Master in Chemical Engineering

New insights in microencapsulation of antioxidants - preparation and characterisation of propolis-loaded microparticles

A Master's dissertation

of

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“I can’t change the direction of the wind, but I can adjust my sails to always reach my destination.”

Jimmy Dean

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Abstract

Dietary antioxidants are essential components that help prevent health issues, such as cancer, diabetes and cardiovascular diseases, and guarantee the well-being of each individual. The human body utilizes these substances as a defence mechanism to neutralise the effects of reactive oxygen species. However, antioxidants are susceptible to oxidation, heat, light and pH, and can also present poor availability and some reactivity. Hence, microencapsulation comes as a possible solution to protect antioxidants and mask possible taste and odour. Propolis is a mixture with a high antioxidant power, derived from apiculture, with biological and pharmacological activities, which can act as antimicrobial, antiviral, anti-inflammatory and anticarcinogenic agent, among others.

The aim of this work was to prepare and characterise propolis microparticles, by double emulsion/solvent evaporation technique using ethyl cellulose as encapsulating material. The resulting microparticles were characterised according to product yield, size distribution, morphology, controlled release in simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), and total phenolic and antioxidant capacity. Four formulations were studied, altering only the propolis:polymer ratio, being the ratio of 1:6, 1:4, 1:2 and 3:4, for formulation 1, 2, 3 and 4, respectively. Two analytical methods for the quantification of phenolic and antioxidant content were validated. For the quantification of phenolic content, Gallic acid calibration curve was linear from 0 to 500 mg/L and, with a limit of detection of 29.8 mg/L and a limit of quantification of 99.4 mg/L. For the quantification of antioxidant content, Trolox calibration curve was linear from 0 to 20 nmole/well, with a limit of detection of 0.9 nmole/well and a limit of quantification of 3.1 nmole/well. The microencapsulation yield ranged from 83.9 ± 4.1% to 63.1 ± 4.3%, with the formulation 1 obtaining the highest yield and formulation 3 obtaining the lowest yield. The loading ranged from 17.0 ± 0.8% to 55.1 ± 4.1%, with the formulation 4 obtaining the highest loading and formulation 1 obtaining the lowest loading. The particle size distribution showed to be very similar for all formulations, ranging from 3.6 ± 0.1 μm to 5.4 ± 0.6 μm and the span value of all formulations verified a narrow distribution around the mean value. The photomicrographs showed that microparticles in all formulations had an increase in roundness and smoothness of the surface when the ratio propolis:polymer increased. The propolis sample had a concentration of phenolic and antioxidant content of 64.8 ± 1.8 mg of Gallic acid equivalent/g of Propolis and 759.0 ± 36.9 mg of Trolox equivalent/g of Propolis, respectively. This revealed an encapsulation efficiency of phenolic and antioxidant compounds around 92% and 99% for all formulations, respectively. The controlled release studies showed that, in SSF there was a substantial release of formulation 4. In SGF all formulations released approximately the same percentage of propolis. In SIF formulation 3 demonstrated a higher percentage release, with about 95% of phenolic content and 20% of antioxidant content, meaning that after 2 hours, 80% of the antioxidants are available for a sustained release.

Keywords: microparticles, microencapsulation, bioactive compounds, controlled release studies under digestion conditions, double emulsion, propolis, ethyl cellulose.

Resumo

Antioxidantes dietéticos são compostos essenciais que ajudam a prevenir problemas de saúde, como cancro, diabetes e doenças cardiovasculares, e garantem o bem-estar pessoal. O corpo humano utiliza estas substâncias como um mecanismo de defesa para neutralizar os efeitos de espécies reativas oxigénio. No entanto, os antioxidantes são suscetíveis à oxidação, calor, luz e pH, e também podem apresentar baixa disponibilidade e alguma reatividade. Assim, a microencapsulação surge como uma solução para proteger os antioxidantes e mascarar possíveis sabores e odores. O própolis é uma mistura com alto poder antioxidante, derivado da apicultura, com actividades biológicas e farmacológicas, que atua como agente antimicrobiano, antiviral, anti-inflamatório, anticarcinogênico, entre outros.

O objetivo deste trabalho foi preparar e caracterizar micropartículas de própolis, através da técnica de dupla emulsão/evaporação de solvente, utilizando etil celulose como material encapsulante. As microparticles foram caracterizadas de acordo com o rendimento, distribuição de tamanho, morfologia, libertação controlada em meio salivar simulado (SSF), meio gástrico simulado (SGF) e meio intestinal simulado (SIF), e capacidade fenólica e antioxidante total. Quatro formulações foram estudadas, alterando apenas o rácio própolis:polímero, sendo estes de 1:6, 1:4, 1:2 e 3:4, para as formulações 1, 2, 3 e 4, respetivamente. Dois métodos analíticos para a quantificação do teor fenólico e antioxidante foram validados. Para a quantificação do teor fenólico, a curva de calibração de ácido Gálico foi linear de 0 a 500 mg/L, com limite de deteção de 29,8 mg/L e limite de quantificação de 99,4 mg/L. Para a quantificação do teor antioxidante, a curva de calibração de Trolox foi linear de 0 a 20 nmole/poço, com limite de detecção de 0,9 nmole/poço e limite de quantificação de 3,1 nmole/poço. O rendimento da microencapsulação variou de 63,1 ± 4,3% a 83,9 ± 4,1%, com a formulação 1 a obter o maior valor e a formulação 3 o menor valor. A quantidade experimental de propolis encapsulado variou de 17,0 ± 0,8% a 55,1 ± 4,1%, com a formulação 4 a obter o maior valor e a formulação 1 a obter o menor valor. O tamanho de partícula foi semelhante para todas as formulações, variando de 3,6 ± 0,1 μm a 5,4 ± 0,6 μm e o valor de *span* de todas as formulações verificou uma distribuição estreita em torno do valor médio. As fotomicrografias mostraram que as micropartículas tiveram um aumento da esfericidade e suavidade da superfície quando o rácio própolis:polímero aumentava. O própolis apresentou um teor fenólico e antioxidante de 64,8 ± 1,8 mg de equivalentes de ácido Gálico/g de Própolis e 759,0 ± 36,9 mg de equivalentes de Trolox/g de Própolis, respetivamente. Revelando uma eficiência de encapsulação de compostos fenólicos e antioxidantes de cerca de 92% e 99% para todas as formulações, respetivamente. Os estudos de libertação controlada mostraram que, em SSF existiu uma libertação substancial da formulação 4. Em SGF todas as formulações libertaram aproximadamente a mesma percentagem de própolis. Em SIF a formulação 3 demonstrou ter a maior percentagem de libertação, com cerca de 95% de teor fenólico e 20% de teor antioxidantes, significando que após 2 horas, 80% dos antioxidantes estão disponíveis para uma libertação prolongada.

Palavras-chave: micropartículas, compostos bioativos, estudos de libertação controlada em condições digestivas, dupla emulsão, própolis, etil celulose.

Declaration

I hereby declare, on my word of honour, that this work is original and that all non-original contributions were properly referenced with source identification.

July 2, 2018

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(Pedro Manuel Alves de Castro Oliveira)

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Notation and Glossary

Abs	Absorbance	AU
C	Concentration	g of Propolis/L mg of GAE/g of Propolis mg of GAE/L mg of TE/g of Propolis nmole of TE/μL
D	Particle diameter	μm
M	Molar mass	mg/nmole
m	Mass	mg

List of Acronyms

a	Sensitivity
AU	Absorbance Units
b	Intercept of the regression
DCM	Dichloromethane
DNA	Deoxyribonucleic Acid
EC	Ethyl Cellulose
EE	Encapsulation Efficiency
EU	European Union
FDA	Food and Drug Administration
GAE	Gallic Acid Equivalent
GC-MS	Gas Chromatography-Mass Spectrometry
GRAS	Generally Recognized As Safe
HPLC	High Performance Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
MP	Microparticle(s)
N/D	Not Defined
O	Oil Phase
OSA	Octenyl Succinic Anhydride
PCL	Polycaprolactone
PEG	Polyethylene glycol
PGA	Polyglycolide
PLA	Polylactide
PLGA	Poly(lactide-co-glycolide)
PVA	Polyvinyl Alcohol
PVD	Physical Vapor Deposition
PY	Product Yield
R	Correlation Coeficient
RNA	Ribonucleic Acid
RSD	Relative Standard Deviation
sd	Standard Deviation
SEM	Scanning Electron Microscopy
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SSF	Simulated Salivary Fluid
TAC	Total Antioxidant Capacity
TE	Trolox Equivalent
UPW	Ultra Pure Water
UV	Ultra Violet
UV-Vis	Ultra Violet-Visible
W_1	Internal Aqueous Phase
W_2	External Aqueous Phase

1 Background motivation and project guidelines

1.1 Background motivation

Market trends nowadays, in both food and cosmetic industry, are looking for unique ingredients with health-beneficial properties (Hyde et al., 2010; Netzel et al., 2007). As such, the study of antioxidants is of great interest due to their health-related assets. However, synthetic antioxidants are labelled by the consumer as unnatural, unhealthy and a public health risk (Chen, 2017) due to the formation of harmful by-products (Kirschweng et al., 2017), and are currently being substituted by natural antioxidants (Lozano-Vazquez et al., 2015).

Antioxidants, such as phenolic compounds present in natural sources, are gathering more attention because of their potent activity against oxidative damage (Qiu et al., 2018). Thus, they are associated with the prevention of oxidative stress-related chronical diseases, such as cancer (Qiu et al., 2018), diabetes, melanin abnormalities, cardiovascular (e.g., angina and hypertension) and neurological disorders (e.g., Alzheimer and Parkinson's diseases) (Carocho et al., 2018).

Natural sources of antioxidants include fruits and vegetables (Chen et al., 2018) but can also be found in the form of a natural resinous mixture such as propolis (Busch et al., 2017) which is collected by honeybees from various plant sources and mixed with beeswax and salivary enzymes (Bruschi et al., 2017). Propolis has been the subject of study in several fields such as cosmetics, pharmaceuticals and food which has been used as a natural preservative and a source of bioactive compounds, although the inclusion of this extract alters organoleptic properties in the final product due to its intense characteristic aroma and flavour (Osés et al., 2016). However natural antioxidants are susceptible to oxidation, heat, light and pH and can also have an unpleasant taste or smell, presents poor availability and high reactivity which can limit their use in industry (Aguiar et al., 2017). Hence, microencapsulation comes as a possible solution.

Microencapsulating techniques allow the protection of these bioactive compounds from external environmental factors (Lozano-Vazquez et al., 2015) while ensuring high stability, controlled and targeted release (Aguiar et al., 2017). It also prevents deterioration and delayed evaporation of volatile compounds whereas at the same time masking undesirable properties such as taste and odour (Aguiar et al., 2017).

Hence, the use of microparticles of propolis shows a potential interest for research due to their antioxidant power and medicinal properties adequately protected.

1.2 Thesis objectives

The primary objective of the thesis is to microencapsulate propolis using ethyl cellulose as wall material by double emulsion solvent evaporation technique and characterise the microparticles. Four different formulations were prepared in which only the ratio propolis:polymer changed and, posteriorly, these microparticles were characterised in terms of encapsulation efficiency, product yield, size and morphology. Their controlled release was studied *in vitro* using salivary, gastric and intestinal simulated fluids in order to simulate the human body. Also, to quantify the controlled release of antioxidants and polyphenols from propolis, two analytical methods were validated (UV-Vis spectrophotometry). And finally, antioxidant and polyphenol activity were evaluated to verify if the microencapsulation process can influence the antioxidant and polyphenol capacity.

1.3 Organisation and structure

The present dissertation is divided in 7 chapters. The first one, *"Background motivation and project guidelines"*, provides some insight on the background of microencapsulation of antioxidants, main objective and structure of the dissertation. In the second chapter, "*Introduction*", a theoretical description of the concepts of antioxidants, microencapsulation techniques and controlled release is provided. The *"Context and State of the art"* chapter is a review of the processes that were found regarding propolis microencapsulation, with a focus on results, operating conditions and industry. The fourth chapter describes the materials and methods used in this project. The fifth chapter is a compilation of the main results acquired throughout this work with an emphasis on microparticles product yield, characterisation, encapsulating efficiency and morphology, validation of analytical methods, antioxidant and polyphenol activity and controlled release profiles in three different simulated fluids. Chapter 6 covers the main conclusion of this project and chapter 7 expresses some limitations and suggestion to future work. Finally, the bibliography used can be consulted in the references section and additional information that can shed some light on a certain subject can be accessed in the appendix section.

2 Introduction

2.1 Antioxidants

Society is increasingly more aware of the fact that having an equilibrated lifestyle can help prevent health issues and guarantee the well-being of each individual. Antioxidants can act as essential dietary component as they can avoid certain diseases such as cancer, cardiovascular diseases, diabetes and premature ageing (Yashin et al., 2012). However, only about 25 years ago began the search for others dietary components (e.g., fruits, berries, vegetable oils, cocoa, wine, honey, tea, juices and coffee) with the ability to act as antioxidants (Weichselbaum and Buttriss, 2010; Yashin et al., 2012).

The human body uses antioxidants as a defence mechanism to neutralise the effects of reactive oxygen species (Mulgund et al., 2015). These antioxidants can be divided into nonenzymatic (e.g., vitamin C, vitamin E, selenium, zinc, ß-carotene, carotene, taurine, hypotaurine and glutathione) and enzymatic (e.g., superoxide dismutase, catalase, glutaredoxin and glutathione reductase), with the latest being produced by the human body (Mulgund et al., 2015). The intake of dietary antioxidants is therefore vital for a proper function of the body and well-being (Verhein et al., 2014).

2.1.1 Important classes of antioxidants

Plant tissues are a natural source of different types of antioxidants. These antioxidants can be divided into some major classes such as vitamins, carotenoids, flavonoids, phenolic acids and phenolic alcohols (Cömert and Gökmen, 2018).

Vitamins like ascorbic acid (vitamin C) and α-tocopherol (vitamin E) act as antioxidants. Vitamin E is a group of plant lipids containing tocopherols and tocotrienols, being the α-tocopherol its most biologically active form (Ulatowski and Manor, 2015). It is responsible in neutralising unstable lipid peroxy-radicals produced from polyunsaturated acids (Ulatowski and Manor, 2015) and is also related with a reduced risk of coronary heart disease and colon cancer (Vrolijk et al., 2015). Ascorbic acid is a water-soluble compound important in the synthesis of catecholamines, carnitine and collagen (Pallauf et al., 2013), and can transform the oxidised tocopheroxyl radical in tocopherol (Ulatowski and Manor, 2015). The effect it provides on health is due to its biological function as a cofactor and preventing proteins and other compounds from oxidation (Pallauf et al., 2013).

Carotenoids are a class of isoprenoid pigments that are synthesised by photosynthetic organisms and non-synthetic bacteria and fungi (Saini and Keum, 2018). Animals need to consume these compounds in order to receive its benefits that can be expressed in significant roles such as ornamentation, protection against lung, head, neck and prostate cancer, modulation of the immune system, growth factors and intracellular signaling pathways, regulation of cell differentiation, cell cycle and apoptosis, protection against UV radiation, and as precursors of retinol (vitamin A) (Saini and Keum, 2018). This class of antioxidants can be divided into two groups; xanthophylls which contain oxygen as a functional group (e.g., astaxanthin, zeaxanthin and lutein); and carotenes which have a hydrocarbon chain with no functional groups (e.g., β-carotene and lycopene) (Saini and Keum, 2018).

Flavonoids are a large class of secondary polyphenolic fungi and plant metabolites (Raffa et al., 2017). These compounds exhibit high nutraceutical and therapeutic properties such as antioxidant, anti-cancer, anti-inflammatory, hormonal bioactivities, weight management, cardiovascular and neurodegenerative diseases protection (Bugel and Tanguay, 2018; Raffa et al., 2017). However, the consequences of exposure to high concentrations of flavonoids are still unclear to human health (Bugel and Tanguay, 2018). Some of its most known compounds are apigenin, quercetin, catechin and leucocyanidin for example (Raffa et al., 2017).

Phenolic compounds are phytonutrients present in plants and can be a parameter of quality, nutritional value, aroma and flavour of fruits (Elmastaş et al., 2017). These metabolites confer protection against environmental factors and physical damage (Elmastaş et al., 2017). Inside the polyphenols group, the phenolic acids show interest due to its potential protective roles in human nutrition and health (De Paula et al., 2017). Their biological activity allows them to act as antioxidants, anti-inflammatory, anti-carcinogenic, immune system modulation, cardiovascular diseases protection agent (De Paula et al., 2017; Walters et al., 2018). Phenolic acids are also classified as benzoic derivatives (e.g., gallic acid) and cinnamic derivatives (e.g., caffeic acid) (Ajiboye et al., 2018). Figure 1 represents que chemical structure of caffeic acid and gallic acid.

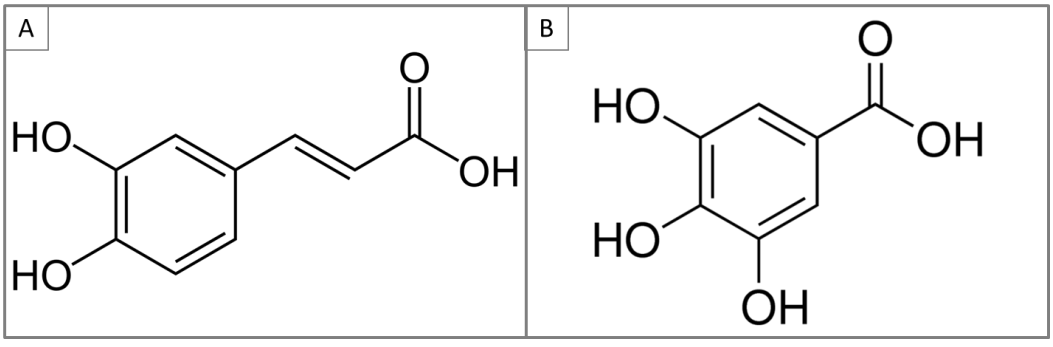

Figure 1 - Chemical structure of: A) caffeic acid and B) gallic acid

2.1.2 Mechanism of action

As the body grows old, the equilibrium between antioxidants and free radicals is disrupted, leading to increased oxidative stress (Mulgund et al., 2015). Antioxidants are stable molecules but when in the presence of free radicals these highly reactive compounds capture electrons from antioxidants in order to stabilise themselves, in a process that includes three steps; initiation, propagation and termination, which are represented by the mechanism in Figure 2 (Carocho et al., 2018).

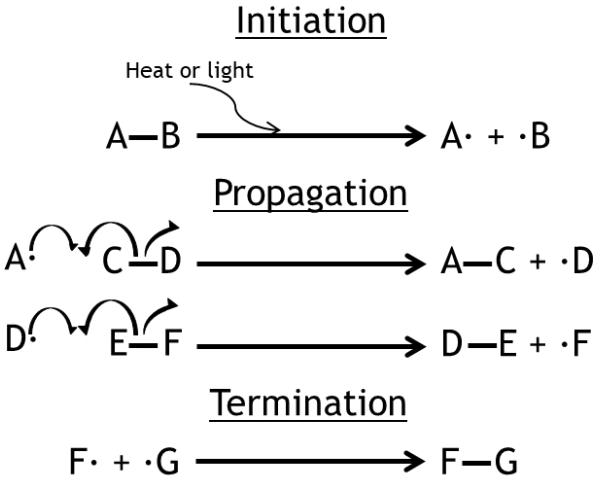

Figure 2 - Three steps of radical chain reaction: initiation, propagation and termination

There are seven mechanisms of action that antioxidants follow: sequestration of free radicals from the medium; chelation of metallic ions; inhibition of free radical producing enzymes; activation of endogenous antioxidant enzymes; prevention of lipid peroxidation; prevention of DNA damage; prevention of protein modification and sugar destruction (Carocho et al., 2018).

The radical sequestration comprises of antioxidants, such as ascorbic acid, tocopherols and polyphenols, that gives an electron or hydrogen atom from their hydroxyl group to stabilise the radical (Carocho et al., 2018).

In the chelation of metallic ions, Fe^{2+} and Cu^{+} react with H_2O_2, which is produced by the superoxide dismutase enzyme, leading to the formation of ·OH and Fe^{3+} or Cu^{2+} (Lü et al., 2010). When reducing agents, like ascorbic acid, are present, these oxidised metal ions are reduced, which in turn allows the reaction with another molecule of H_2O_2 and liberation of ·OH radical (Lü et al., 2010). Eventually, all H_2O_2 will be consumed, inhibiting the formation of more radicals through this molecule (Carocho et al., 2018).

Enzymatic reactions can also produce free radicals (Lobo et al., 2010). Such is the case of xanthine oxidase, for example, which catalyses the oxidation of hypoxanthine to xanthine, producing O_2^{-}, H_2O_2 and uric acid (endogenous antioxidant) (Carocho et al., 2018; Lobo et al., 2010). In this context, the neutralisation of the superoxide radical and hydrogen peroxide is

performed by superoxide dismutase and catalase, respectively, which are antioxidant enzymes (Carocho et al., 2018). Thus, superoxide radical yield molecular oxygen and hydrogen peroxide, and hydrogen peroxide yield oxygen and hydrogen (Carocho et al., 2018).

The prevention of lipid peroxidation can be carried out by antioxidants like vitamin E which in turn have a synergetic relationship with ascorbic acid allowing its regeneration (Ulatowski and Manor, 2015). Lipid peroxidation consists of the destruction of the unsaturated lipid's double bond by reactive oxygen species (Carocho et al., 2018).

Superoxide radical and nitric oxide can also damage DNA and RNA, which can, in turn, translate in neurodegenerative diseases and cancer (Carocho et al., 2018; Lobo et al., 2010). In the same context, proteins are also attacked not only by these radicals but also by chlorination and can be altered through processes of oxidative modification of amino acids, peptide cleavage and formation of protein cross-linkage (Carocho et al., 2018; Lobo et al., 2010).

Sugars are also targets of free radicals during the early stages of glycosylation, which can lead to the formation of mutagenic compounds like α and β-dicarbonyls (Carocho et al., 2018).

2.1.3 Propolis

Propolis is a natural resinous complex mixture collected by honey bees (*Apis mellifera* L.) from different parts of plants, buds and exudates (Busch et al., 2017) which is then mixed with pollen and enzymes secreted by these bees (Kalogeropoulos et al., 2009). This substance is used in beehives as a form of protection against predators and as a thermal isolator, as explicit by its name, of Greek origin, where *pro* means defence and *polis* means city (Bruschi et al., 2017). Ancient civilisations, Assyrian, Greek, Roman, Inca and Egyptian, described the uses of propolis for folk medicine (Bruschi et al., 2017) and nowadays is widely used in pharmaceutical applications (Mascheroni et al., 2014).

Regarding composition, raw propolis is a mixture of more than 300 substances from different chemical classes and is composed of 50% plant resins, 30% waxes, 10% essential and aromatic oils, 5% pollen and 5% other organic compounds (Huang et al., 2014). Phenolic compounds are mainly responsible for bestowing propolis with its biological and pharmacological activities (Escriche and Juan-Borrás, 2018) which include antioxidant, antimicrobial, anti-inflammatory, antiviral, anticarcinogenic, anticariogenic, anaesthetic and healing (Nori et al., 2011). It has also been proposed that flavonoids could be used as a quality indicator for propolis (Gardana et al., 2007).

Due to the flora present in different geographic locations, the chemical composition of propolis may change and, by extension, its properties (Catchpole et al., 2018). According to Bruschi et al. (2017) there are seven types of propolis that have its botanical origins from different species of plants characteristic to a specific location in the globe: Poplar (Europe, North America, New Zealand and nontropical regions of Asia), Green (Brazil), Betula (Russia, Poland and Hungary), Red (Cuba, Brazil and México), Mediterranean (Sicily, Greece, Crete and Malta), Clusia (Cuba and Venezuela) and Pacifica (Pacific region). The colouration of propolis is another aspect that is heavily influenced by phytogeographic characteristics such as vegetation, season of the year and environmental conditions (Bruschi et al., 2017), as shown in some examples in Figure 3.

Figure 3 - Examples of three types of propolis showing an emphasis in colouration differences. A) Red propolis, B) Green propolis and C) Poplar propolis

The use of propolis in food industry is known to increase the shelf life of products, but its intense and unpleasant flavour proves to be a challenge, which limits the application of this substance (Reis et al., 2017). Also, propolis is classified as having a sensitising action which can cause dermatitis reactions due to the presence of caffeic acid, eugenol, anethole, methyl isogenol and elemicina (Bruschi et al., 2017). The ethanolic extract may additionally cause an irritating effect on the mucosa (Bruschi et al., 2017).

Some studies have been done, comparing the total phenolic content of Portuguese propolis with Chinese, Korean, Brazilian and Thai propolis and results show that Portuguese propolis has one of the highest concentration of phenolic content (Moreira et al., 2008; Paula et al., 2017). Also, Falcão et al. (2013b) did a phenolic quantification of Portuguese propolis by high performance liquid chromatography (HPLC) and found that propolis from the north region of Portugal can have a concentration of Caffeic Acid, Ferulic Acid, Caffeic Acid Phenylethyl Ester, Pinocembrin and Chrysin as high as 14.9, 6.1, 22.7, 93 and 68 mg/g of extract, respectively.

Despite all the advantages propolis can provide, its unpleasant taste and odour makes propolis a suitable candidate for microencapsulation as this process can mask these unwanted characteristics.

2.2 Microencapsulation

Microencapsulation is a set of processes that can store an active ingredient or mixture of substances within a shell or coating which provides protection for a later release (Sobel et al., 2014). Small particles, liquid or gas can be encapsulated within a coating layer or matrix (Sobel et al., 2014).

Microparticles have a size ranging from 1 μm to 1000 μm and are composed by a core, where the active ingredient is, and a shell, which is made of natural or synthetic polymer (Martins et al., 2014). In terms of morphology, four possible structures are proposed as shown in Figure 4: reservoir, double shell, matrix and polynucleated (Martins et al., 2014).

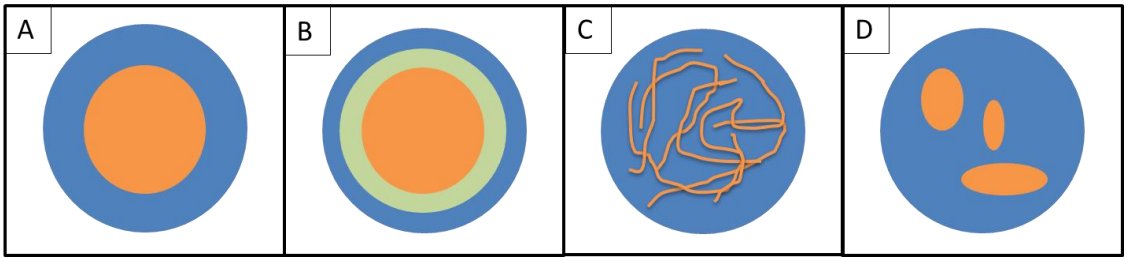

Figure 4 - Microcapsules morphologies: A) Reservoir type, B) Double Shell, C) Matrix, D) Polynucleated

Much like to the most critical functions of a living cell, such as protecting the interior material (core) and control the flow of materials across the membrane, the produced microparticles can be compared to these cells as they also protect the active substance and control their release in target specific sites (Calderón-Oliver et al., 2017; Fanger, 1974). Encapsulating the active substance can also provide other advantages besides protection from external factors such as water, oxygen, light and heat; it can also improve shelf-life and avoid interference with product performance by masking undesirable odour and flavour (Sobel et al., 2014).

In terms of industrial application, this technology is majority explored within the pharmaceutical field but also within the food, cosmetic, textile, biomedical, agricultural and electronic (Paulo and Santos, 2017).

For the purpose of this work, due to its natural properties, propolis was microencapsulated within a polymeric shell. Then, the microparticles produced were characterised and their controlled release was studied through means of simulated salivary, gastric and intestinal fluids. Figure 5 represents a schematic representation of this work.

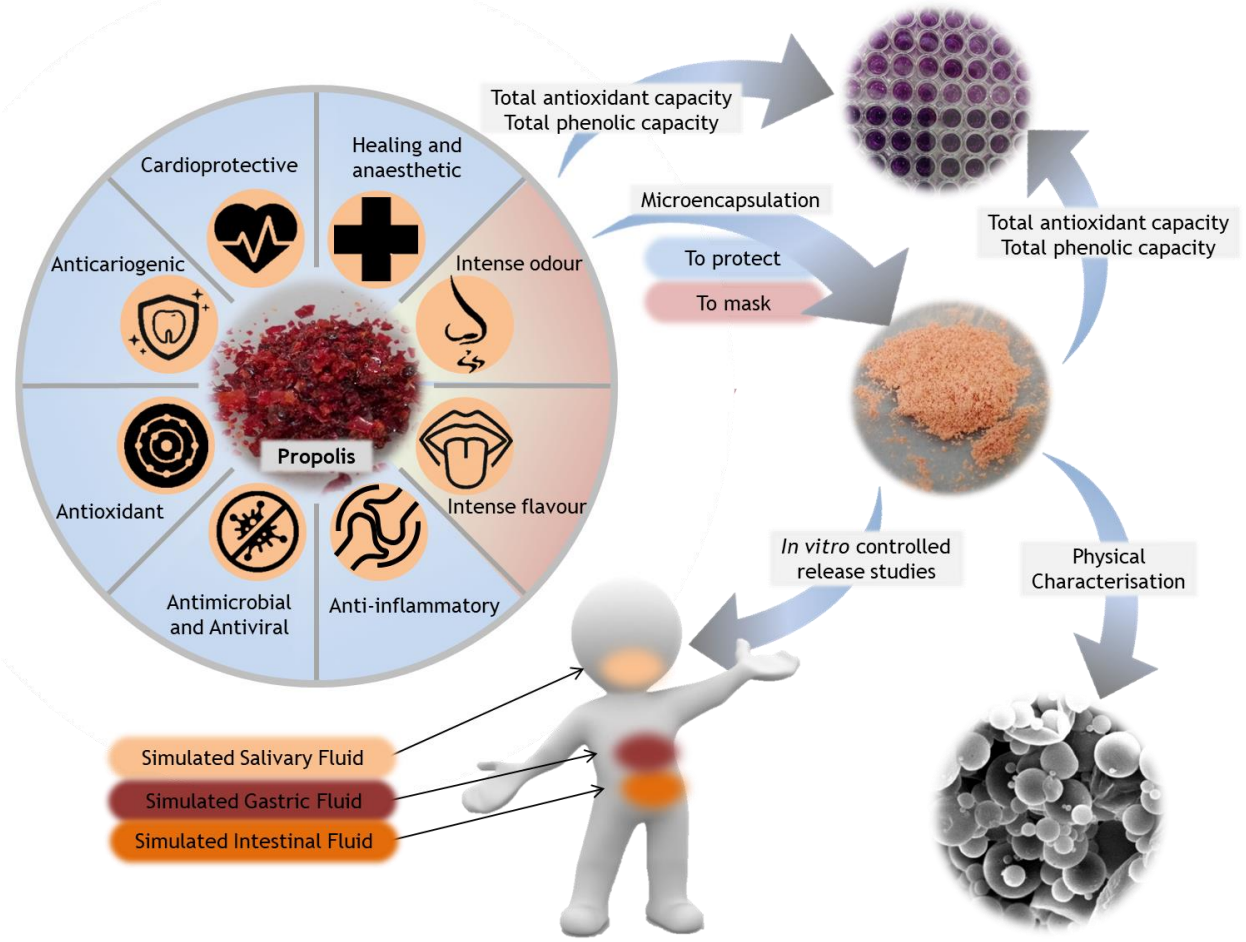

Figure 5 - Schematic representation of the aim of this dissertation regarding propolis microencapsulation

2.2.1 Encapsulating materials

The microencapsulation process is comprised of some steps. One of the first steps is choosing an adequate wall material. This material should be chosen from a wide diversity of natural and synthetic polymers that must be compatible with the core material and must have the desired characteristics (Jafari et al., 2008) and above all, should be biocompatible and/or biodegradable (Djerafi et al., 2015). In food industry, the wall material should be a good film former, have good emulsifying properties, low viscosity and hygroscopicity, bland in taste, stable without reacting with the core material while providing protection to it and be able to release the core material in the desired conditions (Jafari et al., 2008). It should also be noted that the wall material must be listed or affirmed as generally recognised as safe (GRAS) (Vasisht, 2014).

Shell materials used in microencapsulation processes can be divided into natural and synthetic polymers. In the food industry, the majority of encapsulating materials are natural polymers (Nedovic et al., 2011). These polymers are produced during the growth cycle of all living organisms and can be proteins derived from animal or plant, polysaccharides (e.g.,

starch and cellulose) and lipids (Campos et al., 2013; Vroman and Tighzert, 2009). They have remarkable features in terms of biocompatibility and biodegradability as well as an easy processing and chemical modifications (Campos et al., 2013). Table 1 provides some examples of natural encapsulating agents used in the food sector.

Table 1 - Examples of natural polymers with its corresponding class and origin. Adapted from (Nedovic *et al.*, 2011)

Class	Origin	Polymer examples
Polysaccharides	Starch and derivates	Amylose, amylopectin, dextrins, maltodextrins, polydextrose and cellulose
	Plant exudates and extracts	Gum Arabic, gum tragacanth, gum karaya, mesquite gum, galactomannans and pectin
	Marine extracts	Carrageenans and alginate
	Microbial and animal	Dextran, chitosan, xanthan and gellan
Protein	Milk and whey	Casein, gelatine and gluten
Lipid	Waxes	Beeswax, carnauba wax and candellia wax

In another way, synthetic polymers are produced from petroleum resources and can be aliphatic polyesters, aromatic copolyesters, polyamides and poly(ester-amide)s, polyurethanes, polyanhydrides and vinyl polymers (Vroman and Tighzert, 2009). These polymers can be prepared in a controlled process, resulting in a known composition, molecular weight and accurately predictable properties (e.g., solubility and degradability) (Campos et al., 2013). In this synthetic class, aliphatic polyesters (e.g., Polyglycolide (PGA), Polylactide (PLA), Poly(lactide-co-glycolide) (PLGA) and Polycaprolactone (PCL)) are the most used in food industry due to its biodegradability and extensive research available (Vroman and Tighzert, 2009).

2.2.1.1 Ethyl cellulose

For this work, ethyl cellulose (EC) was used as encapsulating material. This material is a semi-synthetic polysaccharide food grade polymer with a similar backbone as cellulose but with a replacement of some of the hydroxyl end groups with ethyl end groups, as shown in Figure 6 (Davidovich-Pinhas et al., 2015).

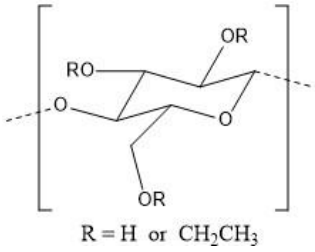

Figure 6 - Polymer structure of ethyl cellulose

Commercially, EC is semi-crystalline polymer with a 2.5 degree of substitution and is FDA approved as a food additive under regulation 21 CFR 172.868 (O'Sullivan et al., 2016) and is also an authorised food additive in the EU as it is listed in Commission Regulation (EU) No 231/2012 (Younes et al., 2018). Furthermore, it is known to be the only polymer with the ability to be directly dispersed in an oil phase (Gravelle et al., 2017) as with the appropriate degree of substitution (ranging from 2.4-2.5) it is insoluble in water, non-toxic, biodegradable, tasteless and with excellent water resistance and barrier properties. Thus making it an optimal polymer for microencapsulation uses in the food industry (Davidovich-Pinhas et al., 2015; Wu et al., 2018). Additionally, when used in microencapsulation, this polymer is capable of providing protection to the core in the harsh conditions of the stomach and is also able to release its contents in the intestinal tract for absorption (Prasertmanakit et al., 2009).

2.2.2 Microencapsulation techniques

The number of microencapsulation techniques is currently increasing as companies develop their products using innovative methods and technologies (Carvalho et al., 2016). The process selection is heavily influenced by the chemical and physical properties of the core and shell material (Aguiar et al., 2016) since not all methods are compatible to the materials used and also to the desired final characteristics of the microparticles (e.g., system stability, biocompatibility, particle size and distribution) (Carvalho et al., 2016). These processes can be divided into three categories which are chemical, physiochemical and physical-mechanical processes and in Table 2 are represented some techniques with its advantages and disadvantages (Aguiar et al., 2016; Carvalho et al., 2016).

Table 2 - Microencapsulation techniques with their respective advantages and disadvantages

Class	Technique	Advantages	Disadvantages
Chemical	Interfacial and *in situ* polymerisation	High encapsulation efficiency and easy to scale-up	Production of high quantity of residual solvent
Physiochemical	Coacervation	High encapsulation efficiency and control of particle size	Expensive, difficult to scale-up and dissolution of active compounds into the solvent
	Emulsification	Slim particle size and suitable for a large range of wall and core materials	Expensive, low encapsulation efficiency and production of high quantities of residual solvent
Physical-mechanical	Spray drying	Easy to scale-up, cheap and simple	Particles are not uniform, require further processing and loss of low boiling point aromatic compounds

2.2.2.1 Double emulsion/solvent evaporation technique

For this work, double emulsion/solvent evaporation technique was used to microencapsulate propolis.

Water/oil/water double emulsion, is classified as an emulsification technique alongside its other related techniques such as water/oil or oil/water emulsions (Nedovic et al., 2011). In short, oil/water emulsification is used to encapsulate water insoluble, or low-soluble, substances (Campos et al., 2013). But the microparticles that originate from this technique have low efficiency encapsulation and its release profiles are characterised by a burst release (Campos et al., 2013). In order to counter these unwanted characteristics, other techniques have been proposed such as water/oil/water double emulsion (Campos et al., 2013).

The most commonly method employed in double emulsion is double emulsion/solvent evaporation (water-in-oil-in-water) technique (Iqbal et al., 2015). This process has the advantage of being capable of encapsulating both lipophilic and hydrophilic compounds, gives a polydisperse particle size and a higher encapsulating efficiency when compared to other emulsification techniques (Iqbal et al., 2015).

In this technique, which is explained by Iqbal et al. (2015), the compound is merged into the inner aqueous phase (W_1), a soluble mean for this substance (e.g., water and ethanol), and the polymer is added into the oil phase (O), which is an organic solvent, (e.g., dichloromethane, methylene chlorine and ethyl acetate). After the preparation of both phases they are combined, by proper agitation, forming a primary emulsion (W_1/O). Then the primary emulsion is once again emulsified with an external aqueous phase (W_2) holding a stabilizer (e.g., Polyvinyl Alcohol (PVA), Span 80 and Tween 80) in its composition. The stabilizer will help maintain the physiochemical properties of the dispersion and prevent the separation of phases, allowing the creation of a double emulsion ($W_1/O/W_2$). The next step in this technique is the evaporation of the organic solvent which can be done by stirring at room temperature or under reduced pressure, depending on its boiling point. When the organic solvent evaporates from the dispersed phase, the polymer will harden with the active ingredient inside, forming the desired microparticles, which are then obtained through a process of filtration and posterior drying. Figure 7 has a representation of a diagram of this technique.

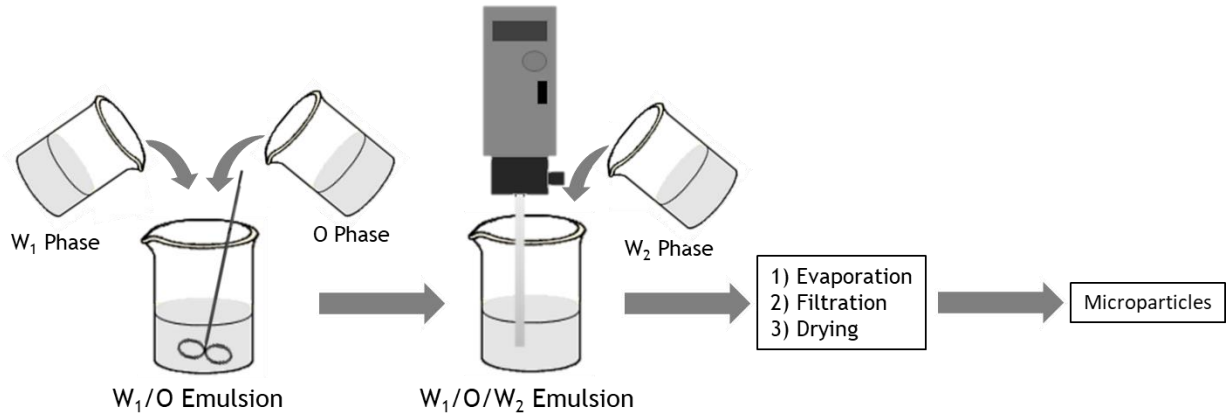

Figure 7 - Diagram of double emulsion solvent evaporation technique

2.3 Controlled release

An understanding of the microparticles' controlled release mechanisms are essential to deliver the active ingredient at recommended rates and also in an efficient, safe and convenient way to the absorption site (Martins et al., 2014). The active ingredient can be release from the microparticles through various actions and factors such as pressure, temperature, diffusion, biodegradation, dissolution and pH (Martins et al., 2014). The diffusion mechanism comprehends two types: reservoir and matrix. In the reservoir type, the active ingredient slowly diffuse through the wall material, thus the release rate depends on the physical and chemical properties of both active ingredient and wall material. In the matrix type, since the active ingredient is scattered in the wall material, its release rate depends mainly on its diffusion rate through the wall material. In the biodegradation mechanism the active ingredient is release through the degradation of the wall material, made by proteins or lipids, by enzymes. The solvent-activated release is controlled by either osmotic pressure, when the solvent flows to the inside of the microparticles and the saturated solution containing the active ingredient exits, or swelling phenomena, when the wall material is able to absorb water before dissolving, thus both release rates are influenced by the water absorption rate. The pH mechanism is caused by a change of pH in the mean that can make the wall material soluble. The temperature mechanism is caused by heat variations that can alter the physical state of the wall material leading to its collapse or distension. The pressure mechanism happens when pressure is applied in the surface of the microparticles (Aguiar et al., 2016).

The release rate of the active ingredient from the microparticles can have variations throughout time. Examples of possible release profiles are represented in Figure 8 in which four different antibiotics were microencapsulated using PLGA as encapsulating material.

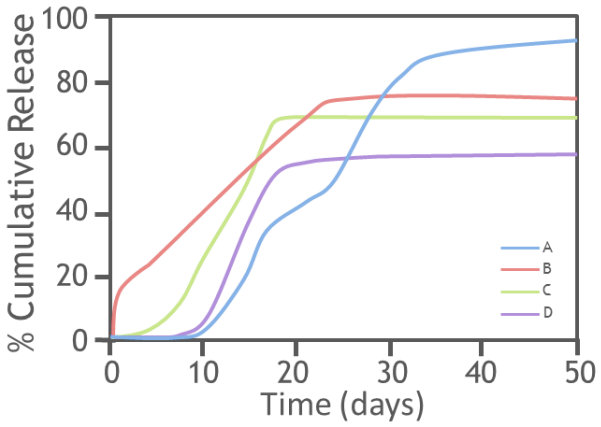

Figure 8 - Release profiles of vancomycin (A), ciprofloxacin (B), cefazolin (C) and doxycycline (D) microencapsulated in PLGA. Adapted from (Iqbal *et al.*, 2015)

In general the profiles are comprised of three or four phases. Phase 1 is characterised by an initial burst release of active ingredient which is associated with its presence on the microparticles surface, clearly depicted between days 0-1 of release profile B. Phase 2 is associated with a steady release of active ingredient, better portrayed once again between days 1-21 of release profile B. Phase 3 can be represented as the last phase in some profiles, as seen between days 21-49 in release profile B, or be described by yet another burst with a faster release profile, as seen between days 24-38 in release profile A. Phase 4 can only be represented in some profiles and is seen as the last phase of these profiles, as shown between days 38-49 of release profile A (Iqbal et al., 2015).

In food industry, the most common mechanisms for a controlled release are solvent-activated and thermal release. In addition, the usage of pH, enzymes and grinding triggers are also employed in this industry (Aguiar et al., 2016).

3 Context and State of the art

Microencapsulation of compounds has been increasing over the years. But the encapsulation of mixtures of natural substances such as propolis has only recently been getting more attention. In Table 3 an overview of recent studies regarding propolis microencapsulation is presented along with its technique, objectives, encapsulating material, operatory conditions and main results. For this research only published articles after the year 2000 were taken in consideration. In these articles, the microparticles obtained were studied through its physical and chemical characterisation, encapsulation efficiency, antibacterial activity, antioxidant quantification (total antioxidant, total phenolic and flavonoid), thermal behavior, *in vitro* release profiles and stability. Some other evaluations, such as anti-inflammatory and anticarcinogenic assays, were performed in order to verify if the microparticles still had some of the medicinal characteristics present in propolis. Furthermore, in some cases, propolis extracts were even analysed by HPLC or gas chromatography mass spectroscopy (GC-MS) in order to quantify and compare some of its most known compounds (e.g., caffeic acid, quercetin and cinnamic acid) with the produced microparticles. Figure 9 shows statistical analysis of the published articles found in terms of industry, technique used, type of polymer and polymers used.

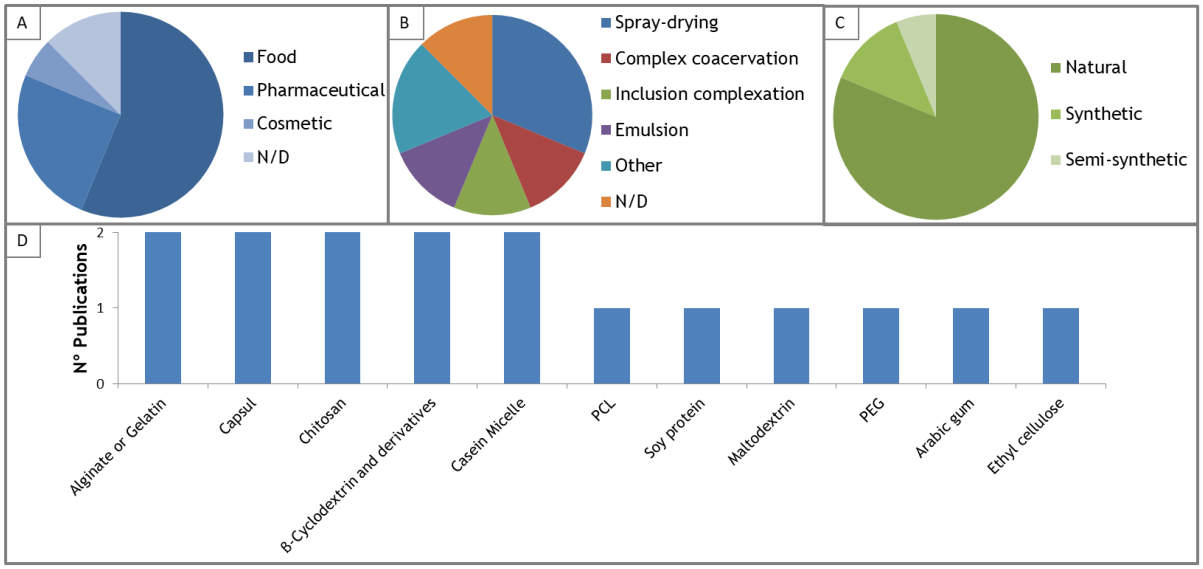

Figure 9 - Statistical analysis of published articles found, since the year 2000, regarding propolis microencapsulation: A) type of industry; B) technique used; C) type of polymer; and D) polymer used

By analysing Figure 9A it is possible to see that food industry is the main focus of propolis microencapsulation research. Although propolis is vastly applied in pharmaceutical industry, its use in food industry, in the form of microparticles, represents a potential component of functional food (Busch et al., 2017).

Figure 9B, which represents the techniques that were used to microencapsulate propolis, the spray-drying technique shows predominance over other techniques. In fact, spray-drying is extensively used in microencapsulation processes, can be applied to a wide diversity of materials and, as mentioned before, it is simple, inexpensive and easy to scale-up (Bruschi et al., 2003). Other methodologies found in these articles, such as complex coacervation, inclusive complexation and emulsification, also show very promising results of applicability.

Regarding Figure 9C and Figure 9D, the microencapsulation of propolis is still very recent and encapsulating materials used throughout these studies are always different as more information regarding this subject is still being investigated. But one common parameter is that the vast majority of articles use natural polymers instead of synthetic ones.

In general, almost all articles show good results regarding encapsulating efficiency and masking of taste and odour. The antibacterial activity was maintained or increased, being more effective against Gram positive bacteria (Bruschi et al., 2003; Durán et al., 2007; Mascheroni et al., 2014; Nori et al., 2011), its anticarcinogenic activity was also verified to have been improved (Catchpole et al., 2018), and was also able to improve the sun protection factor and stability (Sahlan et al., 2017). Furthermore, (Busch et al., 2017) proved that the addition of gums improved the particles integrity and size homogeneity. Finally, one of the most important factor, the antioxidant activity, was either able to be improved (Busch et al., 2017) or preserved (Nori et al., 2011). Moreover, Avanço and Bruschi (2008) found that ethyl cellulose microparticles of propolis are viable for develop aqueous dosage forms without the taste, odour and high concentration of ethanol in propolis extracts, although this publication only has results regarding morphology, particle size distribution and entrapment efficiency.

Table 3 - An overview of recent studies deploying encapsulating techniques of propolis

Method	Objectives	Encapsulating material	Operatory conditions	Results	Reference
Spray-drying	Obtain propolis in a powder, alcohol-free, water-dispersed and shelf-stable form	Arabic gum and octenyl succinic anhydride (OSA) starch	Inlet temperature: 120 ºC; Outlet temperature: 91 ºC; Airflow: 0.60 m^3/min; Feed flow: 0.60 m^3/min; Nozzle diameter: 1.3 mm	Preservation of antioxidant activity, stability for storage at room temperature and high dispersibility in cold water. Particle mean size: 15 - 24 µm	Da Silva et al., 2013
	Optimization of the spray-drying operating conditions and the proportions of gelatin and mannitol	Gelatin with mannitol (20% (w/w)) and gelatin without mannitol	Inlet temperature: 160 ºC; Feed rate (%): 6; Aspiration (%): 80; Pressure (%): 3	Mannitol reduced the microparticles agglomeration. Propolis microparticles maintained antibacterial activity against *Staphylococcus aureus* ATCC 25 923. Particle mean size: 2.70 µm without mannitol and 2.50 µm with mannitol	Bruschi et al., 2003
	Study the impact of added gums in propolis microencapsulation	Maltodextrin with or without added gums (vinal gum and Arabic gum)	Inlet temperature: 120 ºC; Outlet temperature: 70 - 74 ºC; Flow rate: 8 mL/min; Air pressure: 3.2 kPa; Nozzle diameter: 1.5 mm	Addition of gums shows improved particle integrity, size homogeneity, higher degree of polyphenols encapsulation, higher dispersibility, better physical stability towards humidification, higher glass transition temperatures and less broken particles	Busch et al., 2017
	Evaluate the antioxidant activity, physico-chemical characteristics, effects on oxidative stability and sensory acceptability of burger meat during storage	Capsul ®	Inlet temperature: 150 ºC; Outlet temperature: 100 ºC	Process efficiency was 76.86%. Addition of propolis co-product extract provided greater lipid stability	Reis et al., 2017
	Enhance the antioxidant properties of fish burgers	Gum Arabic and Capsul ®	Inlet temperature: 120 ºC. Outlet temperature: 88 ºC. Aspiration rate of 100% and pump flow rate of 25%	Capsul with a mass ratio of 1:20 of core to wall material can retain a greater amount of propolis and mask the characteristic smell	Spinelli et al., 2015
Emulsion solvent evaporation	Evaluate the antibacterial activity	Poly(ε-caprolactone)	Solvent: chloroform Aqueous phase only has PVA as the stabiliser. Stir for 24 h at 1060 rpm. Centrifuge for 30 min at 10000 rpm	A 10-fold lower concentration of microencapsulated propolis has a similar antibacterial activity as free propolis extract against *Streptococcus mutans* (GS5). Particle mean diameter: 5–10 µm	Durán et al., 2007
	Characterisation propolis microparticles	Ethyl cellulose	Propolis extract was dried. Solvent: ethyl acetate. Aqueous phase only has Span 80. Stir until complete evaporation of solvent at 800 rpm	Regular particle morphology without amorphous or sticking characteristics was achieved with a ethyl cellulose:dry extract ratio of 1:0.25 and 1.0% of Span 80. Microparticles mean diameter: 85.83 µm. Entrapment efficiency of 62.99 ± 0.52%	Avanço and Bruschi, 2008

Table 3 - An overview of recent studies deploying encapsulating techniques of propolis (Cont.)

Method	Objectives	Encapsulating material	Operatory conditions	Results	Reference
Inclusion complexation	Assess the use of β-cyclodextrin to increase the solubility of propolis extracts in water	ß-cyclodextrin	Sonication of suspension for 4 h; filtration through 0.45 µm PVDF filter; lyophilisation (freeze dryer)	Microencapsulation resulted in an increase of water solubility for several bioactive compounds of propolis extract	Kalogeropoulos *et al.*, 2009
	Measure the anti-gastrointestinal cancer activity of cyclodextrin-encapsulated propolis	γ-cyclodextrin, α-cyclodextrin and β-cyclodextrin	Mix propolis solution containing 25, 27 or 40% propolis solids with cyclodextrin and then add water with vigorous stirring. The resultant suspension was then freeze-dried	Cyclodextrin complexes inhibited the proliferation of 4 human gastrointestinal cancer cell lines. The complexes were also strongly anti-inflammatory	Catchpole *et al.*, 2018
Complex coacervation	In this study, cytotoxicity of ethanol extract of propolis was screened against cancer cell lines by MTT assay	Gelatin:alginate with a ratio of 1:1	Gelatin:alginate ratio of 1:1 at a stirring rate of 1400 rpm with 2 ml of 1 % (w/v) sodium carboxymethyl cellulose (Na-CMC)	Exhibits cytotoxic nitric oxide inhibition activities with an IC50 value of 0.1 ± 0.1 µg/ml. Mean particle size of 22.62 µm	Onbas *et al.*, 2016
Complex coacervation and freeze-drying	This study aimed to encapsulate propolis extract by complex coacervation using isolated soy protein and pectin as encapsulant agents	Isolated soy protein and pectin	Homogenized for 2 min at 8000 rpm (Ultra Turrax IKA-T25, Germany); Temperature during coacervation: 40 ºC; Freeze-drying: condenser temperature -60 ºC, pressure 1-0.1 kPa and final temperature of 25 ºC	Microencapsulation showed to preserve the antioxidant activity and the antimicrobial activity against Staphylococcus aureus, for both wall materials	Nori *et al.*, 2011
Membrane-encapsulating	Study an alternative encapsulation approach for production of active chitosan–propolis beads	Chitosan	Continuous aqueous solution phase made of acetic acid solution (1% wt.) with propolis concentration of 0%, 2% and 10% wt., and genipin as cross-linker (0.023% wt.). Wet beads were left in the mixture under stirring (150 rpm) for 24 h. The beads were dried at 30 °C for 24 h	Beads proved to be active against *Bacillus cereus*, *Escherichia coli*, *Listeria innocua*, *Pseudomonas fluorescens*, *Yarrovia lipolytica* and three moulds strains; the highest effect was found against Staphylococcus aureus (minimal inhibitory concentration of 0.8 mg beads mL^{-1})	Mascheroni *et al.*, 2014

Table 3 - An overview of recent studies deploying encapsulating techniques of propolis (Cont.)

Method	Objectives	Encapsulating material	Operatory conditions	Results	Reference
Ionic gelation	Formulation of nanoparticles with antibacterial activity against *Enterococcus faecalis* biofilms	Chitosan	Ultracentrifugation at 25000 rpm for 20 minutes for sedimentation of the nanoparticles	formulation inhibited *E. faecalis* biofilm formation and reduced bacteria in the biofilm by ~90% at 200 µg/ml concentration. In pre-formed biofilms, the formulation reduced bacterial number in the biofilm by ~40% and ~75% at 200 and 300 µg/ml, respectively	Ong *et al.*, 2017
anti-solvent co-precipitation	Investigate the pressurised carbon dioxide anti-solvent co-precipitation on encapsulation of propolis with water soluble polyethene glycol	polyethene glycol	20 L/min of CO_2 flowed into a precipitator. Feed solutions pumped into the precipitator at a flow rate of 0.1 ml/min. Operating pressures: 15 MPa, 17.5 MPa, 20 MPa. Operating temperature was 293 K	Total yield reached 88% and recovery of propolis flavonoids achieved 97%	Yang *et al.*, 2014
Not defined	Improve handling properties of Indonesian propolis by casein micelle encapsulation	Casein micelle	The mixture was sonicated for 5 mins and separated by microfiltration. Ultrafiltrate the permate by 10 kDa cut off	Encapsulation efficiency was 94% and 67% for flovonoids and polyphenols, respectively. Particle mean size of 1.3 µm and 300 nm	Sahlan and Supardi, 2013
Not defined	Improve photoprotection activity in sunscreen cream	Casein micelle	Precipitated propolis capsules are made into nanoparticles by High Pressure Ball Mill Homogenizer	Encapsulation efficiency was 80%. Mean particle size: 80 nm. Encapsulation improves photoprotection activity	Sahlan *et al.*, 2017

4 Materials and Methods

4.1 Materials

4.1.1 Reagents

The propolis sample was provided by the Polytechnic Institute of Bragança (IPB). Ethyl cellulose (Ref: 433837-250G, viscosity of 46 cP, CAS 9004-57-3), polyvinyl alcohol (Ref: P8136-250G, 87-90% hydrolysed, average molecular weight of 30,000-70,000, CAS 9002-89-5), Total Antioxidant Capacity Assay Kit (Cat No. MAK187), Folin & Ciocalteu's phenol reagent (Ref: F9252-100ML), Gallic Acid (Ref: 91215-100MG, CAS 149-91-7, linear formula $(HO)_3C_6H_2CO_2H$, molecular weight 170.12), α-Amylase from porcine pancreas (Ref: A6255-10MG, ≥1000 units/mg protein), Bile Salts (Ref: 48305-50G-F), Pepsin from porcine gastric mucosa (Ref: P7000-25G, ≥250 units/mg solid) and Pancreatin from porcine pancreas (Ref: P3292-25G) were purchased from Sigma Aldrich Chemical (St. Louis, MO, USA). The salts potassium chloride (Ref: 1049380050, KCl, CAS 7447-40-7), potassium dihydrogen phosphate (Ref: 104871001, KH_2PO_4, CAS 7778-77-0), sodium hydrogen carbonate (Ref: 1063290500, $NaHCO_3$, CAS 144-55-8), sodium chloride (Ref: 1370170001, NaCl, 7647-14-5), magnesium chloride hexahydrate (Ref: 1023675000, $MgCl_2.6H_2O$, CAS 7791-18-6), sodium carbonate (Ref: 1063920500, Na_2CO_3, CAS 497-19-8), and ammonium carbonate (Ref: 1011369051, $(NH_4)_2CO_3$, CAS 10361-29-2) were purchased from Merck kGaA (Darmstadt, Germany). Dichloromethane (Ref: 83665.320, CH_2Cl_2, ≥99.8% stabilised, CAS 75-09-2) was obtained from VWR International (Leuven, Belgium). Ethanol (Ref: 121085.1212, CH_3CH_2OH, 96% v/v PA, CAS 64-17-5) Panreac (Barcelona, Spain). Water was deionized and double-distilled in the laboratory using a MilliporeTM water purification system (Massachusetts, USA). All the reagents were either chromatographic or analytical grade and used as received.

4.1.2 Equipments

The weight measurements were performed on an analytical scale Mettler Toledo AG245 balance (Columbus, OH, USA). For the microencapsulation technique some equipments were used: an ultrasonic bath (J. P. Selecta, Barcelona, Spain), a vortex shaker (IKA VORTEX GENIUS 3, Staufen, Germany), a high-performance liquid homogenizer (IKA T18 Digital ULTRA-TURRAX®, Staufen, Germany), a stirring plate (AREX Digital, VELP Scientifica, Monza, Italy), vacuum filtration system (KNF Neuberger, Breisgau, Germany), 0.2 µm syringe filter (Ref: 514-0070, VWR International, Fontenay-sous-Bois, France) and a bench top freeze-dryer (SP Scientific, NY, USA). For the *in vitro* release studies of propolis, a horizontal shaker (Orbital

IKA KS 130 basic, Germany) and a Lovibond incubator (Amesbury, United Kingdom) at 37 °C were used. The morphology of the microparticles, which had been previously sputtered using a vacuum-sputtering coater (Leica, EM SCD 500, Wetzlar, Germany), were evaluated using PHENOM XL scanning light microscope (Eindhoven, The Netherlands), and the size distribution was evaluated by Laser Granulometry technique using a Particle Size Distribution Analyser named Coulter Counter-LS 230 (Coulter Corporation, FL, USA). The detection, quantification, validation of analytical methods and sustained release of propolis were performed by UV-Vis Spectrophotometry analysis, using a V-530 (JASCO, OK, USA), a microtiter plate reader (Synergy HT, Biotek, USA) and 96 well plate (Cat# 4430200, Orange Scientific, Belgium).

4.2 Methods

In this work, propolis was microencapsulated through double emulsion/solvent evaporation technique. Figure 10 represents a schematic representation of all the analysis performed on the microparticles during this work, which include product yield, loading, particle size distribution, morphology, encapsulation efficiency and controlled release. It also represents the formulations used, as well as the polymer, surfactant and solvent used.

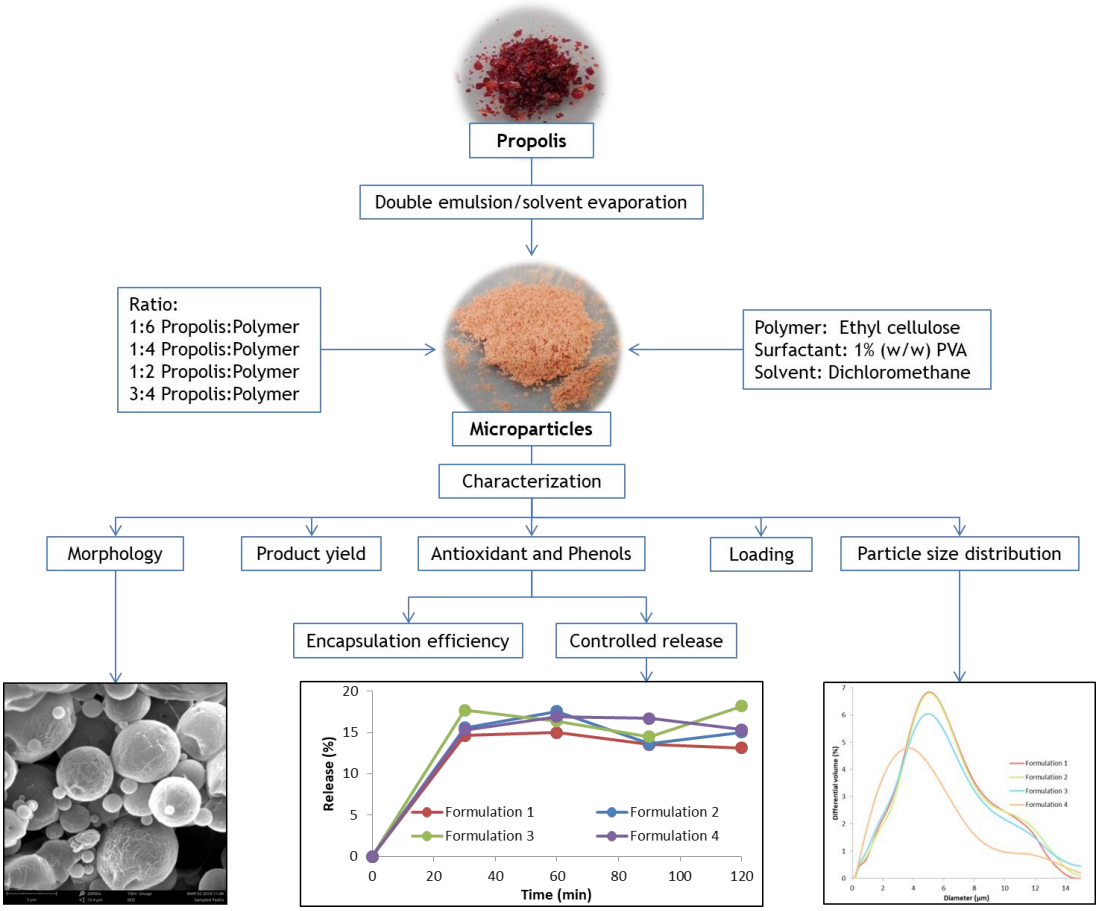

Figure 10 - Schematic representation of the analysis carried out in this work

4.2.1 Analytical method development

The validation of the analytical methods were performed according to Skoog et al. (1992).

In order to quantify the phenolic and antioxidant content of propolis samples, a UV-Vis spectrophotometric method was used to measure total phenolic content and total antioxidant capacity. For total phenolic content, 6 standards of Gallic Acid were prepared in 96 well plate (from 0 to 500 mg/L) and measured at an absorbance of 765 nm, according to Waterhouse (2012). For Total Antioxidant Capacity, 6 standards of Trolox were prepared in 96-well plate (from 0 to 20 nmole/well) and measured at an absorbance of 570 nm, according to the technical bulletin of TAC Assay Kit.

To validate the calibration curves, 5 parameters must be complied with: 5 different standard solutions; linearity range factor greater than 10; R>0.995; $\frac{s_a}{a} \leq 5\%$; $b - ts_b < 0 < b + ts_b$ (where R is correlation coefficient, a is the sensitivity, b is intercept of the regression, s_a and s_b are the correspondent standard deviations). Limits of detection (LOD) and limit of quantification (LOQ) were also determined according to Equation 4.1 and Equation 4.2, respectively.

$$\mathrm{LOD} = \frac{3s_b}{a} \tag{4.1}$$

$$\mathrm{LOQ} = \frac{10s_b}{a} \tag{4.2}$$

4.2.1.1 Total phenolic content

The total phenolic content analysis was performed according to Zhang et al. (2006). The Folin-Ciocalteu method is widely used to quantify total phenolic content and is based on a colour reaction between phenols (sample) and phosphotungsten-polymolybdic acid (reagent). Under basic conditions, provided by the sodium carbonate solution, the phenolic compounds undergo a dissociation process forming a phenolate anion which reduces the Folin-Ciocalteu reagent. This reaction will generate a blue coloured compound (chromogen). The Folin-Ciocalteu reagent also has lithium sulfate in order to prevent precipitation in the reaction and increase the sensibility (Jadhav et al., 2012; Zhang et al., 2006)

For the measurement, 20 μL of each sample or standard (Gallic Acid) was loaded into a well of a 96-well plate. 100 μL of Folin-Ciocalteu reagent was added, mixed and incubated in the dark for 5 minutes. Next, 80 μL of Na_2CO_3 (75 g/L) was added, mixed and incubated in the dark for 2 hours at room temperature. The absorbance was then measured at 765 nm.

The encapsulation efficiency of phenolic compounds was calculated. It is expressed as the ratio between the concentration of phenolic compounds present in lyophilized

microparticles and the concentration of phenolic compounds present in propolis, as shown in Equation 4.3. Where $EE_{phenolic}$ is the encapsulation efficiency of phenolic compounds, $C_{phenolic\ in\ MP}$ is the concentration of phenolic compounds in the microparticles and $C_{phenolic\ in\ Propolis}$ is the concentration of phenolic compounds in propolis. This method was also used in the *in vitro* release studies, in order to study the release profile of phenolic compounds.

$$\mathrm{EE_{phenolic}}\,(\%) = \frac{C_{phenolic\ in\ MP}(\mathrm{mg\ of\ GAE/g\ of\ Propolis})}{C_{phenolic\ in\ Propolis}(\mathrm{mg\ of\ GAE/g\ of\ Propolis})} \times 100 \qquad (4.3)$$

4.2.1.2 Total antioxidant capacity

The total antioxidant capacity analysis was performed according to the technical bulletin of Total Antioxidant Capacity Assay Kit. This kit contains a Cu^{2+} reagent. The Cu^{2+} ion is converted to Cu^{+} by small antioxidant molecules. Then the Cu^{+} ion chelates with a colorimetric probe, giving an absorbance peak at 570 nm, which is proportional to the total antioxidant capacity.

For the measurement of Trolox standards, 0, 4, 8, 12, 16 and 20 μL of the 1 mM Trolox standard was added, and for the measurement of samples, 50 μL of a sample was added. Next, the volume is made up to 100 μL with ultra-pure water. Then, 100 μL of Cu^{2+} working solution was added, mixed and incubated in the dark for 90 minutes. Afterwards, the absorbance was measured at 570 nm.

The encapsulation efficiency of antioxidant compounds was calculated. It is expressed as the ratio between the concentration of antioxidant compounds present in lyophilized microparticles and the concentration of antioxidant compounds present in propolis, as shown in Equation 4.4. Where $EE_{antioxidant}$ is the encapsulation efficiency of antioxidant compounds, $C_{antioxidant\ in\ MP}$ is the concentration of antioxidant compounds in the microparticles and $C_{antioxidant\ in\ Propolis}$ is the concentration of antioxidant compounds in propolis. This method was also used in the in vitro release studies, in order to study the release profile of antioxidant compounds.

$$\mathrm{EE_{antioxidant}}\,(\%) = \frac{C_{antioxidant\ in\ MP}(\mathrm{mg\ of\ TE/g\ of\ Propolis})}{C_{antioxidant\ in\ Propolis}(\mathrm{mg\ of\ TE/g\ of\ Propolis})} \times 100 \qquad (4.4)$$

4.2.2 Preparation of microparticles by Double Emulsion Solvent Evaporation Technique

For the preparation of the microencapsulation process, a propolis standard solution of 7.5 g/L and a PVA solution of 1% (w/w) were prepared in the previous day. The propolis standard solution was prepared by weighting 1.875 g of propolis into 250 mL of a solution with ethanol and water in a volume proportion of 70:30 (ethanol:UPW) and using an ultrasound bath until complete dissolution. The PVA solution was prepared by weighting 9 g of PVA into a

991 g of UPW, under stirring (650 rpm) at 120 ºC until total dissolution. After the dissolution, the PVA solution was cooled down at room temperature, weighted again and UPW was poured until the total mass, of UPW and PVA, of 1000 g.

This work involves the study of four sets of formulation, each done in triplicate, which only variable was the ratio of propolis:polymer. Formulation 1, 2, 3 and 4 have a ratio of propolis:polymer of 1:6, 1:4, 1:2 and 3:4, respectively. For each formulation, three phases were prepared, W_1, O and W_2, according to Table 4. For the preparation of the oil phase (O), 100 mg of ethyl cellulose was weighted in a vial and then 10 mL of dichloromethane were pipetted, then agitated in a vortex shaker for 1 minute and put in an ultrasound bath for 15 minutes.

Table 4 - Summary of the four experiences performed for propolis microencapsulation

Phase		Formulation 1	Formulation 2	Formulation 3	Formulation 4
W_1	Propolis (7.5 g/L) (mL)	2.2	3.3	6.7	10.0
O	Ethyl cellulose (mg)	100.0	100.0	100.0	100.0
	DCM (mL)	10.0	10.0	10.0	10.0
W_2	PVA (1% (w/w)) (g)	100.0	100.0	100.0	100.0

For the microencapsulation of these formulations, first, the internal aqueous phase (W_1) was pipetted into the vial of the oil phase, which was then manually shaken for approximately 5 seconds and agitated in a vortex shaker for 3 minutes. Next, the newly made mixture was quickly poured into a 250 mL beaker containing the external aqueous phase (W_2). This final mixture was then mixed using a high performance liquid homogenizer for 5 minutes at 5000 rpm. Afterwards, the mixture was put in an agitation plate for 3 hours at 650 rpm. Then the microparticles formed during this process were filtered using a 0.45 μm filter and were collected into a vial which was then freeze dried for 24 hours (a study was made to determine the time needed for lyophilifation and can be consulted in Appendix 1). Finally, the microparticles were weighted and transferred to a smaller vial and stored in a desiccator.

4.2.3 Microparticles characterisation

Some of the microparticles parameters were evaluated such as product yield, loading, total antioxidant encapsulation efficiency, total phenolic encapsulation efficiency, particle size distribution, morphology and release profiles of total antioxidant and total phenolic.

4.2.3.1 Product yield

The product yield (PY) is the ratio between the mass of lyophilized microparticles and the sum of the initial masses of ethyl cellulose and propolis solution (7.5 g/L), as expressed in

Equation 4.5 (where m_{MP} is the mass of microparticles, m_{EC} is the mass of ethyl cellulose and $m_{Propolis}$ is the mass of propolis), and was calculated for all four formulations.

$$\text{PY}\ (\%) = \frac{m_{MP}\ (\text{mg})}{m_{EC}\ (\text{mg}) + m_{Propolis}\ (\text{mg})} \times 100 \tag{4.5}$$

4.2.3.2 Loading

The loading, Equation 4.6, is the ratio between the mass of propolis and the mass of lyophilized microparticles and reflects the ability of the polymer to entrap the active ingredient. The theoretical loading, Equation 4.7, is the ratio between the mass of propolis and the sum of the masses of propolis and ethyl cellulose. Both loading were calculated for all formulations.

$$\text{Loading}\ (\%) = \frac{m_{Propolis}(\text{mg})}{m_{MP}(\text{mg})} \times 100 \tag{4.6}$$

$$\text{Theoretical Loading}\ (\%) = \frac{m_{Propolis}(\text{mg})}{m_{EC}(\text{mg}) + m_{Propolis}(\text{mg})} \times 100 \tag{4.7}$$

4.2.3.3 Particle size distribution

The particle size distribution was analysed by means of a Laser Granulometry technique, in which a Particle Size Distribution Analyser named Coulter Counter-LS 230 was used. Each sample was suspended in distilled water before the analysis and three runs were made. The polydispersity degree was measured in terms of span, as shown in Equation 4.8.

$$\text{span} = \frac{D_{v,90}(\mu\text{m}) - D_{v,10}(\mu\text{m})}{D_{v,50}(\mu\text{m})} \tag{4.8}$$

where the $\text{D}_{\text{v,10}}$, $\text{D}_{\text{v,50}}$ and $\text{D}_{\text{v,90}}$ represent respectively, the maximum particle diameter below which 10% of the sample volume exists, the maximum particle diameter below which 50% of the sample volume exists and the maximum particle diameter below which 90% of the sample volume exists.

4.2.3.4 Particle morphology

First, through a method of physical vapour deposition (PVD) designated by sputtering, the microparticles were coated with a thin layer of gold for 20 seconds using a vacuum-sputtering coater. Then, the morphology was evaluated using a PHENOM XL scanning light microscope at an accelerating voltage of 10 kV. Freeze-dried microparticles were placed on an aluminium stub with a carbon double-sided adhesive tape. Parameters such as sphericity, agglomerates, smoothness and porosity were analysed.

4.2.3.5 *In vitro* release studies

In order to mimic the human body physiologic conditions, three different solutions were prepared, according to Table 5, to simulate the salivary, gastric and intestinal fluids, with a

pH of 7, 3 and 7, respectively. But first, several stock solutions with the necessary constituents of these simulated fluids were made. This step was performed according to Minekus et al. (2014).

Table 5 - Preparation of simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) using several stock solutions. Adapted from (Minekus *et al.*, 2014)

		SSF	SGF	SIF
Constituent	**Stock concentration (g/L)**	**Stock volume (mL)**	**Stock volume (mL)**	**Stock volume (mL)**
KCl	37.3	15.1	6.9	6.8
KH_2PO_4	68	3.7	0.9	0.8
$NaHCO_3$	84	6.8	12.5	42.5
NaCl	117	-	11.8	9.6
$MgCl_2$	30.5	0.5	0.4	1.1
$[NH_4]_2CO_3$	48	0.06	0.5	-

After the preparation of the simulated fluids, the controlled release studies could start.

For the salivary simulation, 10 mg of microparticles were weighted and added into a vial. Then 750 μL of ultra-pure water, 750 μL of SSF and 1.16 μL of α-amylase were added into the same vial. The vial was then vortexed for 30 seconds and put inside a Lovibond incubator (Amesbury, United Kingdom) at 37 ºC and on top of a horizontal shaker (Orbital IKA KS 130 basic, Germany) at 170 rpm. Samples were taken every 0, 1 and 2 minutes and filtered into another vial using a 0.2 μm filter, in order to measure only the active ingredient in future analysis.

For the gastric simulation, 10 mg of microparticles were weighted and added into a vial. Then 750 μL of ultra-pure water, 750 μL of SGF and 6 mg of pepsin were added into the same vial. The vial was then vortexed for 30 seconds and put inside a Lovibond incubator (Amesbury, United Kingdom) at 37 ºC and on top of a horizontal shaker (Orbital IKA KS 130 basic, Germany) at 170 rpm. Samples were taken every 0, 30, 60, 90 and 120 minutes and filtered into another vial using a 0.2 μm filter, in order to measure only the active ingredient in future analysis.

For the intestinal simulation, 10 mg of microparticles were weighted and added into a vial. Then 750 μL of ultra-pure water, 750 μL of SIF, 18.75 mg of pancreatin and 375 mg of bile salts were added into the same vial. The vial was then vortexed for 30 seconds and put inside a Lovibond incubator (Amesbury, United Kingdom) at 37 ºC and on top of a horizontal shaker (Orbital IKA KS 130 basic, Germany) at 170 rpm. Samples were taken every 0, 30, 60, 90 and 120 minutes and filtered into another vial using a 0.2 μm filter, in order to measure only the active ingredient in future analysis.

4.2.4 Waste treatment

All the residues of this work were collected in closed containers and labelled for further treatment by the Environmental Management System of FEUP – EcoFEUP.

5 Results and discussion

The results obtained throughout this dissertation are presented in this chapter. In section 5.1 the analytical method is validated regarding Trolox and Gallic Acid; in section 5.2 the product yield, loading and encapsulation efficiency are analysed; in section 5.3 the size and morphology of the microparticles are discussed; and finally, section 5.4 presents the control release studies performed *in vitro* using salivary, gastric and intestinal fluids. In addition, Table 4 has the information regarding the formulation of each experience.

5.1 Analytical Method Development

To validate the analytical methods employed, a UV-Vis spectrophotometry was used to quantify both Trolox and Gallic Acid. In turn, these methods were used in the determination of encapsulation efficiencies of the microparticles, and phenolic and antioxidant content determination of the propolis sample.

For Trolox, the calibration curve, represented in Figure 11A and Equation 5.1, was obtained using 6 standards and validated according to the five parameters specified in chapter 4.2.1. As shown in Table 6, regarding the Trolox column, this method was validated as it satisfies all five parameters.

$$\text{Abs (AU)} = (0.0384 \pm 0.0027)\, C\ \text{(nmole/well)} + (-0.0054 \pm 0.0331) \qquad (5.1)$$

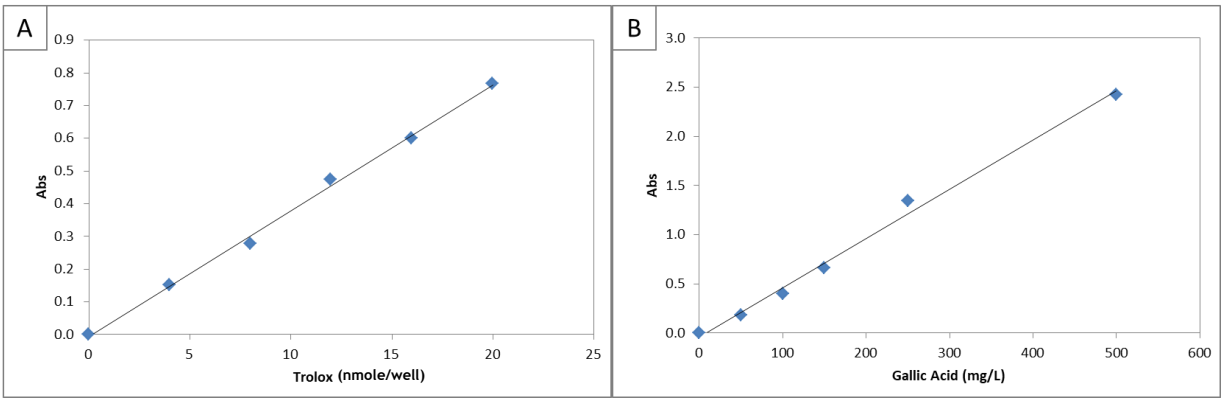

Figure 11 - Calibration curve of Trolox (A) and Gallic Acid (B).

For Gallic acid, the calibration curve obtained, represented in Figure 11B and Equation 5.2, was obtained using 6 standards and validated according to the five parameters specified in chapter 4.2.1. As shown in Table 6, regarding the Gallic Acid column, this method was validated as it satisfies all five parameters.

$$\text{Abs (AU)} = (0.0050 \pm 0.0006)\, C\text{(mg/L)} + (-0.0408 \pm 0.1380) \qquad (5.2)$$

Table 6 - Linearity parameters for the validation of UV-Vis Spectrometry calibration curve of Trolox and Gallic Acid

Parameters	Validation condition	Trolox	Gallic Acid
Number of standards	≥ 5	6	6
Linearity range	≥ 10	0.0 - 20.0 nmole/well	0.0 - 500.0 mg/L
Correlation coefficient (R)	> 0.995	0.999	0.997
Determination coefficient (R^2)	-	0.997	0.993
Slope error (%) (s_a/a)	≤ 5	2.564	4.130
Intercept confidence interval	$b-ts_b < 0 < b+ts_b$	-0.017 < 0 < 0.007	-0.091 < 0 < 0.009
LOD	-	0.9 nmole/well	29.8 mg/L
LOQ	-	3.1 nmole/well	99.4 mg/L

5.2 Microparticles Characterisation

5.2.1 Product yield

The product yield is an important parameter in the microencapsulation process as it can determine the amount of microparticles that is possible to obtain from the initial raw materials. Table 7 has the mean results of product yield of all four formulations.

Table 7 - Product yield of the four formulations

	Formulation 1 /RSD%	Formulation 2 /RSD%	Formulation 3 /RSD%	Formulation 4 /RSD%
PY (%)	83.9 ± 4.1 /4.9	81.0 ± 3.5 /4.3	63.1 ± 4.3 /6.9	78.2 ± 5.6 /7.1

Formulation 1 is shown to have obtained the best results of product yield (83.9% ± 4.1%) compared to the other three formulations. Since the only difference between the formulations is the ratio propolis:polymer, then, by analysing the product yield results, it is possible to understand that low ratios of propolis:polymer can increase the product yields. Nevertheless, in general, the product yields achieved were very satisfactory. It should also be noted that throughout the process of microencapsulation there are losses of mass that happen mainly during the filtration due to the fact that particles with lower diameter than 0.45 μm are discarded and also during the transfer of the microparticles from the filter to a vial there is always a loss of mass.

Yang et al. (2014) microencapsulated propolis with PEG utilizing a pressurized carbon dioxide anti-solvent co-precipitation process and, through a few preliminary runs, also found that product yield would increase with the decrease of the ratio propolis:polymer. Using the same operation conditions between experiences, for the ratios of 2, 1 and 0.67, a product yield of 37%, 86% and 88% was obtained, respectively.

5.2.2 Loading

The loading represents the ability of ethyl cellulose in entrapping propolis. Table 8 has the values of loading and theoretical loading for all four formulations.

Table 8 - Loading and theoretical loading for all four formulations

	Formulation 1 /RSD%	Formulation 2 /RSD%	Formulation 3 /RSD%	Formulation 4 /RSD%
Loading (%)	17.0 ± 0.8 /4.9	24.7 ± 1.0 /4.2	52.8 ± 3.7 /7.1	55.1 ± 4.1 /7.5
Theoretical loading (%)	14.3 ± 0.0 /0.1	20.0 ± 0.0 /0.2	33.2 ± 0.0 /0.1	42.9 ± 0.1 /0.2

As expected, the values of theoretical loading are inferior to the values of loading. This is because the mass of the microparticles is less than the sum of the masses of propolis and ethyl cellulose and also because of the losses of mass throughout all of the process. Nonetheless, the mass both loadings increase as the ratio propolis:polymer increases and formulation 4 has the best result of loading (55.1% ± 4.1%) meaning that 55.1% of the microparticle weight is propolis weight. Also, the different loading resulted in different coloration of the propolis microparticles. With the increase in loading, the colour of propolis changed to darker and intense shades of orange, as shown in Figure 12.

Mascheroni et al. (2014) encapsulated propolis in chitosan beads and also stated that the higher the loading, the darker the colour would be.

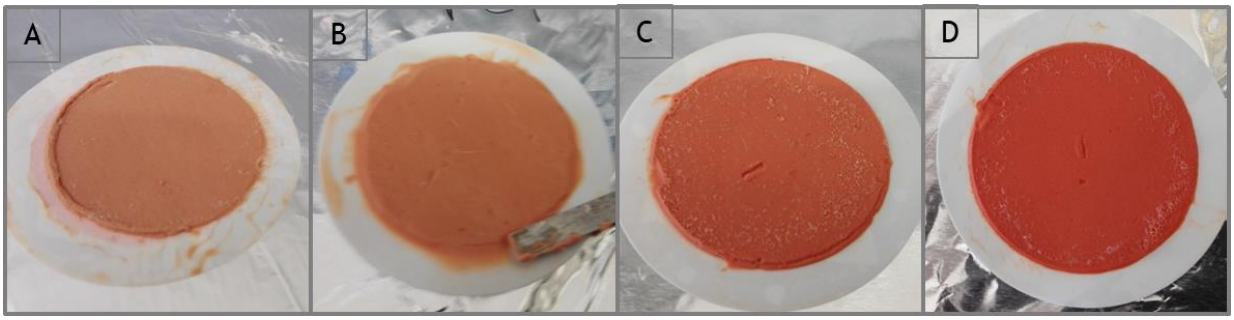

Figure 12 - Change in coloration of wet propolis microparticles with the increase of loading. In order, A) formulation 1, B) formulation 2, C) formulation 3 and D) formulation 4

5.2.3 Particle size distribution

The particle size is an important factor in food industry. These particles can change the perception of the costumer since the smallest particles can affect the texture and mouthfeel of the product. The particles size distribution was analysed, in triplicate, by a Laser Granulometry technique and the results for mean diameter, D_{10}, D_{50}, D_{90} and span are represented in Table 9 for all formulations. Also, the size distribution by volume is represented in Figure 13.

Table 9 - Results of particle mean diameter, D_{10}, D_{50}, D_{90} and polydispersitivity for all formulations

	Formulation 1 /RSD%	Formulation 2 /RSD%	Formulation 3 /RSD%	Formulation 4 /RSD%
Mean diameter (μm)	4.9 ± 0.2 /4.5	5.4 ± 0.6 /11.1	4.8 ± 0.2 /3.6	3.6 ± 0.1 /3.9
D_{10} (μm)	1.4 ± 0.0 /1.9	1.3 ± 0.0 /0.6	1.1 ± 0.2 /16.0	0.8 ± 0.1 /7.1
D_{50} (μm)	4.6 ± 0.2 /4.9	4.7 ± 0.2 /5.4	4.3 ± 0.5 /10.7	2.9 ± 0.0 /1.4
D_{90} (μm)	8.9 ± 0.5 /5.9	9.5 ± 0.8 /8.0	9.2 ± 0.6 /6.7	7.1 ± 0.4 /6.1
span	1.6 ± 0.0 /2.9	1.8 ± 0.1 /7.3	1.9 ± 0.4 /18.8	2.1 ± 0.1 /5.1

In general, all formulations showed similar results in terms of mean particle diameter, between 3.6 ± 0.1 μm and 5.4 ± 0.6 μm, suggesting that an increase in loading does not affect in any substantial way the particle mean size and also confirming that the structures produced were microparticles.

In terms of span, with the increase of the ratio propolis:polymer, so does the span value increases. Since the value of span is small, bellow 2 for all formulations except formulation 4, this indicates a narrow dispersion around the particle mean size.

Regarding the particle size distribution, all formulations are represented by two peaks, indicating a bimodal behaviour. This can happen due to particle aggregation forming bigger clusters and affecting the final particle size. Da Silva et al. (2013) used gum Arabic and OSA in a spray-drying process to microencapsulate propolis and also obtained a bimodal behaviour, indicating that smaller particles could penetrate in the interstices of larger ones.

Avanço and Bruschi (2008) microencapsulated propolis using ethyl cellulose and obtained higher mean particle size, 85.83 μm, through a process of emulsification, indicating that stirring method and emulsifier concentration can influence the particle size. Also, the particle size distribution followed unimodal behaviour. Durán et al. (2007) used an emulsification solvent evaporation process to microencapsulate propolis using poly(ε-capralactone) and reported a range of mean particle size between 5 and 10 μm. Bruschi et al. (2003) used gelatine in a spray-drying technique and reported to have microencapsulated propolis with a mean diameter between 2.5 and 2.7 μm.

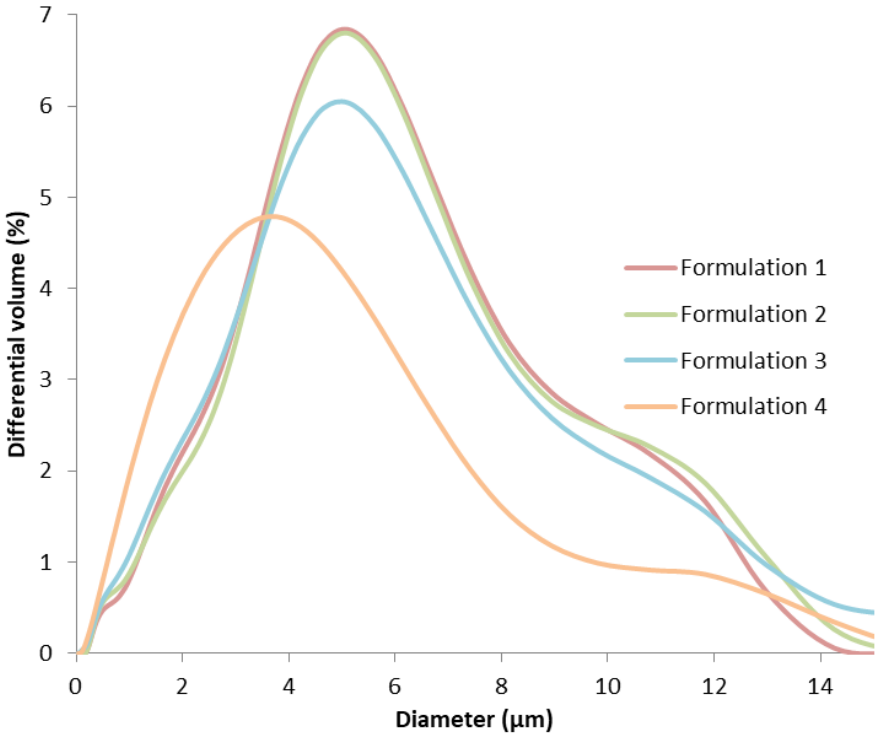

Figure 13 - Particle size distribution of the microparticles by volume for each formulation

5.2.4 Particle morphology

The morphology of the propolis microparticles was analysed through the photomicrographs obtained by SEM, represented in figures 14, 15 and 16 with an amplification of 5 000, 10 000 and 20 000 times, respectively.

In all formulations, the microparticles are small and present a spherical shape without pores, although some of them have dents. In some of these dents, it is possible to see some microparticles allocated in it, as shown in Figure 16C. The microparticle surface has some subtle differences throughout all formulations. With the increase of the ratio propolis:polymer, the surface of the microparticles becomes increasingly smooth and uniform. Figure 15A can also provide some insight in the type of morphology of the microparticles because in the upper right corner it is possible to see a burst microparticle. By analysing this particle, it can be determined that the microparticles produced are from reservoir types.

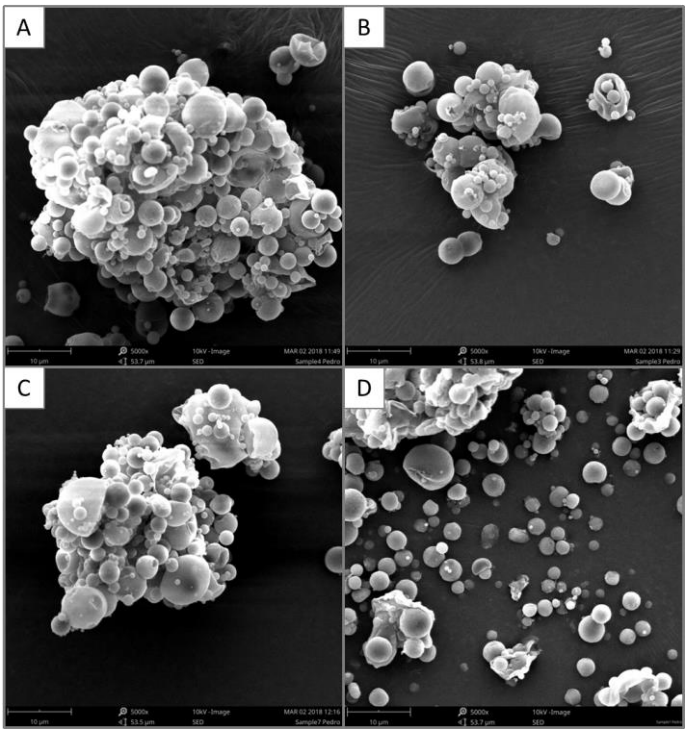

Figure 14 - SEM photomicrographs of propolis microparticles, in which represents the microparticles from formulation: A from 1, B from 2, C from 3 and D from 4. These photomicrographs have an amplification of 5 000 times

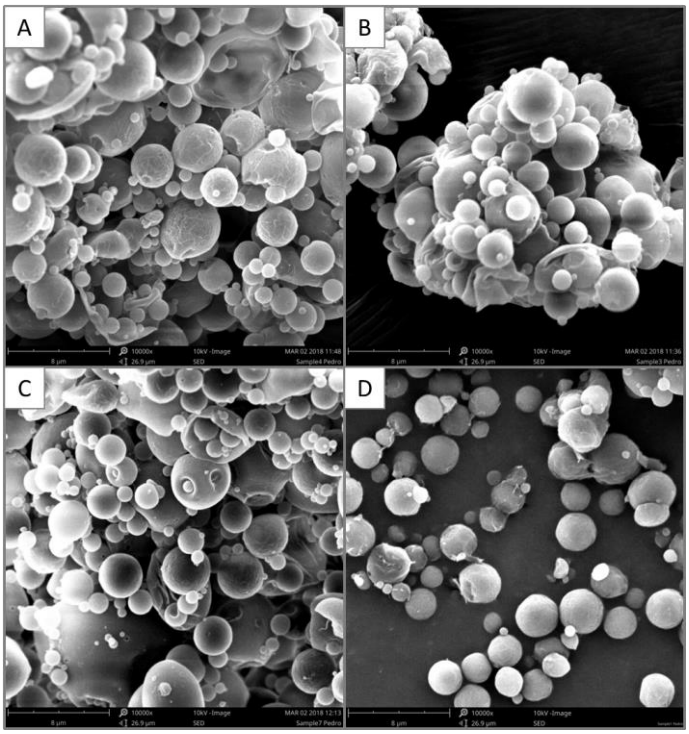

Figure 15 - SEM photomicrographs of propolis microparticles, in which represents the microparticles from formulation: A from 1, B from 2, C from 3 and D from 4. These photomicrographs have an amplification of 10 000 times

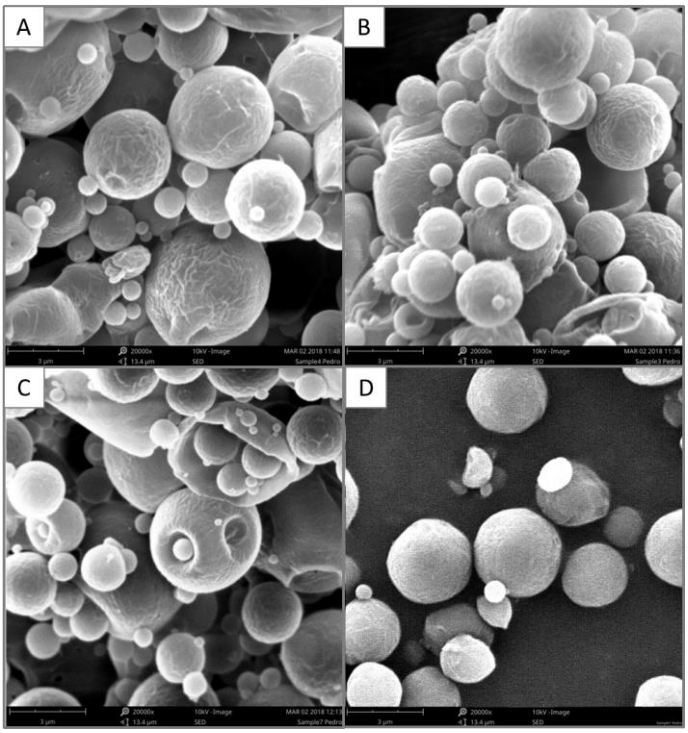

Figure 16 - SEM photomicrographs of propolis microparticles, in which represents the microparticles from formulation: A from 1, B from 2, C from 3 and D from 4. These photomicrographs have an amplification of 20 000 times

Avanço and Bruschi (2008) used ethyl cellulose as encapsulating material and also obtained spherical and uniform microparticles. Durán et al. (2007) produced spherical and smooth microparticles using PCL as polymer and reported that PVA can help obtain the spherical form and a low distribution size profile.

5.2.5 Encapsulation efficiency

Both total phenolic and total antioxidant encapsulation efficiency for all formulations of propolis microparticles were calculated. But first, the phenolic and the antioxidant quantities of propolis were determined. These results for propolis are presented in Table 10 and they had to be converted in order to be compared with the results of other authors. Such conversions can be consulted in Appendix 2 and Appendix 3 for total phenolic content and total antioxidant content, respectively. Table 11 represents the encapsulation efficiency of total phenolic content and total antioxidant capacity for all four formulations that were studied.

Table 10 - Concentration of phenolic content and antioxidant content in propolis sample

	Propolis /RSD%
Total phenolic content (mg of GAE/g of Propolis)	64.8 ±1.8 /2.7
Total antioxidant content (mg of TE/g of Propolis)	759.0 ± 36.9 /4.9

Propolis is known to have a high concentration of phenolic content and antioxidant content. But these concentrations are different comparing different types of propolis and comparing different geological locations of the same type of propolis. The propolis used in this study has a phenolic content of 64.8 ± 1.8 mg of GAE/g of Propolis and an antioxidant content of 759.0 ± 36.9 mg of TE/g of Propolis.

Falcão (2013a) did a quantification of total phenolic content in Portuguese propolis samples from the north of Portugal, and found a concentration between 7.07 ± 0.01 and 33.24 ± 0.01g/100g of raw propolis. In fact the geographical location of propolis plays an important role in its composition and also the season of the year the samples are collected can affect its composition. Also, according to an experience conducted by Paula et al. (2017), the optimal conditions for extraction of phenolic compounds from propolis is pH equal to 3 and a solution with a ratio 20:80 of water:ethanol. Other authors, such as Busch et al. (2017) and Spinelli et al. (2015), used a propolis sample from Tigre, Argentina, and a commercial solution produced and distributed by Dr. Taffi s.r.l. (La California, LI, Italy), and obtained a total phenolic content of 866 ± 22 mg of GAE/g of Propolis and 13.95 ± 0.06 mg of GAE/g of Propolis, respectively.

Table 11 - Encapsulation efficiency for the total phenolics content and total antioxidant content of all four formulations

	Formulation 1 /RSD%	Formulation 2 /RSD%	Formulation 3 /RSD%	Formulation 4 /RSD%
Total phenolic encapsulation efficiency (%)	91.4 ± 0.3 /0.3	92.0 ± 1.5 /1.6	92.0 ± 1.6 /1.7	87.5 ± 5.7 /6.5
Total antioxidant encapsulation efficiency (%)	99.8 ± 0.0 /0.0	99.7 ± 0.0 /0.0	99.2 ± 0.1 /0.1	98.5 ± 0.2 /0.2

The encapsulation efficiency for both contents was high. For phenolic content an efficiency ranging from 92.0 ± 1.6% and 87.5 ± 5.7% was achieved and for antioxidant content an efficiency ranging from 99.8 ± 0.0% and 98.5 ± 0.2% was achieved. Although the encapsulation efficiency has a tendency to decrease when the ratio propolis:polymer increases, the decrease in the encapsulation efficiency was not substantial. Also, the structures of the microparticles obtained, which have a smooth and homogeneous surface, can also ensure that the material encapsulated stays within the polymer walls.

Avanço and Bruschi (2008) obtained an encapsulation efficiency of 62.99 ± 0.52, using an emulsification method and using ethyl cellulose, and stated that the drug solubility is an important factor for the encapsulation efficiency. Busch et al. (2017) achieved an encapsulation efficiency of phenolic content between 89 ± 9% and 93 ± 7% and an

encapsulation efficiency of antioxidant content between 81 ± 8% and 86 ± 9%, by using a spray-drying technique and maltodextrin as polymer, and stated that a continuous wall with the absence of fissures could guarantee good encapsulation results. Reis et al. (2017) and Sahlan and Supardi (2013) indicated that the interactions between the polymer and the active compound can affect the encapsulation efficiency.

5.2.6 *In vitro* controlled release studies

The controlled release studies were performed using three simulating fluids: salivary, gastric and intestinal. The main advantage of a controlled release is the ability to release its content under the desired conditions. These studies were performed for both the phenolic content and antioxidant content and the release profiles are represented in Figure 17 and Figure 18, respectively.

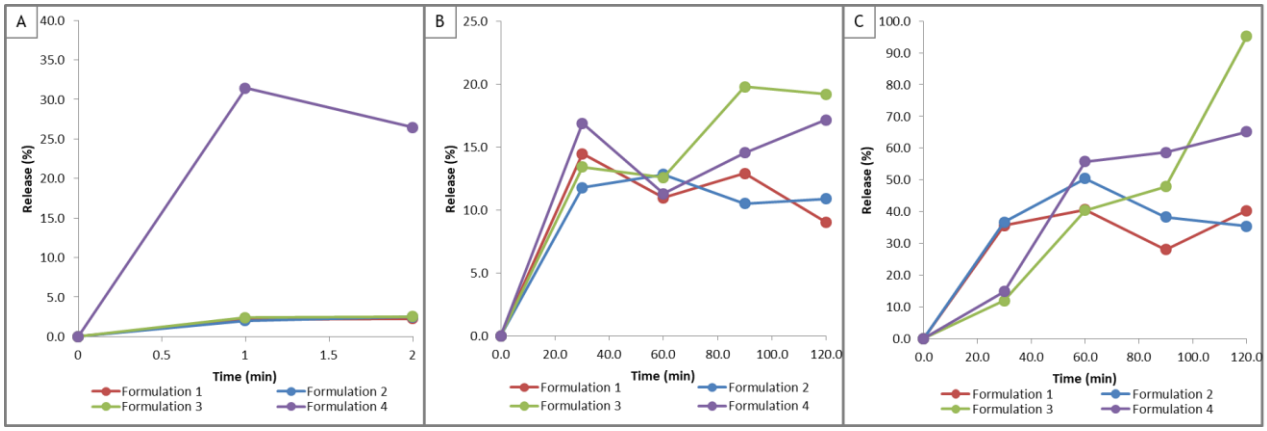

Figure 17 - Release profiles of phenolic compounds of propolis microparticles. A), B) and C) represent the release profile in SSF, SGF and SIF, respectively

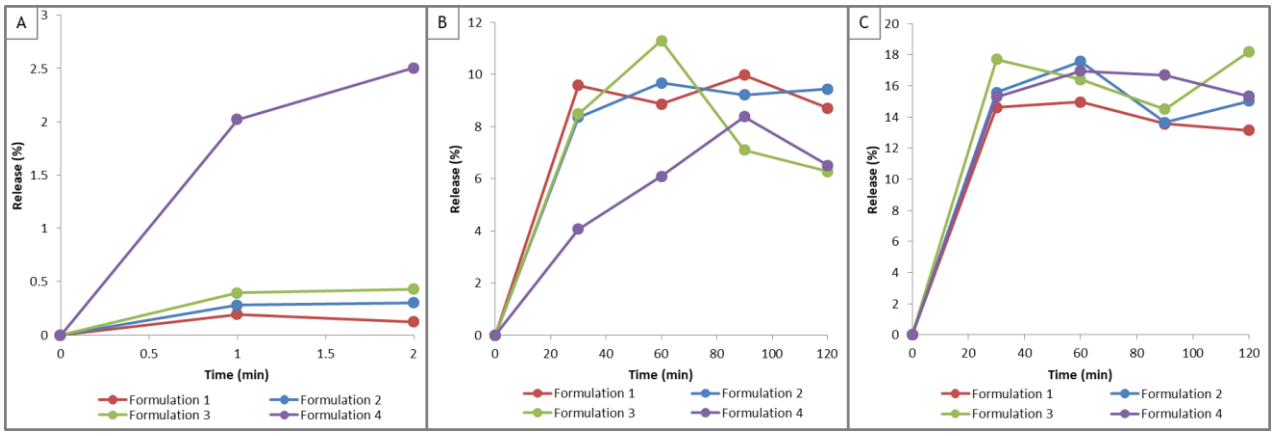

Figure 18 - Release profiles of antioxidant compounds of propolis microparticles. A), B) and C) represent the release profile in SSF, SGF and SIF, respectively

Analysing the release in the simulated salivary fluid, Figure 17A and Figure 18A, it is possible to see that the release of both phenolic content and antioxidant content increases

with the increase of the ratio propolis:polymer. However, formulation 4 has an unusual increase, in the release of both contents, when compared to the other three formulations, achieving releases of 35% and 2.5% for phenolic release and antioxidant release, respectively. This makes formulation 4 interesting for the study of propolis' anticariogenic capability, which is not addressed in this dissertation. Examining the release in the simulated gastric fluid, Figure 17B and Figure 18B, all formulations exhibit a gradual release of the encapsulated propolis. Polyphenol release has a minimum of 10% for formulation 1 and a maximum of 25% for formulation 3, and antioxidant release has a range between 6% and 10%. Finally, for the release in the simulated intestinal fluid, Figure 17C and Figure 18C, all formulations showed, in overall, good results of release. For the phenolic release, formulation 3 released almost all of its content during the first two hours. Formulation 1 and 2 showed similar results, having a release of 35%. For the antioxidant release, all formulation showed similar results, having a release between 14% and 18% after two hours. This means that around 80% of all the microparticles that arrived to the intestinal are still available to release its contents after two hours, providing a sustained release of antioxidants to the human body.

Kalogeropoulos et al. (2009) stated that the less efficient encapsulated compounds are more easily released, indicating that the rate of dissociation overwhelms the rate of association with the polymer. This fact can account for the increase in the release of the formulations with the highest ratio propolis:polymer, which were also the ones with the lowest encapsulation efficiency. More recently, Paulo and Santos (2018) studied the *in vitro* release under digestion conditions of hydroxytyrosol microparticles using the same conditions for the preparation of the simulated fluids and the same polymer as this work. They reported that an erosion-controlled release could be the reason for the release improvement in SIF.

6 Conclusion

In this work, propolis was microencapsulated through a method of double emulsion/solvent evaporation, using ethyl cellulose as polymer, with the aim to characterise the microparticles regarding product yield (PY), loading, particle size distribution, morphology, encapsulation efficiency (EE) and study its controlled release in simulated salivary (SSF), gastric (SGF) and intestinal fluids (SIF).

For the quantification of phenolic and antioxidant content, two analythical methods were validated. For phenolic quantification, Gallic acid calibration curve presented a limit of detention (LOD) of 29.8 mg/L and a limit of quantification (LOQ) of 99.4 mg/L. For antioxidant quantification, Trolox calibration curve presented a LOD of 0.9 nmole/well and a LOQ of 3.1 nmole/well. Regarding microparticles characterisation, the PY achieved a maximum of 83.9 ± 4.1% for formulation 1 and a minimum of 63.1 ± 4.3% for formulation 3. The loading reached a maximum of 55.1 ± 4.1% for formulation 4 and a minimum of 17.0 ± 0.8% for formulation 1. For the particle size distribution all formulations showed similar mean particle size which ranged from 3.6 ± 0.1 μm to 5.4 ± 0.6 μm, and all span values showed that there was a narrow distribution around the mean value. In terms of morphology, the microparticles were round with a continuous and smooth surface, which got smoother when the ratio propolis:polymer increased. Both size distribution and morphology were affected by the polyvinyl Alcohol since it can help in the spherical form and low distribution size. The phenolic content was determined for propolis sample, which had 64.8 ± 1.8 mg of GAE/g of Propolis revealing to be lower than expected when compared to other propolis samples from the north of Portugal, with a minimum reported in literature of 70.7 ± 0.1 mg/g of raw propolis, and thus the area where propolis is collected plays an important role in its composition. The antioxidant content revealed to be high, with 759.0 ± 36.9 mg of TE/g of Propolis. The EE of phenolic and antioxidant content was around 92% and 99% for all formulations, respectively, indicating that the continuous surface of the microparticles is capable of protecting propolis. In SSF, formulation 4 revealed a great release when compared to other formulations. In SGF, all formulations released around the same percentage of propolis. In SIF, formulation 3 showed the highest release percentage of around 95% and 20% for phenolic and antioxidant content after 2 hours, respectively. Thus, 80% of antioxidant content that arrived to the intestine is available for a sustained release.

In short, the studies performed in this work revealed that the produced propolis-loaded microparticles have the potential to be used as carriers to deliver a sustained release of antioxidants while maintaining and protecting its beneficial activities.

7 Assessment of the work done

7.1 Objectives Achieved

In this dissertation, the main objective was to microencapsulate four different ratio formulations of propolis:polymer by double emulsion/solvent evaporation technique using ethyl cellulose as wall material. This objective was successfully completed. Then the next objective was its characterisation in terms of encapsulation efficiency, product yield, size, morphology, phenolic content and antioxidant capacity, which was also completed. Finally, the controlled release of the microparticles was evaluated using simulated salivary, gastric and intestinal fluids and successfully performed.

7.2 Limitations and Future Work

During the performance of this work, some of the limitations included the availability of some equipments such as bench top freeze-dryer (SP Scientific, NY, USA) and PHENOM XL scanning light microscope (Eindhoven, The Netherlands) due to an existence of a long waiting list. Also, the delivery of the Total Antioxidant Capacity Assay Kit (Cat No. MAK187) took several months. Some of the equipments and material were shared with other students and investigators, which in turn caused the work plan to be postponed a day or two due to equipment/material unavailability. Another important limitation was the time available for the completion of this work. With more time, it would be possible to repeat some assays in order to attain more reliable results.

For future work, it would be interesting to quantify some of the phenolic or antioxidant compounds present in the propolis samples by HPLC or GC-MS in order to have a better understating of its properties and composition. Since propolis is also an antimicrobial agent, its activity could be analysed against gram-positive and gram-negative bacteria. Finally, a study regarding propolis microparticles economic viability in industrial symbiosis could be performed in order to improve apiculture products like honey and royal jelly.

7.3 Final Assessment

The development of this dissertation allowed the deepening of my knowledge regarding the thematic of antioxidants, microencapsulation techniques and characterisation methods of microparticles while also promoting a more independent laboratory work.

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Appendix 1 Freeze drying study

The freeze drying study was performed to determine the optimal time the microparticles need to be lyophilized using a bench top freeze-dryer (SP Scientific, NY, USA).

After the filtration process, the microparticles were collected into vials. These microparticles were then lyophilised in those vials, without the lid but with a parafilm in its place with five holes made with a needle. After the first 24 and 48 hours of lyophilisation, all the vials were weighted and the absolute value of the weight of the difference between those two instances of time was calculated in order to determine the standard deviation and relative standard deviation, as shown in Table A1.

These studies were performed until the masses of latest measurements were equal to the masses weighted 24 hours previously. For each formulation, a triplicate was made. In formulation 1 it was obtained a mean of $6.7x10^{-5} \pm 5.8x10^{-5}$ (RSD 86.6%); in formulation 2 it was obtained a mean of $1.3x10^{-4} \pm 5.8x10^{-5}$ (RSD 43.3%); in formulation 3 it was obtained a mean of $3.7x10^{-4} \pm 1.5x10^{-4}$ (RSD 41.7%); and in formulation 4 it was obtained a mean of $1.0x10^{-4} \pm 1.0x10^{-4}$ (RSD 100.0%). For all formulations a time of 24 hours is enough to dry the microparticles.

Table A1 - Freeze drying study performed on all four formulations with mass of vial and microparticles weighted 24 hours and 48 hours after the start of the lyophilisation process. The absolute value of the differences of both masses is also presented and used to calculate the standard deviation and the relative standard deviation

	Formulation 1			Formulation 2			Formulation 3			Formulation 4		
	F1.1	F1.2	F1.3	F2.1	F2.2	F2.3	F3.1	F3.2	F3.3	F4.1	F4.2	F4.3
a) m vial + MP (24h) (g)	17.4809	15.9593	15.9609	17.1490	17.2079	17.4547	17.4280	17.2055	17.4152	17.7317	11.3221	11.2949
b) m vial + MP (48h) (g)	17.4810	15.9594	15.9609	17.1491	17.2080	17.4549	17.4285	17.2059	17.4154	17.7317	11.3222	11.2947
|a) - b)| (g)	0.0001	0.0001	0.0000	0.0001	0.0001	0.0002	0.0005	0.0004	0.0002	0.0000	0.0001	0.0002
Mean |a)-b)| (g)	6.6667×10^{-5}			1.3333×10^{-4}			3.6667×10^{-4}			1.0000×10^{-4}		
sd	5.7735×10^{-5}			5.7735×10^{-5}			1.5275×10^{-4}			1.0000×10^{-4}		
RSD (%)	86.60			43.30			41.66			100.00		

Appendix 2 Unit conversion for propolis assay of total phenolic content

The total phenolic content assay was performed on a 96 well plate. The results obtained from the reading of the 96 well plate are expressed in absorbance unities. Then through the calibration curve of Gallic acid, the concentration is expressed in mg of GAE/L. In order to compare this value to the results of other authors, a conversion needs to be made from mg of GAE/L to mg of GAE/g Propolis. This conversion is expressed in Equation A2.1 where C_1 is the concentration obtained from the calibration curve, C_2 is the concentration of propolis standard used and C_3 is the concentration in the desired units.

$$C_3\left(\frac{\text{mg of GAE}}{\text{g of Propolis}}\right) = C_1\left(\frac{\text{mg of GAE}}{\text{L}}\right) \times \frac{1}{C_2\left(\frac{\text{g of Propolis}}{\text{L}}\right)} \quad \text{(A2.1)}$$

Appendix 3 Unit conversion for propolis assay of total antioxidant capacity

The total antioxidant capacity assay was performed on a 96 well plate. The results obtained from the reading of the 96 well plate are expressed in absorbance unities. Then through the calibration curve of Trolox, the concentration is expressed in nmole of Trolox/μL. In order to compare this value to the results of other authors, a conversion needs to be made from nmole of Trolox/μL to mg of Trolox/g of Propolis. This conversion is expressed in Equation A3.1, where C_4 is the concentration obtained from the calibration curve, C_2 is the concentration of propolis standard used, C_5 is the concentration in the desired units and M is the molar mass of Trolox (250.29 g/mol).

$$C_5\left(\frac{\text{mg of TE}}{\text{g of Propolis}}\right) = C_4\left(\frac{\text{nmole of TE}}{\mu\text{L}}\right) \times M\left(\frac{\text{mg}}{\text{nmole}}\right) \times \frac{1}{C_2\left(\frac{\text{g of Propolis}}{\mu\text{L}}\right)} \quad \text{(A3.1)}$$