U.PORTO
FEUP FACULDADE DE ENGENHARIA
UNIVERSIDADE DO PORTO

Master in Bioengineering

Co-encapsulation of an Antioxidant and a Vitamin: Preparation, characterization, and controlled release

Master's dissertation

of

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Developed within the course of dissertation

held in

Laboratory for Process Engineering, Environment, Biotechnology and Energy (LEPABE)

Department of Chemical Engineering, Faculty of Engineering, University of Porto

lepabe
Laboratory for Process Engineering, Environment, Biotechnology and Energy

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July 2022

Acknowledgment

E assim concluo este que caminho que foi longo, mas cheio de experiências, altos e baixos e muita aprendizagem. Teria sido impossível sem a ajuda, orientação e suporte de todos os envolvidos de alguma maneira neste meu trajeto.

Em primeiro lugar quero agradecer à minha orientadora, Drª Berta Estevinho, pelo voto de confiança, por esta oportunidade de trabalhar num projeto tão enriquecedor e gratificante e pelo carinho ao longo de todos estes meses.

Agradecer a todos as pessoas que me acompanharam diariamente no laboratório e que fizeram os meus dias mais alegres e diferentes. Um especial agradecimento ao Raphël e à Maéna por me terem dado uma ajuda fundamental. Às minhas amigas, Joana e Inês, por nunca me deixarem desistir, por estarem sempre lá em cada passo e pela incrível amizade. Ao meu namorado, João, pela paciência infinita e por estar sempre presente de uma maneira ou de outra. Por nunca me deixar desistir e por me fazer sorrir nos momentos mais difíceis.

Um obrigada muito especial e sentido à minha família – pais, irmãos, avós, primas, tios e bisavô – por todo o apoio, por todo o carinho, por toda a preocupação, por terem sempre uma palavra de conforto e por me estenderem a mão sempre. Não consigo expressar a gratidão que tenho por ter crescido numa família tão especial, muito obrigada!

Como não é possível mencionar toda a gente, deixo o meu obrigada a todas as pessoas que de alguma maneira contribuíram para o meu sucesso e que marcaram a minha vida de alguma forma.

Por fim, mas não menos importante agradecer à Francisca de há uns anos por nunca ter desistido e ter enfrentado os seus medos da melhor forma para que fosse possível estar aqui hoje, a acabar o Mestrado em Bioengenharia.

This work was financially supported by LA/P/0045/2020 (ALiCE), UIDB/00511/2020 and UIDP/00511/2020 (LEPABE), funded by national funds through FCT/MCTES (PIDDAC); Project POCI-01-0145-FEDER-028715 (MicroDelivery - Development of controlled delivery functional systems by microencapsulation of natural and active compounds with therapeutic, nutritional and technological interest), funded by FEDER funds through COMPETE2020 – Programa Operacional Competitividade e Internacionalização (POCI) and by national funds (PIDDAC) through FCT/MCTES.

Abstract

Nowadays there has been a greater interest in products that include key substances such as vitamins, antioxidants, and minerals. Epigallocatechin-3-gallate (EGCG) is a catechin present in green tea known for its antioxidant, anti-inflammatory, and antibacterial properties. In addition, Vitamin B12 is an essential vitamin that is present only in products of animal origin and that the lack of it causes several problems such as anemia and neurological disorders. However, the effectiveness of catechin and vitamin depend on its stability and bioavailability, which in industrial processes can be lost by degradation. Thus, it is necessary to respond with fast, efficient, and practical solutions for the incorporation of these agents in food products, cosmetics, and supplements. A meaningful solution is the microencapsulation of the active substances in order to protect them from external agents. The main goal of the present work is the microencapsulation of two compounds, an antioxidant (EGCG) and a vitamin (Vitamin B12), separately and simultaneously in a polymer matrix of biological origin. To achieve this goal, microencapsulation was performed by the electrospinning technique and two encapsulating agents, zein and pullulan were used. Different concentrations of each biopolymer (1-30%) and of each active substance (0.5-5%) were tested. The microstructures obtained were analyzed and characterized by their morphology, profile and release kinetics and encapsulation efficiency. By Scanning Electron Microscope (SEM) it was found the production of microparticles (lower concentrations of encapsulating agent), fibers (higher biopolymer's concentrations) or the mixture of both. High encapsulation efficiencies were obtained for both encapsulating agents, and the highest were found in the samples with 1% of active substance and 30% of zein or 10% pullulan. Controlled release studies, which were conducted in deionized water, revealed that in the microparticles of zein the release was immediate, unlike the fibers, where the release is prolonged. Pullulan microparticles have a release profile similar to zein fibers. Five kinetic models were applied to the release profiles, and it was found that the model that best fit the greater number of results was the Weibull model.

Keywords: Vitamin B12; EGCG; electrospinning; zein, pullulan; microencapsulation

Resumo

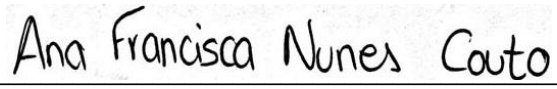
Nos tempos que correm tem-se observado um maior interesse em produtos que incluam substâncias chave como vitaminas, antioxidantes e minerais. A Epigalocatequina-3-galato (EGCG) é uma catequina presente no chá verde conhecida pelo seu poder antioxidante, anti-inflamatório e antibacteriano. Adicionalmente, a Vitamina B12 é uma vitamina essencial que está presente apenas em produtos de origem animal e que a falta desta origina diversos problemas como anemia e desordens do foro neurológico. No entanto, a eficácia da catequina como da vitamina dependem da sua estabilidade e biodisponibilidade, o que nos processos industriais se pode perder por degradação das mesmas. Assim, é necessário dar resposta com soluções rápidas, eficientes e práticas para a incorporação destes agentes em produtos alimentares, cosméticos e suplementos. Uma boa solução é a microencapsulação das substâncias ativas de forma a proteger os compostos de agentes externos. O principal objetivo deste trabalho é a microencapsulação de dois compostos, um antioxidante (EGCG) e uma vitamina (Vitamina B12), em separado e em simultâneo numa matriz polimérica de origem biológica. Para atingir este objetivo, a microencapsulação foi realizada pela técnica de *electrospinning* e usou-se dois agentes encapsulantes, a zeína e o pululano. Foram testadas diferentes concentrações de cada biopolímero (1-30%) e de cada substância ativa (0,5-5%). As microestruturas obtidas foram analisadas e caracterizadas pela sua morfologia, perfil e cinética de libertação e eficiência de encapsulação. Recorrendo ao Microscópio Eletrónico de Varrimento (*SEM*) foi constatou-se a formação de micropartículas (concentrações de agente encapsulante mais baixas), fibras (concentrações de biopolímero mais altas) ou a mistura de ambas. Obtiveram-se eficiências de encapsulação altas para ambos os agentes encapsulantes, sendo que as mais altas se verificaram nas amostras com 1% de substância ativa e 30% de zeína ou 10% pululano. Os estudos de libertação controlada, que foram realizados em água desionizada, revelaram que nas micropartículas de zeína a libertação é imediata, ao contrário das fibras, onde a libertação é prolongada. As micropartículas de pululano apresentam um perfil de libertação semelhante às fibras de zeína. Aos perfis de libertação foram aplicados cinco modelos cinéticos e verificou-se que o modelo que melhor ajustava ao maior número de resultados era o modelo de Weibull.

Palavras-chave: Vitamina B12; EGCG; *electrospinning*; zeína, pululano; microencapsulação

Declaration

I hereby declare, under word of honor, that this work is original and that all non-original contributions is indicated and due reference is given to the author and source

Porto, July of 2022



(Ana Francisca Nunes Couto)

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Notation and Glossary

M_w	Molecular Weight
K	Baker-Lonsdale constant
n	Release exponent
K_k	Korsmeyer-Peppas constant
β	Weibull model shape parameter
R^2	Correlation coefficient

List of acronyms

Cbl	Cobalamin
EE	Encapsulation Efficiency
EGCG	Epigallocatechin-3-gallato
EHD	Electrohydrodynamic
RDA	Recommended Daily Allowances
SEM	Scanning Electron Microscopy
UV	Ultraviolet
VitB12	Vitamin B12

Introduction

Framing and presentation of the work

Nowadays, has been a growing concern about health and the type of consumed products by the society. The demand for food products, supplements and cosmetics containing bioactive ingredients such as antioxidants and vitamins is increasing (Peanparkdee et al., 2016).

The catechin, EGCG, has been incorporated into products since has interesting characteristics like antioxidant, anticarcinogenic and antibacterial activities (Cerbin-Koczorowska et al., 2021). Also, vitamins have been gaining importance in food fortification, namely vitamin B12. This vitamin is only obtained through the consumption of animal-derived meals and is part of essential functions such as normal growth and development, red blood cells production and DNA synthesis (Silverman & Brauer, 2008). The lack of this vitamin can cause anemia and neurological disorders (Coelho et al., 2021). The process of addition of these compounds to foods or supplements and their storage often leads to the degradation by external factors such as the presence of oxygen, pH, temperature, presence of metals, among others (Krupkova et al., 2016; Mukherjee & Sen, 2011). This way the compounds may no longer be bioactive and lose their functionality.

Microencapsulation, defined by the entrapment of small particles in a polymeric matrix, can overcome some of the problems. This technology has several advantages like the protection of the core from external factors, stabilization of compounds, improve shelf-life and mask unpleasant textures or flavors (Castro Coelho et al., 2021). The electrohydrodynamic techniques (electrospinning and electrospray) are an appealing alternative since do not use high temperatures or strong reagents.

Taking into account the aforementioned, the present work has as main goal the encapsulation of an antioxidant, EGCG, and a vitamin, Vitamin B12, in two different biopolymers, zein and pullulan, through electrospinning in order to produce different microstructures with micro/nano characteristics and high encapsulation efficiencies. Is also an objective of the work the evaluation on how the type and concentration of encapsulating agent and bioactive compound influence the morphology and release profiles.

Organization of the dissertation

The present work is organized in 8 different main chapters:

Chapter 1: **Introduction**, highlights the project's aims and objectives, as well as the organization.

Chapter 2: **Context and state of the art**, a theoretical introduction about the main components of the work (EGCG, Vitamin B12 and microencapsulation) and an overview of some studies.

Chapter 3: **Materials and methodologies**, enumerates the materials used during the experimental work and the methodologies adopted, as well as the conditions of each operation.

Chapter 4: **Results and discussion**, contains the descriptions of the obtained results, their respective analysis and discussion, and comparisons with the literature.

Chapter 5: **Conclusions**, describes the main conclusions of the results obtained.

Chapter 6: **Assessment of the work done**, contains an overview of the work, which objectives were achieved, limitations and future works.

Chapter 7: **Bibliography**, references used for the construction of the dissertation.

Chapter 8: **Appendix**, contains additional information of the work done in order to better understand some concepts discussed over the dissertation.

Context and State of the art

1. Catechins

Catechins are natural polyphenolic compounds (polyphenols). These compounds are a highly diversified group, synthesized by plants, with at least two phenyl rings and one or more hydroxyl substituents (Singla et al., 2019). Polyphenols can be classified into different classes according to several features. Therefore, two main groups can be identified: flavonoids and non-flavonoids. Within the flavonoids, six subgroups can be distinguished, namely the flavanols (or flavan-3-ols) that in general contain a saturated heterocyclic ring, no double bond between C2 and C3 and a hydroxyl group at the C3 position (Singla et al., 2019). Flavanols can be found as monomeric units known as catechins (isomer with *trans* configuration) and epicatechins (isomer with *cis* configuration), as well as polymeric forms known as tannins (Singla et al., 2019; Tsao, 2010).

Catechins and epicatechins have two stereoisomers each, *i.e.*, (+)-catechin, (−)-catechin, (+)-epicatechin and (−)-epicatechin. In addition to these isomers, there are gallic acid esters of catechin/epicatechin: epigallocatechin (EGC), epigallocatechin gallate (EGCG), epicatechin gallate (ECG), and gallocatechin (GC) (Habauzit et al., 2014).

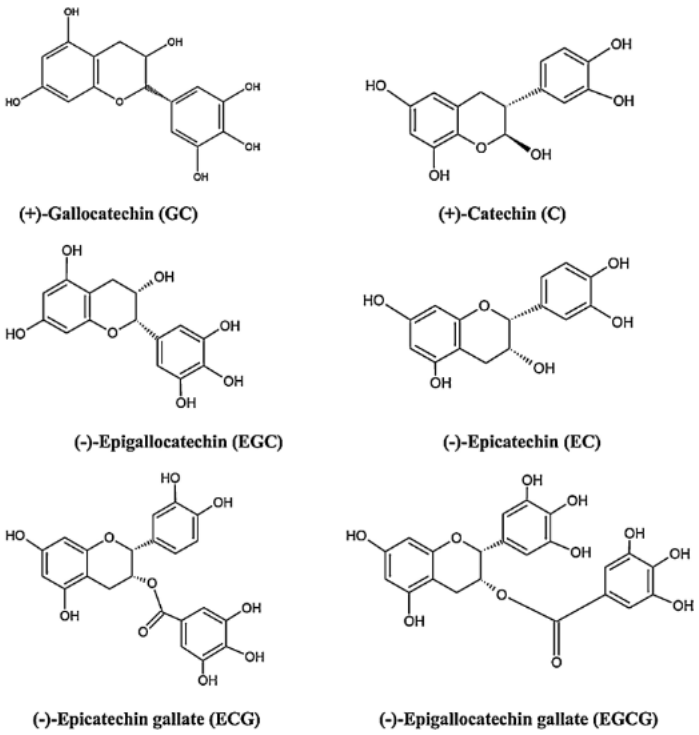


Figure 1 - Catechin and Epicatechin structures. Retrieved from (Gadkari & Balaraman, 2015)

Catechins are mainly found in green tea which is associated with several health benefits that are generally attributed to the presence of the catechins (Chacko et al., 2010; Singh et al., 2011). A cup of green tea, made with one gram of tea leaves in 100 ml of boiling water, normally contains 250–350 mg of dry materials with 30–42% catechins (Braicu et al., 2013). The extraction process to obtain the purified catechins is a difficult process since they occur naturally bounded to sugars, proteins or may create polymerized derivatives, but also because are highly susceptible to oxidation (Gadkari & Balaraman, 2015).

These polyphenols act like powerful antioxidants that scavenges free radicals and regulates the activity of several types of oxidases in the body, preventing and treating diseases (Yan et al., 2020). Furthermore, the catechins antioxidant activity is strongly affected by the molecular structure, starting conditions and the microenvironment of the reaction media (Colon & Nerín, 2016; Yan et al., 2020).

1.2. Epigallocatechin-3-gallate (EGCG)

Epigallocatechin-3-gallate (EGCG) is the most abundant and bioactive catechin, making up to 59% of total catechin in green tea (Yan et al., 2020), with a molecular formula of $C_{22}H_{18}O_{11}$ and molecular weight of 458 g/mol (Gadkari & Balaraman, 2015). Catechins are usually colorless crystalline substances but EGCG, when extracted from green tea, has an appearance of a white to pink hydrophilic powder and has a melting point of around 224 °C (Agrawal et al., 2019; Gadkari & Balaraman, 2015). This compound is highly soluble in polar solvents (water, ethanol and methanol) but insoluble in organic solvents and moderately soluble in grease (Gadkari & Balaraman, 2015; Yan et al., 2020). The identification and quantification of EGCG can be done based on UV light absorption since the maximum absorbance of this compound is approximately at 275 nm (Snitsarev et al., 2013).

EGCG is an esterified catechin that is highly unstable to changes in pH and temperature and easily transformed into non epi-structure (Gadkari & Balaraman, 2015). This catechin is stable at low pH (<4) but unstable in alkaline solutions, also studies have confirmed that EGCG is unstable to heat and easily oxidated (Agrawal et al., 2019; Widyaningr et al., 2015). Zeng et al. (2017) conducted a study on tea polyphenols (including EGCG) stability and concluded that was dependent on temperature, pH and oxygen concentration. Also, Hong et al. (2002) reported that a number of factors such as

pH, concentration of proteins, antioxidant concentrations and the presence of metal ions could affect the EGCG stability.

Especially in the recent years, studies have confirmed that EGCG has benefits for human health including antioxidant, antitumor, anti-inflammatory, antibacterial, antiviral and protection against excessive UV radiation (Cerbin-Koczorowska et al., 2021; Chu et al., 2017; Dai et al., 2020; Singh et al., 2011). However, these features depend on the bioavailability and stability of the EGCG in the human body and, in general, catechins present a low bioavailability after oral administration (Dai et al., 2020). Zhang et al. (2013) reported that the highest plasma concentration of EGCG was only 0.15 μM after the ingestion of two cups of green tea.

Oral EGCG is usually absorbed into the blood through the gastrointestinal tract, particularly in the small intestine. Although large quantities of the compound can reach the large intestine due to the low absorption rate in the small intestine, probably due to the high intestine pH and microbiota that may lead to the degradation of the catechin (Dai et al., 2020; Shirakami et al., 2016). The poor transcellular transport and fast biliary excretion also contribute to a low EGCG bioavailability (Zagury et al., 2019).

Due to EGCG appealing characteristics and human health benefits, the interest in its application as a dietary supplement and food preservative has been increasing. So, green tea extracts containing EGCG may be found in a variety of foods such as ready-to-eat meals, cereals, bakery goods, salad dressings, margarine, oils and beverages (Cerbin-Koczorowska et al., 2021). However, studies on food products containing EGCG have revealed that they are unstable (EGCG degradation due to epimerization and/or autooxidation) during storage which leads to a low bioavailability (Dettlaff et al., 2017; Krupkova et al., 2016; Shi et al., 2018).

Given the foregoing information, it is critical to ensure the bioactivity and bioavailability of EGCG when added to food products or supplements, as well as to manage the release to ensure that it reaches the target. Additionally, since EGCG has an astringent and bitter taste, it is necessary to mask the EGCG flavor as it that can be unpleasant when consumed. (Fang & Bhandari, 2010; Gadkari & Balaraman, 2015).

2. Vitamins

Vitamins are a very heterogenous and diverse group of compounds that are required for appropriate physiological function (micronutrient), but most are not generated by the body, at least not enough to meet human daily needs (Kennedy, 2016; Silverman & Brauer, 2008). Therefore, they must be acquired from exogenous sources, mostly through the diet. To this day are known 13 different vitamins who don't share common structural features neither perform the same functions so, empirically, are divided into two groups according to their solubility: nine water-soluble vitamins, that comprises vitamin C and the B-complex - thiamine (B_1), riboflavin (B_2), niacin (B_3), pantothenic acid (B_5), vitamin B_6, folate (B_9) and vitamin B_{12} - and four fat-soluble vitamins (vitamins A, D, E and K) (Kennedy, 2016).

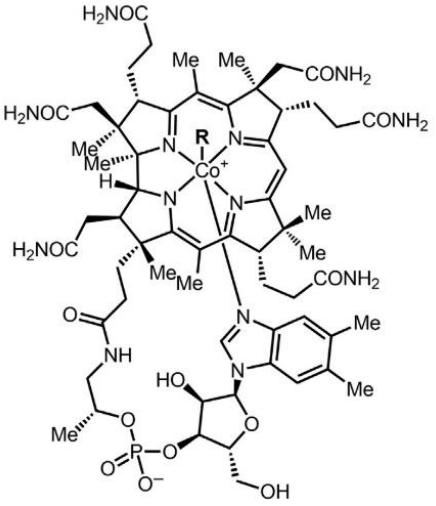
The majority of vitamins are sourced from plants, although they are commonly consumed indirectly from higher up the food chain in meals of animal origin (meat, eggs and dairy) (Kennedy, 2016). Vitamin B12, the vitamin with the biggest structure, being the exception, is a product from bacterial fermentation and is normally sequestered from animal-based meals (Silverman & Brauer, 2008). A healthy adult should be able to acquire all their vitamin requirements from their regular diet (consisting of high consumption of fruit, vegetables, legumes, complex instead of simple carbohydrates, olive oil, and moderate consumption of fish and white meat) (Kennedy, 2016). However, populations with a risk of vitamin deficiency, such as the elderly, pregnant women, and vegans, may not meet the Recommended Daily Allowances (RDA) and therefore vitamin supplementation may be necessary.

In comparison to other types of nutrients, vitamins breakdown (catabolism) does not give considerable energy or structural functions to the organism. Instead, very small amounts of vitamins are required to fulfill specific and distinct activities of health maintenance and metabolic integrity (Ofoedu et al., 2021).

2.1. Vitamin B12

Belonging to the B-complex, Vitamin B12 is a water-soluble vitamin, discovered in 1926, also known as Cobalamin (Cbl). This vitamin has a very complex structure and is the only vitamin containing metal – central cobalt atom (Ofoedu et al., 2021; Vandamme & Revuelta, 2016). In the environment, vitamin B12 occurs in many forms, namely: hydroxocobalamin, cyanocobalamin, nitritocobalamin, methylcobalamin and 5-

deoxyadenosylcobalamin, depending on the on the groups attached to the cobalt (O'leary & Samman, 2010).



R	Colalamin isoform
-OH	Hydroxocobalamin
-CN	Cyanocobalamin
$-CH_4$	Methylcobalamin
$-C_{10}H_{13}N_5O_3$	Deoxyadenosylcobalamin

Figure 2 – Vitamin B12 structure. Retrieved from (Rizzo & Laganà, 2019)

As previously stated, vitamin B12 is synthesized by certain bacteria in the gastrointestinal tract of animals and subsequently absorbed by the host animal, therefore it can only be found animal-derived foods, with the liver being the most abundant source (Rizzo & Laganà, 2019). There are no bioactive forms of vitamin B12 from plant sources (O'leary & Samman, 2010). Cyanocobalamin and hydroxocobalamin are the only isoforms suitable for pharmaceutical and food applications, although cyanocobalamin is preferred for products like additives, supplements, fortified foods, or medical products, since it has better stability (Kumar et al., 2010; Ofoedu et al., 2021).

Vitamin B12 plays an essential role in various body functions such as normal growth and development (especially in children), normal production of red blood cells (conjunction with folate), nerve system function, DNA synthesis, processing and burning of fat and carbohydrate, and participates in the conversion of homocysteine to methionine (Ofoedu et al., 2021; Silverman & Brauer, 2008; Vandamme & Revuelta, 2016). The lack or malabsorption of this vitamin can cause health problems such as anemia (Coelho et al., 2021).

The amount and kind of protein ingested, as well as the individual's gastrointestinal absorption ability (directly linked with ageing), have been found to affect vitamin B12 absorption and bioavailability in normal adults (O'leary & Samman, 2010). Cbl is escorted through the organism by a complex system of carriers from the oral cavity to the

ultimate cellular location. In the stomach is synthesized a binding protein (Intrinsic factor) so that vitamin B12 can be absorbed in the terminal ileum (Rizzo & Laganà, 2019; Silverman & Brauer, 2008). Since Cbl is a water-soluble vitamin, the excess is excreted with urine.

Regarding the stability, aqueous solutions and crystalline forms of Cbl are considered stable when protected from light exposure (Cârlan, 2021). However, part of vitamin B12 is degraded and loses activity during cooking (when reach temperatures higher than 120 ºC) and storage (Coelho et al., 2021). Also, in the presence of compounds like ascorbic acid, thiamine, niacin, strong acids, metals and UV light, VitB12 is susceptible to degradation (Mukherjee & Sen, 2011).

Considering the aforementioned, when vitamin B12 cannot be acquired naturally or is insufficient (risk populations), synthetic versions are required for dietary and medicinal purposes (fortified food and supplements). Microencapsulation is a suitable way to make these products since it has various benefits such as enhanced stability, protection from external factors, easier handling and storage, and ensuring organoleptic qualities, among others (Carlan et al., 2017).

3. Microencapsulation

Microencapsulation is an emerging and promising technology that is defined as the entrapment of small particles (gases, liquids or solids), known as “core material”, in a polymeric matrix, named “wall material”, “shell”, “coating”, “carrier” or “encapsulant”, resulting in microstructures with a size distribution ranging from 1-5000 μm, different morphology and different internal structure (Huang et al., 2021; Peanparkdee et al., 2016).

The microencapsulation technique is used in various fields, including food, nutraceutical, pharmaceutical, agricultural, cosmetics and textile industry (Choudhury et al., 2021; Huang et al., 2021; Peanparkdee et al., 2016). The main purposes of this technique are to protect the core material/active ingredients from degradation from various unfavorable factors (pH, light, temperature, moisture, enzymes and oxygen) to enhance their bioavailability, preservation and stability (Castro Coelho et al., 2021; Drosou et al., 2017), extend shelf-life (Peanparkdee et al., 2016), improve the organoleptic characteristics by masking unpleasant aroma, texture and flavor (Choudhury et al., 2021) and control the release behavior in order to deliver the bioactive compounds

to the target site, at a particular time and under particular conditions (Castro Coelho et al., 2021; Choudhury et al., 2021).

3.1. Microencapsulation techniques

Various methods are available for the encapsulation of core materials and can be divided into three main types: chemical, physical and physicochemical (Peanparkdee et al., 2016; Venkata et al., 2010). The commonly used chemical methods include cross-linking, polymerization, etc. Physical microencapsulation methods comprise spray-drying, freeze-drying, fluidized bed coating, extrusion, etc. And physicochemical methods are ionotropic gelation, supercritical fluids, simple and complex coacervation, electrohydrodynamic techniques - electrospinning and electrospray, etc (Huang et al., 2021).

3.1.1. Electrohydrodynamic techniques: Electrospinning and Electrospray

Electrohydrodynamic processes (EHD) are appealing alternatives of encapsulation due to its versability and aim to improve microstructures/nanostructures production. EHD arises as an option to overcome some disadvantages associated with conventional encapsulation techniques such as high temperatures and the use of reagents with extreme pH (Castro Coelho et al., 2021; Silva et al., 2021).

As described in Huang et al., 2021, electrospinning has been reported to encapsulate a wide range of bioactive compounds, just like other microencapsulation techniques, such as hydrophobic compounds (lipids, vitamins, curcumin, essential oils, etc), hydrophilic compounds (anthocyanin, gallic acid, etc), enzymes and probiotic. Regarding antioxidants many have been reported in studies such as caffein, green tea polyphenols, β-carotene, folic acid and many others (Aguiar et al., 2016; Li et al., 2009).

EHD processes use high-voltage electrostatic fields to charge the surface of a polymer solution jet, ensuring microstructures with a large area to volume ratio and high porosity (Castro Coelho et al., 2021; Mendes & Chronakis, 2021) and can be operated in two basic and similar methods: electrospinning, that produces microfibers; electrospray, that similarly produces microparticles (Silva et al., 2021). The main difference between electrospinning and electrospray is the polymer concentration since electrospray occurs when the polymer concentration is low enough to destabilize the jet and form fine charged droplets (Castro Coelho et al., 2021; Mendes & Chronakis, 2021).

The typical setup for these two methods is identical and consists of four main components, the schematic setup is represented in Figure 3: (i) a metal spinneret that usually consists in a blunt ended stainless-steel needle of varying diameters, (ii) syringe pump to control the flow rate, (iii) a high voltage power supply (1-30 kV) that applies high fields to the tip of the needle and usually operated in direct current mode and (iv) a grounded collector, that can be a metal flat plate or rotating drum (Akdere & Schneiders, 2021; Anu Bhushani & Anandharamakrishnan, 2014; Silva et al., 2021).

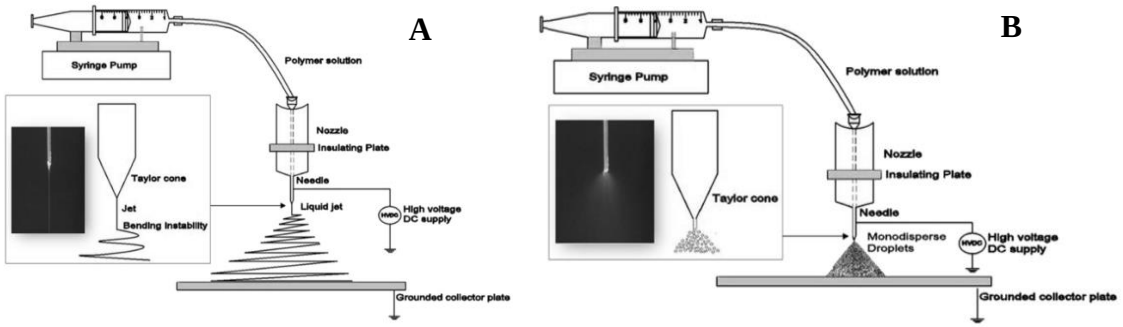


Figure 3 - Schematic illustration of the basic setup of electrospinning (A) and electrospray (B). Retrieved from Anu Bhushani & Anandharamakrishnan, 2014.

The solution (only the polymer or polymer plus the core material) is fed through to a syringe needle (spinneret) at a specific flow rate, by the pump, until a droplet of solution collects at the tip. As shown in Fig. 3, a high voltage is applied to the tip of the spinneret and generates an electric field between the needle and the grounded collector (Davoodi et al., 2021; Silva et al., 2021).

Electrospinning fundamentals

In electrospinning, when the droplet on the tip of the syringe is exposed to the high voltage potential it induces the accumulation of free charges in the polymer's solution surface. Due to two major electrostatic forces, particularly electrostatic repulsion of like charges and the Coulombic force of external electric field, the surface of the droplet is distorted into a conical shape and the so-called Taylor cone is formed, named after Geoffrey Ingram Taylor, the first scientist to describe this phenomenon. The development of the Taylor cone is essential but only occurs in a limited set of operational conditions (Akdere & Schneiders, 2021; Anu Bhushani & Anandharamakrishnan, 2014; Castro Coelho et al., 2021; Davoodi et al., 2021). Once the electrostatic force overcomes the surface tension, the charged polymer is ejected from the tip of the Taylor cone towards the collecting plate, as in Fig. 1. During the course of the jet from the nozzle to the

collector, the unevenly distributed charges cause whipping or bending motion of the jet causing elongation and the solvent quickly evaporates resulting in the depositions of fibers, with a micro/nano diameter range, in the collector (Akdere & Schneiders, 2021; Anu Bhushani & Anandharamakrishnan, 2014; Davoodi et al., 2021; Silva et al., 2021).

Electrospray fundamentals

As for the electrospray, the setup and the basic operation is the same as the electrospinning technique, but the fundamentals are different since electrospray is a process of liquid atomization by electrical forces (Anu Bhushani & Anandharamakrishnan, 2014). In electrospray, as said before, the solution concentration is low causing jet destabilization and so the elongation doesn't take place. Thus, instead of fibers, fine droplets are formed resulting in polymeric micro/nano particles deposited in the collector (Anu Bhushani & Anandharamakrishnan, 2014; Castro Coelho et al., 2021).

Factors affecting electrohydrodynamic processes

Given the complexity of these processes there are several conditions and parameters that can be manipulated and adjusted in order to obtain microstructures with different characteristics. These factors can influence the jet movement and can be categorized into three different groups: solution parameters, process variables and environmental conditions; with the first two being the most important and easier to control (Akdere & Schneiders, 2021; Castro Coelho et al., 2021). Not all parameters are independent but rather exert an influence on other factors.

- Solution parameters

Solution parameters that affect EHD processes include polymer's molecular weight (M_w), concentration and conformation of the chain, type or mixture of solvents, viscosity, conductivity and surface tension (Castro Coelho et al., 2021; Davoodi et al., 2021; Silva et al., 2021).

Polymer concentration and M_w significantly influence the solution viscosity and are important parameters to manipulate to optimize the products obtained by EDH processes since low-M_w polymers are more adequate to produce particles while high-M_w polymers tend to produce fibers. Normally, an increase in the viscosity (increasing polymer's concentration or M_w) of the solution corresponds to formation of electrospun fibers with

bigger diameters and more uniform, however there is an optimum range of polymer concentration, since the resistance of the polymer jet to deformation by elongation in also increased (Castro Coelho et al., 2021; Silva et al., 2021).

The solvent choice can greatly influence essential parameters such as viscosity, surface tension and conductivity. Appropriate solvents should be chosen in order to show a good polymer solubility, low surface tension that allows the formation of Taylor cone, since the applied voltage must be able to overcome it, but sufficient to stabilize the process (addition of surfactants to lower the surface tension is a current practice), and display an adequate volatility allowing a proper evaporation during the polymer path from the needle tip to the collector which will determine the porosity of the obtained structures (Castro Coelho et al., 2021; Davoodi et al., 2021; Silva et al., 2021).

In general, solution conductivity determines the charge density along the polymer jet, by affecting the surface free charges at the fiber surface, that will determine if the solution has viable spinning characteristics. Extreme conductivity levels, low or high, can interfere and block the EHD processes (Davoodi et al., 2021; Silva et al., 2021).

- Process variables

The process variables or operating parameters that have influence in EDH processes include applied voltage, tip-to-collector distance, solution flow rate and needle diameter (Castro Coelho et al., 2021).

An adequate solution flow rate is needed to have proper jet velocity and maintain the electrospinning or electrospray process. Usually, the employment of a high flow rate is associated with the formation of beaded fibers from electrospinning processes since the porosity of the fibers increase, on the other hand low flow rates (but sufficiently high to maintain the process) are commonly used in electrospray as they produce more spherical particles (Castro Coelho et al., 2021; Davoodi et al., 2021; Silva et al., 2021).

A crucial element is the voltage that is applied to the solution. The variation of this parameter controls the electrical field strength between the spinneret and the collector and so the strength of the drawing force. The applied voltage is strictly associated with the flow rate since an increase of the flow rate may require an increase in the applied voltage in order to stabilize the Taylor cone (Ghorani & Tucker, 2015). The manipulation of the applied voltage is also a crucial parameter to the microstructure diameter since an increase in the applied voltage contribute to smaller diameters, until an optimal point (Castro

Coelho et al., 2021). The applied voltage and the tip-to-collector distance are closely related. For instance, when the distance is increased, the electric field intensity decays and generates a need to increase the applied voltage (Davoodi et al., 2021; Ghorani & Tucker, 2015; Silva et al., 2021).

The needle diameter also plays an important role in this type of microencapsulation processes. As the needle diameter increases, clogging may occur at the needle tip as the result of the surface tension effects. With the needle diameter decrease the surface tension of the droplet increases and consequently the initial acceleration of the jet and the average velocity decreases resulting in a slower solvent evaporation (Ghorani & Tucker, 2015).

- Environmental conditions

The environmental conditions, or ambient parameters, are the more challenging factors that affect EDH processes to control but have an unquestionable impact in these processes. These include temperature, humidity and air flow/velocity in the process chamber. All these factors affect the morphology and the diameter of the microstructures. The temperature has an important effect on the rate of solvent evaporation, viscosity and surface tension (Davoodi et al., 2021; Ghorani & Tucker, 2015; Haider et al., 2018).

3.2. Encapsulating agents

The encapsulating agents used in microencapsulation techniques range from biopolymers, synthetic polymers and non-polymeric systems (Anu Bhushani & Anandharamakrishnan, 2014). Some examples of biopolymers are carbohydrates (starch, cellulose, pectin, guar gum, carrageenan, alginate, dextran, etc), lipids, proteins (whey protein isolate, soy protein, egg albumen, collagen, casein, zein), etc (Anu Bhushani & Anandharamakrishnan, 2014; Choudhury et al., 2021; Ghorani & Tucker, 2015; Kai et al., 2014).

To choose the appropriate encapsulating agent it is necessary to have in account some requirements, such as:

- To be non-reactive with the core material;
- Provide the desired coating specifications;
- Allow controlled release under different and certain conditions;
- Protect the core from external conditions.

In the present study, the encapsulating agents chosen were Zein (protein) and Pullulan (polysaccharide). The choice was made considering previous studies and taking into account the advantages and disadvantages of these biopolymers.

3.2.1. Zein

Zein is a prolamin protein extracted from the corn endosperm and represents 35-60% of total proteins in corn and 44-79% of all endosperm proteins (Silva et al., 2021). The zein amino acid profile is made almost from hydrophobic types, including leucine (19%), alanine (12%) and proline (10%). It also contains glutamic acid (20%) and glutamine residues but is low in lysine and tryptophan (Giteru et al., 2021). Based on solubility and conformational arrangements, zein can be classified into 4 different types: α-zein (19 and 22 kDa), β-zein (14 kDa), g-zein (16 and 27 kDa) and d-zein (10 kDa). Off all types, α-zein is the main and accounts for 75-85% of the whole zein in corn, also is the most abundant type in commercial zein (Silva et al., 2021; X. Zhang et al., 2021).

This hydrophobic protein is widely used for the processing and encapsulation of compounds due to its characteristics such as low moister absorption, high thermal resistance, oxygen barriers and hydrophobicity (Coelho et al., 2022; Giteru et al., 2021). Due to the high content in hydrophobic amino acids, zein is insoluble in water and is only soluble in limited types of solvents such as aqueous alcohols, alkaline solutions and aqueous solution with the addition of high concentrations of urea or/and anionic detergents (Silva et al., 2021; X. Zhang et al., 2021).

Regarding the applications, zein has been used in a wide range of industries, especially in pharmaceuticals and food since it is considered a safe food ingredient by the Food and Drug Administration (FDA) (Coelho et al., 2022). Moomand & Lim, 2015 suggested that zein can be a promising matrix to encapsulate omega-3-rich-fish-oil through electrospinning/electrospray. Also, the potential of electrospray zein microstructures to increase bioaccessibility of carotenoids after digestion was reported by Gómez-Mascaraque et al., 2017.

3.2.2. Pullulan

Pullulan is an extracellular linear polysaccharide synthesized by *Aureobasidium pullulans*, yeast-like fungi, from starch (Qian et al., 2013). It consists of maltotriose units (three glucopyranose units linked by α-1,4 glycosidic bonds) connected each other by an α-1,6 glycosidic bond (Lochhead, 2017).

The combination of linkages (1→4 and 1→6) is relatively distinctive and results in unique characteristics, such as adhesiveness, structural flexibility, enhanced solubility, oxygen permeability and electrospinnability (Angel et al., 2022; Qian et al., 2013). Pullulan differs from most polysaccharides in that it is easily soluble in water, as a consequence of the low degree of hydrogen bonding in its crystal form, but is also soluble in formic acid and DMSO (Angel et al., 2022; Lochhead, 2017). This polysaccharide is stable regardless of pH and temperature changes (Wilk & Benko, 2021).

Pullulan demonstrates promising potential in pharmaceuticals, cosmetics, food packaging, biomedical purposes, and electronics (Angel et al., 2022). Is widely used in the food industry (FDA accepted as a safe compound) due to its appealing characteristics, namely low digesting, no flavor neither odor, hence is a low-calorie food additive that provides bulk and texture. Additionally, is employed in pharmaceuticals and electronics, particularly in tissue engineering, drug delivery systems, as coating agent and film/fiber-forming agent (Nasrollahzadeh et al., 2019).

Regarding encapsulation and delivery systems, pullulan is a desirable encapsulating agent because it is blood compatible, non-toxic, elicits a low immune system response, is not mutagenic or carcinogenic and it is not attacked by the digestive enzymes in the human gut (Angel et al., 2022). Lee et al., 2017 reported that pullulan used as an encapsulating agent to encapsulate rutin-Pluronic SDs may be a promising alternative for enhancing the water-solubility and UV stability of the active agent.

Materials and Methods

4. Chemical compounds and reagents

Zein (grade Z3625) and Pullulan powder were purchased from Sigma-Aldrich and used as received, without further purification. EGCG from green tea and Vitamin B12 were purchased from Sigma-Aldrich. The 70% aqueous ethanol solution was purchased from VWR BDH-chemicals and used as solvent. Deionized water - Milli Q water, resistivity of 18.2 MΩ/cm at room temperature (~23 °C) – was used for the *in vitro* release assays and the construction of calibration curves.

5. Microstructure production

5.1. Sample preparation

Two types of solutions were prepared: the biopolymers solutions (Zein and Pullulan) and the active compounds solutions (EGCG and VitaminB12).

Zein and pullulan solutions were prepared by dissolving each powder in aqueous ethanol solution and deionized water, respectively, until completely dissolved (at room temperature, ~23 °C). Solutions with different zein concentrations (w/v, weight of zein/ volume of solvent) were prepared in 70% (v/v) ethanol, ranging from 1% to 30% w/v (Table 1). For the solution with pullulan, two different pullulan concentrations (w/v, weight of pullulan/volume of solvent) were prepared in deionized water, 10% and 15% w/v (Table 1).

The EGCG solutions were prepared by dissolving the powder in 70% (v/v) aqueous ethanol (for the samples with zein) and in deionized water (for the samples with pullulan). The EGCG concentration used range between 0.5%, 1% and 5% (w/w, weight of EGCG/weight zein or pullulan)), represented in Table 1. The Vitamin B12 solutions were prepared the same way as the EGCG solutions, and the conditions are represented in Table 1. Were also made samples with a mixture between EGCG and VitB12 (co-encapsulation), the concentrations are presented in Table 1.

For the electrospinning process, EGCG, VitaminB12 and mixture solutions were added to the polymer's solutions, according to what is shown in Table 1, and stirred to ensure a good mixture.

The samples are identified according to a code so that it is easier to understand: biopolymer : concentration of the biopolymer : active ingredient(s) : concentration of the active ingredient(s).

Table 1 - Description of the solution conditions tested in electrospinning process for the samples containing EGCG and the samples containing Vitamin B12.

Process	Biopolymer	Active ingredient	Biopolymer concentration (% w/v)	Active ingredient concentration (% w/w)	SAMPLE
Electrospinning	Zein	EGCG	1	1	**Z:1:E:1**
			5	1	**Z:5:E:1**
			15	1	**Z:15:E:1**
			30	1	**Z:30:E:1**
			30	5	**Z:30:E:5**
			30	0.5	**Z:30:E:0.5**
		Vitamin B12	1	1	**Z:1:B:1**
			5	1	**Z:5:B:1**
			15	1	**Z:15:B:1**
			30	1	**Z:30:B:1**
			30	5	**Z:30:B:5**
			30	0.5	**Z:30:B:0.5**
		EGCG + Vitamin B12	1	0.5+0.5	**Z:1:EB:1**
			5	0.5+0.5	**Z:5:EB:1**
			15	0.5+0.5	**Z:15:EB:1**
			30	0.5+0.5	**Z:30:EB:1**
			30	2.5+2.5	**Z:30:EB:5**
			30	0.25+0.25	**Z:30:EB:0.5**
	Pullulan	EGCG	10	1	**P:10:E:1**
			15	1	**P:15:E:1**
		Vitamin B12	10	1	**P:10:B:1**
			15	1	**P:15:B:1**

5.2. Production of microstructures by electrospinning

Different microstructures were produced (particles, fibers and films) using electrospinning technique. The electrospinning experimental set-up, equipped with a variable high-voltage 0-20 kV power supply, was supplied by Spraybase® (Dublin, Ireland), Figure 4.

The biopolymer solutions were electrosprayed under a steady-state flow rate using a stainless-steel needle situated towards a metal collector plate. The optimal process conditions were considered (needle diameter, tip-to-collector distance, flow rate and applied voltage) and described in Table 2. The process occurred at room temperature (~23 °C).

In the end of the process, the microstructures obtained were scraped from the collector and placed in plastic boxes. The samples were kept in the refrigerator (~4 °C) to preserve their characteristics and to be able to analyze them later.

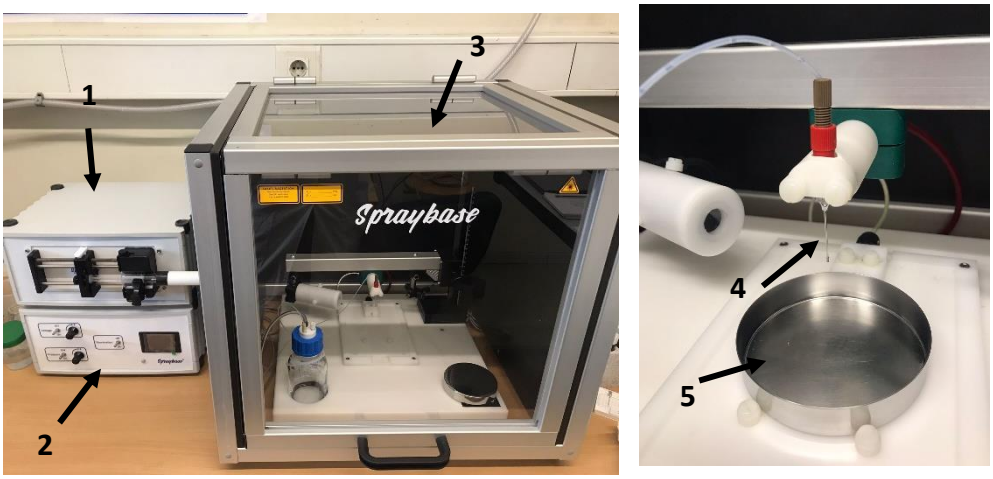


Figure 4 - Experimental set-up for the production of zein microstructures containing EGCG. 1-Syringe pump; 2-high voltage supply; 3-electrospinning chamber; 4-needle; 5-collector plate.

Table 2 - Description of the process conditions tested in electrospinning for all the samples.

Process	SAMPLE	Flow rate (mL/h)	Applied voltage (kV)	Needle (Gauge)	Tip-to-collector distance (cm)
Electrospinning	**Z:1:E:1**	0.6	20	22	5
	Z:5:E:1	0.3			
	Z:15:E:1	0.6			
	Z:30:E:1	3			
	Z:30:E:5	1.5			
	Z:30:E:0.5	1.5			
	Z:1:B:1	0.6			
	Z:5:B:1	0.3			
	Z:15:B:1	0.6			
	Z:30:B:1	3			
	Z:30:B:5	1.5			
	Z:30:B:0.5	1.5			
	Z:1:EB:1	0.6			
	Z:5:EB:1	0.3			
	Z:15:EB:1	0.6			
	Z:30:EB:1	3			
	Z:30:EB:5	1.5			
	Z:30:EB:0.5	1.5			
	P:10:E:1	0.25	10		
	P:15:E:1	0.5	15		
	P:10:B:1	0.25	10		
	P:15:B:1	0.5	15		

6. Microstructure characterization

The morphology and shape of the microstructures was evaluated in different areas of the prepared sample by scanning electron microscopy (SEM). Also, the release profiles of the samples in water was performed.

6.1. Scanning electron microscopy (SEM)

SEM was performed with a Fei Quanta 400 FEG ESEM/EDAX Pegasus X4M equipment (Eindhoven, The Netherlands) at Centro de Materiais da Universidade do Porto (CEMUP), Porto, Portugal. A small portion of each sample was fixed on a brass stub using double-side adhesive tape followed by the vacuum coating with a thin gold layer.

SEM is a method to image surfaces of samples (micro and nano structures) with a high resolution, accuracy and is a very versatile tool. Through the collection and processing of signals generated by a sharp electron probe, SEM provides information about the composition and topography of the samples (Henning & Adhikari, 2017).

Many advantages can be enumerated, such as the vast range of adjustable magnifications that can be used, capability to produce images with high plasticity and almost stereoscopic appearance ("image to imagination") and the power to obtain information about the composition of samples by choosing the signals that originate from specific interactions (Henning & Adhikari, 2017).

6.2. *In vitro* release assays

The *in vitro* release assays were performed for all samples in deionized water. Water was selected as a solvent in order to simulate industrial aqueous formulations that are the most common medium applied on food, cosmetic and pharmaceutical products.

The *in vitro* release assays were carried out by placing an amount of each sample in 3 mL of water that would correspond to a concentration of 0.005 mg/mL of active ingredient (EGCG and/or B12), in a cuvette with magnetic stirring, and measured the absorbance at a wavelength of 274 nm (EGCG) and 361 nm (VitB12) using a NanoDrop One-C spectrophotometer (Themo Fisher Scientific USA). The release of the core was determined by continuous absorbance measurements (reading intervals of 30s until 1500s and then intervals of 120s) until the value of absorbance was stable. All the assays were carried out at room temperature (~23 °C) and in triplicate.

The EGCG and VitB12 concentration were evaluated by using linear calibration curves.

6.2.1. Calibration curve

In order to be able to relate the absorbance measured and the concentration of EGCG and VitB12 in the samples, linear calibration curves were made for the release in deionized water.

The EGCG calibration curve was prepared with eight standard solutions with concentrations ranging from 0.0001 mg/mL and 0.1 mg/mL. The absorbance was measured at 274 nm. The calibration curve of Vitamin B12 was made with ten standard solutions with concentrations between 0.002 mg/mL and 0.05 mg/mL and the absorbance measured at 361 nm. Both correlation coefficients obtained were above 0.999 and the measurements were made in triplicate. More details about the calibration curves are presented in Appendix A.

7. Microencapsulation efficiency

The success of the microencapsulation process was determined by the encapsulation efficiency. During the encapsulation process, not all the active ingredient will get inside of the microstructures, some part will remain outside or at the surface.

The encapsulation efficiencies (EE) of the EGCG and/or Vit B12 into the zein and pullulan microstructures were estimated taking into account the release and a mass balance and is defined as the percentage ratio of the total quantity of catechin and/or vitamin contained inside the microstructures and the total amount of active ingredient used during the electrospinning method (Equation 1).

The EGCG or VitB12 released immediately (time zero) in the release assays was assumed to be the amount of EGCG/VitB12 that was not encapsulated, and the total EGCG/VitB12 released in the end of the release assays corresponds to the total amount of EGCG/VitB12 (inside and outside the microstructures). Thus, it is possible to determine the amount of EGCG entrapped inside the microstructures.

From the graph that represents the amount of active substance released during the release assay, it is possible to calculate the EE by the difference between the absorbance in the end of the release test and the absorbance in time zero, according to Equation 2.

$$EE\ (\%) = \frac{Total\ amount\ released - Amount\ released\ in\ time\ zero}{Total\ amount\ released} \times 100 \quad (1)$$

$$EE\ (\%) = \frac{Final\ Abs - Initial\ Abs}{Final\ Abs} \times 100 \quad (2)$$

Results and Discussion

The main goal of the present work was the production of biopolymeric microstructures containing a catechin - EGCG, and a vitamin - vitamin B12, through electrospinning technique and with high encapsulation efficiency in order to evaluate their potential for application in the food, pharmaceutical and cosmetic industries. To achieve this objective, two different encapsulating agents of biological origin were used (zein and pullulan) to produce different microstructures, with different characteristics. An aspect that was evaluated was the effect of the zein, pullulan, EGCG and Vitamin B12 concentration on the microstructures.

The zein microstructures were done with Vitamin B12, EGCG and a mixture of both and the pullulan microstructures were done with Vitamin B12 and EGCG. In the end, this results in twenty-two distinct structures: eight with EGCG, eight with VitB12, six with mixture of EGCG+VitB12.

The procedures taken to create the microstructures throughout this experimental study were identical for all the active agents previously mentioned. It began with the production of the solutions, both active component and polymer solutions. Immediately before the electrospinning process, both solutions (active agent and biopolymer) were mixed. Then, the mixture was fed to the electrospinning and, using pre-defined conditions (the applied voltage, needle and tip-to-collector distance were kept constant; the flow-rate and concentrations were adjusted in accordance with the desired microstructure), the microstructures were produced for a certain amount of time.

The microstructures obtained were characterized by size, morphology and release profiles by the application of kinetic models. The encapsulation efficiency was also assessed.

The validation of the methods is described in detail in Appendix A.

In the present section, the results of all the characterizations made during this study are stated and discussed, which allows a final assessment of the encapsulation procedure, as well as of the microstructures production.

8. ZEIN

8.1. Scanning Electron Microscopy (SEM)

Zein microstructures, produced by electrospinning, containing EGCG, VitB12 and the mixture of both were evaluated using scanning electron microscopy, SEM, Figure 5. The images obtained were useful to characterize the morphology and size of the microparticles and electrospun fibers, since are crucial factors to evaluate when considering industrial applications since there must be a guarantee of consistency, similarity and replicability of the products.

As observed in Figure 5, as the zein concentration increases, regardless of the active agent, the morphology of the structures tend to be different. The higher concentration of zein (30% w/v) used formed fibers, the intermediate concentration (15% w/v) presents a mixture between fibers and particles and the lower concentrations (1 and 5% w/v) formed different particles. Coelho et al., 2021 studied the production of zein microstructures containing Vitamin B12 by electrospinning, the parameters used were similar to the ones used in the present work, and higher zein concentration promoted the formation of fibers and the formation of microparticles was verified in lower zein concentrations. These results are in consistence with Hosseini et al., 2021 that reported solution concentration as one of the most important parameters in the morphology control during electrospinning process.

Samples with 1% (w/v) zein, Fig. 5A, 5G and 5M, have a peculiar shape since appears to be small and flattened particles, thin and porous, forming structures similar to small films. A heterogenous matrix for microstructures of 1% (w/v) zein in 70% ethanol was reported by Coelho et al., 2022, which was not the case. The samples produced with 5% (w/v) zein, Fig. 5B, 5H and 5N, shows small round particles with rough surface. These microstructures were produced with a low polymer concentration and for that reason the process works as electrospray instead of electrospinning forming fine droplets instead of fibers (Castro Coelho et al., 2021). As the zein concentration increases, the formation of fibers instead of microparticles is visible since in Fig. 5C, 5I and 5O, it is possible to observe the formation of a mixture between microparticles connected with fibers due to the encapsulation operation being a hybrid between electrospinning and electrospray, since is an intermediate polymer concentration.

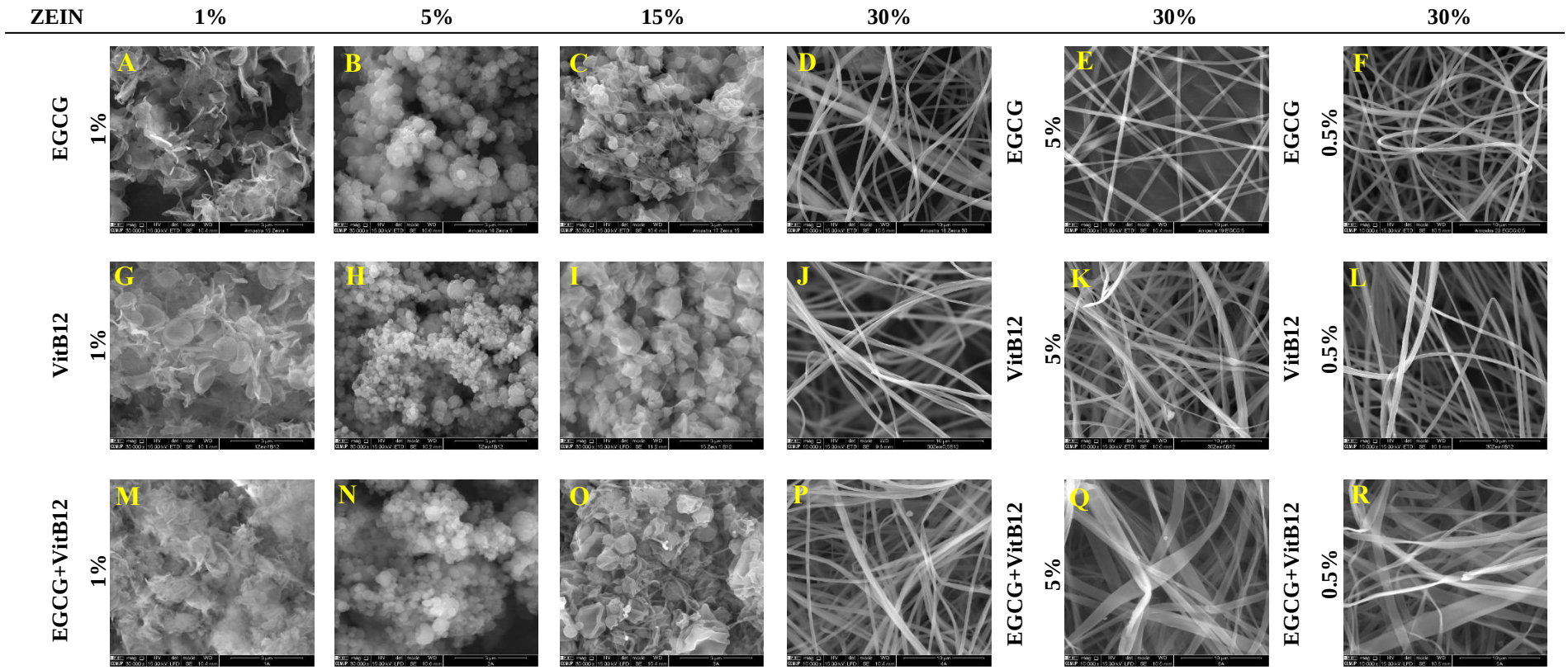


Figure 5 – SEM images of electrospinning microstructures containing different concentrations of zein, EGCG and VitB12. Magnification of 30 000x for A, B, C, G, H, I, M, N and O and 10 000x for D, E, F, J, K, L, P, Q and R, beam intensity (HV) 15.00 kV, distance between the sample and the lens (WD) less than 10.6 mm, scale bar of 3 μm for A, B, C, G, H, I, M, N and O, and 10 μm for D, E, F, J, K, L, P, Q and R.

The microparticles presented in figures 5C, 5I and 5O have an irregular surface, as if possesses an outer skin that has imploded inwards as the solvent evaporates (Li et al., 2009). The samples with 30% (w/v) zein, Fig. 3D-F, 3J-L and 3P-R, presented the formation of homogeneous and continuous fibers, with a slightly rough surface and shape between tubular (30% w/v zein + EGCG or VitB12) and ribbon-like shape (30% w/v zein + (EGCG+VitB12)).

The switch from the formation of microparticles to fibers is probably due to the greater resistance of the high concentration solutions to be stretched towards the collector or due to the electrical conductivity of the solution (Hosseini et al., 2021). Also, higher polymer concentrations result in a low intermolecular entanglement between polypeptide chains, which would lead to the formation of fibers instead of spherical structures after solvent evaporation (Erdogan et al., 2019). Additionally, the increased polymer concentration will possibly increase the interaction between the zein and the 70% ethanol, reducing the propensity to form droplets.

The morphology of the samples was not affected by the type or concentration of the core material, confirming that the type and concentration of encapsulating agent is the key to form different structures through electrospinning. However, the co-encapsulating microfibers appear to have a width larger than the fibers produced with only one component.

8.2. *In vitro* Release Assays

Protection and controlled release are the biggest advantages of microencapsulation. The bioactive compound release at appropriate time and under desired environments can condition the microstructures effectiveness according to their final application. The behavior of the release is mainly affected by the interactions between the polymeric matrix, the solvent and/or the core material, as well as by other factors such as microstructure type (particles, fibers or films). There are several release mechanisms that differ depending on the encapsulating agent, the technique of preparation, and the environment in which the release happens. It can be based on a single or a mixture of release mechanisms, including processes of diffusion, degradation and biodegradation, dilatation (gel formation and swelling), melting and osmosis (Estevinho & Rocha, 2017).

In the present work, the release assays of the zein microstructures containing EGCG, VitB12 and (EGCG+VitB12) were performed in deionized water, in order to simulate

industrial formulations that are mainly aqueous (food and pharmaceutical products) and because is considered as a hydrophilic food simulant.

Figure 6 displays the obtained release profiles in deionized water of the samples containing different concentrations of zein and core material, showing the release percentage (amount release at a time t normalized by total release amount) over time. The assays were performed during 30 min to 3 hours, depending on the sample. Some measures were deleted due to errors.

In a first approach it is possible to observe that almost all samples present the same release behavior, except the EGCG samples with 1% and 5% (w/v) zein where the release is done immediately. In all other samples is possible to distinguish two different zones: the release zone, which is identified by the increase of the core release, and the stabilization zone that is characterized by a stabilization of this release. The total time to release the core completely is the sum of these two stages.

The accompanying errors between the three measurements performed on each sample, as reflected on each release profile, are more obvious in the release zone, but they are nearly non-existent in the stabilization level. Also, is important to note that the release is unstable and therefore the release profiles fluctuate, especially in the microparticles where zein interfered most with the measurements.

By the interpretations of the graphs, it is possible to observe that as the zein concentration increases, the release time tends also to increase, regardless the type of core material. Thus, the microparticles have a faster release than the fibers in deionized water. This result may indicate that as the zein concentration increases, and consequently the type of microstructure changes, the amount of core released in the first moments decreases and tends to be prolonged and slower. Coelho et al., 2021 reported the same fact with zein microstructures loaded with vitamin B12 released in ethanol.

Comparing release profiles of the samples with different EGCG concentration, it is interesting to note that as the EGCG concentrations decrease, the time necessary to release all the content from the microstructures increases. So, in the samples with 0.5% (w/w) EGCG the release time will be longer than the ones with 1% and 5% (w/w). The same phenomenon also happens with the samples loaded with VitB12 and loaded with the mixture of catechin and vitamin. This result is in accordance with Hosseini et al., 2021

that reported the same behavior with zein fibers loaded with rosemary essential oil, leading to a large initial burst.

When comparing the single samples with the co-microencapsulation ones, it is interesting to note that the release time is longer when the catechin is mixed with the vitamin than when are alone.

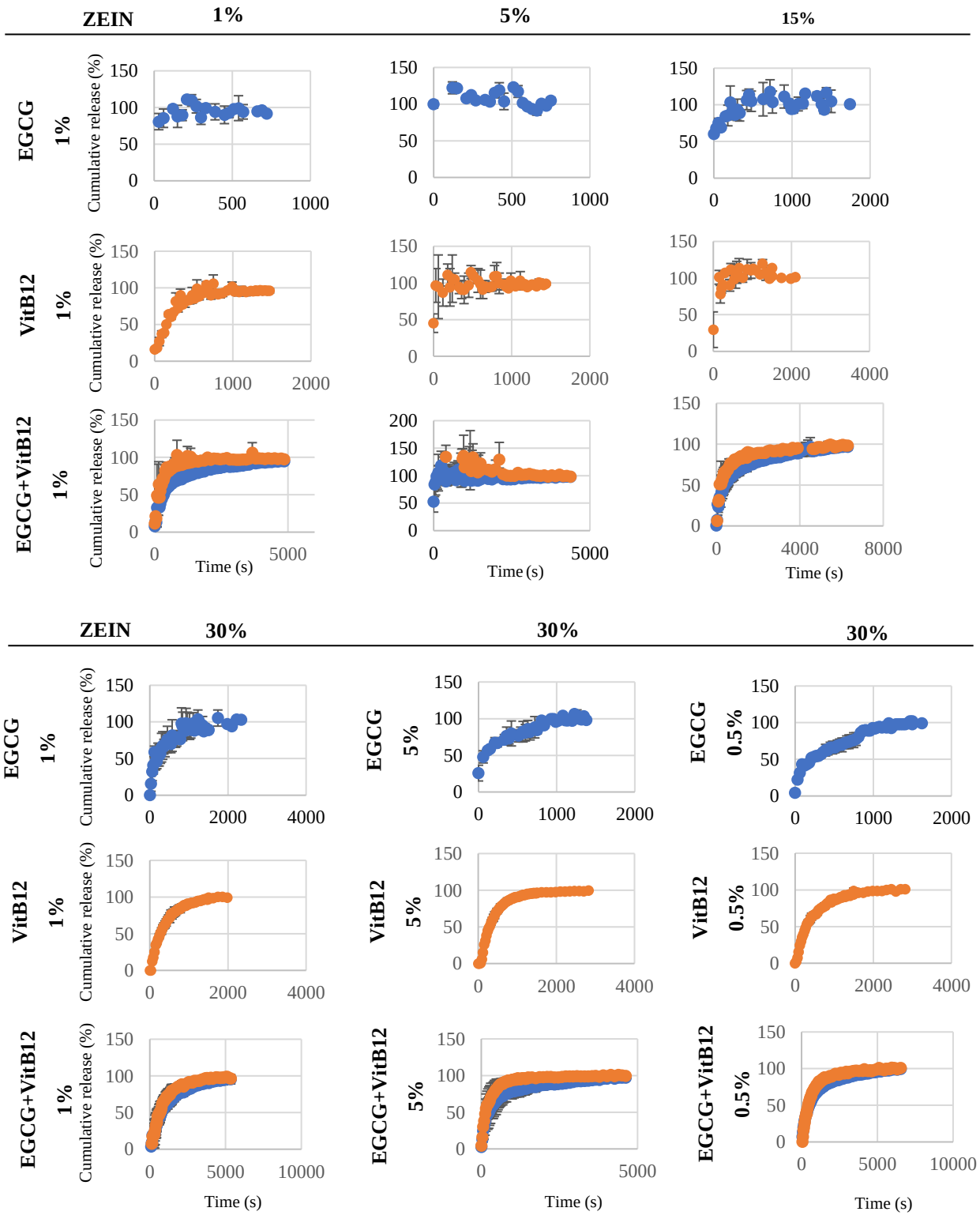


Figure 6 - In vitro release profiles, % of EGCG/VitB12/(EGCG+VitB12) normalized by the total amount released, in H_2O of the microstructures loaded with 1/, 5% and 0.5% of active compounds and 1%, 5%, 15% and 30% zein.

Kinetic models

To the release profiles, obtained for each sample, was adjusted several mathematical models in order to better understand the mechanisms behind the release of the core from the encapsulating agent and evaluate which models better fits the experimental results. The application of these mathematical models helps to design structures with particular characteristics and optimize the process parameters (Estevinho et al., 2013). There are several mathematical models that can be applied to the release profiles, mostly used in food and pharmaceutical industries. In the present work five were fitted to the experimental results: Zero Order, First Order, Korsmeyer-Peppas, Weibull and Baker-Lonsdale model. The different equations of each model are present in Table 8 in Appendix B. The parameters and the correlation coefficients of the five equations fitted to the zein samples are displayed in Table 3 and Table 4. The graphs of the adjustments of the Weibull model to the experimental data is also available in Appendix B. It is important to note that was not possible to fit any model to all the samples with 5% (w/v) zein and the samples with 1% (w/v) zein+EGCG and with 1% (w/v) zein+VitB12 because of the immediate release of the core.

By the analysis of Table 3 and Table 4, it is possible to conclude that the kinetic models that showed a better fit to the experimental results were the Weibull model and the Baker-Lonsdale model, with correlation coefficients that range from 0.761 to 0.994 and 0.845 and 0.998, respectively. However, from these two models, the one that adjusts to a greater number of results is the Weibull model (Baker-Lonsdale model only adjusts to measurements up to 80% of total release). This conclusion applies to the majority of the samples, with exception of Z:30:0.5, where the models with a greater fit are the Korsmeyer-Peppas and Baker-Lonsdale, and Z:30:B:5 (Zero order and Baker-Lonsdale model).

*Table 3 – Parameters and correlation coefficients of each equation from the kinetic models of the zein microstructures loaded with EGCG or VitB12. To samples marked with * was not possible to fit any model due to the fast core release.*

	Zero Order		**First Order**		**Baker-Lonsdale**	
SAMPLE	**K_0**	**R^2**	**K_1**	**R^2**	**K**	**R^2**
Z:1:E:1	*					
Z:5:E:1	*					
Z:15:E:1	5.69E-02	0.765	-6.99E-02	0.738	9.74E-02	0.845
Z:30:E:1	9.91E-02	0.779	-0.804	0.442	1.86E-02	0.970
Z:30:E:5	7.13E-02	0.861	-0.143	0.776	2.35E-02	0.966
Z:30:E:0.5	9.41E-02	0.824	-0.381	0.660	1.34E-02	0.996
Z:1:B:1	1.60E-03	0.889	-0.198	0.750	3.27E-02	0.926
Z:5:B:1	*					
Z:15:B:1	0.144	0.780	-0.249	0.664	6.26E-02	0.992
Z:30:B:1	1.80E-03	0.970	-1.167	0.462	1.61E-02	0.981
Z:30:B:5	0.109	0.985	-0.999	0.587	1.34E-02	0.993
Z:30:B:0.5	9.37E-02	0.956	-0.600	0.536	1.72E-02	0.978

	Korsmeyer-Peppas			**Weibull**		
SAMPLE	**K_k**	***n***	**R^2**	**τ_d (min)**	**β**	**R^2**
Z:1:E:1	*					
Z:5:E:1	*					
Z:15:E:1	0.738	3.16E-02	0.634	0.6	0.403	0.761
Z:30:E:1	0.147	0.956	0.789	5.1	0.519	0.897
Z:30:E:5	0.570	0.124	0.917	3.0	0.439	0.959
Z:30:E:0.5	0.342	0.310	0.990	6.7	0.518	0.972
Z:1:B:1	0.446	0.203	0.729	3.4	0.991	0.918
Z:5:B:1	*					
Z:15:B:1	0.756	0.135	0.877	1.4	0.681	0.765
Z:30:B:1	6.76E-02	1.115	0.919	6.5	1.051	0.994
Z:30:B:5	8.83E-02	0.970	0.905	8.0	0.970	0.979
Z:30:B:0.5	8.66E-02	1.046	0.933	6.9	0.959	0.959

*Table 4 - Parameters and correlation coefficients of each equation from the kinetic models of the zein microstructures loaded with (EGCG+VitB12). To samples marked with * was not possible to fit any model due to the fast core release.*

	Zero Order				**First Order**				**Baker-Lonsdale**			
	Ko		**R2**		**K1**		**R2**		**K**		**R2**	
SAMPLE	**EGCG**	**B12**	**EGCG**	**B12**	**EGCG**	**B12**	**EGCG**	**B12**	**EGCG**	**B12**	**EGCG**	**B12**
Z:1:EB:1	0.056	0.0726	0.887	0.850	-0.168	0.159	0.765	0.725	0.008	0.026	0.976	0.969
Z:5:EB:1	*											
Z:15:EB:1	0.109	0.096	0.849	0.847	-0.872	0.350	0.557	0.705	0.006	0.014	0.978	0.991
Z:30:EB:1	0.037	0.0413	0.974	0.975	-0.139	0.144	0.867	0.893	0.005	0.007	0.992	0.978
Z:30:EB:5	0.147	0.1863	0.969	0.976	-0.807	0.763	0.775	0.516	0.011	0.03	0.989	0.998
Z:30:EB:0.5	0.105	0.0465	0.930	0.933	-0.313	0.285	0.559	0.516	0.007	0.007	0.997	0.987
	Korsmeyer-Peppas						**Weibull**					
	Kk		**n**		**R2**		**Td (min)**		**B**		**R2**	
SAMPLE	**EGCG**	**B12**	**EGCG**	**B12**	**EGCG**	**B12**	**EGCG**	**B12**	**EGCG**	**B12**	**EGCG**	**B12**
Z:1:EB:1	0.316	0.428	0.282	0.238	0.943	0.83	10.7	3.5	0.545	0.526	0.957	0.883
Z:5:EB:1												
Z:15:EB:1	0.169	0.186	0.495	0.605	0.912	0.88	13.6	6.5	0.528	0.488	0.977	0.958
Z:30:EB:1	0.063	0.058	0.766	0.863	0.929	0.950	21.8	16.6	0.894	0.929	0.977	0.979
Z:30:EB:5	0.291	0.375	0.364	0.339	0.973	0.964	7.7	3.2	0.509	0.610	0.987	0.971
Z:30:EB:0.5	0.048	0.038	0.961	1.128	0.860	0.898	15.5	11.2	0.707	0.784	0.978	0.977

The Zero Order model (kinetics with a constant release rate) is applied to releases where the core is a pure material and is release from the system as a pure material (Fickian diffusion) (Estevinho & Rocha, 2017). In the present work, only in the sample Z:30:B:5 the Zero Order model presented a good fit to the results, meaning that the majority of the samples have releases associated with more complex mechanisms. The First Order model is applied to kinetics associated to the core release of solutions, which was not the case in any of the samples.

The Baker-Lonsdale model, the model with higher correlation coefficients, is a model created from the Higuchi model. This model is applied for linearization of the release data from formulations of microcapsules, spherical matrices, and is associated with releases based on a diffusion mechanism (Padmaa Paarakh et al., 2018). However, it fitted well the releases of all type of microstructures, from spherical microparticles to fibers. The constant obtained from this model, K, acquires lower values to slower releases, i.e., in the samples with the mix active agents (EGCG+VitB12).

From the Korsmeyer-Peppas model fitting to the experimental data, it is possible to retrieve the values of the parameter *n* (release exponent) and the values of the constant

K_k. The parameter *n* defines the mechanism responsible for the core release (Estevinho et al., 2013). In the present work, the release profiles of the microstructures obtained are associated with one of the three release mechanisms (based on the *n* obtained from the model fitting):

- "Fickian Diffusion" – molecular diffusion of the core due to a chemical potential gradient ($n<0.43$ for the microparticles; $n<0.45$ for the fibers).
- "Super Case-II Transport" – influenced by the swelling of the matrix ($n>0.85$ for the microparticles; $n>0.89$ for the fibers).
- "Anomalous Transport" – result of the combination of diffusion mechanisms and swelling release ($0.43<n<$ for the microparticles; $0.45<n<0.89$ for the fibers).

The samples that followed a "Fickian Diffusion" were Z:1:B:1, Z:1:EB:1, Z:15:E:1, Z:15:B:1, Z:30:E:0.5, Z:30:E:5 and Z:30:EB:0.5. The remaining samples followed a "Super Case-II transport" except for the Z:15:EB:1 and Z:30:EB:1 that followed an "Anomalous Transport". With these results, it is possible to conclude that the mechanism associated with the core release from the microparticles is mostly the "Fickian Diffusion" and the fibers is "Super Case-II transport".

Lastly, the Weibull model is the most adequate to evaluate the matrix-type (active compound distributed through the encapsulating agent) profile release, which is the case of the structures obtained in the present work. The parameter β, related with the release curve shape, was lower than 1 for all samples except Z:30:B:1 in which the β value was 1.051. So, in the cases that $\beta<1$, the curves showed shape near exponential but with a stepper increase when compared to the ones with $\beta=1$; for $\beta>1$ the profile shows a sigmoidal shape.

Many authors have been applying the kinetic models to the release of active substances from encapsulating agents. Coelho et al., 2021 studied the encapsulation of vitamin B12 in zein, by electrospinning and spray drying techniques, find similar results like the fact that the Weibull model showed, in general, the better fitting for the experimental data and the $\beta<1$ for almost all the cases. Like the results from the present work, the Baker-Lonsdale model also showed high correlation coefficients for some samples.

9. PULLULAN

9.1. Scanning Electron Microscopy (SEM)

Similarly to zein microstructures, pullulan microstructures loaded with EGCG or VitB12 were evaluated using scanning electron microscopy, Figure 7. From the images obtained was possible to characterize the morphology of the microstructures produced with different pullulan concentrations.

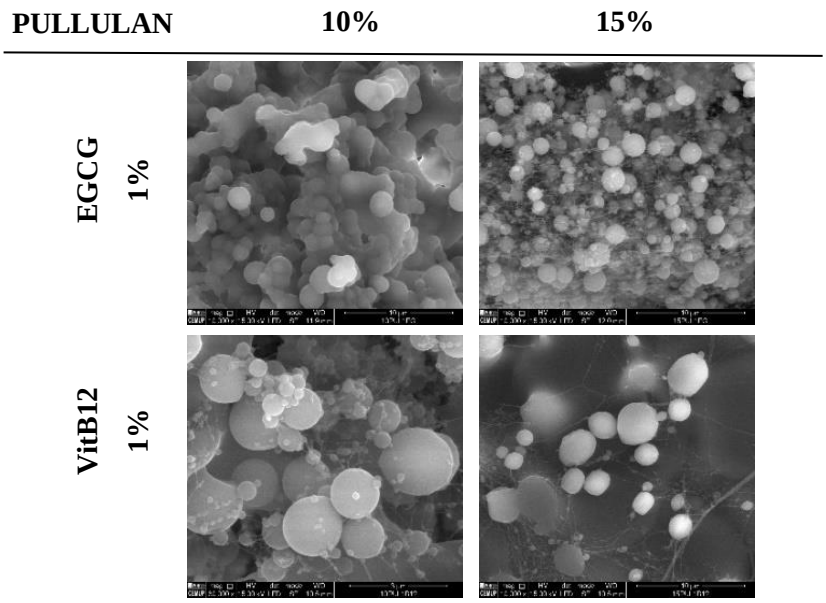


Figure 7 - SEM images of electrospinning microstructures containing different concentrations of pullulan, loaded with 1% EGCG and 1% VitB12. Magnification of 30 000x for the sample with 10% pullulan+1% Vit B12 and 10 000x for samples with 1%EGCG and 15%pullulan+1%VitB12, beam intensity (HV) 15.00 kV, distance between the sample and the lens (WD) less than 10.6 mm, scale bar of 3 μm for the sample with 10% pullulan+1% Vit B12, and 10 μm for samples with 1%EGCG and 15%pullulan+1%VitB12.

As observed in Figure 7, for all the active compounds and pullulan concentrations were produced spherical microparticles, and fibers in some samples. In terms of size, it is possible to find that all samples present a heterogenous size distribution.

In the case of the 10% samples, the microparticles loaded with the vitamin presents a homogeneous and smooth surface. However, the ones loaded with the catechin exhibit the formation of a structure that appears to be aggregated microparticles. This phenomenon could have been caused by the insufficient evaporation of the solvent, and not by the type of core material, since the day that the microstructures where produced the relative humidity of the air was high and may have influenced the water evaporation during the electrospinning process.

As for the 15% ones, the microparticles produced show a smooth and homogeneous surface, whatever the active substance. In this concentration, along with microparticles, was produced some thin and beaded fibers. This also occurred in the same concentration of zein, where a mix of particles and fibers was produced which means a transition between the electrospinning and electrospray process.

Lee et al., 2017 studied the production of 17% pullulan microstructures containing rutin-Pluronic SD and reported the formation of complex fibers with a smooth surface with porous structures. These results reinforce the conclusion that the increase of the pullulan concentration would lead to the formation of fibers instead of particles.

9.2. *In Vitro* Release Assays

Just like the zein samples, the release studies of the pullulan microstructures containing EGCG and VitB12 were performed in deionized water and the obtained release profiles are displayed in Figure 8. All the assays were performed for 50 minutes.

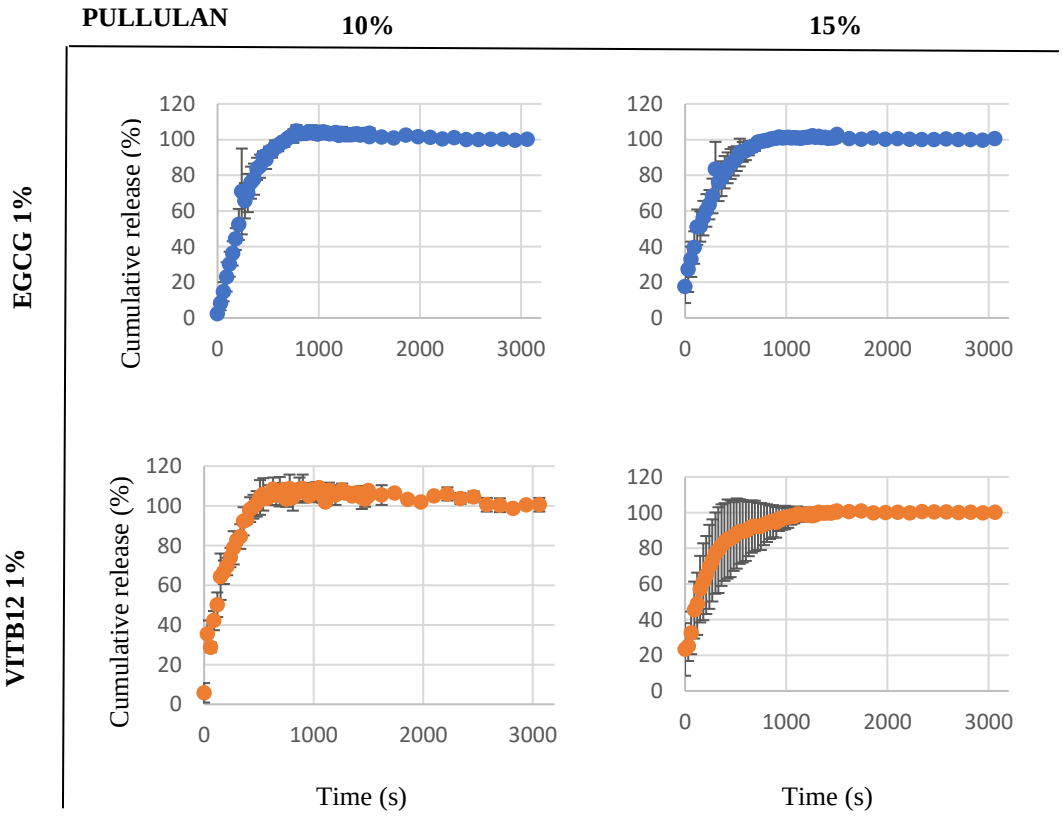


Figure 8 - In vitro release profiles, % of EGCG/VitB12 normalized by the total amount released, in H_2O of the microstructures loaded with 1% of active compounds in 10% and 15% pullulan.

As opposed to the zein microparticles, where the release was immediate, the pullulan microparticles showed a release profile similar to the zein fibers. Therefore, is possible to distinguish two different zones in the release profile: the release zone and the stabilization zone. Also, the associated errors are higher in the release zone.

By the interpretation of the graphs, is possible to note that the higher pullulan concentration is associated with longer release times, regardless of the core (EGCG or VitB12). This occurs probably due the greater presence of the thin fibers in the highest concentration, since fibers are associated with prolonged releases, as previously stated.

In the 15% (w/v) pullulan microstructures, it is possible to not that at time zero the percentage of compound is higher than the amount detected in the 10% (w/v) pullulan, which means that with the lowest concentration of encapsulating agent it is possible to have a better protection of the core.

Kinetic models

Similar to the zein microstructures release profiles, five mathematical models (Zero Order, First Order, Korsmeyer-Peppas, Weibull and Baker-Lonsdale model) were adjusted to the release profiles of the pullulan microstructures. The parameters and the correlation coefficients of the five equations fitted to the pullulan experimental data are displayed in Table 5.

Table 5 - Parameters and correlation coefficients of each equation from the kinetic models of the pullulan microstructures loaded with EGCG or VitB12.

	Zero Order		**First Order**		**Korsmeyer-Peppas**			**Weibull**			**Baker-Lonsdale**	
SAMPLE	**K_0**	**R^2**	**K_1**	**R^2**	**K_k**	***n***	**R^2**	**T_d (min)**	**β**	**R^2**	**K**	**R^2**
P:10:E:1	0.121	0.986	0.372	0.807	0.263	0.413	0.893	4.2	1.344	0.996	0.024	0.930
P:15:E:1	0.081	0.957	0.155	0.869	0.457	0.168	0.783	3.4	0.898	0.978	0.027	0.993
P:10:B:1	0.126	0.921	0.307	0.782	0.434	0.304	0.910	2.8	1.043	0.982	0.037	0.985
P:15:B:1	0.092	0.941	0.217	0.823	0.431	0.222	0.866	4.2	0.758	0.990	0.024	0.997

It was possible to fit all the kinetic models and obtain the respective parameters and constants for all the samples.

By the analysis of the Table 5, it is possible to conclude that the models that presented higher correlation coefficients (in most of the samples) were the Baker-Lonsdale and the Weibull model, with R^2 from 0.930 to 0.997 and 0.978 to 0.996,

respectively. However, from these two models, the one that adjusts to a greater number of results is the Weibull model since the Baker-Londsdale is limited to 80% of the total core release. The similar outcome was reached in the zein samples. The sample P:10:E:1 is the only exception, since the highest correlation coefficients were obtained in the Baker-Lonsdale and Zero Order model (R^2=0.986).

Unlike the zein samples, the pullulan samples present high correlation coefficients in the Zero Order model. This model, as previously said, is applied to release profiles where the core is released as a pure material from the encapsulating agent and is associated with simple diffusion releases. Regarding the First Order model, does not have significant interest since it is applied to the release of solutions.

From the Baker-Lonsdale model, the model with higher correlation coefficients, it is possible to obtain the constant K that, in the same way as zein samples, increases with faster releases.

The parameter *n*, obtained from the adjustment of the Korsmeyer-Peppas, as formerly mentioned, gives information about the mechanism associated with the release of the core from the encapsulating agent. In the case of the pullulan samples, all the release profiles presented a *n* lower than 0.43 (spherical microparticles) indicating that the release followed a "Fickian Diffusion". This result is in accordance with the one obtained in the adjustment of the Zero Order model, as the good fitting of the model to the experimental data reflects the association of the core release to a simple diffusion mechanism.

Assuming that the microparticles obtained through electrospinning of pullulan solutions are matrix-type, the Weibull model is the most suitable model to evaluate the profile release of the encapsulated catechin and vitamin. Zhu et al., 2019 studied and evaluated the *in vitro* and *in vivo* delivery of pullulan-based nanoparticles for delivery of Adriamycin into the brain and came to the same conclusion that the best kinetic model to fit the results was also the Weibull model. The parameter β, related to the release curve shape, was lower than 1 for the 15% (w/v) pullulan microstructures and higher for the 10% (w/v) pullulan microstructures. Meaning that the microstructures produced with the highest pullulan concentration showed release profile with a shape near exponential and the ones produced with the lowest concentration a sigmoidal shape.

10. Entrapment Efficiency

One of the most important quality parameters in microencapsulation of active compounds is the encapsulation/entrapment efficiency (EE). EE indicates the amount of substance (bioactive compound) that is successfully entrapped within the particles or fibers (Budinčić et al., 2021). The encapsulation efficiencies of EGCG, VitB12 and EGCG+Vit12 in electrospun zein and pullulan microstructures from blend solutions of encapsulating agent and active ingredient are described in Table 6. There are different methods and approaches to determine it (Bucurescu et al., 2018): in the case of the present work, and considering the specifications of the experiment, the encapsulation efficiency can be calculated considering the release profiles as described in Materials and Methods.

Table 6 - Entrapment efficiency for all the microstructures produced by electrospinning.

SAMPLE	**EE (%)**	SAMPLE	**EE (%)**	SAMPLE		**EE (%)**	SAMPLE	**EE (%)**
Z:1:E:1	19.26±0.08	**Z:1:B:1**	108.7±0.4*	**Z:1:EB:1**	**EGCG**	92.4±0.4	**P:10:E:1**	97.7±0.4
					B12	70.5±0.3		
Z:5:E:1	0.309±0.001	**Z:5:B:1**	89.7±0.4	**Z:5:EB:1**	**EGCG**	47.6±0.3	**P:15:E:1**	82.4±0.3
					B12	86.6±0.4		
Z:15:E:1	39.75±0.16	**Z:15:B:1**	81.1±0.3	**Z:15:EB:1**	**EGCG**	99.7±0.4	**P:10:B:1**	94.2±0.4
					B12	78.6±0.3		
Z:30:E:1	100.0±0.4	**Z:30:B:1**	93.9±0.4	**Z:30:EB:1**	**EGCG**	100.0±0.4	**P:15:B:1**	86.9±0.3
					B12	100.0±0.4		
Z:30:E:5	74.3±0.3	**Z:30:B:5**	79.6±0.3	**Z:30:EB:5**	**EGCG**	97.2±0.4		
					B12	105.1±0.4*		
Z:30:E:0.5	95.6±0.4	**Z:30:B:0.5**	77.1±0.3	**Z:30:EB:0.5**	**EGCG**	103.2±0.4*		
					B12	96.6±0.4		

These results are highly susceptible to errors because of numerous variables such as the immediate release in zein microparticles, the interference of the encapsulating agent in the measure of the absorbance (leading to a lower EE value) and the fluctuation of the absorbance measurements during the release assays.

The encapsulating efficiencies range from 0.309% to 100% (the EE with * are above 100% due to the encapsulating agent interference in the absorbance measurement). In almost all the samples, the EE was above 70% which is considered a high efficiency.

In the zein microstructures, the highest EE is seen in the samples containing 30% (w/v) zein and 1% of core material. These results may indicate that zein fibers were the type of microstructure produced with zein with a better ability to encapsulate either catechin and

vitamin alone or co-encapsulated at all concentrations tested, with the ideal concentration among those tested, being 1% (w/w). When comparing the EE of the samples with simple core and the samples with co-encapsulation the results are similar between samples. Analogous results were found by Bucurescu et al., 2018, while developing a Gum Arabic matrix by spray drying to microencapsulate curcumin, the highest encapsulation efficiencies were greater in the particles prepared with higher amounts of encapsulating agents.

Regarding the pullulan microstructures, the highest EE was achieved in the pullulan concentration of 10% (w/v) for the EGCG and for the VitB12. Meaning that the encapsulating agent concentration, zein and pullulan, is a critical parameter to encapsulation efficiency.

The microstructures that present a very low EE is probably due to the errors and uncertainly of the method used, as mentioned above.

Conclusion

EGCG and Vitamin B12 are compounds with interesting characteristics for the incorporation into food products (and others) but have bioavailability and bioactivity problems during industrial processes and storage. These challenges can be overcome with microencapsulation techniques. The main goal of the present work was to produce microstructures loaded with EGCG, vitamin B12 and the mixture of both and characterized them according to the morphology, release profiles and encapsulation efficiency.

With zein, different structures were obtained. From microparticles, lowest zein concentrations (1% and 5%), to fibers in the highest zein concentrations (30%). This evidence that the encapsulating agent concentration has a great impact in the microstructure's morphology since can alter the solution conductivity or increase the interaction between the biopolymer and the solvent. On the other hand, the type of bioactive compound or concentration did not affect the morphology.

The microstructures produced with pullulan were all small and spherical microparticles (except for the sample P:10:1:E) but in the higher pullulan concentration some fibers started to form.

In all samples was possible to distinguish two different zones: release and stabilization zones. Except for the microparticles produced with zein loaded with EGCG where the release was immediate. It was possible to observe that the slower releases were noted in the fibers and the pullulan microparticles, which can suggest that increasing the polymer concentration is associated with an increasing of the release time. As opposed, the samples with higher bioactive compound concentration are the ones with faster release.

Five kinetic models were adjusted to the release profiles of each sample. In general, the best model to fit the greater number of results was the Weibull model. In addition, by the analysis of the release exponent, *n*, obtained from the fitting of the Korsmeyer-Peppas model, was possible to determine that the pullulan samples followed a "Fickian Diffusion" mechanism of release and the zein samples followed either a Fickian Diffusion", a "Super Case-II transport" or an "Anomalous transport".

In conclusion, the main goals of the dissertation were achieved successfully.

Assessment of the work done

Other Work Carried Out

In parallel with the experimental work proposed for the dissertation, other work has been carried out. In addition to the release of EGCG and Vitamin B12 from the zein and pullulan microstructures in deionized water, the release was also performed in 50% ethanol as it simulates alcoholic beverages and oil in water emulsions. Also, both active substances were encapsulated in the same agents (with the same concentrations) but by an electrospray technique with pressure instead of a steady flow controlled by a pump.

Considering the large volume of results, three scientific articles are expected to be published. Also, a Scientific Poster with results from the present work was presented in the **11th International Colloids Conference**, 12-15 June 2022, Lisbon – Portugal entitled *Preparation of Different Food-Grade Microstructures Containing Epigallocatechin Gallate (EGCG) by an Electrospinning* with the authorship of Berta Estevinho and Ana Couto and is presented in Appendix C.

Limitations and Future Work

During the elaboration of the dissertation several limitations were found, restricting the amount and quality of the results. Still, practically all the proposed experimental work was successfully performed. These limitations were associated with the limited time available, the fact that the equipment (electrospinning) was inoperative for a considerable amount of time and the limited amount of EGCG and pullulan that led to the impossibility to produce the pullulan samples with the mixture of catechin and vitamin.

For future work, and in order to add important knowledge to this study, more analysis can be performed, as the evaluation of the antioxidant activity and perform Fourier Transform Infrared Spectroscopy (FTIR) to better understand the interaction between the core and the encapsulating agent. Also, would be interesting to use different biopolymers and mixtures, so it would be possible to assess which is the best encapsulating agent for different purposes.

Final Assessment

As a final appreciation, this work brought me an important knowledge about the science behind the processes of microencapsulation and delivery systems, especially when it comes to the encapsulation of polyphenols and vitamins. With the development

of this work, I developed and perfected some skills as critical thinking, laboratory experience, bibliographic research, among others.

Furthermore, I believe this study has contributed significantly to the scientific community and, given the rising demand for fortified goods and controlled release systems, is an important addition for the industry.

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Appendix A – Method validation

In order to establish a relation between the absorbance and the active ingredient concentration in deionized water, was necessary to trace two different types of calibration curves: one for EGCG and another for Vitamin B12. In both curves was used UV-Vis spectrometry to trace the absorbance values as a function of the concentration of the respective substance (EGCG or VitB12).

EGCG Calibration Curve

In the construction of the catechin calibration curve, Figure 9, eight standard solutions were prepared with concentrations ranging from 0.0001 mg/mL and 0.1 mg/mL. The eight different solutions were prepared from a "mother solution", with a concentration of 0.1 mg/mL. The absorbance of each standard solution was measured in triplicate by UV-Vis spectrometry, with detection of 274 nm (corresponding to the highest peak of the EGCG spectrum). The results presented a variation coefficient lower than 10%.

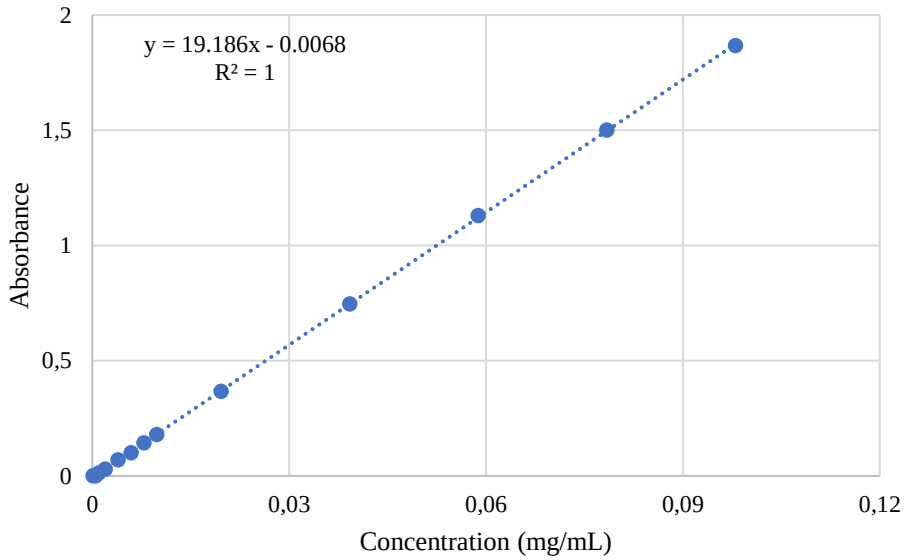


Figure 9 - EGCG calibration curve.

A linear adjustment was done to the obtained results in order to obtain Equation 3. The correlation coefficient, R^2, was equal to 1.

$$y = 19.186x - 0.0068 \quad (3)$$

Vitamin B12 Calibration Curve

For the construction of the calibration curve for the VitB12 (Figure 10) ten standard solutions were made with concentrations from 0.002 mg/mL to 0.05 mg/mL. The solutions were prepared from an initial solution with a concentration of 0.2 mg/mL. The absorbance of each standard solution was measured in triplicate by UV-Vis spectrometry, with detection of 361 nm (corresponding to the highest peak of the VitB12 spectrum). The results presented a variation coefficient lower than 5%.

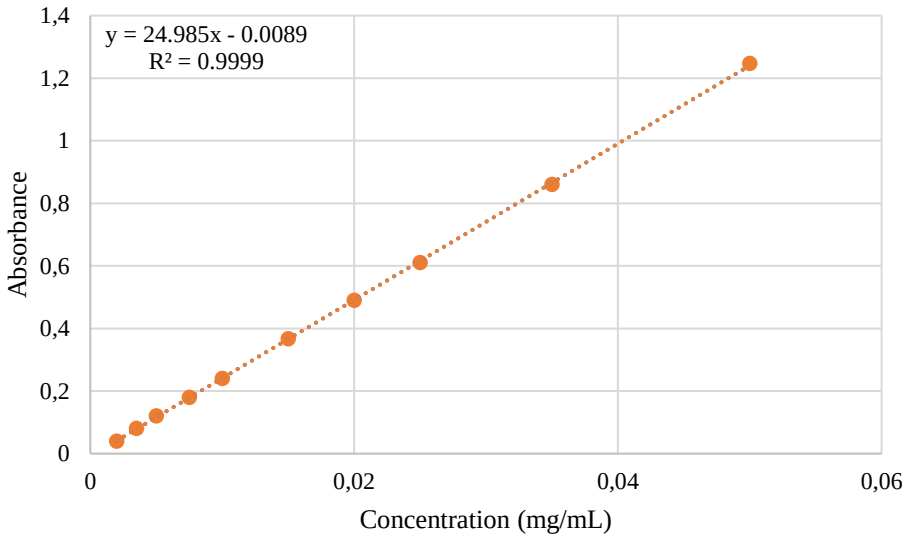


Figure 10 - Vitamin B12 Calibration Curve.

A linear adjustment was done to the obtained results in order to obtain Equation 4. The correlation coefficient, R^2, was equal to 0.999.

$$y = 24.985x - 0.0089 \quad (4)$$

Validation

The validation of the analytical method involves acquiring a collection of statistical parameters that characterize it and enables the method to be proven valid. Parameters of characterization, quantification and reliability of the method were determined. The characterizations parameters give information about what the methos is for (specificity). Quantification methods include the study of linearity and associated statistical parameters (slope, interception, correlation coefficient, detection and quantification limits). As for the reliability, it demonstrates if the method is suitable for the obtained results (precision, accuracy).

The Detection Limit (LOD) is the minimum concentration at which the presence of the component of interest can be detected. On the other hand, Quantification Limit (LOQ) is the lowest concentration of the desired compound that is possible to measure. Both parameters were calculated according to Equation 5 and 6, respectively.

$$LOD = \frac{3 \times S_b}{a} \quad (5)$$

$$LOQ = \frac{10 \times S_b}{a} \quad (6)$$

Where a is the slope of the calibration curve and S_b the standard deviation of the intercept.

The degree of proximity between results is evaluated through precision parameter. This parameter was obtained regarding repeatability. The repeatability parameter is calculated by the standard deviation of the same sample, at the same conditions. This parameter was obtained by the application of Equation 7.

$$CV\ (\%) = \frac{S \times 100}{c} \quad (7)$$

Where S is the standard deviation and c is the concentration (mg/mL).

In Table 7 is presented the values of the parameters mentioned above, as well as the LOD and LOQ.

Table 7 – Values of the validation parameters for EGCG and VitB12.

	EGCG	**VitB12**
Number of standarts	8	10
Correlation Coefficient	1	0.999
LOD (mg/mL)	0,001	0,001
LOQ (mg/mL)	0,002	0,003

Appendix B – Kinetic Models

As mentioned, some mathematical models were adjusted to the release profiles in order to choose, by the analysis of the parameters, which model presents the best correlation and fitting to the experimental results (Padmaa Paarakh et al., 2018). The five different equations used in the present work are presented in Table 8.

Table 8 – Mathematical kinetic Models for controlled release of substances.

EQUATIONS	***LEGEND***
Zero Order $Q_t = Q_0 + K_0 t$ First Order $Q_t = Q_0 e^{-K_1 t}$ Korsmeyer-Peppas $\frac{Q_t}{Q_\infty} = K_k t^n$ Weibull $\frac{Q_t}{Q_\infty} = 1 - e^{-\left(\frac{t-t_0}{\tau_d}\right)^\beta}$ Baker-Lonsdale $f_1 = \frac{3}{2}\left[1 - \left(1 - \frac{M_t}{M_\infty}\right)^{\frac{2}{3}}\right] - \frac{M_t}{M_\infty} = kt$	Q_t – Amount of the active compound release at time *t* Q_0 – Initial amount in solution (normally Q_0=0) K_0 – Zero order constant *t – Time* K_1 – First order constant K_k – Korsmeyer constant *n* – Release exponent (defines the release mechanism) t_0 – Initial time τ_d – Time at which 63.4% of material had been released β – shape factor M_t – % of compound release at time *t* M_∞ – % of total release k – Baker-Lonsdale constant

After the fitting of each model to each sample, was possible to obtain the parameters and correlation coefficients presented in the Results.

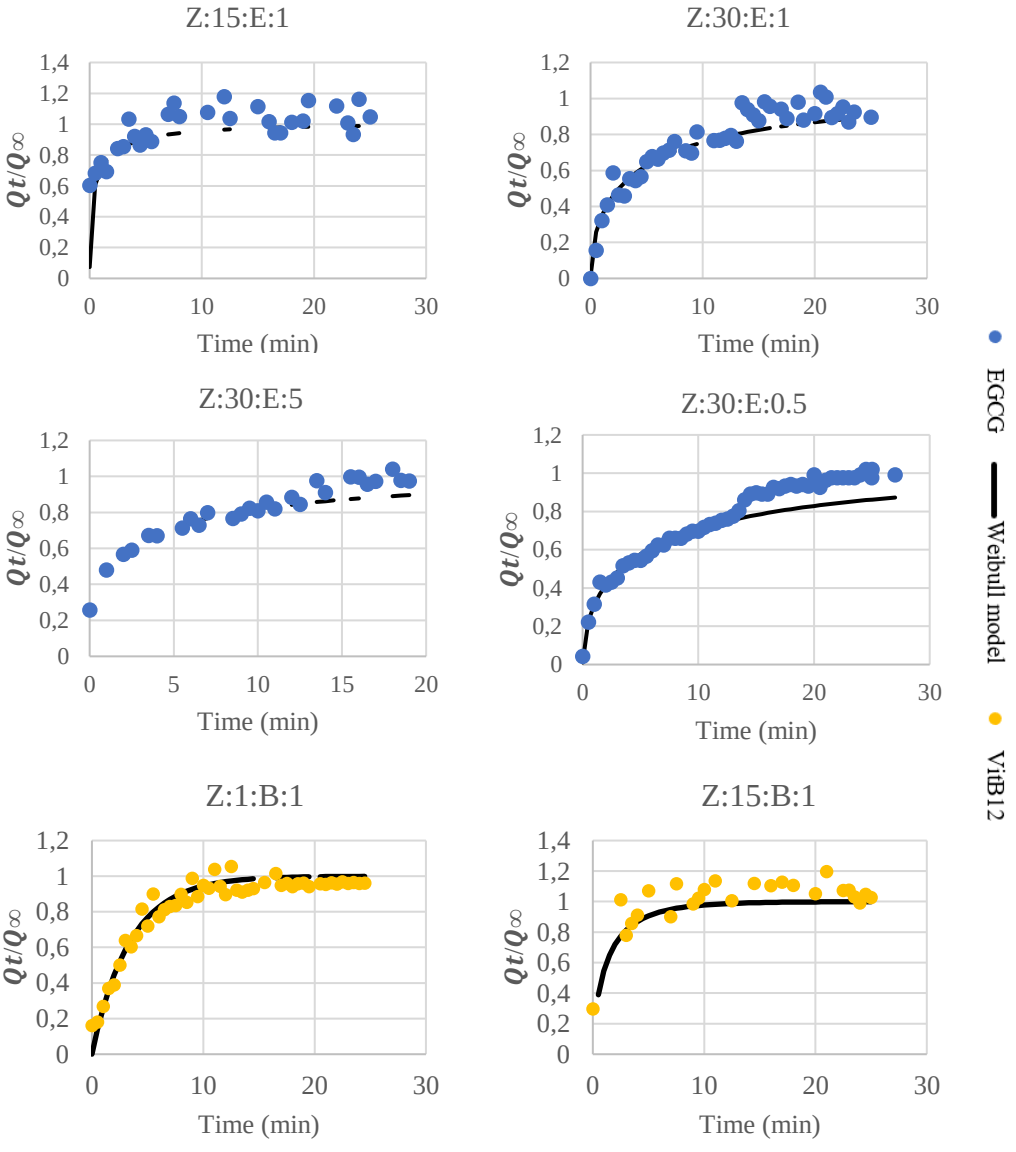
In the specific case of Korsmeyer-Peppas model, the parameter *n* defines the mechanism associated with the release profile of the active ingredients. The values for

the release exponent, *n*, are attributed according to the type of structure (film, cylinder or sphere), as presented in Figure 11.

Form	Release exponent (n)	Drug transport mechanism	Rate as a function of time
Film	<0.5	Fickian diffusion	$t^{-0.5}$
	$0.5 < n < 1.0$	Anomalous transport	t^{n-1}
	1.0	Case-II transport	Zero order release
	>1.0	Super case-II transport	t^{n-1}
Cylinder	<0.45	Fickian diffusion	$t^{-0.55}$
	$0.45 < n < 0.89$	Anomalous transport	t^{n-1}
	0.89	Case-II transport	Zero order release
	>0.89	Super case-II transport	t^{n-1}
Sphere	< 0.43	Fickian diffusion	$t^{-0.57}$
	$0.43 < n < 0.85$	Anomalous transport	t^{n-1}
	0.85	Case-II transport	Zero order release
	>0.85	Super case-II transport	t^{n-1}

Figure 11 - Interpretation of the release exponent (n) in the Korsmeyer-Peppas (extracted from Estevinho et al., 2013).

As said previously, the model that best fit better and a great number of results is the Weibull model. For that reason, the graphs with the model adjust for all samples are presented in Figure 12 (zein microstructures) and Figure 13 (pullulan microstructures).



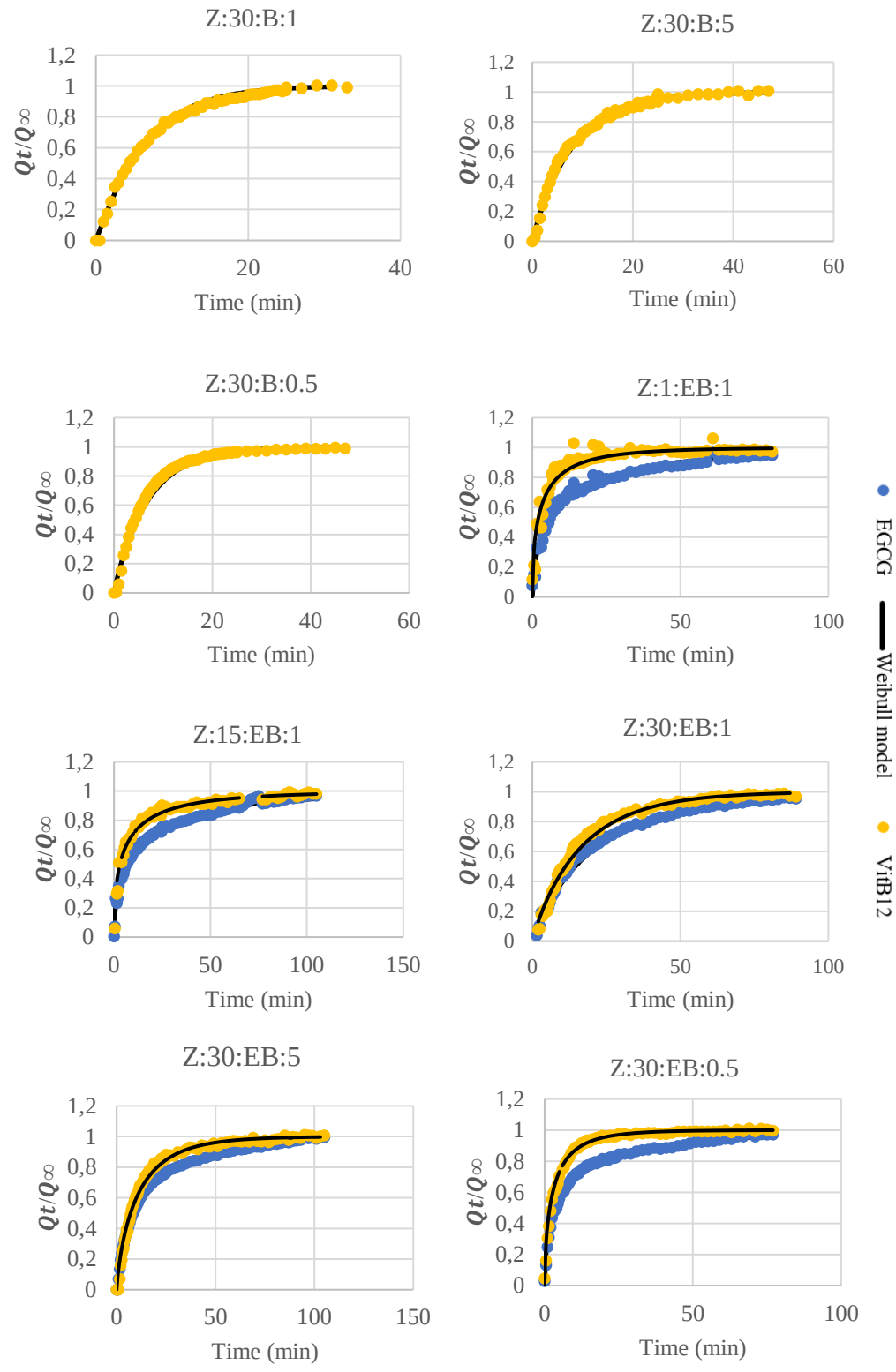


Figure 12 – Adjustment of the Weibull model to the zein microstructures loaded with EGCG, VitB12 and (EGCG+VitB12).

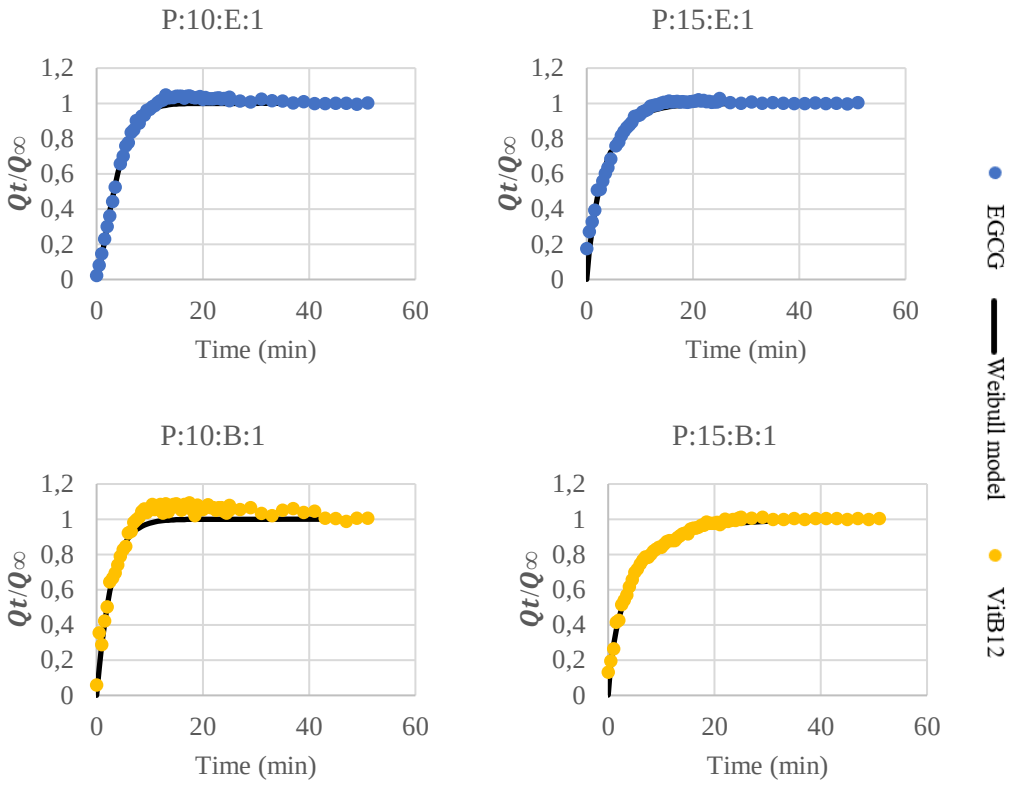


Figure 13 - Adjustment of the Weibull model to the pullulan microstructures loaded with EGCG, VitB12 and (EGCG+VitB12).

Appendix C – Poster

PREPARATION OF DIFFERENT FOOD-GRADE MICROSTRUCTURES CONTAINING EPIGALLOCATECHIN GALLATE (EGCG) BY AN ELECTROSPINNING TECHNIQUES

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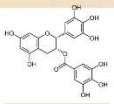
Introduction

GREEN TEA



EGCG

Anti-oxidative
Anti-bacterial
Anti-mutagenic
Anti-inflammatory
Anticarcinogenic

The effectiveness of EGCG depends on preserving the **stability**, **bioactivity** and **bioavailability**. When in contact with unfavorable factors this properties can be compromised.

Microencapsulation can be a great solution, namely the through **ELECTROSPINNING**.

Microstructures Production

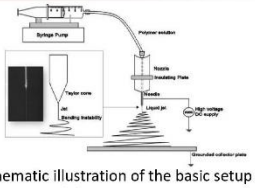
1. Sample Preparation

- **Zein** was chosen as the encapsulating agent and was used in concentrations between **1-30% (w/v)**.
- **EGCG** was used in concentration between **0.5-5% (w/w)**.

Solvent: ethanol 70% (v/v)

2. Electrospinning

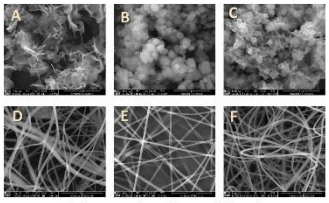
CONDITIONS:
- Needle: 22G
- Applied voltage: 20 kV
- Flow rate: 0.3-3 mL/h
- Tip-to-collector distance: 5 cm



Schematic illustration of the basic setup of electrospinning.

Microstructures Characterization

1. Scanning electron microscopy (SEM)



A) 1%zein+1%EGCG
B) 5%zein+1%EGCG
C) 15%zein+1%EGCG
D) 30%zein+1%EGCG
E) 30%zein+0.5%EGCG
F) 30%zein+5%EGCG

SEM images of the samples obtained by electrospinning.

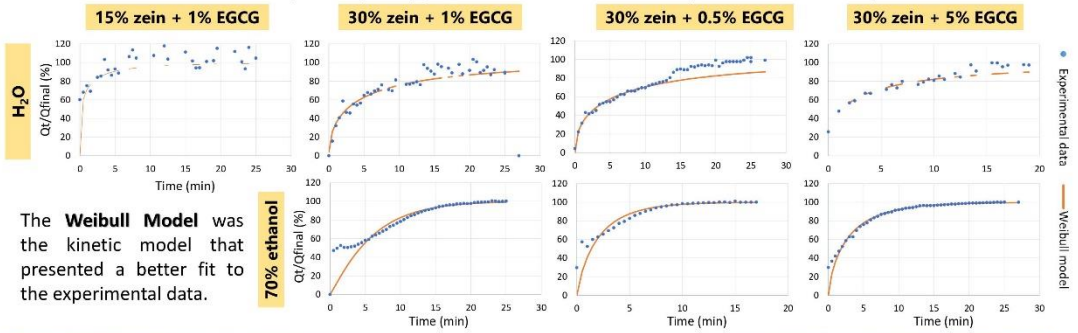
Production of different microstructures:
- **Films** (A)
- **Microparticles** (B,C)
- **Fibers** (D,E,F)

Microparticles diameter:
B 0.22 - 0.98 μm
C 0.17 - 0.83 μm

Fiber width:
D 0.44±0.13 μm
E 0.47±0.11 μm
F 0.36±0.10 μm

2. *In vitro* Release Assays

The release profiles were obtained based on the evaluation of the EGCG absorbance in water and ethanol 70% and as function of time. Kinetic models (Korsmeyer-Peppas, Weibull and Baker–Lonsdale) were fitted to the experimental data.



The **Weibull Model** was the kinetic model that presented a better fit to the experimental data.

Conclusions

- Production of different microstructures according to the % of zein.
- Good quality microstructures with high encapsulation efficiencies (>70%).
- Fast release rates in water and in 70% ethanol.
- The higher the EGCG content, higher the total release time.

Acknowledgments

This work was financially supported by: Base Funding - UIDB/00511/2020 of the Laboratory for Process Engineering, Environment, Biotechnology and Energy – LEPABE - funded by national funds through the FCT/MCTES (PIDDAC); Project POCI-01-0145-FEDER-028715 (MicroDelivery - Development of controlled delivery functional systems by microencapsulation of natural and active compounds with therapeutic, nutritional and technological interest), funded by FEDER funds through COMPETE2020 – Programa Operacional Competitividade e Internacionalização (POCI) and by national funds (PIDDAC) through FCT/MCTES.
Berta Estevinho acknowledges FCT for the contract based on the "Lei do Emprego Científico" (DL 57/2016).

11th International Colloids Conference, 12-15 June 2022 , Lisbon - Portugal