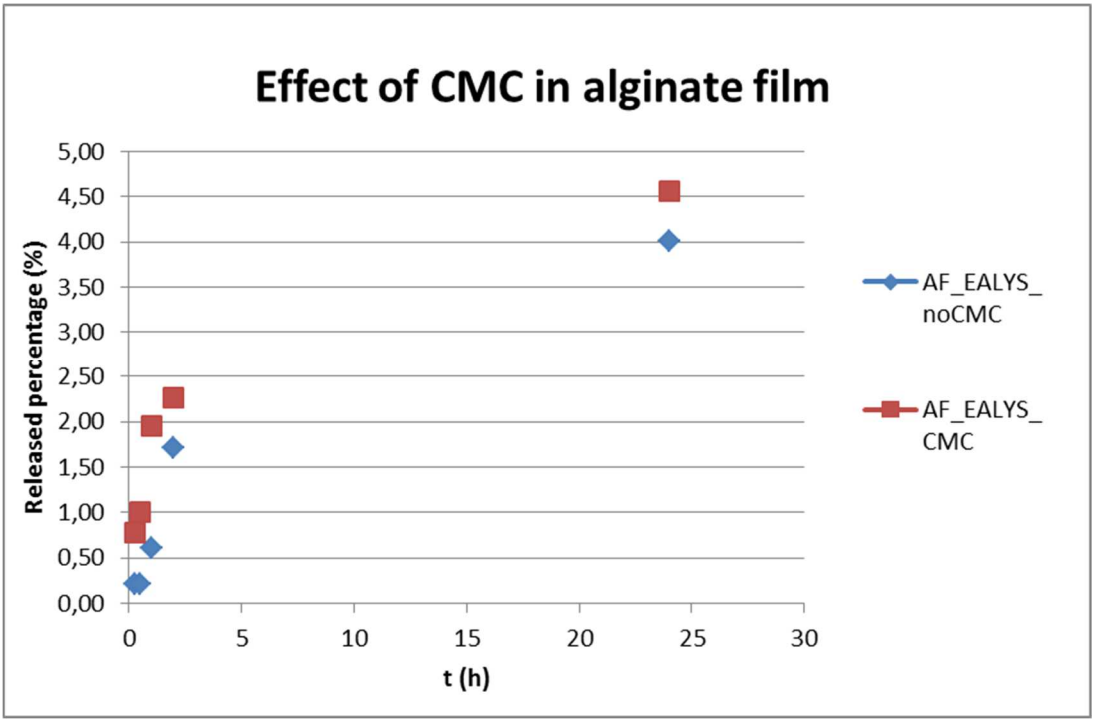


Figure 11.5: Comparison of AF_EALYS_noCMC and AF_EALYS_CMC

Sample	15’	σ	30’	σ	60’	σ	120’	σ	24 h	σ
AF_EALYS_noCMC	0.22%	0.0	0.22%	0.3	0.61%	0.6	1.72%	1.5	4.01%	0.3
AF_EALYS_CMC	0.78%	0.7	1.00%	0.1	1.95%	0.1	2.26%	0.5	4.56%	0.7

Table 11.4 - Release data of AF_EALYS_noCMC and AF_EALYS_CMC

It can be observed that the trends of the two formulations are not significantly different. However, the formulation without CMC started to release EALYS after minutes (mostly between 30 and 60 minutes) while the material with CMC started to release it since the beginning of the test. CMC is eliminated from alginate beads or films when the lattice structure is already formed, therefore the solubilization and extraction in water remove the excess of $CaCl_2$ leaving micro holes in the alginate structure: the high porosity structure dramatically increases the surface in contact with the solvent promoting the extraction process.

The film formulation and the bead material were compared and the results are reported in figure 11.6 and table 11.5.

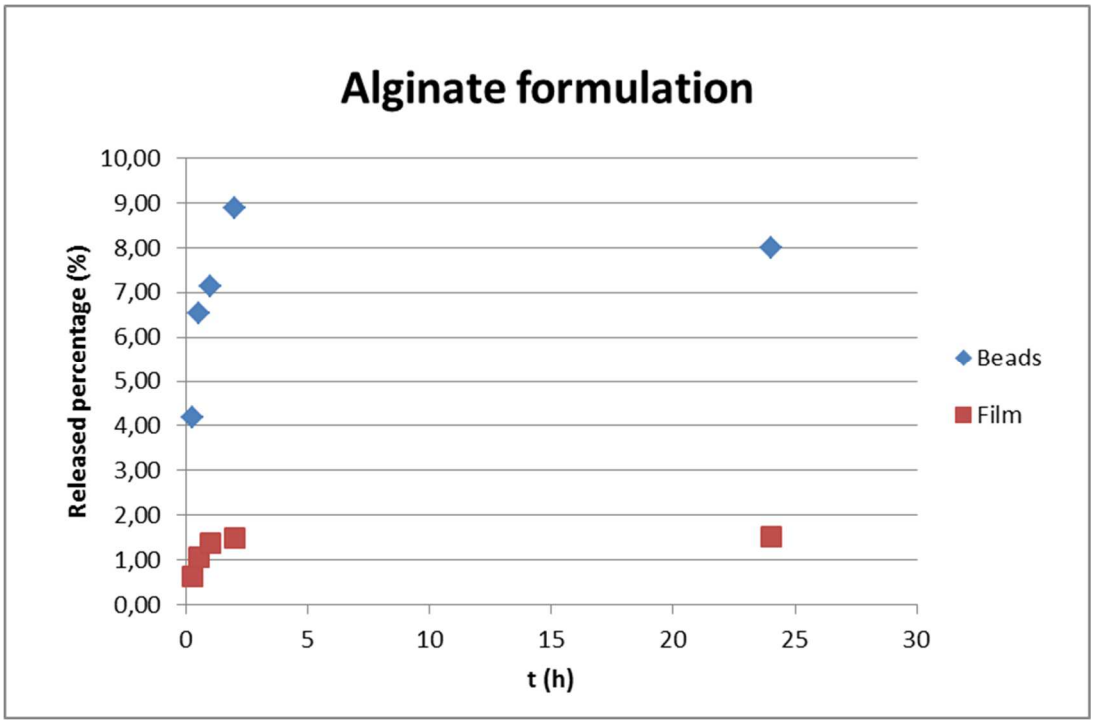


Figure 11.6: Comparison of PAB and AF_EALYS

Sample	15’	σ	30’	σ	60’	σ	120’	σ	24 h	σ
PAB	4.18%	1.0	6.53%	0.7	7.14%	0.6	8.89%	0.1	7.99%	0.3
AF_EALYS	0.62%	0.1	1.07%	0.0	1.37%	0.1	1.48%	0.0	1.52%	0.1

Table 11.5 - Release data of PAB and AF_EALYS

The highest release obtained with beads is entirely due to the higher surface area in contact with the solvent: the surface area of beads is several times higher than the surface area of film.

11. 4 Conclusions

In conclusion, it is possible to identify some characteristics that an alginate material should have to obtain a good release overtime of EALYS:

- Alginate should be produced using CMC to increase the viscosity of the preparative solution and to obtain a release of EALYS starting as soon as the material gets in contact with liquid. However, if the material is designed to release EALYS only after some time no CMC should be used during the film preparation process.
- Beads formulation has a better control on the release of EALYS if compared with film. In addition, CMC is needed to obtain a bead material; in fact, without CMC, the $CaCl_2$ solution, dropped in the alginate solution, does not have a viscosity high enough to obtain a spherical shape (see “Beads production procedure” paragraph in the experimental part) leading to an irregular and non-homogeneous material.
- Beads have to be produced in “blank” form and then EALYS has to be incorporated making the beads swelling in a water solution of EALYS.
- Alginate gels are able to release up to 86% of the loaded quantity of EALYS within 24 hours, the largest amount of EALYS being released within 2 hours; in comparison to pectin, alginate beads are able to release EALYS over a longer period of time and in greater amounts.

Since the release of EALYS should occur at colon level in the GUT, the alginate formulation should be protected against strong acidic environment in stomach that can alter the EALYS form. Although alginates are shown to undergo an almost immediate hydration that creates a hydrocolloidal layer at high viscosity, which acts as diffusion barrier decreasing the migration rate of drugs (200), EALYS will start to be released as soon as a water environment will make the alginate swelling.

To prevent the release before the formulation reaches the colon, a proper coating should be used in order to block the release of EALYS until the intestine, where the coating layer will be disrupted by microbial action or basic environment (pH= 7.5-8.1).

12. Acrylic derivative of Ellagic Acid

EA is a very stable and, consequently, very low reactive polyphenol, with a melting point higher than 360°C. Few examples concerning the EA reactivity are provided in literature; however, the four hydroxyl moieties can react with highly reactive molecules (i.e. anhydride) as was reported by Ren et al. (201), describing the preparation of a tetra-acetyl derivative.

The aim of the third part of the project was to obtain a derivative of EA where the phenolic groups are bound to moieties available for further polymerization but, at the same time, can be rapidly hydrolyzed in weak acid or basic environment in order to release EA. The moiety with these features is an ester: aromatic ester can be obtained with the reaction of anhydride or acyl chloride, and is prone to hydrolysis releasing the active molecules. This approach is completely different from the approach used for EALYS delivery: in this case the release kinetic is not regulate, as in the case of pectin or alginate, by diffusion phenomena through the polymer but it is dependent by ester bond hydrolysis kinetic. Therefore, the interaction with water of the polymer and the presence of acid/base moieties along the polymer chains are the triggers of the release and their tuning permits to fine control the release kinetic. pH is not the only parameter which can influence the release, since enzyme catalyzed hydrolysis normally occurs inside human body: esterase enzymes are able to cleave the ester bonds releasing the EA.

EA was modified in order to be further reactive with vinyl monomers; free radical polymerization was chosen due to it features:

- low temperature required for the reaction that avoids possible monomer degradation
- very fast kinetics and well-known polymerization scheme (possibly to speed up a future scale up)
- availability of several biocompatible monomers with different features (water compatibility, ionization, acid or base behavior etc.) that permits to tailor the properties of the material

Copolymers produced with this approach could be used not only for delivering EA in the gastrointestinal tract as food supplement formulations, but also for the production of transdermal patches and subcutaneous implants able to release EA over long period of time, due to the slow kinetic of hydrolysis of the polymeric matrix.

The synthesis of a modified moiety of EA with methacryloyl chloride was carried out, as reported in the experimental part. The tetra-carboxylate derivative (EAMAC), whose structure is shown in figure 12.1, contains four reactive vinyl groups and can be used as crosslinking agent in radical polymerizations.

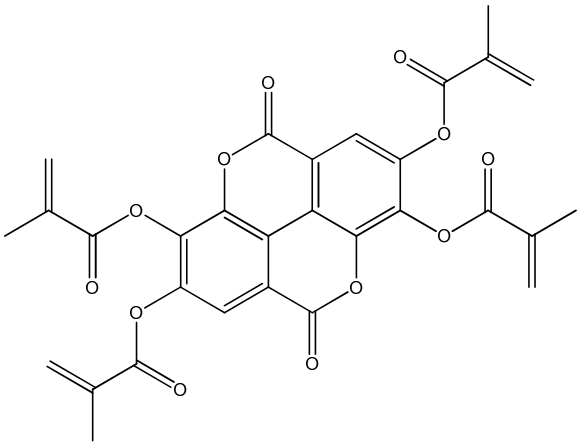


Figure 12.1: EAMAC chemical structure

EAMAC was fully characterized via ^{1}H-NMR (spectrum is reported in figure 12.2) and ESI-Q-Tof mass spectrum and the thermal properties were assessed via DSC.

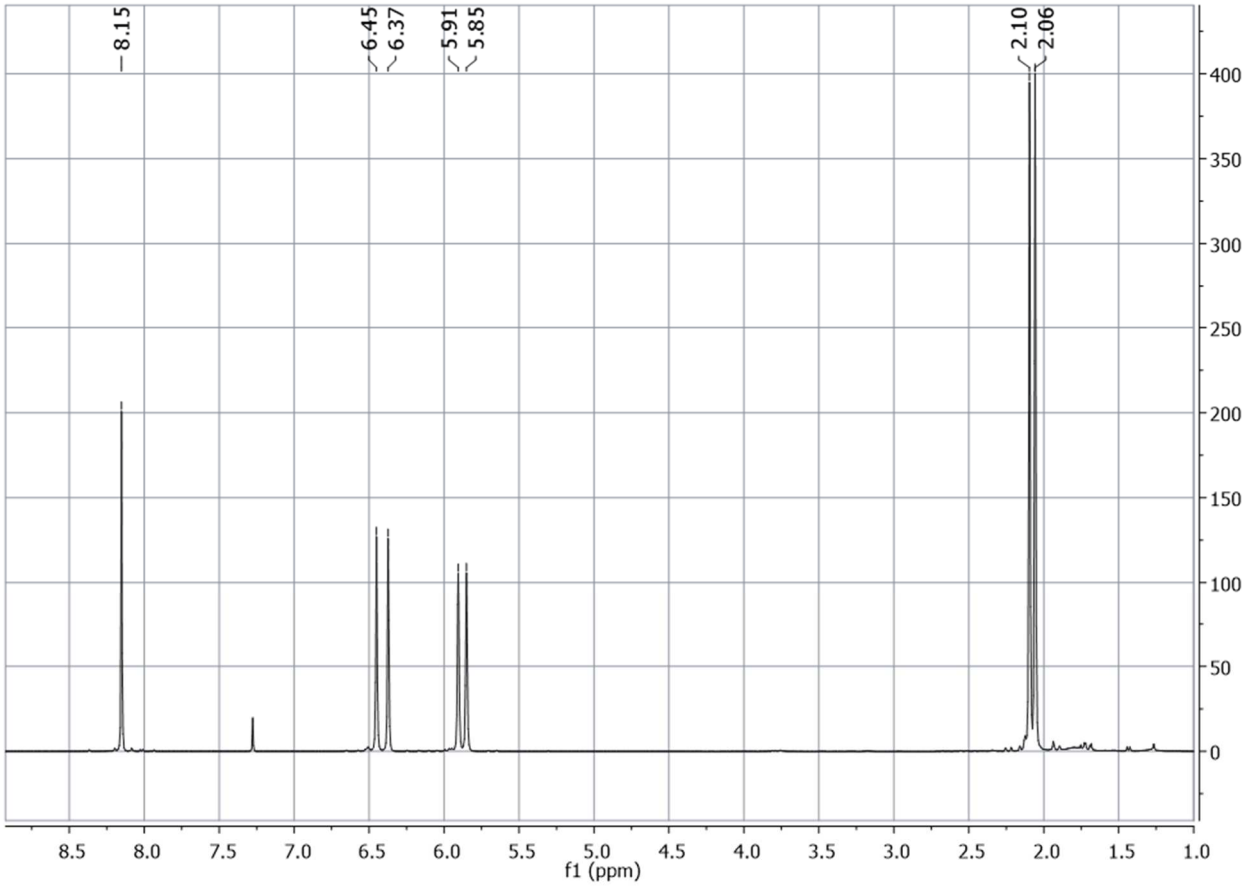


Figure 12.2: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of EAMAC

In figure 12.3 are reported the attributions of the hydrogen atoms of EAMAC.

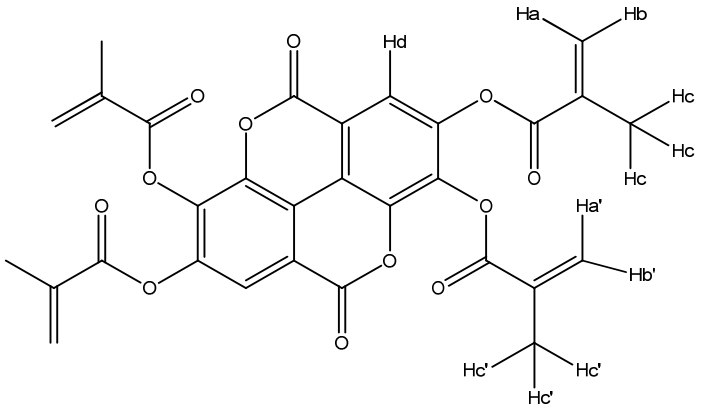


Figure 12.3: Representation of EAMAC protons

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (1H, s, Hd), 6.45 (1H, s, Ha or Ha'), 6.37 (1H, s, Ha' or Ha), 5.91 (1H, s, Hb or Hb'), 5.85 (1H, s, Hb' or Hb), 2.10 (3H, s, Hc or Hc'), 2.06 (3H, s, Hc' or Hc). EAMAC molecule presents a symmetry over the central axis, therefore two different types of methacrylic substituents are present on EAMAC, having different chemical shifts. In addition, methacrylic substituents place themselves in order to obtain the lowest possible steric hindrance thus rendering unsymmetrical the molecule. Geminal constants cannot be identified in the spectrum and therefore all hydrogen atoms give singlet signals.

The theoretical molecular weight of EAMAC is 575.29 g/mol and this value was confirmed by ESI-Q-Tof mass analysis.

The DSC thermogram of EAMAC displays a melting transition from 261°C to 266°C. After melting the compound starts to degrade.

12.1 NVP-EAMAC copolymer

Poly(vinyl pyrrolidone) (PVP) is a biocompatible, easily obtainable and low cost synthetic polymer widely used for biomedical application. N-vinyl pyrrolidone (NVP) was chosen for copolymerization with EAMAC since PVP is largely used as matrix in pharmaceuticals application and as excipient in drug release formulations. Moreover, the absence of acid or basic moieties along the side chains avoids the hydrolysis of ester bonds of EAMAC permitting a regular growth of polymeric lattice: for instance, acrylic acid (another highly biocompatible monomer) can catalyze the hydrolysis of esters bond compromising the formation of a polymeric lattice.

One of the most diffused radical initiators is azobisisobutyronitrile (AIBN), however, many studies have demonstrated that the reaction products of radical decomposition of AIBN are toxic and therefore it cannot be used for biomedical application. In order to obtain a biocompatible material, Vazo-67©, 2,2′-Azobis(2-methylbutyronitrile), whose structure is shown in figure 12.4, was chosen as biocompatible radical initiator: Vazo-67® does not present toxicity issue and reacts in the same way of AIBN.

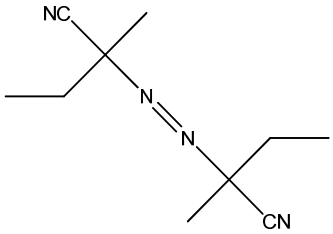


Figure 12.4: Vazo-67© chemical structure

Bulk polymerization approach was used to obtain crosslinked copolymers of NVP and EAMAC with molar ratio of EAMAC to NVP of 0.5% and 1%, as reported in the experimental part. The weights of obtained materials are reported in table 12.1.

Sample	Obtained weight	Yield
PVP_EAMAC_0.5%	1351.8 mg	43.6%
PVP_EAMAC_1%	1534.4 mg	56.5%

Table 12.1: Obtained weights of NVP co EAMAC

EAMAC, due to its structure, acts as a crosslinking agent; however, solubility tests of PVP_EAMAC_0.5% and PVP_EAMAC_1% were performed in order to verify the formation of polymeric lattices. Both poly(NVP-co-EAMAC) synthetized were insoluble in water and methanol; it is possible to conclude that they are crosslinked polymers.

The hydrolysis of EAMAC was verified leaving the polymers in NaOH water solution: the hydrolysis of ester bonds makes the polymer chains soluble. On the other hand, strong alkali solutions, as aqueous NaOH or KOH, are able to solubilize EA obtaining a dark-orange solution due to its partial degradation via quinone formation (187). This qualitative tests shows the ability of poly(NVP-co-EAMAC) to release EA after the hydrolysis of ester bonds of EAMAC under hydrolytic conditions.

12.2 Release of Ellagic Acid

PVP_EAMAC_0.5% and PVP_EAMAC_1% were submitted to release studies, as described in the experimental part, making hydrolysis experiments in basic environment. The tests were performed in D_2O in order to evaluate the hydrolysis products of the materials via NMR. In addition, ^1H NMR spectrum of PVP_blank, which is reported in figure 5.18, was registered to evaluate the differences between poly(NVP-co-EAMAC) materials and normal PVP.

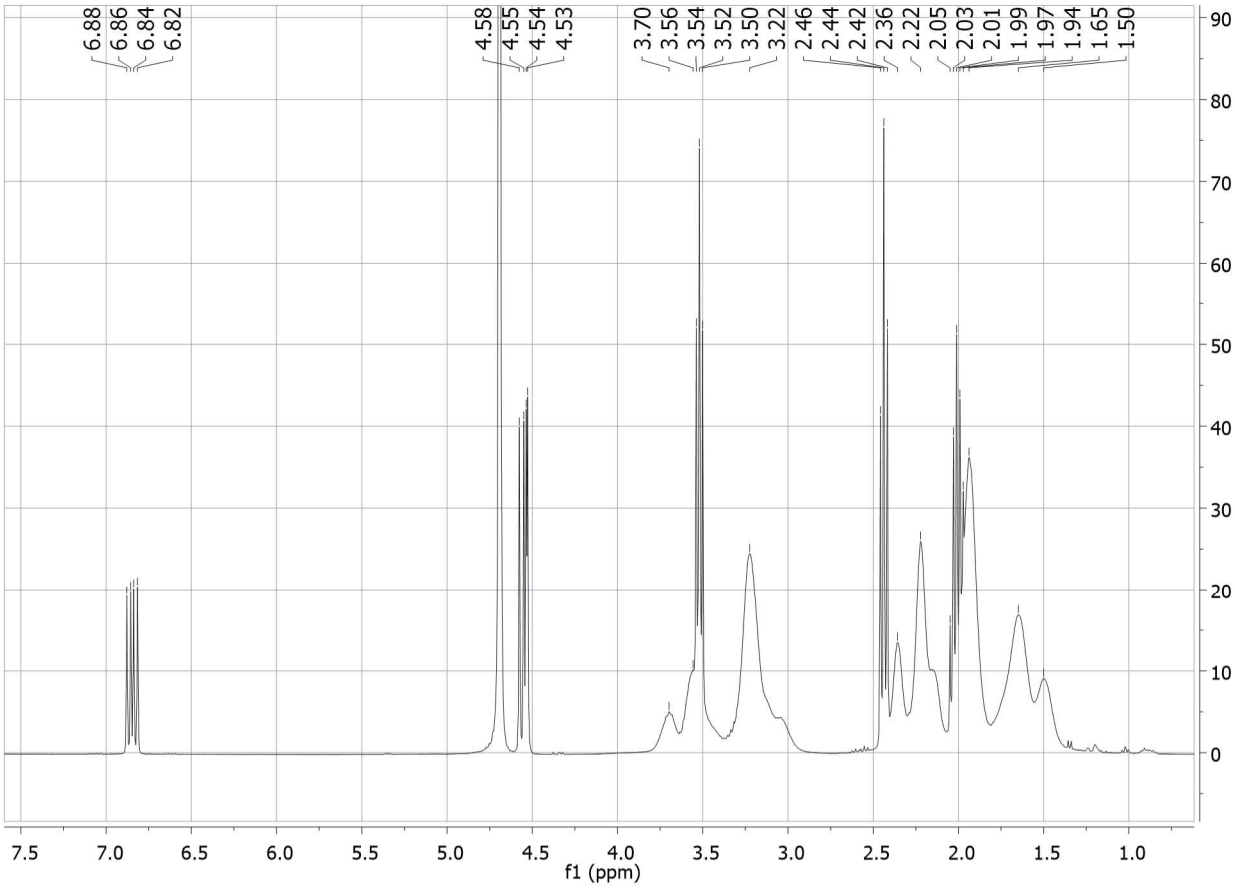


Figure 12.5: ^1H-NMR (400 MHz, D_2O) spectrum of PVP_blank

In figure 5.19 the attributions of the hydrogen atoms of unreacted NVP and PVP are reported.

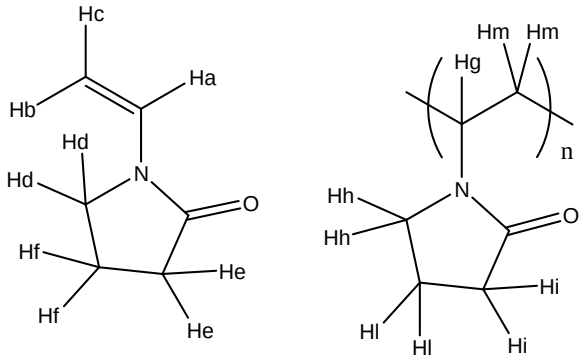


Figure 12.6: Representation of NVP and PVP protons

^{1}H NMR (400 MHz, D_2O) δ (ppm) of NVP: 6.85 (1H, dd, J=8 Hz and 10 Hz, Ha), 4.57 (1H, d, J=12 Hz, Hb), 4.54 (1H, d, J=4 Hz, Hc), 3.52 (1H, t, J=8 Hz, Hd), 2.44 (1H, t, J=8 Hz, He), 2.01 (1H, qui, J=8 Hz , Hf). Unreacted NVP is present in PVP_blank material, since the material was not submitted to washing procedure in water because PVP is water soluble itself.

^{1}H NMR (400 MHz, D_2O) δ (ppm) of PVP: 3.70 (s, Hg), 3.56 (s, Hg), 3.22 (s, Hh), 2.36 (s, Hi), 2.22 (s, Hi), 1.94 (s, Hl), 1.65 (s, Hm), 1.50 (s, Hm). The signals of PVP chains are very broad; this behaviour is typical of vinylic polymers.

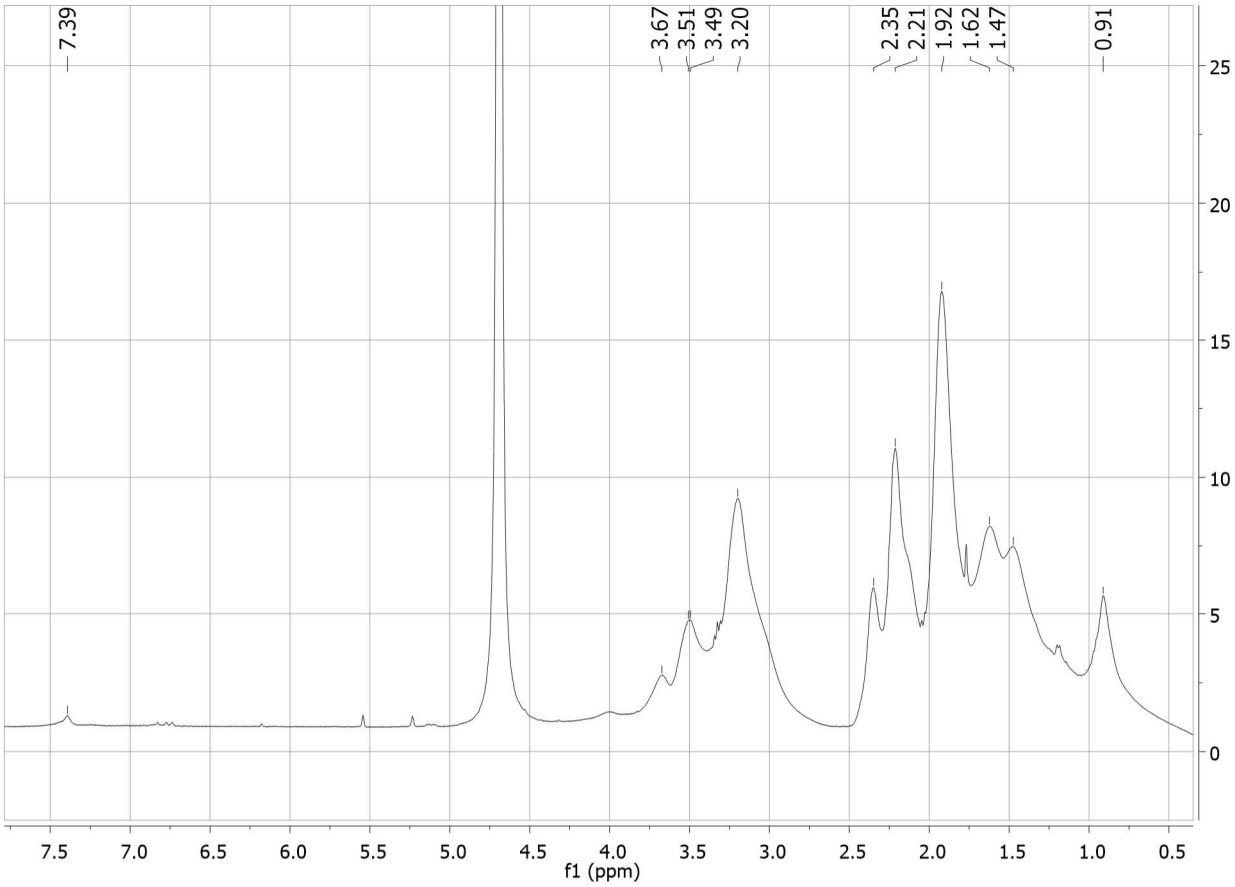


Figure 12.7: ^{1}H-NMR (400 MHz, D_2O) spectrum of PVP_EAMAC_1%

Signals of unreacted NVP are not present in the spectra of final poly(NVP-co-EAMAC) materials, since they were submitted to a washing procedure in water to remove the unreacted NVP. In addition to signals of PVP polymer chains, the signals of EA appeared after the hydrolysis experiments, conducted on PVP_EAMAC_0.5% and PVP_EAMAC_1%, as it can be noticed from figure 12.7: ^{1}H NMR (400 MHz, D_2O) δ (ppm): 7.39 (s).

Spectra of poly(NVP-co-EAMAC) present signals that can be attributed to methyl groups present on PVP chain terminals, i.e. methyl groups in α position to the acid residues obtained after the hydrolysis of EAMAC ester bonds: ^{1}H NMR (400 MHz, D_2O) δ (ppm): 0.91 (s).

These experiments assessed the ability of poly(NVP-co-EAMAC) to release EA when submitted to hydrolytic environment. The ester bonds of EAMAC can be hydrolysed and this represents the rate determining step of the release of EA.

12.3 Conclusion

A tetra-vinylic derivative (EAMAC) of EA was successfully synthetized and purified. This derivative was copolymerized with a vinylic monomer, under free radical reaction conditions, and acts as cross-linking agent. Several poly(NVP-co-EAMAC) were synthesized using different amounts of EAMAC and qualitative NMR experiments were carried out to assess the ability of these materials to release EA through hydrolysis of the ester bonds of EAMAC.

Conclusion

13. Conclusion

The project aims to supply natural antioxidants using polymeric matrixes like poly(lactic acid), poly(vinylpyrrolidone) and polysaccharides. The attention focused on the use of bio-based polymers both synthetic and natural using environmentally friendly materials.

Biopolymers were investigated for possible applications in two different fields, food packaging and biomedicine (active substances delivery), using the same approach:

1. to Identify the best polymer for the application required
2. to Tailor the synthesis or the preparation and formulation in order to achieve the best results
3. to Verify the activity in terms of antioxidant power of the material, assessing also how the antioxidant molecules have been supplied

This approach allows to develop new smart materials using the polymerization process that could be in a future easily scaled up.

The first part of the thesis was devoted to the development of an intrinsic antioxidant polymer for food packaging. The world of natural antioxidant is very wide therefore the first step was to identify a molecule with antioxidant power able to react with PLA: an aliphatic alcoholic moiety is required for the reaction with PLA and a phenolic moiety as radical scavenger. These features narrow a lot the range of possible antioxidants of natural origin; moreover, the antioxidant has to be stable at polymerization temperature (many natural antioxidants with very high radical scavenger power were rejected due to their poor thermal stability even in inert atmosphere). Two molecules were actually identified: Tyrosol, as model molecule used to discover how a phenolic moiety interact with PLA polymerization process, and Vanillyl Alcohol as antioxidant molecules.

Vanillyl Alcohol modified PLA has interesting antioxidant activity and it is obtained with a very low quantity of VA ($0.1\%_{mol/mol}$). The polymer for active packaging are normally produced adding active substance (antioxidant or other additives); in this work, the approach used is completely different: the active molecule is bond to polymer chains avoiding the migration during the contact with food.

This strategy permits to overcome two important issues related to the migration of active substance:

- the loss of activity during the time;
- the pollution of food

The antioxidant power was measured through *in-vitro* test as DPPH reduction; the interesting results obtained allow to perform shelf life test in order to verify the activity of VA modified PLA

on a real food matrix: VA modified PLA sheets were used as interlayer between two salami slice. The preliminary results were interesting:

- Antioxidant power, measured during the assay, did not decrease confirming that the VA is effectively bonded to polymer chains;
- Polymeric sheets reduce the oxidation rate in food: the oxidation is delayed of about 14 days in comparison to control samples packaged without interlayer.

In conclusion, antioxidant material is obtained using standard industrial conditions (Temperature and time), moreover, VA modified PLA is obtained using very low quantity of VA that does not negatively affect the mechanical properties of the polymer and permits to achieve interesting antioxidant activity. These features allow to hypothesize a rapid scale up for the production of antioxidant films based on VA modified PLA.

The second part of the project aims to supply and to deliver antioxidants for biomedical applications: the idea was to use *standard* polymer for production of devices for controlled release. The industrially-oriented approach of this project has promoted the choice of *standard* materials in order to avoid problems related to the study of effects of new material on human body: in this way it is possible to predict a rapid industrialization of the devices.

Both synthetic and natural polymer were chosen for the production of release devices: PLA, normally used in biomedical field, and Alginate and Pectin, natural polysaccharides intrinsically biocompatible already used for the production of drug release device. These material were used for two very different applications:

- PLA was used for the production of solid scaffolds used for the delivery of OligoTyr (ossification enhancer).
- Pectin and Alginate were used for the production of edible hydrogels for the release of Ellagic Acid in gastrointestinal tract.

The PLA is widely used for the production of biomedical devices therefore the focus of the work was to tailor the morphology of the scaffolds in order to control the release of OligoTyr. OligoTyr is a mixture of oligomeric species of Tyrosol, obtained by oxidative coupling reaction catalyzed by horseradish peroxidase: oligomerization reduce the water solubility of OligoTyr therefore for the release from a polymer, a very high surface area is required in order to improve the contact with the extractive media (PBS at pH 7.4). Two morphologies can satisfy this request: nanoparticles and hierarchical scaffolds; on the other hand, the problem related to nanoparticles safety (many studies

have been conducted but their effects on human health are not clear yet) leads to prefer hierarchical scaffolds, always looking for a future industrial scale up.

Two different methods for the production of the scaffolds were set up:

- the method A permits to obtain very high porosity of the material but it has reduced reliability on the morphology and on the load of OligoTyr;
- the method B, on contrary, leads to obtain less porous material but it has good reliability and homogenous dispersion of OligoTy

The kinetics of the release were assessed using PBS at pH 7.4 for 70h; the reduced solubility of OligoTyr, in comparison to Tyrosol, and the poor water compatibility of PLA lead to obtain very slow release: a continuous release of low quantity of OligoTyr for a long time is required for the ossification process that normally takes at least 30 days. It was also verified that the morphology triggers the release.

Besides the development of PLA scaffolds, polysaccharides based delivery system were studied. Pectin and alginate, intrinsically biocompatible acid polysaccharides, were used for the production of hydrogel: the gel structure is due to the ionic interaction with calcium (II) of acid moieties along polymeric chains. Even though they have similar structure, the physical properties of pectin gels and alginate gels are very different: rheological studies allow to identify the mechanical properties of the formulation and to address formulation for best application.

Both alginate and pectin are water soluble polymers whereas Ellagic Acid (a natural antioxidant from pomegranate) is not; the poor water solubility of EA dramatically reduces its possible application as antioxidant supplement, beyond reducing its bioavailability. Therefore, a part of the project was devoted to enhance the water solubility of EA: the formation of L-lysine salt allows to increase the water solubility up to 400 times (EA water solubility is 10µg ml^{-1} whereas the EA lysine salt solubility is 13mg ml^{-1}). Pectin and alginate hydrogels loaded with EA lysine salt were prepared: different release profiles were obtained able to target different scopes (fast or slow release, resistance to low or high pH, high or low EA load).

The last part of the thesis was dedicated to the study of the reactivity of the Ellagic Acid; only one literature example is reported about its reactivity: the acetylation of phenolic moieties in order to enhance EA lipophilicity. Following the same scheme, a tetra acrylic ester of ellagic acid was synthesized: the reaction proceeds at room temperature and does not require purification after the work up. Ellagic acid derivative is a tetra-functional acrylate able to react with other acrylic or vinyl monomer via free radical polymerization; the idea was to use it as co-monomer for the production

of scaffolds and act as delivery system for EA. The release is achieved not by extraction of EA from the polymer but it is due to the hydrolysis of ester bonds. The kinetic of the release is not regulated by the Fick's law but the erosion of the matrix determines the release.

The EA derivative was used as cross-linking agent for the polymerization of N-vinylpyrrolidone. Poly(vinylpyrrolidone) is a hydrophilic biocompatible polymer widely used for biomedical application. A crosslinked material insoluble either in water or in organic solvents was obtained. The release was verified in hydrolytic environment: in 24h at 37°C and basic pH, ester bonds are hydrolyzed leading to complete solubilization of polymers as verified via 1H-NMR.

In conclusion, different fields of antioxidant application were studied and new materials able to supply antioxidants were developed and tested understanding the advantage and also the limits related to the use of natural antioxidant.

Materials and methods

14. Materials and Methods

14.1 Materials

Ellagic Acid (EA) (Fluka, ≥96.0%), L-lysine (crystallized, ≥98.0%), Calcium chloride (anhydrous, ≥99.9%, 40 mesh), Pectin from apple (Poly-D-galacturonic acid methyl ester), Pectin from citrus peel (Galacturonic acid ≥74.0 %), Potassium hydroxide (Fluka, ≥85%, pellets), Sodium chloride (Fluka, ≥99.0%) Alginic acid sodium salt (powder), Methacryloyl chloride (≥97.0%, contains ~ 0.02% of 2,6-di-tert-butyl-4-methylphenol as stabilizer), triethylamine (≥ 99%), Hydrochloric acid (Fluka, 37%), Sodium bicarbonate (Fluka, ≥99.7%), Sodium sulphate (Fluka, ≥99.0%, anhydrous), 2,2′-Azobis(2-methylbutyronitrile) (VAZO-67©, ≥98.0%), Sodium hydroxide (Fluka, ≥97.0%, pellets) were purchased from Sigma-Aldrich Co. and they were used without further purification. Sodium carboxymethyl cellulose (medium viscosity) was purchased from A.C.E.F. SPA and was used without further purification. 1-Vinyl-2-pyrrolidone (≥ 99.0% stabilized with N,N'-Di-sec-butyl-p-phenylenediamine) was purchased from TCI chemicals. NVP was distilled under reduced pressure in order to remove N,N'-Di-sec-butyl-p-phenylenediamine polymerization inhibitor. Water (CHROMASOLV® Plus, for HPLC), Dichloromethane (ACS reagent, ≥99.5%, contains 40-150 ppm amylene as stabilizer), Hexane (anhydrous, 95%), Ethyl acetate (ACS reagent, ≥99.5%), Acetonitrile (CHROMASOLV® Plus, for HPLC, ≥99.9%) were purchased from Sigma Aldrich Co.,

L-lactide, Puralact L (polymer grade), purchased from Purac Biomaterials (Gorinchem, The Netherlands) was used for the synthesis of PLAs. An industrial PLA, trademark Natureworks® 4043D (D-isomer content z 4.3% as declared by the producer) was purchased from Resinex Srl, Italy. Methylene chloride, methanol (HPLC purity), tin octanoate (SnOct2), 2-(4-Hydroxyphenyl)ethanol, Tyrosol (Tyr, purity >98%) were purchased from Sigma-Aldrich Co. 4-(hydroxymethyl)-2-methoxyphenol, Vanillyl Alcohol (VA, purity > 98%) was purchased from TCI Europe NV. All reagents were used without further purification and no drying process was performed.

14.2 Sample characterization

UV-Vis spectra were recorded on a UV-Vis spectrophotometer Jasco V-630. Ten absorbance measures were collected using by the instrument and the average value was calculated automatically by the instrument's software. Differential scanning calorimetry (DSC) analyses were conducted under nitrogen flow using a Mettler Toledo DSC 1 instrument. NMR spectra were

recorded using a Bruker NMR-400. IR spectra were recorded using a PerkinElmer ATR FT- IR Spectrum 100. Solution's pH values were monitored using a pH-meter "Titrino 751 GPD". Rheological tests were performed using an Anton-Paar MCR 300 rotational rheometer equipped with a 50 mm diameter conic plate and 2° slope using a frequency sweep program between 0.01±20 Hz at 3% strain. Mass spectra were recorded on ESI-Q-Tof Micro-Waters (Wates Corporation, Milford, MA) in the data dependent acquisition and positive ion mode.

14.2.1 UV spectrum of EA

EA (30 mg) was weighted in a 50 mL round bottom flask and was dispersed in of chromasolv© water (20 ml) under magnetic stirring at 30°C overnight. The suspension was allowed to cool at room temperature and was filtered using a syringe equipped with a 0.45 μm Teflon® filter. In this way, a saturated EA solution was obtained and, by using a UV-Vis spectrophotometer, the spectrum of EA in water was recorded between 700 nm and 220 nm wavelength. EA presents two maximum of absorbance: one around 360 nm and one around 280 nm. 280 nm was used for performing further UV analysis due to higher intensity.

14.3 Ellagic Acid – L-lysine Salts

EA and L-lysine were weighted in a 100 mL round bottom flask and solubilized in 20 mL of distilled water under magnetic stirring at room temperature. After 30 minutes, water was removed using rotary evaporator at 50°C till obtaining a powder, then the residual water was removed under vacuum (2 x 10^{-2} kPa) for 10h at room temperature. The result is a yellowish powder. The used amounts of EA and L-lysine used for the preparation of salts are reported in table 14.1.

Molar ratio of salt	EA	L-lysine
1:1	0.4985 g (1.65 mmol)	0.2414 g (1.65 mmol)
1:2	0.5009 g (1.66 mmol)	0.4829 g (3.30 mmol)
1:3	0.4982 g (1.65 mmol)	0.7231 g (4.95 mmol)
1:4	0.4978 g (1.65 mmol)	0.9770 g (6.68 mmol)

Table 14.1 - Weights of reagents used in EA salification reactions

14.3.1 Water solubility

EA-L-lysine salt (EALYS) (20 mg) was weighted in a 15 mL glass vial and aliquots of 0.1 mL of chromasolv© water were added, under magnetic stirring at room temperature (T=18°C) until homogenous solution was obtained. The results are provided in table 14.2.

Salt (EA to lysine molar ratio)	Weight of salt (mg)	Used water	Water solubility (mg/ml)
1:1	20.1	15	Insoluble
1:2	23.6	15	Insoluble
1:3	19.8	15	Insoluble
1:4 (EALYS)	22.4	1.7	12.99

Table 14.2 – Weights and volumes used in solubility tests

14.3.2 Solubility in $CaCl_2$ water solution

EALYS was weighted in a 50 ml conic flask and 30 ml of chromasolv water was added. The flask was shaken in a water shaking bath for 60 minutes at 36°C. After the solution was filtered using a syringe equipped with a 0.45 μm Teflon® filter and the resulting solution was analyzed using an UV-Vis spectrophotometer.

The same procedure was slavishly followed using a $CaCl_2$ water solution in order to assess the interaction between EALYS and Ca(II). $CaCl_2$ and of EALYS were dissolved in 50 ml of chromasolv water in a 100 ml round bottom flask. The amount of EALYS was determined using the calibration obtained using EALYS as standard in water. Both tests were made in triplicate in order to validate the results, which are provided in table 14.3.

Sample	EALYS weight (mg)	CaCl2 weight (mg)	Water (ml)	EALYS released (%)
W1	18.5	0.0	30	100.83
W2	18.2	0.0	30	98.48
W3	18.7	0.0	30	99.39
$CaCl_2$1	11.2	8.0	50	2.31
$CaCl_2$2	11.7	8.7	50	2.51
$CaCl_2$3	11.6	8.9	50	2.44

Table 14.3 – Results of solubilization tests in water and in $CaCl_2$ solution

14.3.3 Differential Scanning Calorimetry

Samples of EA and all EA-lysine salts were prepared weighting about 6 mg of sample and analyzed using a dynamic scanning program from 25°C to 500°C at 10°C/min in order to assess their melting temperature and their thermal stability.

14.3.4 NMR

NMR spectrum of EALYS was recorded by weighting about 10 mg of EALYS in 1 ml of deuterium oxide (D_2O).

14.3.5 Calibration curve of EALYS

EALYS (22.51 mg) was weighted in a 100 ml volumetric flask and solubilized in water. 20 ml of this solution was taken with a 10 ml pipette and transferred in a 100 ml volumetric flask, which was filled with chromasolv© water (sol. A). 10 ml of sol. A was transferred in a 25 m volumetric flask and was filled with chromasolv© water (sol. B). 10 ml of sol. B was transferred in a 25 ml volumetric flask and was filled with chromasolv© water (sol. C). 10 ml of sol. C was transferred in a 25 ml volumetric flask and was filled with chromasolv© water (sol. D). 10 ml of sol. D was transferred in a 25 ml volumetric flask and was filled with chromasolv© water (sol. E). The absorbances of these solutions were registered using a UV-Vis spectrophotometer setting the instrument at 280nm. The absorbance values and the concentration of each solution are reported in table 14.4.

Sample	Concentration (mg/ml)	Absorbnce λ=280
Sol. A	0,04502	1,9665
Sol. B	0,01801	0,8432
Sol. C	0,00720	0,3244
Sol. D	0,00288	0,1466
Sol. E	0,00115	0,0658

Table 14.4 - Absorbance values of EALYS calibration curve

A calibration curve, shown in figure 14.1, was obtained using these data: concentration 0 corresponds to a 0 absorbance. The linear regression was calculated using Microsoft Excel software obtaining the equation 1:

$$C_{EALYS}(mg/ml) = 44.1644 \times Absorbance(\lambda = 280nm) \quad (1)$$

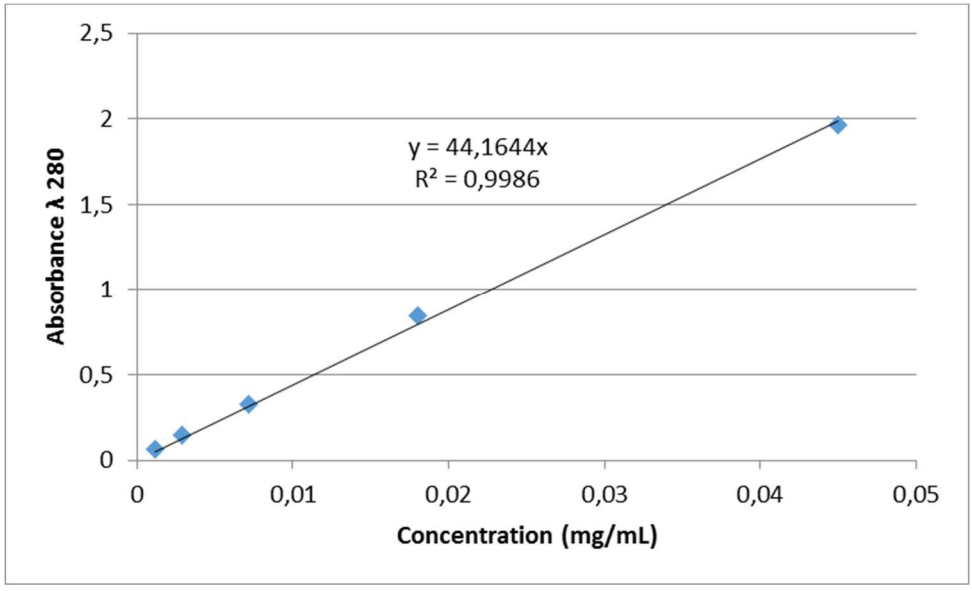


Figure 14.1 - Calibration curve for EALYS in water

14.4 Pectin

14.4.1 Degree of Esterification Determination

Degrees of Esterification were determined according to the method developed by Manrique et al. (117). Pectin was dissolved in water (concentration of 5 g/l) under magnetic stirring at room

temperature overnight; then the pH was adjusted at 6.0 using KOH 1M water solution. Water was removed by freeze-drying in order to obtain solid powders that can be analyzed using an ATR FT-IR.

14.4.2 Gels

Pectin gels were prepared following the procedure described by Fu et al. (202) and Tibbits et al. (203):

1. Pectin was weighted in a 100 ml round bottom flask and dissolved in NaCl 0.1 M water solution under magnetic stirring overnight.
2. pH was adjusted between 6.5 and 7.0 by using 1 M KOH water solution.
3. The solution was mechanically stirred at 80°C for 15 minutes in order to obtain a homogeneous temperature.
4. Solid $CaCl_2$ was added keeping the solution under mechanical stirring for 10 minutes at 80°C in order to obtain a homogenous solution. The high temperature inhibits the gelling process.
5. The solution was left at room temperature for 48h in order to complete the gelling process.

The amount of $CaCl_2$ required for gelling process was calculated on the degree of esterification of each pectin (see paragraph 10.1). For each pectin several samples were prepared using concentrations of 3 g/L, 5 g/L, 7.5 g/L, 10 g/L, 20 g/L, 30 g/L and 40 g/L.The amount of $CaCl_2$, used for the preparation of each gel, was calculated using equation 2.

$$R = \frac{2[Ca^{2+}]}{[COO^{-}]} \quad (2)$$

Where R is a predetermined parameter already used in the procedure described in literature, $[COO^-]$ is the concentration on carboxylic acid moieties (determined knowing the DE previously obtained using the IR-based methodology) and $[Ca^{2+}]$ is the calcium concentration. The amount of pectin and $CaCl_2$ are provided in table 14.5.

Sample	Pectin type	Pectin weight	$CaCl_2$ weight
3 g/l	LM	0.1512 g	12.03 mg
5 g/l	LM	0.2558 g	24.70 mg
7.5 g/l	LM	0.3756 g	36.12 mg
10 g/l	LM	0.5001 g	43.37 mg
20 g/l	LM	0.9989 g	86.70 mg
30 g/l	LM	1.5142 g	126.86 mg
40 g/l	LM	2.0516 g	169.60 mg
3 g/l	HM	0.1506 g	8.10 mg
5 g/l	HM	0.2518 g	13.91 mg
7.5 g/l	HM	0.3939 g	19.72 mg
10 g/l	HM	0.5064 g	25.53 mg
20 g/l	HM	1.0018 g	50.46 mg
30 g/l	HM	1.5185 g	74.04 mg
40 g/l	HM	1.9963 g	99.20 mg

Table 14.5 – Pectin and $CaCl_2$ weights used for the choice of pectin formulation

A similar procedure was used for the preparation of pectin gels with EALYS and the amount of pectin, calcium chloride and EALYS used are provided in table 14.6:

1. Pectin was weighted in a 100 ml round bottom flask and dissolved in NaCl 0.1 M water solution under magnetic stirring overnight.
2. pH was adjusted between 6.5 and 7.0 by using 1 M KOH water solution.
3. EALYS was added and the solution was mechanically stirred for 10 minutes till obtaining a homogenous solution.
4. The solution was mechanically stirred at 80°C for 15 minutes in order to obtain a homogeneous temperature.

5. Solid $CaCl_2$ was added keeping the solution under mechanical stirring for 10 minutes at 80°C in order to obtain a homogeneous solution. The high temperature inhibits the gelling process.
6. Solution was left in the closed flask avoiding the evaporation of water at room temperature for 48h in order to complete the gelling process.

Sample	Pectin type	Pectin weight	EALYS weight	$CaCl_2$ weight
LM_10%EALYS_30g/l	LM	1.5228 g	152.1 mg	128.7 mg
HM_10%EALYS_40g/l	HM	2.0194 g	200.3 mg	102.0 mg

Table 14.6 - Pectin, EALYS and $CaCl_2$ weights used for rheological study of pectin formulation with EALYS

14.4.3 Pectin Film Casting

Both LM and HM pectin were used for the incorporation of EALYS using a concentration of 30 g/l of pectin in water.

Procedure

The procedure described in paragraph “Pectin gels” was used for the preparation of pectin gel. On the other hand, pectin-based film was required for further release assays therefore the procedure previously described was modified. After point 5, instead of leaving pectin for 48h in order to obtain a gel, the obtained solution was poured into a plastic Petri dish, having 8.5 cm of diameter and 1 cm of depth. The film was obtained by solvent evaporation after 48h under suction hood. After that time, the film was overturned and left under the suction hood for other 24h in order to obtain a dry material.

Amounts of pectin and the type, $CaCl_2$, EALYS used for the preparation of each sample are reported in the table 14.7.

Sample	Pectin type	Pectin weight	EALYS weight	$CaCl_2$ weight
LM_10%_100	LM	1.5228 g	152.1 mg	128.7 mg
HM_10%_100	HM	1.5531 g	156.3 mg	76.4 mg
LM_2%_100	LM	1.5273 g	32.7 mg	127.1 mg
HM_2%_100	HM	1.5032 g	31.6 mg	74.9 mg
LM_10%_50	LM	1.5105 g	151.2 mg	63.8 mg
LM_10%_0	LM	1.5033 g	150.5 mg	0 mg
HM_10%_50	HM	1.5067 g	150.8 mg	38.3 mg
HM_10%_0	HM	1.5063 g	156.1 mg	0 mg

Table 14.7 - Pectin, EALYS and $CaCl_2$ weights used for pectin formulation

14.4.4 Rheology

Rheological tests were performed in order to evaluate toughness and mechanical stability of pectin gels.

14.5 Alginate

14.5.1 Beads

Alginate beads were prepared following the procedure described by Nigam et al. (204)and Blandino et al. (198):

1. Aqueous solution at 0.5% $_{w/w}$ of alginate (Solution A) and aqueous solution of 1.3% $_{w/w}$ of $CaCl_2$ and 3% $_{w/w}$ carboxymethyl cellulose (CMC) (Solution B) were prepared leaving reagents under magnetic stirring for 15h in order to obtain homogenous solutions.
2. Solution B (7 ml) was slowly dropped in solution A (100 ml) using a disposable syringe equipped with a 20G needle (inner diameter of 0.6 mm), inside a 250 ml beaker under magnetic stirring in order to avoid beads coupling.
3. The obtained mixture was diluted with 400 ml of water
4. Beads were filtrated using a metal net colander.
5. Beads were transferred into a $CaCl_2$ aqueous solution at 1.3%$_{w/w}$ and left there for 15 minutes in order to complete gelling process.

6. Beads were filtered with a colander and washed several times with water in order to remove the excess of $CaCl_2$.
7. Beads were freeze-dried to avoid microbial attack.

The amounts of alginic acid sodium salt, $CaCl_2$ and CMC, which were used for the preparation of White Alginate Beads (WAB), are reported in the table 14.8.

Sample	Weight of alginic acid	Weight of $CaCl_2$	Weight of CMC
WAB	0.5146 g	0.3335 g	0.7525 g

Table 14.8 – Alginic acid sodium salt, $CaCl_2$ and CMC weights used for alginate bead production

14.5.2 Beads swelling degree

Beads (86.1mg) were dipped in water (100 ml) at room temperature and their weight changing was monitored at different time (after 15, 30, 60, 90, 120 minutes and after 24 hours). Alginate beads were wiped using filtering paper in order to remove the excess of water presents on their surface and then weighted. The weight changes were transformed to a percentage using the equation 3:

$$Swelling\ Degree\ (\%) = \frac{m_w - m_D}{m_D} \times 100 \quad (3)$$

Where m_w is the wet weight and m_D is the dry weight of beads samples.

The same experiment was repeated in order to study how the presence of 5 mg/ml EALYS water solution affects the swelling behavior of alginate beads.

14.5.3 EALYS beads

Alginate-EALYS samples were prepare using two different strategies.

A) Weighted amount of WAB was transferred in a round bottom flask and a water solution of EALYS (C_{EALYS}= 5 mg/ml) was added using the ratio 1 ml of solution for 20 mg of WAB. The swelling process took 15h and, at the end, water was removed by freeze-drying. The used amounts of freeze-dried alginate beads and EALYS are reported in the table 14.9.

Sample	Weight of white alginate beads	Weight of EALYS
SAB	201.7 mg	51.19 mg

Table 14.9 – Freeze-dried alginate beads and EALYS weights used SAB production

B) EALYS was added in the solution A in order to obtain a concentration of 5 mg/ml and then the alginate beads were made using the same procedure reported above. The used amounts of alginic acid sodium salt, EALYS, $CaCl_2$ and CMC used are reported in the table 14.10.

Sample	Weight of alginic acid	Weight of EALYS	Weight of $CaCl_2$	Weight of CMC
PAB	0.5135 g	0.5010 g	0.3351 g	0.7586 g

Table 14.10 – Alginic acid sodium salt, $CaCl_2$ and CMC weights used for alginate produced bead production

14.5.4 Film

The previously described procedure for the preparation of alginate beads was modified in order to obtain alginate films.

1. The solutions A and B were prepared using the same concentration of alginate, CMC and $CaCl_2$.
2. Solution A (25 ml) was poured into a plastic Petri dish, having a diameter of 8.5 cm and a depth of 1 cm.
3. The solution B was firstly sprayed on the surface of solution A, in order to obtain a superficial gel formation, and then the Petri dish was sunk in the solution B in a crystallizer leaving the material inside the solution B overnight in order to complete the gelling process.
4. Petri dish was removed from solution B and the material was washed several times with distilled water removing the excess of $CaCl_2$.
5. The obtained films were dried at room temperature for 48 hours and, after this time, the material was overturned and left to dry for another 24 hours.

The amounts of alginic acid sodium salt and CMC used are reported in table 14.11.

Sample	Weight of alginic acid	Weight of CMC
WAF	0.1320 g	0.7642 g

Table 14.11 – Alginic acid sodium salt, $CaCl_2$ and CMC weights used for alginate film production

EALYS-Alginate films were obtained using a similar procedure: EALYS was added in solution A then the procedure previously followed for the non-filled film was slavishly followed. The amounts of alginic acid sodium salt, EALYS and CMC used for the preparation of each sample are reported in the table 14.12.

Sample	Weight of alginic acid	Weight of EALYS	Weight of CMC
AF_EALYS	0.1263 g	0.1244 g	0.7604 g
AF_EALYS_noCMC	1.0085 g	0.1248 g	0 g
AF_EALYS_CMC	1.0041 g	0.1246 g	0.7495 g

Table 14.12 – Alginic acid sodium salt, EALYS and CMC weights used for the production of alginate film within EALYS

14.5.5Rheology

Rheological analyses of the samples reported in table 12 were registered after step 4 of the production procedure, before the drying process. The amounts of alginic acid sodium salt, EALYS and CMC used for the preparation of film are provided in the table 4.13.

Sample	Weight of alginic acid	Weight of EALYS	Weight of CMC
WAF	0.1320 g	0 g	0.7642 g
AF_EALYS	0.1263 g	0.1244 g	0.7604 g

Table 14.13 - Alginic acid sodium salt, EALYS and CMC weights for production of alginate films submitted to rheological study

14.6 Release studies

Pectin or alginate (10mg < m(SAMPLES) < 30mg) within EALYS was weighed in a 15 mL flat bottom vial and dispersed in Phosphate Buffered Saline (PBS) at pH 7.4 (10 ml). The release assay was developed in order to study the release in the time range of 24h sampling at 15, 30, 45, 60, 120 minutes and after 24h. Each sample was prepared in triplicate, therefore 18 vials were prepared, using the previously described procedure. The vials were placed in a horizontal water shacking bath (Dubnoff), showed in figure 1, set at 36°C with a shacking ratio of 90 strokes per minute.

Figure 14.2 - Dubnoff horizontal water shacking bath

At fixed time three samples were removed from bath, they were hand-shaken in order to obtain a homogeneous solution and then filtered using a syringe equipped with a 0.45 μm Teflon® filter. This procedure was repeated on the other samples after 30, 60, 120 minutes and 24h after the starting of the test.

The filtered solutions were analyzed by using a UV-Vis spectrophotometer in order to evaluate the amount of EALYS present in them.

14.6.1 Blank tests

The solubility of EALYS was previously verified in water solution; on the other hand, the release assays were performed in PBS solution therefore a solubility check is required (the ionic strength solution changing can affect the solubility of EALYS). The same amount of EALYS filled in pectin and alginate samples was used for the solubility test; therefore, EALYS was dissolved in 50 ml of PBS in order to prepare two solutions:

2 mg/ml (theoretical concentration obtainable if the 10% sample is able to completely release EALYS)

0.04 mg/ml (theoretical concentration obtainable if the 2% sample is able to completely release EALSY)

The samples were shaken at 36 °C for one hour, filtered using a syringe equipped with a 0.45 μm Teflon® filter and then analyzed via UV-Vis spectrophotometer.

14.7 Ellagic acid reaction with methacryloyl chloride

14.7.1 Reaction scheme:

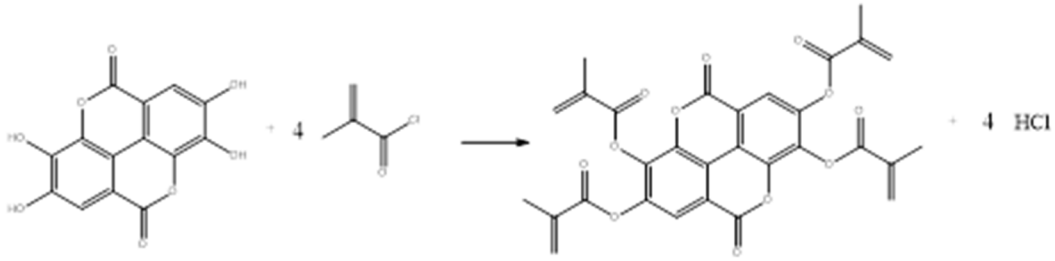


Figure 14.3 - Synthesis of EAMAC

14.7.2 Procedure

Ellagic acid (500,00mg, 1.6545mmol) was dispersed in 100 ml CH_2Cl_2 in a round 2 necks bottom flask under nitrogen atmosphere at room temperature. Triethylamine (669.70mg, 6,6181mmol) was added in the feed in order to neutralize the hydrochloric acid produced from the reaction between methacryloyl chloride and EA (the presence of the amine led to obtain a partial solubilization of EA into CH_2Cl_2 where it is not) then the methacryloil chloride (6918.00 mg, 66.1819mmol) was added dropwise. After the adding was completed (30min) the reaction was stirred for another 3h and the reaction proceeding was checked with TLC (reported in figure 3) using EA as reference (eluent n-hexane/ethyl acetate 1: 1). The reaction mixture was cooled a 0°C and first washed with a cold solution at 5% of hydrochloric acid, then with a solution at 10% of $NaHCO_3$ and finally with water, dried with anhydrous Na_2SO_4 and then the solvent was removed under vacuum at room temperature. The product obtained was a pale yellow powder ^1H NMR (400 MHz, $CDCl_3$) δ 8.15 (1H, s, Hd), 6.45 (1H, s, Ha or Ha'), 6.37 (1H, s, Ha' or Ha), 5.91 (1H, s, Hb or Hb'), 5.85 (1H, s, Hb' or Hb), 2.10 (3H, s, Hc or Hc'), 2.06 (3H, s, Hc' or Hc). EAMAC. ESI-Q-Tof MS= 587.48 m/z (EAMAC + Na^+).

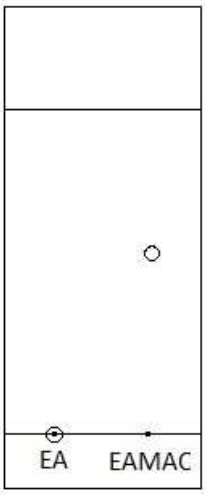


Figure 14.5 - TLC of EAMAC (hexane: EtOAc, 1:1), Rf=0.56

14.7.3 NMR

^{1}H-NMR spectra was collected solubilizing about 10 mg of product in 1 ml of deuterated chloroform.

14.7.4 Mass spectrum

Mass spectrum was recorded using AcCN as solvent.

14.7.5 Differential Scanning Calorimetry

Sample was prepared weighting about 6 mg of EAMAC and analysed using a dynamic scanning program from 25°C to 400°C at 10°C/min in order to assess the melting temperature and the thermal stability.

14.8 Poly(NVP-co-EAMAC)

The procedure for bulk polymerization of PVP was designed based on a literature work (205).

EAMAC, NVP and VAZO-67 were weighed in a 15 ml flat bottom vial and magnetically stirred at room temperature for 30 minutes in order to obtain a homogeneous dispersion. In fact, EAMAC is slightly soluble in NVP at room temperature whereas the solubility rapidly increases when the temperature rise. The reaction was conducted under nitrogen atmosphere at 60°C for 8h leaving the reactor cooling at room temperature for 15h: a solid pale yellow glassy material was obtained. The unreacted NVP was removed under vacuum. The polymer was finally crushed and washed overnight in water (50 ml of water for 2.5 g of material) in order to remove unreacted monomer and linear fractions. Later, the mixture was filtered and dried under vacuum (0.1 mPa for 8h). The PVP

synthetized without EAMAC was not submitted to the washing procedure in water (PVP is soluble in water.

The amounts of NVP, EAMAC and VAZO-67 used for the preparation of each sample are provided in the table 14.14.

Sample	Weight of NVP	Weight of EAMAC	Weight of VAZO-67
PVP_blank	5.0090 g	0 mg	35.7 mg
PVP_EAMAC_0.5%	3.0044 g	77.7 mg	20.0 mg
PVP_EAMAC_1%	2.5711 g	128.6 mg	17.6 mg

Table 14.14 - NVP, EAMAC and VAZO-67 weights used for PVP samples synthesis

14.8.1 Release studies of EA

About 25 mg of poly(NVP-co-EAMAC) was weighted in a 3 ml flat bottom vial and 1 ml of D_2O was added. The mixture was magnetically stirred at room temperature for 30 minutes to obtain a homogeneous dispersion. 40 µl of NaOH solution in D_2O (30 mg/ml) was added using a micro syringe and the mixture was placed in an ultrasonic bath at 40°C until complete dissolution. The solution was transferred into a NMR tube and ^{1}H-NMR spectrum was recorded. The same analysis was performed also on PVP_blank sample in order to asses' differences between linear materials.

14.9 Poly(lactic acid) synthesis

14.9.1 Bulk polymerization

PLA was synthesized in bulk using a 250 ml three-neck glass flask: 50 grams of L-lactide was added in the feed together with tin octanoate (0.3% w/w) used as catalyst, and Tyrosol or Vanillyl Alcohol, that acted as initiator of lactide polymerization. The mixture was allowed to react under slow nitrogen flow at 180 °C for 1.5 h using mechanical stirring (50 rpm). At the end of the reaction, the polymer was left in the flask under nitrogen flow and cooled at room temperature; a white solid polymer was obtained. After the syntheses, all samples underwent Solid State Polymerization (SSP) at 150°C for 12 hours under vacuum (about 4 mbar); all the analyses were conducted on the samples obtained after SSP.

14.9.2 Solution Polymerization

PLA was synthetized in tetrahydrofuran solution using a 100ml two-neck round bottom flask: approximately 10g of L-lactide was solubilized in 50ml of anhydrous THF together with Vanillyl Alcohol, used as lactide ROP initiator, and tin octanoate (0.3% w/w) used as catalyst. The mixture was allowed to react at 67°C for 165h under magnetic stirring in nitrogen atmosphere. At the end of the reaction, the solution was allowed to cool at room temperature; the polymer was poured in cool methanol in order to remove the unreacted monomer and catalyst; a white powder was obtained. The polymer was dried under vacuum (room temperature; 15h; 4 mBar) (156).

14.9.3 Thermal Analysis

Differential Scanning Calorimetry (DSC) analyses were performed using a Mettler Toledo DSC1 instrument, with 40µl Aluminum pan and under nitrogen atmosphere. An empty pan was used as reference. Samples were prepared weighting about 6 mg of Tyr and VA sample and analyzed using a dynamic scanning program from 25°C to 200°C at 10°C/min in order to assess their melting temperature and their thermal stability.

Polymers samples were prepared weighting about 6mg of polymers and they were analyzed via DSC in order to obtain information about their thermal behavior and to assess crystalline content. PLA Ingeo 4043D, poly(lactic acid) with 0,1%$_{mol/mol}$ of tyrosol (PLA+0,1%Tyr) and poly(lactic acid) with 0,1%$_{mol/mol}$ of vanillyl alcohol (PLA+0,1%VA) were analyzed under nitrogen flow using a Mettler Toledo DSC 1 instrument. The samples were first heated from 25°C to 200°C and left at 200°C at 10°C/min, left for 5 minutes, then cooled from 200°C to 25°C at -10°C/min, left for 2 minutes and then re-heated with a second thermal cycle from 25°C to 200°C at 10°C/min.

The crystalline weight fraction (Xc) of the sample was determined as previously described according to eq n(1):

$$1) \quad X_c = \frac{\Delta H_m - \Delta H_{cc}}{\Delta H_0} \times 100$$

where ΔH_m is the heat of fusion measured on the second heating, ΔH_{cc} is the cold crystallization heat and ΔH_o m is the melting enthalpy of the 100% crystalline polymeric matrix (93 J g-1) (165).

Thermo gravimetrical analysis (TGA) were performed with a TGA 4000 under nitrogen flux at 20ml/min with a temperature ramp from 30°C to 600°C at 20°C/min on 6mg of samples. Isothermal analyses were performed at 80°C for 120min under nitrogen flux of 20ml/min.

14.9.4 Gel Permeation Chromatography

Gel permeation chromatography (GPC) was performed using a size exclusion chromatography (SEC) system based on a Waters 1515 Isocratic HPLC pump and a four Phenomenex Phenogel column set (10^3Å-10^4Å-10^5Å-500Å) using a flow rate of 1 mL/min and 20 μL as injection volume. The detector was a Waters 2487 Dual λ Absorbance Detector, set at 230 nm. Samples were prepared dissolving 30 mg of polymer in 1 mL of anhydrous CH_2Cl_2; before the analysis, the solution was filtered with 0.45 μm filters. Given the relatively high loading, a check was performed using lower concentrations of polymer (5 mg/mL), in order to verify that no column overloading could be observed. Anyway, higher loadings were preferred as the UV absorption of PLA is relatively weak. O-dichlorobenzene was used as internal standard (peak appears at 46 minutes in the chromatograms). Molecular weight data were obtained using a linear polystyrene standard calibration in the range (1600000 – 106) Da.

14.9.5 Film Casting

Films were obtained via casting solubilizing 10g of polymer in 50g of CH_2Cl_2. The solution was cast on a glass surface and the solvent was evaporated at room temperature and pressure overnight. The absence of residual solvent in the films was checked via isothermal TGA (120min at 80°C under nitrogen flow). Film thickness was measured using a Digimatic micrometer (Mitutoyo, Japan). The value of film thickness was obtained by averaging 10 measurements, the average value obtained being 74 ± 18mm.

14.9.6 Determination of antioxidant capacity of films in vitro

The radical scavenging capacity of commercial PLA films was measured as an indicator of the antioxidant capacity of films. The radical scavenging capacity was evaluated through the reaction with the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). 0.05 g of film were cut and placed in a tube with 2 ml of 0.21 mM solution of DPPH in methanol. VA modified PLA, Tyr modified PLA and standard PLA were stored at room temperature for 15 days after the solvent casting deposition. The tubes were vigorously vortexed for 1 min to assure full contact between the film and the solution. After vortexing, the tubes were left in the dark for 30 min at room temperature. The absorbance was then measured against methanol at 515 nm in 1 ml cuvettes using a spectrophotometer (UV-1700 Pharma Spec, Shimadzu, Milton Keynes). As the DPPH is reduced by the antioxidants present in the sample, the solution colour fades in a way that is proportional to the antioxidant concentration (206).

According to Trombino et al.(2012) the radical scavenging capacity was expressed as:

Percentage of inhibition = (1- A_{PLA}/A_{blanc}) x 100

Where A_{PLA} is the absorbance of the solution in the presence of PLA films (Ingeo 4043D, PLA+0.1%VAand PLA+0,1%Tyr), and A_{blanc} is the absorbance of the DPPH solution.

The radical scavenging capacity of the films was analysed during shelf life of salami. At each sampling time the interleavers were removed from the package, cleaned with n-hexane in order to remove any food residue and analyzed.

14.9.7 1H-NMR of PLAs

^{1}H-NMR spectra was collected solubilizing about 10 mg of product in 1 ml of deuterated chloroform.

^{1}H-NMR (400 MHz, $CDCl_3$) ppm 1H NMR (400 MHz, CDCl3) δ ppm: 6.90 - 6.82 (m; 3H); 5.18 (q; J = 5.16; 1H); 4.37 (q; J= 4.36; 1H); 3.89 (s, 3H); 1.59 (d; J=1.58; 3H).

14.10 Stability Test

14.10.1 Sample preparation

Salami (79% pork lean, 21% subcutaneous back fat, and additives, in g/kg of batter: salt, 29; sodium nitrite, 0.15; dextrose, 10; lactose, 20; black pepper, 3.0; sodium erythorbate, 0.5; carmine, 0.05; sodium caseinate, 10; soy protein, 12; decalcified water, 63, and starter culture consisting of lyophilized Lactobacillus sakei at levels of 107 CFU/g) was sliced to 1 mm thick samples in a white chamber refrigerated at 1ºC. Samples were vacuum packed in PA/PE bags (Sacoliva, Spain) and stored at 4 ± 2ºC in a display cabinet. The samples were subjected to light (fluorescent lamp) 12h and 12h to darkness (simulating retail conditions). Two batches were obtained: a control batch (C) without interleave and an active batch packed with 8x8 cm PLA films containing 0.1%$_{mol/mol}$ VA as interleaves to separate salami slices (A).

Three samples of each batch were removed at 0, 7, 14, 43, and 51 days of storage for analysis. The upper slice was used for the color measurement, and after color measurement all the slices were minced for further analysis.

14.10.2 Water activity and pH analysis

The pH of the minced samples was measured directly with a Crison penetration 52-32 probe connected to a Crison Basic 20 pH-meter (Crison Instruments S.A., Alella, Spain). The mean of three measurements was recorded for each sample. The water activity of the minced samples was

measured using a water activity meter AquaLab™ Series 3 (Decagon Devices, Inc., Pullman, WA, USA).

14.10.3 Color measurement

Instrumental color measurement of films was performed using a Konic Chroma Meter CR-410 (Minolta, Osaka, Japan). C illuminant and 28 standard observers were chosen. L* (lightness), a* (redness, greenness), and b* (yellowness, blueness) color values were determined in the 1976 CIELAB system. The chromameter was calibrated before each series of measurements using a white ceramic plate. The mean of 6 measurements was recorded for each film. Three different points from each film type were tested.

14.10.4 Lipid oxidation analysis

Thiobarbituric acid reactive substances (TBARS) was determined following an adaptation of the method based on Buege and Aust (207). Two grams of minced salami were homogenized with 20 ml of 1.2 M HCl solution containing 0.1% (w/v) propyl gallate and 0.1%, w/v EDTA for 30s using an ULTRA-TURRAX® blender. The homogenate was centrifuged at 5,000 rpm for 10 min. The supernatant was injected in a continuous flow analyser Futura System (Alliance Instruments, Frepillon, France). A solution of 1.2 M HCl containing 0.327% thiobarbituric acid and 0.5 % Brij-35 was also injected in the system. The system consists of a bath at 90oC were the reaction is accelerated and a colorimeter set at 531 nm to detect the reaction product malondialdehyde (MDA). The calibration curve was prepared using 1,1,3,3-tetraethoxypropane (Sigma-Aldrich, Madrid, Spain) as a standard. The results were expressed as mg MDA/kg salami.

14.10.5 Statistical analysis

Statistical analysis was performed using the General Linear Model from SAS 9.2 software (Statistical Analytical Systems Institute, Cary, NC, USA). The batch (control and PLAc), the storage time (0, 7, 14, 43, 51 days) and their interaction were included in the model as fixed effects. Differences between effects were assessed by the Tukey test ($p < 0.05$).

14.11 Preparation of PLA scaffolds

All scaffolds were prepared using PLA after purification. PLA was dissolved in dichloromethane and then re-precipitated in methanol to remove processing additives e.g. antioxidants.

Scaffolds were prepared according to two different methods:

- **Method A.** PLA (0.1 mg ml^{-1}) and OligoTyr (5%$_{w/w}$ on PLA) were dissolved in THF in a 100 mL glass flask. Methanol was added at room temperature under mechanical stirring up to 95/5 v/v THF/methanol ratio. The solution was frozen by immersion of the flask into liquid nitrogen and was then poured in warm water. The solid PLA scaffolds that separated were recovered after removal of the solvent by filltration.
- **Method B**. PLA (0.1 mg ml^{-1}) and tyrosol or OligoTyr (5%$_{w/w}$ on PLA) were dispersed in 1,4-dioxane in a 100 mL glass flask at room temperature and taken under mechanical stirring overnight. The resulting homogeneous dispersion was frozen by immersion of the flask into liquid nitrogen and the solvent was removed by sublimation at room temperature under vacuum (2.5 h; 10^{-3} bar). In other experiments PLA scaffolds containing b-tricalcium phosphate (β-TCP) (5% $_{w/w}$ on PLA, porosimetry d50 ¼ 100 nm) with or without OligoTyr were prepared.

14.12 Porosity and swelling measurements

The porosity of PLA scaffold prepared according to method B was determined using two different gravimetric methods. In the first one, related porosity was calculated by equation (1) using the density of the dry scaffold (ρ_s) and the density of raw PLA (ρ_{PLA}), determined as the ratio between the dry mass and the volume of the scaffolds:

1) $\text{Porosity}(\%) = [1-(\rho_s/\rho_{PLA})] \times 100$

The dry weight of the scaffolds was determined using a high precision balance CPA225D Sartorius, the volume was evaluated by geometrical calculation using a caliper. In the second method the porosity was evaluated using equation 2:

2) $\text{Porosity}(\%)=\{(m_w-m_D)/[(m_D/\rho_{PLA}) + m_w]\} \times 100$

where m_w is the wet weight of the scaffold (contact time between scaffold and water: 96 h) and m_D is the dry mass. In this case, the porosity was evaluated as the total amount of water absorbed by the scaffold. Swelling of the wet sample was also evaluated using equation 3

3) $\text{Swelling}(\%)=[(m_w-m_D)/m_D] \times 100$

References

(1) D'Orazio, J.; Jarrett, S.; Amaro-Ortiz, A.; Scott, T. International Journal of Molecular Sciences2013, 14, 12222-12248.

(2) Alamed, J.; Chaiyasit, W.; McClements, D.; Decker, E. Journal of Agricultural and Food Chemistry2009, 57, 2969-2976.

(3) Valko, M.; Jomova, K.; Rhodes, C.; Kuča, K.; Musílek, K. Archives of Toxicology 2015, 90, 1-37.

(4) Kadiiska, M.; Mason, R.; Dreher, K.; Costa, D.; Ghio, A. Chemical Research in Toxicology 1997,10, 1104-1108.

(5) Foldes, E.Lohmeijer, J. Journal of Applied Polymer Science 1997, 65, 761-775.

(6) Pfaendner, R. Polymer Degradation and Stability 2013, 98, 2430-2435.

(7) Oral, E.; Neils, A.; Yabannavar, P.; Muratoglu, O. Journal of Orthopaedic Research 2014, 32, 757-761.

(8) Marwick, J.; Ito, K.; Adcock, I.; Kirkham, P. Expert Opinion on Therapeutic Targets 2007, 11, 745-755.

(9) Bart, J. Polymer additive analytics; 1st ed.; Firenze University Press: Firenze, 2006.

(10) Scheirs, J. Compositional and failure analysis of polymers; 1st ed.; Wiley: Chichester, 2000.

(11) Singh, R.; Tomer, N.; Bhadraiah, S. Polymer Degradation and Stability 2001, 73, 443-446.

(12) Crowley, M.; Zhang, F.; Koleng, J.; McGinity, J. Biomaterials 2002, 23, 4241-4248.

(13) Rex, I.; Graham, B.; Thompson, M. Polymer Degradation and Stability 2005, 90, 136-146.

(14) Fang, Y.; Sadeghi, F.; Fleuret, G.; Carreau, P. The Canadian Journal of Chemical Engineering2008, 86, 6-14.

(15) Dellarosa, N.; Laghi, L.; Martinsdóttir, E.; Jónsdóttir, R.; Sveinsdóttir, K. LWT - Food Science and Technology 2015, 62, 746-752.

(16) Evans, G.; de Challemaison, B.; Cox, D. Appetite 2010, 54, 557-563.

(17) Scott, K.; McGlasson, W.; Roberts, E. Australian Journal of Experimental Agriculture 1970, 10, 237.

(18) Harman, D. Journal of Gerontology 1956, 11, 298-300.

(19) Harman, D. Biogerontology 2009, 10, 773-781.

(20) Ishikawa, K.; Takenaga, K.; Akimoto, M.; Koshikawa, N.; Yamaguchi, A.; Imanishi, H.; Nakada, K.; Honma, Y.; Hayashi, J. Science 2008, 320, 661-664.

(21) Mishra, A.; Kumar, S.; Pandey, A. The Scientific World Journal 2013, 2013, 1-8.

(22) Halliwell, B.Gutteridge, J. Free radicals in biology and medicine; 1st ed.; Clarendon Press: Oxford, 1985.

(23) Send Press Releases with GlobeNewswire http://globenewswire.com/

(24) Gram, L.; Ravn, L.; Rasch, M.; Bruhn, J.; Christensen, A.; Givskov, M. International Journal of Food Microbiology 2002, 78, 79-97.

(25) Pokorny, J.; Réblova, Z.; Nguyen Thi Thu Huong,; Korczak, J.; Janitz, W. Food Chemistry 1996,57, 59.

(26) Dainelli, D.; Gontard, N.; Spyropoulos, D.; Zondervan-van den Beuken, E.; Tobback, P. Trends in Food Science & Technology 2008, 19, S103-S112.

(27) US96871 A 1869

(28) Vermeiren, L.; Devlieghere, F.; van Beest, M.; de Kruijf, N.; Debevere, J. Trends in Food Science & Technology 1999, 10, 77-86.

(29) Malhotra, B.; Keshwani, A.; Kharkwal, H. Frontiers in Microbiology 2015, 6.

(30) Olejar, K.; Ray, S.; Kilmartin, P. Journal of the Science of Food and Agriculture 2015, 96, 2825-2831.

(31) Marcos, B.; Sárraga, C.; Castellari, M.; Kappen, F.; Schennink, G.; Arnau, J. Food Packaging and Shelf Life 2014, 1, 140-150.

(32) Licciardello, F.; Wittenauer, J.; Saengerlaub, S.; Reinelt, M.; Stramm, C. Food Packaging and Shelf Life 2015, 6, 1-6.

(33) Domenek, S.; Louaifi, A.; Guinault, A.; Baumberger, S. Journal of Polymers and the Environment2013, 21, 692-701.

(34) Chen, X.; Lee, D.; Zhu, X.; Yam, K. Journal of Agricultural and Food Chemistry 2012, 60, 3492-3497.

(35) Ramos, M.; Jiménez, A.; Peltzer, M.; Garrigós, M. Food Chemistry 2014, 162, 149-155.

(36) Arrieta, M.; Castro-López, M.; Rayón, E.; Barral-Losada, L.; López-Vilariño, J.; López, J.; González-Rodríguez, M. Journal of Agricultural and Food Chemistry 2014, 62, 10170-10180.

(37) Tullo, A. c&en. 2016, pp. 32-37.

(38) Jamshidian, M.; Tehrany, E.; Imran, M.; Jacquot, M.; Desobry, S. Comprehensive Reviews in Food Science and Food Safety 2010, 9, 552-571.

(39) Drieskens, M.; Peeters, R.; Mullens, J.; Franco, D.; Lemstra, P.; Hristova-Bogaerds, D. Journal of Polymer Science Part B: Polymer Physics 2009, 47, 2247-2258.

(40) Regulation No (EU) 1169/2011 on the provision of food information to consumers. Union, European. 2011

(41) Jovanovic, S.; Steenken, S.; Hara, Y.; Simic, M. Journal of the Chemical Society, Perkin Transactions 2 1996, 2497.

(42) Jørgensen, L.; Madsen, H.; Thomsen, M.; Dragsted, L.; Skibsted, L. Free Radical Research 1999,30, 207-220.

(43) Bensalah,; Gadri,; Cañizares, P.; Sáez, C.; Lobato, J.; Rodrigo, M. Environmental Science & Technology 2005, 39, 7234-7239.

(44) Enache, T.Oliveira-Brett, A. Journal of Electroanalytical Chemistry 2011, 655, 9-16.

(45) Antenucci, S.; Panzella, L.; Farina, H.; Ortenzi, M.; Caneva, E.; Martinotti, S.; Ranzato, E.; Burlando, B.; d'Ischia, M.; Napolitano, A.; Verotta, L. RSC Advance 2016, 6, 2993-3002.

(46) Miró-Casas, E.; Covas, M.; Fitó, M.; Farré-Albadalejo, M.; Marrugat, J.; de la Torre, R. European Journal of Clinical Nutrition 2003, 57, 186-190.

(47) Fragopoulou, E.; Nomikos, T.; Karantonis, H.; Apostolakis, C.; Pliakis, E.; Samiotaki, M.; Panayotou, G.; Antonopoulou, S. Journal of Agricultural and Food Chemistry 2007, 55, 80-89.

(48) Je, I.; Kim, D.; Kim, S.; Lee, S.; Lee, H.; Park, E.; Khang, D.; Kim, S. PLOS ONE 2015, 10, e0129829.

(49) Fabiani, R. Food & Funcion. 2016, 7, 4145-4159.

(50) Arias, L.; Delbem, A.; Fernandes, R.; Barbosa, D.; Monteiro, D. Journal of Applied Microbiology 2016, 120, 1240-1249.

(51) Behling, R.; Valange, S.; Chatel, G. Green Chemistry 2016, 18, 1839-1854.

(52) Priefert, H.; Rabenhorst, J.; Steinbüchel, A. Applied Microbiology and Biotechnology 2001, 56, 296-314.

(53) Reimer, K.Tiemann, F. Berichte der deutschen chemischen Gesellschaft 1876, 9, 1268-1278.

(54) Sheldon, R. Green Chemistry 2014, 16, 950-963.

(55) Behrens, M.Datye, A. Catalysis for the conversion of biomass and its derivatives; 1st ed.

(56) Furukawa, H.; Wieser, M.; Morita, H.; Nagasawa, T. Bioscience, Biotechnology, and Biochemistry 1999, 63, 1141-1142.

(57) Naimi-Jamal, M.; Mokhtari, J.; Dekamin, M.; Kaupp, G. European Journal of Organic Chemistry 2009, 2009, 3567-3572.

(58) Ko, J.; Kim, Y.; Ximenes, E.; Ladisch, M. Biotechnology and Bioengineering 2014, 112, 252-262.

(59) Lu, Z.; Hu, C.; Guo, J.; Li, J.; Cui, Y.; Jia, Y. Organic Letters 2010, 12, 480-483.

(60) Hernandez, E.; Bassett, A.; Sadler, J.; La Scala, J.; Stanzione, J. ACS Sustainable Chemistry & Engineering 2016, 4, 4328-4339.

(61) Kwon, B.; Kang, C.; Kim, J.; Yoo, D.; Cho, B.; Kang, P.; Lee, D. International Journal of Pharmaceutics 2016, 511, 1022-1032.

(62) Veličković, D.; Dimitrijević, A.; Bihelović, F.; Bezbradica, D.; Knežević-Jugović, Z.; Milosavić, N. Bioprocess and Biosystems Engineering 2012, 35, 1107-1115.

(63) More, S.; Kumar, H.; Kang, S.; Song, S.; Lee, K.; Choi, D. Evidence-Based Complementary and Alternative Medicine 2013, 2013, 1-15.

(64) Verotta, L.; Macchi, M.; Venkatasubramanian, P. Connecting Indian wisdom and western science; 1st ed.

(65) Landete, J. Food Research International 2011, 44, 1150-1160.

(66) Sepulveda, L.; Ascacio, A.; Rodriguez-Herrera, R.; Aguilera-Carbo, A.; Aguilar, C. ChemInform 2012, 43, no-no.

(67) Sarkar, S.; Siddiqui, A.; Mazumder, S.; De, R.; Saha, S.; Banerjee, C.; Iqbal, M.; Adhikari, S.; Alam, A.; Roy, S.; Bandyopadhyay, U. Journal of Agricultural and Food Chemistry 2015, 63, 4988-4998.

(68) Mandal, S.Stoner, G. Carcinogenesis 1990, 11, 55-61.

(69) Li, Z.; Guo, X.; Dawuti, G.; Aibai, S. Phytotherapy Research 2015, 29, 1019-1025.

(70) Wang, Y.; Qiu, Z.; Zhou, B.; Liu, C.; Ruan, J.; Yan, Q.; Liao, J.; Zhu, F. Toxicology in Vitro 2015,29, 1107-1115.

(71) Seeram, N.; Adams, L.; Henning, S.; Niu, Y.; Zhang, Y.; Nair, M.; Heber, D. The Journal of Nutritional Biochemistry 2005, 16, 360-367.

(72) Malik, A.; Afaq, F.; Sarfaraz, S.; Adhami, V.; Syed, D.; Mukhtar, H. The Journal of Urology 2006,175, 1171.

(73) Novamont S.p.A. - Chimica Vivente per la Qualità della Vita http://www.novamont.com

(74) Bioplastics & Biopolymers Market by Type, Application & by Geography - 2021 MarketsandMarket http://www.marketsandmarkets.com/Market-Reports/biopolymers-bioplastics-market-88795240.html

(75) Home http://www.european-bioplastics.org

(76) Qi, X.; Xiu, H.; Wei, Y.; Zhou, Y.; Guo, Y.; Huang, R.; Bai, H.; Fu, Q. Composites Science and Technology 2016, 139, 8-16.

(77) Othman, S.; Hassan, N.; Talib, R.; Kadir Basha, R.; Risyon, N. Journal of Polymer Engineering2016, 0.

(78) Ortenzi, M.; Basilissi, L.; Farina, H.; Di Silvestro, G.; Piergiovanni, L.; Mascheroni, E. European Polymer Journal 2015, 66, 478-491.

(79) Virtanen, S.; Wikström, L.; Immonen, K.; Anttila, U.; Retulainen, E. AIMS Materials Science2016, 3, 756-769.

(80) Halász, K.Csóka, L. Journal of Engineering 2013, 2013, 1-9.

(81) Nam, T. H., Ogihara, S., Kobayashi, S.Advanced Composite Materials 2012, 21, 103-122

(82) Datta, R. FEMS Microbiology Reviews 1995, 16, 221-231.

(83) Garlotta, D. Journal of Polymers and the Environment 2001, 9, 63-84.

(84) Auras, R.; Harte, B.; Selke, S.; Hernandez, R. Journal of Plastic Film and Sheeting 2003, 19, 123-135.

(85) Nam, J.; Sinha Ray, S.; Okamoto, M. Macromolecules 2003, 36, 7126-7131.

(86) Carothers, W.; Dorough, G.; Natta, F. Journal of the American Chemical Society 1932, 54, 761-772.

(87) Flory, P. Principles of polymer chemistry; 1st ed.; Cornell University Press: Ithaca, 1953.

(88) Carothers, W. Transactions of the Faraday Society 1936, 32, 39.

(89) Auras, R.; Harte, B.; Selke, S. Macromolecular Bioscience 2004, 4, 835-864.

(90) Proikakis, C.; Tarantili, P.; Andreopoulos, A. Journal of Elastomers and Plastics 2002, 34, 49-63.

(91) Stanford, M.Dove, American Chemmical Society Review 2010, 39, 486-494.

(92) Saegusa, T.; Fujii, H.; Kobayashi, S.; Ando, H.; Kawase, R. Macromolecules 1973, 6, 26-32.

(93) Zhong, Z.; Dijkstra, P.; Feijen, J. Angewandte Chemie International Edition 2002, 41, 4510-4513.

(94) Kricheldorf, H.; Berl, M.; Scharnagl, N. Macromolecules 1988, 21, 286-293.

(95) Nijenhuis, A.; Grijpma, D.; Pennings, A. Macromolecules 1992, 25, 6419-6424.

(96) Korhonen, H.; Helminen, A.; Seppälä, J. Polymer 2001, 42, 7541-7549.

(97) Zhao, Y.; Cai, Q.; Jiang, J.; Shuai, X.; Bei, J.; Chen, C.; Xi, F. Polymer 2002, 43, 5819-5825.

(88) Dong, C.; Qiu, K.; Gu, Z.; Feng, X. Macromolecules 2001, 34, 4691-4696.

(99) Kricheldorf, H.Serra, A. Polymer Bulletin 1985, 14.

(100) Shinno, K.; Miyamoto, M.; Kimura, Y.; Hirai, Y.; Yoshitome, H. Macromolecules 1997, 30, 6438-6444.

(102) Vouyiouka, S.; Theodoulou, P.; Symeonidou, A.; Papaspyrides, C.; Pfaendner, R. Polymer Degradation and Stability 2013, 98, 2473-2481.

(103) Gao, C.; Gao, J.; You, X.; Huo, S.; Li, X.; Zhang, Y.; Zhang, W. Journal of Biomedical Materials Research Part A 2005, 73A, 244-253.

(104) Yadav, P. Journal of Clinical and Diagnostic Research 2015.

(105) Maharana, T.; Mohanty, B.; Negi, Y. Progress in Polymer Science 2009, 34, 99-124.

(106) Leong, K.Langer, R. Advanced Drug Delivery Reviews 1988, 1, 199-233.

(107) Soares, J.; Moore, J.; Rajagopal, K. ASAIO Journal 2008, 54, 295-301.

(108) Iwata, T. Angewandte Chemie International Edition 2015, 54, 3210-3215.

(109) Castro-Aguirre, E.; Iñiguez-Franco, F.; Samsudin, H.; Fang, X.; Auras, R. Advanced Drug Delivery Reviews 2016, 107, 333-366.

(110) Kasirajan, S.Ngouajio, M. Agronomy for Sustainable Development 2012, 32, 501-529.

(111) Sriamornsak P., Silpakorn University International Journal 2003, 3.1-2, 206-228

(112) Liu, L.; Fishman, M.; Hicks, K. Cellulose 2006, 14, 15-24.

(113) Palmer G. H., Dixon D. G., Am. The American Journal of Clinical Nutrition1966, 18 437-442

(114) Sriamornsak, P. Expert Opinion on Drug Delivery 2011, 8, 1009-1023.

(115) Fraeye, I.; Duvetter, T.; Doungla, E.; Van Loey, A.; Hendrickx, M. Trends in Food Science & Technology 2010, 21, 219-228.

(116) Kastner, H.; Einhorn-Stoll, U.; Senge, B. Food Hydrocolloids 2012, 27, 42-49.

(117) Manrique, G.Lajolo, F. Postharvest Biology and Technology 2002, 25, 99-107.

(118) Lee, K.Mooney, D. Progress in Polymer Science 2012, 37, 106-126.

(119) Pawar, S.Edgar, K. Biomaterials 2012, 33, 3279-3305.

(120) Nandini, V.; Venkatesh, K.; Nair, K. Journal of Conservative Dentistry 2008, 11, 37.

(121) McHugh, D. Production and utilization of products from commercial seaweeds; 1st ed.; Food and Agriculture Organization of the United Nations: Rome, 1987.

(122) CODEX Alimentarius: Home http://www.fao.org/fao-who-codexalimentarius.

(123) Paul W., Sharma C. P. Trends in Biomaterials & Artificial Organs 2004, 18, 18-23

(124) Zhang, Y.; Wei, W.; Lv, P.; Wang, L.; Ma, G. European Journal of Pharmaceutics and Biopharmaceutics 2011, 77, 11-19.

(125) Reppe, W. Angewandte Chemie 1953, 65, 577-578.

(126) Haaf, F.; Sanner, A.; Straub, F. Polymer Journal 1985, 17, 143-152.

(127) Turner, D.Schwartz, A. Polymer 1985, 26, 757-762.

(128) Zabska, M.; Jaskiewicz, K.; Kiersnowski, A.; Szustakiewicz, K.; Rathgeber, S.; Piglowski, J.Radiation Physics and Chemistry 2011, 80, 1125-1128.

(129) Nalwa, H. Encyclopedia of nanoscience and nanotechnology; 1st ed.; American Scientific Publishers: Stevenson Ranch, Calif., 2004.

(130) Mark, H. Encyclopedia of polymer science and technology; 1st ed.; Wiley-Interscience: Hoboken, N.J., 2007.

(131)Polyvinylpyrrolidone (PVP) Market Industry Report, 2024 http://www.grandviewresearch.com/industry-analysis/polyvinylpyrrolidone-market

(132) Teodorescu, M.Bercea, M. Polymer-Plastics Technology and Engineering 2015, 54, 923-943.

(133) Kanagalingam, J.; Feliciano, R.; Hah, J.; Labib, H.; Le, T.; Lin, J. International Journal of Clinical Practice 2015, 69, 1247-1256.

(134) Tyler, B.; Gullotti, D.; Mangraviti, A.; Utsuki, T.; Brem, H. Advanced Drug Delivery Reviews2016, 107, 163-175.

(135) Gokhale, A., Pharmaceutical Technology 2014, 38,

(136) Combination Devices: Particle Sciences - Technical Brief: 2010: Volume 2. s.l. : Particle Sciences, 2010

(137) Tuncer Degim, I.Celebi, N. Current Pharmaceutical Design 2007, 13, 99-117.

(138) Kim, W.Kim, S. Pharmaceutical Research 2008, 26, 657-666.

(139) Seitz, H.; Rieder, W.; Irsen, S.; Leukers, B.; Tille, C. Journal of Biomedical Materials Research Part B: Applied Biomaterials 2005, 74B, 782-788.

(140) Della Rocca, J.; Liu, D.; Lin, W. Accounts of Chemical Research 2011, 44, 957-968.

(141) Arifin, D.; Lee, L.; Wang, C. Advanced Drug Delivery Reviews 2006, 58, 1274-1325.

(142) Lin, C.Metters, A. Advanced Drug Delivery Reviews 2006, 58, 1379-1408.

(143) Oupicky, D.; Bisht, H.; Manickam, D.; Zhou, Q. Expert Opinion on Drug Delivery 2005, 2, 653-665.

(144) Alarcón, C.; Pennadam, S.; Alexander, C. Chem. Soc. Rev. 2005, 34, 276-285.

(145) Wang, N.von Recum, H. Macromolecular Bioscience 2010, 11, 321-332.

(146) Wei, S.Mizaikoff, B. Journal of Separation Science 2007, 30, 1794-1805.

(147) Oral, E.Peppas, N. Journal of Biomedical Materials Research Part A 2006, 78A, 205-210.

(148) Alvarez-Lorenzo, C.; Hiratani, H.; Gómez-Amoza, J.; Martínez-Pacheco, R.; Souto, C.; Concheiro, A. Journal of Pharmaceutical Sciences 2002, 91, 2182-2192.

(149) Ball D.W., Hill J.W., Scott R.J. Introduction to Chemistry: General, Organic, and Biological. 2011. pp. 1009-1075.

(150) Vlatakis, G.; Andersson, L.; Müller, R.; Mosbach, K. Nature 1993, 361, 645-647.

(151) Hiratani, H. Biomaterials 2004, 25, 1105-1113.

(152) Di Maio, I.; Esposto, S.; Taticchi, A.; Selvaggini, R.; Veneziani, G.; Urbani, S.; Servili, M. Food Chemistry 2011, 125, 21-28.

(153) Korhonen, H.; Helminen, A.; Seppälä, J. Polymer 2001, 42, 7541-7549.

(154) Zhu, K.; Xiangzhou, L.; Shilin, Y. Journal of Applied Polymer Science 1990, 39, 1-9.

(155) WO2015/021254

(156) Basilissi, L., Synthesis of PLA homo and copolymers and their nanocomposites for advanced materials, 2011, Ph.D. Thesis.

(157) Lahtinen, M.; Heinonen, P.; Oivanen, M.; Karhunen, P.; Kruus, K.; Sipilä, J. Organic & Biomolecular Chemistry 2013, 11, 5454.

(158) Montilla, F.; Morallón, E.; Vázquez, J. Journal of The Electrochemical Society 2005, 152, B421.

(159) Dorgan, J.; Williams, J.; Lewis, D. Journal of Rheology 1999, 43, 1141.

(160) Kowalski, A.; Duda, A.; Penczek, S. Macromolecular Rapid Communications 1998, 19, 567-572.

(161) Sahoo, S.; Liu, W.; Samuelson, L.; Kumar, J.; Cholli, A. Macromolecules 2002, 35, 9990-9998.

(162) Zaragoza-Gasca, P.; Villamizar-Gálvez, O.; García-Arrazola, R.; Gimeno, M.; Bárzana, E. Polymers for Advanced Technologies 2009, n/a-n/a.

(163) Oguchi, T.; Tawaki, S.; Uyama, H.; Kobayashi, S. Bulletin of the Chemical Society of Japan 2000,73, 1389-1396.

(164) Zhang, L.; Zhao, W.; Ma, Z.; Nie, G.; Cui, Y. European Polymer Journal 2012, 48, 580-585.

(165) Basilissi, L.; Di Silvestro, G.; Farina, H.; Ortenzi, M. Journal of Applied Polymer Science 2012,128, 3057-3063.

(166) Gibson, A.Roberts, T. International Journal of Food Microbiology 1986, 3, 183-194.

(167) Christian JH., Scott WJ., Aust J International Journal of Biological Sciences 1953, 6, 549-564

(168) Scott WJ., Aust J International Journal of Biological Sciences 1953, 6, 549-564

(169) Valík, L.; Baranyi, J.; Görner, F. International Journal of Food Microbiology 1999, 47, 141-146.

(170) Sperber, W. Journal of Food Protection 1983, 46, 142-150.

(171) Fernández, J.; Pérez-Álvarez, J.; Fernández-López, Journal of Food Chemistry 1997, 59, 345-353.

(172) Clericuzio, M.; Burlando, B.; Gandini, G.; Tinello, S.; Ranzato, E.; Martinotti, S.; Cornara, L. Journal of Natural Medicines 2013, 68, 31-37.

(173) Martinotti, S.; Mazzucco, L.; Balbo, V.; Patrone, M.; Mozzati, M.; Ranzato, E.; Burlando, B. Current Pharmaceutical Biotechnology 2014, 15, 120-126.

(174) Rezwan, K.; Chen, Q.; Blaker, J.; Boccaccini, A. Biomaterials 2006, 27, 3413-3431.

(175) Canha-Gouveia , Rita Costa-Pinto A, Martins E, Silva NA, Faria S, Sousa RA, Salgado AJ, Sousa N, Reis RL, Neves NM. 3, 2015.

(176) Liao, S.; Cui, F.; Zhang, W.; Feng, Q. Journal of Biomedical Materials Research 2004, 69B, 158-165.

(177) Martins, A.; Chung, S.; Pedro, A.; Sousa, R.; Marques, A.; Reis, R.; Neves, N. Journal of Tissue Engineering and Regenerative Medicine 2009, 3, 37-42.

(178) Xu, M.; Li, H.; Zhai, D.; Chang, J.; Chen, S.; Wu, C. Journal of Materials Chemistry

B 2015, 3, 3799-3809.

(179) Lebedev, B.; Rabinovich, I.; Lebedev, N.; Sladkov, A.; Vasneva, N. Polymer Science U.S.S.R.1978, 20, 382-386.

(180) Jacobs, C.Parks, G. Journal of the American Chemical Society 1934, 56, 1513-1517.

(181) Liao, S.; Cui, F.; Zhang, W.; Feng, Q. Journal of Biomedical Materials Research 2004, 69B, 158-165.

(182) Song, K.; Ji, L.; Zhang, J.; Wang, H.; Jiao, Z.; Mayasari, L.; Fu, X.; Liu, T. Nanomaterials 2015, 5, 415-424.

(183) George, P.; Quinn, K.; Cooper-White, J. Biomaterials 2010, 31, 641-647.

(185) Yeo, M.; Jung, W.; Kim, G. Journal of Materials Chemistry 2012, 22, 3568.

(186) Yeo, M.; Lee, H.; Kim, G. Biomacromolecules 2011, 12, 502-510.

(187) Weisburger, J. Food and Chemical Toxicology 1999, 37, 943-948.

(188) Bala, I.; Bhardwaj, V.; Hariharan, S.; Kumar, M. Journal of Pharmaceutical and Biomedical Analysis 2006, 40, 206-210.

(189) Zivica, V.Bajza, A. Construction and Building Materials 2002, 16, 215-222.

(190) Kobakhidze, K.Alaniya, M. Chemistry of Natural Compounds 2004, 40, 89-90.

(191) Bagri, P.; Ali, M.; Sultana, S.; Aeri, V. Chemistry of Natural Compounds 2010, 46, 201-204.

(192) Simić, A.; Verbić, T.; Sentić, M.; Vojić, M.; Juranić, I.; Manojlović, D. Monatshefte für Chemie - Chemical Monthly 2012, 144, 121-128.

(193) Morris, G.; Castile, J.; Smith, A.; Adams, G.; Harding, S. Polymer Degradation and Stability 2010,95, 2670-2673.

(194) US 4241186

(195) Losso, J.; Bansode, R.; Trappeyii, A.; Bawadi, H.; Truax, R. The Journal of Nutritional Biochemistry 2004, 15, 672-678.

(196) Sungthongjeen, S.; Sriamornsak, P.; Pitaksuteepong, T.; Somsiri, A.; Puttipipatkhachorn, S. AAPS PharmSciTech 2004, 5, 50-57.

(197) Swelling measurements of Crosslinked Polymers. Group, Cambridge Polymer

(198) Bajpai, S.Sharma, S. Reactive and Functional Polymers 2004, 59, 129-140.

(199) Blandino, A.; Macías, M.; Cantero, D. Journal of Bioscience and Bioengineering 1999, 88, 686-689.

(200) Ahmad, Z.Khuller, G. Expert Opinion on Drug Delivery 2008, 5, 1323-1334.

(201) Ren, Y.; Wei, M.; Still, P.; Yuan, S.; Deng, Y.; Chen, X.; Himmeldirk, K.; Kinghorn, A.; Yu, J. ACS Medicinal Chemistry Letters 2012, 3, 631-636.

(202) Fu, J.Rao, M. Food Hydrocolloids 2001, 15, 93-100.

(203) Tibbits, C.; MacDougall, A.; Ring, S. Carbohydrate Research 1998, 310, 101-107.

(204) Nigam, S.; Tsao, I.; Sakoda, A.; Wang, H. Biotechnology Techniques 1988, 2, 271-276.

(205) Yuan C.M., Trasferitori di catena polifunzionali nella polimerizzazione radicalizza, PhD thesis 1992, University of Milan

(206) Trombino, S.; Cassano, R.; Ferrarelli, T.; Leta, S.; Puoci, F.; Picci, N. Molecules 2012, 17, 12734-12745.

(207) Buege, J. A., & Aust. 1978, S. D, pp. 302-310

(208) WO 2015132087 A1

(209) Cicero, J.; Dorgan, J.; Dec, S.; Knauss, D. Polymer Degradation and Stability 2002, 78, 95-105.

(210) Food and Agriculture Organization of the United Nations http://www.fao.org