Universidad de Navarra

Study of the protective capacity of a β-cyclodextrin polymer with chlorogenic acid (coffee antioxidant)

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Abstract

This study focuses on coffee as a source of antioxidants, hence, protecting from the damage caused by free radicals; which has been demonstrated to have a protective action in numerous diseases. This capacity is attributed to its phenolic compounds among which chlorogenic acid stands out, together with caffeic acid. Besides, cyclodextrins are well known for their capacity of forming inclusion complexes where the host molecule is located in the interior cavity, allowing the transport and storage of it.

My experimental project is focused on the synthesis and characterization of a protective polymeric system formed by β-cyclodextrin, which is then going to be loaded with chlorogenic acid. This inclusion complex as well as the β-cyclodextrin polymer and chlorogenic acid themselves, have been studied through preliminary studies of stability, using principally UV and FTIR spectroscopies as well as thermogravimetric calorimetry, which showed that the polymer prevents the acid from degrading through time, when maintained either at dark or in direct sunlight. The protective effect has been determined through scavenging capacity measurements analyzing the variation in the activity of chlorogenic acid as well as its change when the acid is introduced into β-cyclodextrin. For this, it has first been studied the change in absorbance using a mixture of 2,2-diphenil-1-picrylhydrazyl (DPPH) and chlorogenic acid at different concentrations; leading to the already known fact that, at higher concentrations, the antioxidant capacity increases. Furthermore, the controlled release of the antioxidant from the cavities of the β-cyclodextrin in the polymeric matrices has been studied through Sotax Dissolution tests and applying mathematical models.

This data leads to the conclusion that the CD-chlorogenic acid inclusion complex helps to maintain the integrity and scavenging activity of the bioactive compound especially when the chlorogenic acid is measured over time.

1. Introduction

Coffee is a vegetal origin product endowed with a series of components including vitamins and minerals that have diverse effects on our organism. Among its effects, the ability to stimulate the central nervous system, reducing the sense of fatigue and increasing awareness; as well as its protective effect against numerous diseases implicating oxidative stress, are the best known ones [1]. While its psychostimulant property is gifted mainly due to the presence of caffeine, its protective action is derived from the action of antioxidants coffee is gifted with, caffeine, chlorogenic acid and caffeic acid, principally. These compounds protect the organism against the damage caused by free radicals formed by the organism itself or from external compounds [2]. However, the increase of the scavenging or antioxidant capacity after consuming this drink is mainly attributed to its phenolic compounds, among which caffeic acid stands out, and it can be found also as part of chlorogenic acid. Thus, chlorogenic acid (CGA or 5-O-caffeoylquinic acid) present in coffee is a natural polyphenolic compound [3] formed by caffeic acid and quinic acid, linked by an ester bond (Fig. 1); and is the one founded to be in greater quantity in green coffee [4].

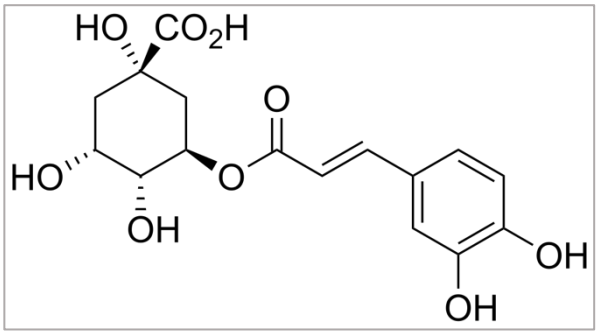

Figure 1. Chemical structure of chlorogenic acid.

Green coffee is basically coffee before it has been roasted, in this form, about 6 to 7 percent of the coffee is chlorogenic acid, however, once the beans are roasted, its molecular structure changes breaking the ester bond and converting into caffeic acid and quinic acid. Thus, despite CGA is absorbed in the small intestine, this bioactive compound is mainly reduced into caffeic and quinic acid, which are then absorbed in the colon [5].

As it has been already stated, one of the properties of coffee is to protect against oxidative stress caused by free radicals, these free radicals are also known as oxygen reactive

compounds (ROS) [6] and are named so because they carry one or more unpaired electrons in the external orbitals [7]. They are generated continuously through aerobic metabolism, the process by which glucose reacts with oxygen to form energy in form of ATP, as a form of hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\cdot}$), leading to the fact that the mitochondrion is the predominant cell organelle in ROS production [8]. These compounds then exert their function on various cellular elements such us in the nucleus, membrane, cytoplasm etc., where they are highly harmful and lead to oxidative stress if they are not eliminated. In a young and healthy person, oxygen reactive compounds are eliminated rapidly in the interior of the cell by natural antioxidants, however, fabricated antioxidants also exist for those who their organism eliminate them too slowly, or does not eliminate them at all. In addition, natural or fabricated antioxidants, can work either by preventing the formation of this biological substrates, preventive antioxidants; or by blocking their further production inhibiting free radical chain reactions, secondary antioxidants [9]. Natural antioxidants are synthesized by plants, and are characterized by the presence of hydroxyl groups (OH) bounded together by benzene rings. The presence of this hydroxyl group is what confers these substances their antioxidant properties, as it works generally by donating an electron to the free radicals, without converting into electron-scavenging substances themselves [10].

Besides, cyclodextrins (CDs) are cyclic oligosaccharides made up of six to twelve rings of units of glucose linked by α (1-4) bonds [11], therefore, their tridimensional structure is represented as a truncated cone or toroid in which the hydrophilic groups are exposed to the outside [12], resulting in a hydrophobic internal cavity.

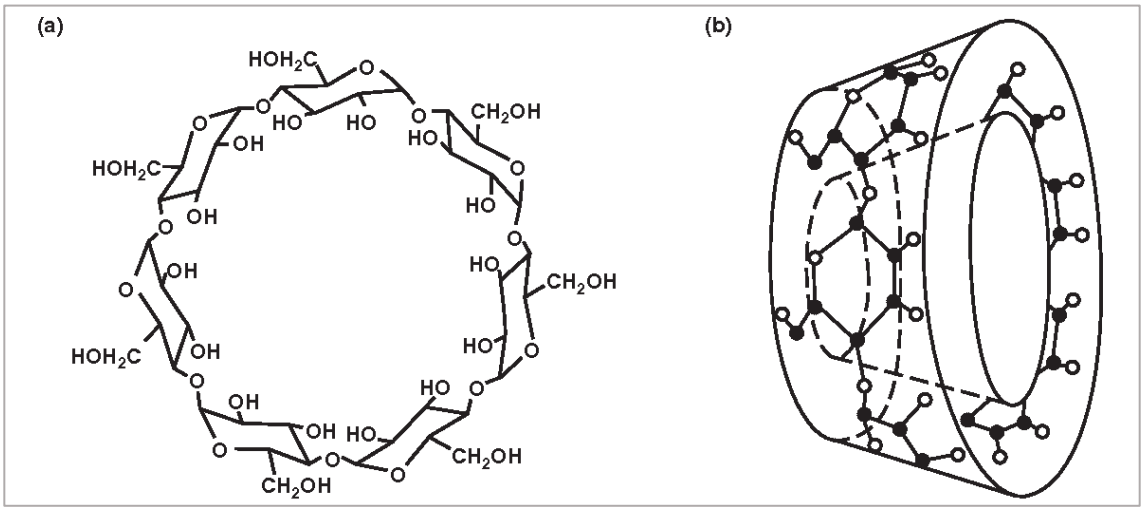

Figure 2. The chemical structure (a) and the toroidal shape (b) of β-cyclodextrin [13].

Among CDs, three of them have been described as natural cyclodextrins, α, β, and γ, formed by 6, 7 and 8 units of glucopyranose, respectively [14]. This study focuses solely on β-cyclodextrin, being this one the least soluble among the three cyclodextrins, with a concentration in aqueous environments of 10^{-2}M due to its ability to form intramolecular hydrogen bonds [12]. However, β-cyclodextrin was chosen because its cavity is of a suitable size to hold the chlorogenic acid. Since their discovery, CDs have been used as inclusion complexes for a variety of bioactive compounds, especially with those that present a low polarity [12]; as they can introduce the host molecule inside their central CD cavity [15], this inclusion improves the stability and solubility of the compounds [16]. Hence, even though neither CDs or CD-drug complexes are able to go beyond biological membranes [17], the complexes can act near these membranes like a reservoir providing high concentrations of the drug indeed, easing the passage of the free bioactive compound by diffusion [12]. Cyclodextrins are quite small and soluble when found free, two characteristics that are exposed as a limitation in certain applications. Therefore, CDs are incorporated in a polymeric structure to increase their effectiveness since when they come across an aqueous solution, they do not dissolve, unlike free CDs [12]. So, cyclodextrin polymers present large applications as drug and bioactive compounds vehicles [18].

Cyclodextrin polymers are referred as such when two or more CD units are covalently bound [19]. This polymerization is gifted by the reaction of hydroxyl groups of CDs with a crosslinking agent like epichlorohydrin (ECH) [20].

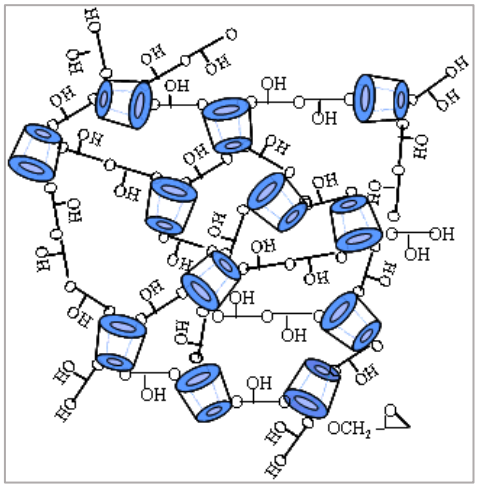

Figure 3. Scheme of the structure of a CD polymer [21].

This can then evolve into the establishment of an hydrogel of cyclodextrin polymers which swells when found in an aqueous solution but without the loss of its components

[22]. Consequently a cyclodextrin-rich microenvironment is created [23] with enough cavities to interact with high concentrations of any bioactive compound or drug and so, the bioactive compounds can be loaded into the cavities of cyclodextrins and also be adsorbed by the interstices of the polymeric net. On top of that, it allows a further control of the release of the drug from the cavities [22], making this polymers applications include the retention and controlled release of different substances. This experimental project is based on the hypothesis that the polymer will protect the chlorogenic acid from oxidation and gift it with stability, in order to be then released with all its properties, as in the water drunken. Thus, the consumer would benefit from the assets of the acid, like its antioxidant property for example, without the need to drink coffee.

Therefore, the formation of CD complexes in the hydrogels has been quite a discovery in the pharmaceutical area, as it allows the control of drug release and the affinity for biological compounds. In this study, as it has been stated before, the attention is drawn to the inclusion complex of β-cyclodextrin with chlorogenic acid, the synthesis of the β-cyclodextrin polymer (βCDP) and the loading of the compound in the polymer. The methods used for this analysis include UV and FTIR spectroscopies, thermogravimetric calorimetry and elemental analysis. In addition to this, the scavenging capacity measurements were also included to study the protective effect of the polymer; and the release of the antioxidant compound indeed, has been examined through Sotax Dissolution tests.

2. Objectives

The main objective of this experimental project is to demonstrate the ability of the β-cyclodextrin polymer to protect the chlorogenic acid when it's inside the cavities of the cyclodextrin. This objective is specified in the following:

- Determination of the stability constant of the CD-chlorogenic acid inclusion complex.
- Synthesis and characterization of the polymeric system of β-cyclodextrin.
- Study of the inclusion of the CGA and its controlled release from the polymeric matrices with mathematical models.
- Study of the protective effect of the polymer analyzing the scavenging activity of the chlorogenic acid in light/dark conditions, and in its solid or liquid state over time.

3. Material and methods

3.1. Material

On one hand, the bioactive compound used in this study is chlorogenic acid, also named as (1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid by IUPAC, and represented with the molecular formula of $C_{16}H_{18}O_9$. This compound was acquired from Sigma-Aldrich, presents a molecular weight of 354.31, with a ≥95% titration and a melting point of 210ºC.

On the other hand, for the synthesis of the β-CD polymer (βCDP), β-CD, $NaBH_4$, H_2O, NaOH and epichlorodydrin (ECH) are needed. Firstly, CAVAMAX® W7 was the β-cyclodextrin used, which is a standard grade beta-cyclodextrin from Wacker Chemie AG that presents seven glucose units. The empirical formula is represented as $C_{42}H_{70}O_{35}$ and presents a molecular weight 1135; in addition to this, its loss on drying is of 12.51%, meaning that this percentage is just H_2O. Secondly, both sodium tetrahydroborate or borohydride and sodium hydroxide were obtained from Panreac, $NaBH_4$ presenting a molecular weight of 37.86 and NaOH of 39.997. Finally, the crosslinking agent used was epichlorohydrin (ECH), also described as 1-Chloro-2,3-epoxypropane, obtained from Sigma-Aldrich; it presents a molecular weight of 92.52 and its empirical formula is C_3H_5ClO.

Furthermore, the determination of the scavenging activity was measured through the free radical method [24] using 2,2-Diphenyl-l-picrylhydrazyl ($DPPH^{\cdot}$) from Sigma-Aldrich, as the free radical, which presents hydrogen acceptor ability towards antioxidant. This compound presents a molecular weight of 394.32, and its empirical formula is $C_{18}H_{12}N_5O_6$.

3.2. Methods

3.2.1. Determination of the stability constant (K_s) for the complex CGA-βCD

In order to study the CD-chlorogenic acid inclusion complex, the stability constant of the inclusion complex between chlorogenic acid and β-CD has to be defined through UV-visible spectrophotometry (Agilent Technologies, Cary 8454 UV-Vis model). For that, the concentration of CGA was maintained constant and increasing concentrations of

cyclodextrin were added measuring each absorbance each time. So, first of all, two stock solutions in water have to be prepared, one for CGA, and another one for β-CD. From those solutions, 3 main solutions had to be made, a solution of chlorogenic acid 2.5 x 10^{-5}M, a solution of β-CD and chlorogenic acid, and a solution of β-CD 4.5 x 10^{-3}M. Following, two tests were carried out, both were performed in 2 different days and repeated 6 times in each, so they were finally 12 experiments. As for the procedure, on one, the solution of CGA was measured in order to obtain the maximum absorbance wavelength (324nm); then, portions of 100μL of the solution of β-CD and CGA were added 10 times (until 1000μ) and measured after each additive. This way, the concentration of CGA was kept constant while varying the concentration of cyclodextrin. The same procedure was used for the other test, but using H_2O as the first measurement and adding portions of the solution of β-CD as reference or blank solution, in order to subtract the absorbance of the cyclodextrin and not of the complex. The temperature was maintained constant during the study being this one the room temperature of the laboratory (20-22℃).

Finally, the absorbances corresponding to the β-cyclodextrin-CGA complex were obtained by subtracting the absorbances obtained in the second test (β-CD alone). Following, the Benesi-Hildebrand method, taking into account the following equation: $\frac{b}{\Delta A} = \frac{1}{[S]K_s\Delta\varepsilon[CD]} + \frac{1}{[S]\Delta\varepsilon}$ (Eq. 1) the representation of $\frac{1}{\text{Absorbance}}$ against $\frac{1}{[CD]}$ was performed in order to extrapolate and then calculate the K_s of the CD-chlorogenic acid inclusion complex [25].

3.2.2. Synthesis and characterization of β-cyclodextrin polymer (βCDP)

The synthesis of β-cyclodextrin polymer was carried out in the laboratory, following the method described in Machín *et al*., 2012 [21]; for that, firstly, a solution of NaOH at 40% which increases the alkalinity of β-CD by the deprotonation of its hydroxyl groups; this was united with a solution containing 24g of β-CD and 24mL of H_2O. Secondly, 60mg of $NaBH_4$ were added to the mixture which prevents the oxidation of cyclodextrin. At last, 18.2mL of epichlorohydrin were added with a 11:1 stoichiometry in relation to β-CD, acting like a crosslinking agent between the cyclodextrins. This product was added slowly into the solution, at 50℃ and with constant agitation, so that it progressively reacts

with the cyclodextrin until the formation of a homogeneous mixture, this takes around 3 hours. It was finally left in the heated bath at 50℃ still, all the way to gelation, resulting in an acidic gel, which was washed with water several times to eliminate the residual reagents.

This final product was then frozen at -50℃ and lyophilized (Telstar Cryodos lyophilizer) in order to eliminate the water of the pores, by the transformation of the frozen solid matter into gas without passing through the liquid state; thus, dehydrating the sample by sublimation. The outcome of this process was ultimately brought up to a sieve analysis by the distribution of the product into sieves of 250µm and 160µm, in order to achieve the desired particle size [21].

Once the polymer was synthesized, it was proceeded to perform its characterization through different methods: elemental analysis and infrared radiation (IR), which were performed with both 250µm and 160µm particle size β-CD; and thermal analysis and mercury porosimetry, which were performed just with 250µm particle size β-CD. The organic elemental analysis (OEA) was carried out with the Thermo Scientific Flash Smart Elemental Analyzer, which performs a flash combustion of CHN and the pyrolysis of oxygen simultaneously; in such way that this analysis provides the information about the composition (C, O and H content) of both 250µm and 160µm β-CD so that the percentage of the resulting cyclodextrin can be calculated. The method is as follows, the combustion reaches a temperature of 950℃ resulting in elemental gases that, after a subsequent reduction, are separated by column chromatography and transferred to a thermal conductivity detector, with a flow of 140mL/min. Secondly, a thermal analysis was performed with a thermogravimetric analyzer, TGA/SDTA (851^e model from Mettler-Toledo), that quantifies the weight change of the synthesized β-cyclodextrin (250µm particle size), in relation to change in temperature, in this case the condition of the maximum temperature was of 300℃. Thus, it depicts the relationship between TGA, thermogravimetric analyzer (weight change) and DTA, differential thermal analysis (thermal change). Besides, the infrared spectrum of β-cyclodextrin polymer was obtained by Fourier transform infrared spectroscopy (FTIR) with the interferometer of IRAfinnity-1S from Shimadzu, in order to determine specific functional groups of the polymer. Finally, the porosity and pore size of the porous structure of β-cyclodextrin polymer were analyzed with Micromeritics Autopore IV Mercury Porosimeter [26] with a range of

pressure of 0.00015-207MPa, by the determination of the following parameters: total intrusion volume (mL/g), total pore area (m^2/g), pore diameter (μm), porosity (%), permeability (mDarcy) and tortuosity. This technique is based on the intrusion of mercury in the polymer matrices, thus, as the mercury atoms are entering through the interstices of the network, the equipment obtains the values of porosity of the hydrogel.

3.2.3. Inclusion of chlorogenic acid into the polymer

Once determined the stability constant for the complex CGA-βCD, it is expected that when the acid is in contact with the polymer, the compound includes itself in the cavities of the cyclodextrin and adsorbed in the interstices of the network. Before the inclusion of chlorogenic acid into the polymer and for its loading and release, a standard curve of the CGA needs to be created following Beer-Lambert's law, which states that the absorbance of the bioactive compound is proportional to its concentration. For this, a calibration curve of CGA that was already created in another work was taken into consideration: $y = 19630x - 0.006$ [27]. Thus, a stock solution of chlorogenic acid 8×10^{-4}M in water was prepared from which 3 solutions were created, in order to confirm that the standard curve that will be used is correct. This was performed at the laboratory's room temperature (20-22℃), and in topaz glass bottles because as CGA is an antioxidant, is sensitive to temperature and light.

In order to include the chlorogenic acid in the polymer, 0.5g of βCDP of a particle size of 250μm, were dissolved in 2.5mL of water for 10 minutes to swell the cavities of the polymer, making them more accessible for the chlorogenic acid. Following, 50mL of a CGA 3×10^{-3}M dissolution was added and left on a magnetic stirrer at 500rpm for 24 hours. Once the inclusion time has passed, as CGA loaded is in 10^{-3}M order, the resulting product was diluted 100 times so that it can be read by the UV-spectrophotometer at 324nm. Taking the solution of CGA without β-CDP as reference, the mass of CGA loaded can be obtained by the subtraction of the sample's absorbance, which is how much CGA has not been able to enter, to the reference absorbances; and then replacing this data in the standard curve equation. Finally, the system went through a process named as vacuum filtration, which is a filtration technique that separates the solid from the liquid. The solid outcome was then frozen at -50℃ and subsequently lyophilized, so that the final product

is like a powder. All these processes were performed 8 times so that different tests can be carried out.

3.2.4. Release of chlorogenic acid from the βCDP

To finally complete the study of the CGA-βCDP system, the release of the bioactive compound from the polymer was studied. Firstly, an initial study of its release was done with manual sampling in order to settle the time at which the chlorogenic acid is completely released so that the procedure can be done in Sotax dissolution test (Sotax AT7 Smart Semi-Automated Dissolution Tester), with automatic sampling. This dissolution testing is used to characterize the properties and the mechanism of release [28] of an active drug, in this case, the antioxidant chlorogenic acid.

The manual sampling was performed by adding 200mL of water to the 50.5mg CGA/g of polymer system and extracting aliquots every 5 minutes for 40 minutes, every 10 for the next 80 minutes, and every 15, 20 and 30 for 60 minutes each, until a final time of 5 hours (300 minutes). Each sample was measured by UV-spectrophotometry at 324nm in order to determine the amount of CGA released at each time and adding the same amount of water that was extracted each time, in this study being this data of 2mL, so that the final volume remains the same over the experiment.

Once the time parameter has been established, two equal dissolution tests were carried out where each sample was given every 2 minutes for the first 10 minutes, every 5 for the next 30 minutes, every 10 for the next 40 minutes and every 20 for the last 80 minutes. The chlorogenic acid release from the CGA-βCDP system was performed following Sotax recommendations [29] by the dissolution testing USP 1, which is the basked method. This equipment is composed of 6 glass vessels that are placed in a thermostatic bath so that their temperature is constant during the whole experiment. In these vessels, a metallic drive shaft which is connected to the basket is introduced, this cylindrical basket is where the sample is deposited and it stirs the mixture as the gastrointestinal tract would do. So, the CGA-βCDP system powder (for test 1, 49.7mg of CGA per gram of polymer and for test 2, 52.0mg/g of polymer) was placed in the basket and introduced in 500mL of water; following, the test parameters were settled. The temperature was programmed to be 37℃, which is the physiological temperature of the intestines; and the

stirring speed was of 50rpm. In this case, the volume that was extracted at each time was also of 2mL, and therefore, another 2 mL of water were added after each extraction. Once the samples have been given by the equipment, the following procedure is the same as in the manual test, thus, the samples were measured with UV-spectrophotometry at 324nm.

The results obtained for the controlled release of the chlorogenic acid from the polymer were adjusted to the following 4 mathematical models: Korsmeyer-Peppas model, Higuchi model and zero and first order kinetic models. All these models relate drug release, CGA, with elapse time [18]:

$M_t/M_\infty = k_{KP}t^n$ Equation 2. Korsmeyer-Peppas model

$M_t/M_\infty = k_H t^{0.5}$ Equation 3. Higuchi model

$M_t/M_\infty = k_0 t$ Equation 4. Zero order kinetics model

$M_t/M_\infty = 1 - e^{-k_1 t}$ Equation 5. Frist order kinetics model

For Korsmeyer-Peppas model, where M_t is the amount of chlorogenic acid delivered at each time (t) measured and M_∞ the total amount of drug released; k_{KP}, is the kinetic constant and n informs of the mechanism of release [18]. This first model gives the corresponding data for k and n, and is the one the rest are based on; thus, the Higuchi model is based on n being 0.5, providing only the parameters for k. Furthermore, zero and first order kinetics models give the information for k_0 and k_1.

3.2.5. Determination of the stability of chlorogenic acid included in the polymer

The stability of the chlorogenic acid was measured by aging; thus, measuring the absorbance of the chlorogenic acid itself and when this bioactive compound is forming an inclusion complex with β-CD, so that the protective action of the polymer can be demonstrated. The cited data were obtained at room temperature, but with the action of sun light taking into consideration because CGA should deteriorate under that circumstance.

So, for that, CGA itself was left at direct sun light, and in the dark for comparison; in addition to this, two solutions of CGA at 2.8 x 10^{-5}M were prepared to undergo the same

conditions. The concentration is the one stated because once the solution of chlorogenic acid 3 x 10^{-3}M is loaded (see section 2.3), the 2.5mL of water that were first added to the polymer have to be taken into consideration, as well as the dilution of 100 that was done afterwards. The same was done for the chlorogenic acid-βCDP system, so that the aging of the product, both at light and in the dark, can be known for it being in powder and in solution. The powder was left in the same conditions and two solutions were prepared knowing that the CGA loaded has to be at 2.8 x 10^{-5}M for the results to be accurate.

When both the CGA itself and included in the polymer were measured in their solid from, depicted in Fig. 10, the measures were taken in the following space of time: time 0, 48h, and then once a week for 4 weeks. However, when they were measured in dissolution, the measures were taken in the space of time stated before, but the second measured was taken in 96 hours, 4 days.

The stability of the chlorogenic acid, was also studied through the methods used to characterize β-cyclodextrin polymer being those thermogravimetric calorimetry and infrared spectroscopy (FTIR). These two methods were carried out the same as they were done for the β-CD polymer itself, but with the system CGA-βCDP and also with CGA in order to determine the protective capacity of the polymer.

3.2.6. Scavenging activity

As it has been previously stated, chlorogenic acid is an antioxidant, and therefore, its scavenging activity can be determined. The method used for this is based on DPPH, this is because a decrease in this stable radical absorbance, measured at 517nm, means that is undergoing a reduction process, either by an antioxidant or a radical species [24]. This practice was developed with just chlorogenic acid in order to obtain the standard curve, and then with the loaded CGA in the β-CDP measured over time, as a means to prove the protective action of the polymer once again.

Firstly, from a stock solution of 50 mL chlorogenic acid at 50µg/mL, 7 solutions were prepared at the following concentrations and in a volume of 10mL: 35, 30, 25, 20, 15, 20 and 5µg/mL [20]. Besides, solution of 0.2mM DPPH in methanol was prepared; however, due to its sensitiveness to light and heat, it must be kept in the dark and freezer until its use. From those 7 solutions 2mL were taken and mixed together with 2mL of the DPPH

solution; following, they were placed on a magnetic stirrer for a couple of minutes and finally kept in the dark for 30 minutes.

Once the 30 minutes passed, the absorbance of each of the 7 solutions was measured at 517nm in the UV-spectrophotometer together with a control solution made of 2mL of DPPH and other 2mL of methanol. Finally, the scavenging activity of each solution was obtained with the following equation:

$$\text{Scavenging activity (\%)} = [(A_{517}\ \text{control} - A_{517}\ \text{sample}) / A_{517}\ \text{control}] \times 100. \quad \text{(Eq. 6)}$$

The same procedure was used for the study of the scavenging activity of the loaded CGA in βCDP; however, instead of measuring the absorbance at different concentration, only one concentration was measured but in the space of time of both the CGA loaded and CGA itself. So, both CGA (free and loaded) were left in their solid form in the dark and in direct sunlight, at room temperature (20-22℃). The loaded CGA was first released in water and measured its scavenging activity before leaving it in the dark and in the light, in order to settle a time 0. This absorbance value was replaced in the standard curve equation of the antioxidant activity given by the chlorogenic acid in the above preparations; and at this concentration was then prepared a solution of CGA each time the measurement was taken. The measurements were taken in the following space of time: at time 0 and in 48 hours; after that, it was measured once a week; for 3 weeks.

4. Results and discussion

4.1. Determination of the stability constant (K_s) for the complex CGA-βCD

Firstly, the maximum absorbance wavelength of the chlorogenic acid was determined, 324nm, which will be the wavelength that it will be used for the measurements; but the spectrum shows another absorbance peaks at 222nm and a shoulder at 240nm (Fig 4).

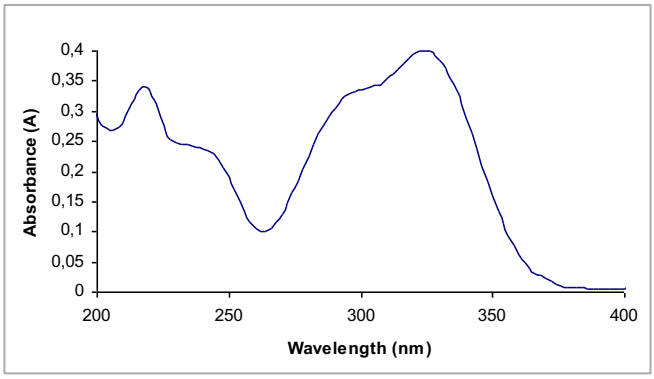

Figure 4. Absorption spectrum of chlorogenic acid in water measured by UV/Vis [27].

Regarding the determination of the stability constant for the inclusion complex in water at room temperature, 12 records were calculated as stated, one for each experiment. However, the final K_s value was obtained taking into consideration only the results from 7 experiments, which were the ones that presented a more alike stability constant. The following graph (Fig. 5) represents the plotting of $\frac{1}{\Delta ABS}$ against $\frac{1}{[CD]}$; from which the K_s can be calculated giving a result of 151 M^{-1} for a 1:1 stoichiometry.

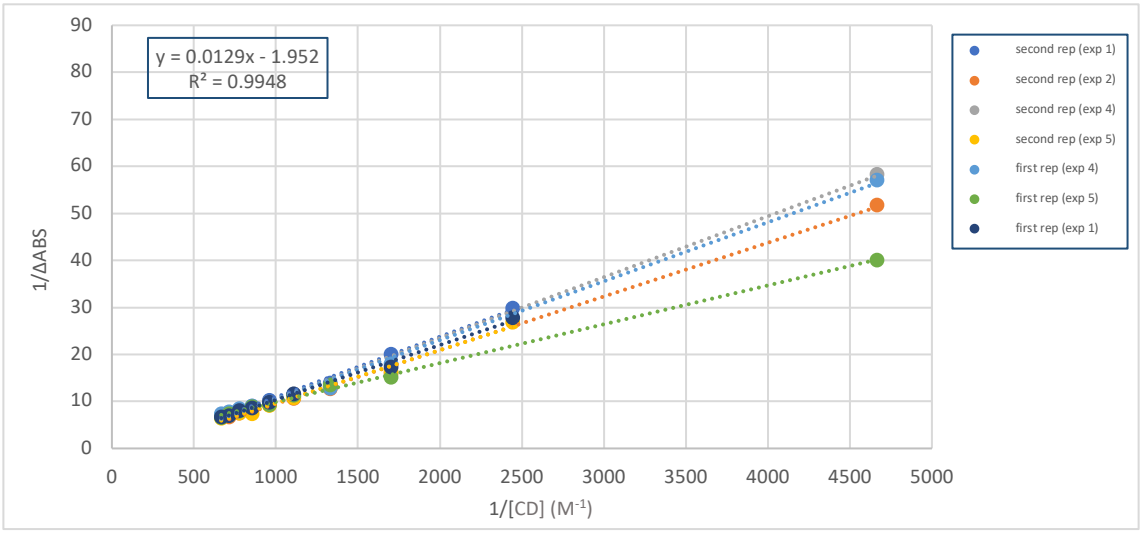

Figure 5. Representation of $\frac{1}{\Delta ABS}$ against $\frac{1}{[CD]}$ for the inclusion complexes between chlorogenic acid and β-CD in water, through UV-visible spectrophotometry at 324nm.

Furthermore, the stability constant of each those 7 experiments on their own was also obtained and then calculated the mean along with its standard deviation, giving a measure of 160±11 M^{-1}; which correlates neatly with the one obtained solely by the above graph.

4.2. Synthesis and characterization of β-cyclodextrin polymer (βCDP)

As for the characterization of β-cyclodextrin polymer; the elemental analysis resulted in a percentage of epichlorohydrin of 11.96% and β-CD of 88.04% for 250µm particle size samples; and a percentage of ECH of 19.29% and β-CD of 80.71% for 160µm particle size samples. Both were prepared exactly the same and with the same products so the most logical explanation for the difference in the percentage is the heterogenicity of the polymers, meaning that not all portions have the same cross linkage and therefore, not the same quantity of cyclodextrin. However, the values obtained were higher than the ones obtained by another study [21], which were of 68% of β-CD.

The thermal analysis depicted in Fig. 6, showed that β-CD polymer first suffers a dehydration process at 90-100°C that changes its weight too, but it is still present after that until its weight decreases at 290-300°C, which is supposed to be the melting point of β-cyclodextrin.

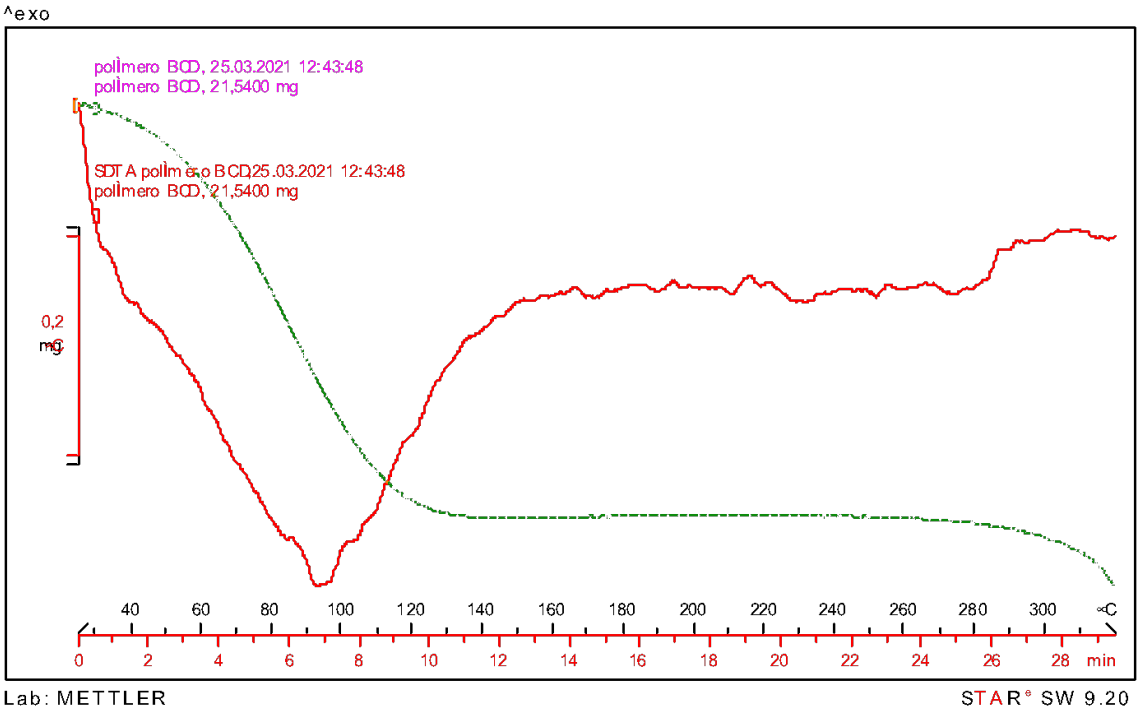

Figure 6. The relationship between TGA and DTA of β-CD polymer measured by thermogravimetric analysis.

The IR spectra of β-cyclodextrin polymer was the same for both 250µm and 160µm particle size β-CD, as supposed, and therefore, only one of them was analyzed (Fig. 7). As a general view, firstly, there is a rounded peak around the 3200-3400cm^{-1} which corresponds to the hydroxyl groups (OH), then, the spectra showed a C-H stretch at 3000cm^{-1} and continuously, at 1500-1600cm^{-1} there was a C=O type of stretch, not that prominent. This functional groups can be observed in the structure of the polymeric structure of any cyclodextrin as depicted in Fig 2. Lastly, the maximum peak is depicted at 1000-1100cm^{-1} and followed by a stretch at 900cm^{-1}; however, 500 to 1000cm^{-1} wavenumbers are generally not useful as it is the "fingerprint" region [30].

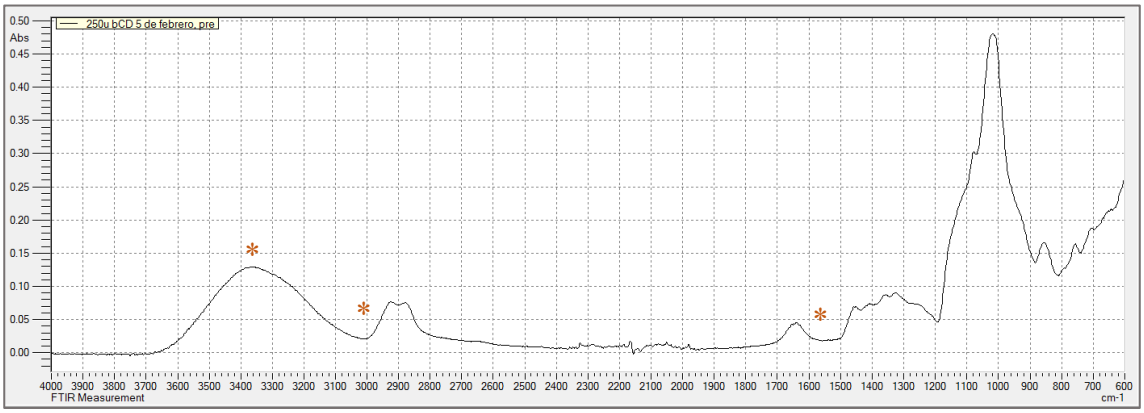

Figure 7. FTIR spectra of β-CDP.

The mercury intrusion porosimetry performed on the porous structure of the β-cyclodextrin polymer revealed the following information: the total intrusion volume was of 0.8372mL/g and the total pore area was of 0.04m²/g; the pore area being a small value indicates a large number of pores and a small average pore size, which is related to a lower value of porosity [26], thus, the percentage of porosity is of 1%. This percentage is very small (this procedure should be repeated) which indicates that the inclusion of the bioactive compound in the polymer is also going to be small. This is not only due to the size of the pore, but also the heterogenicity of the hydrogel plays a role, as not all cyclodextrins are exposed making it difficult the entrance of mercury. This small value of porosity relates to a higher value of permeability [26] with a value of 191.96mdarcy. Finally, the tortuosity is of 22.9, this value represents the difficulty that the hydrogel presents to mercury so that it can reach the structure of the β-CD polymer, and therefore its interior cavity. The representation of log differential intrusion (mL/g) versus the pore size diameter (µm) depicted in Fig. 8 showed two pore populations probably due to the increase in pressure during the test, the first presenting a pore size diameter of around a

100µm, and the second one of around 60µm; however, both can be classified as macropores [31].

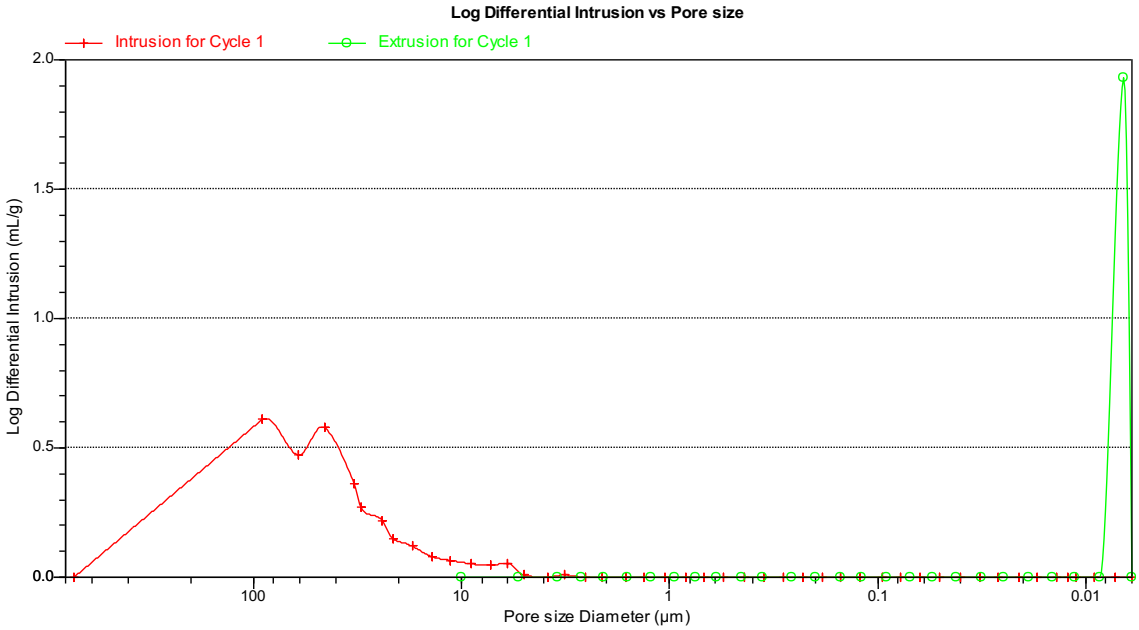

Figure 8. Pore size distribution β-cyclodextrin polymer hydrogels analyzed by mercury intrusion porosimetry.

4.3. Inclusion of chlorogenic acid into the polymer

As for the standard curve, it was confirmed that the one used in another essay was performed correctly [27]; giving the following equation: y = 19630x – 0.006 (Fig. 9).

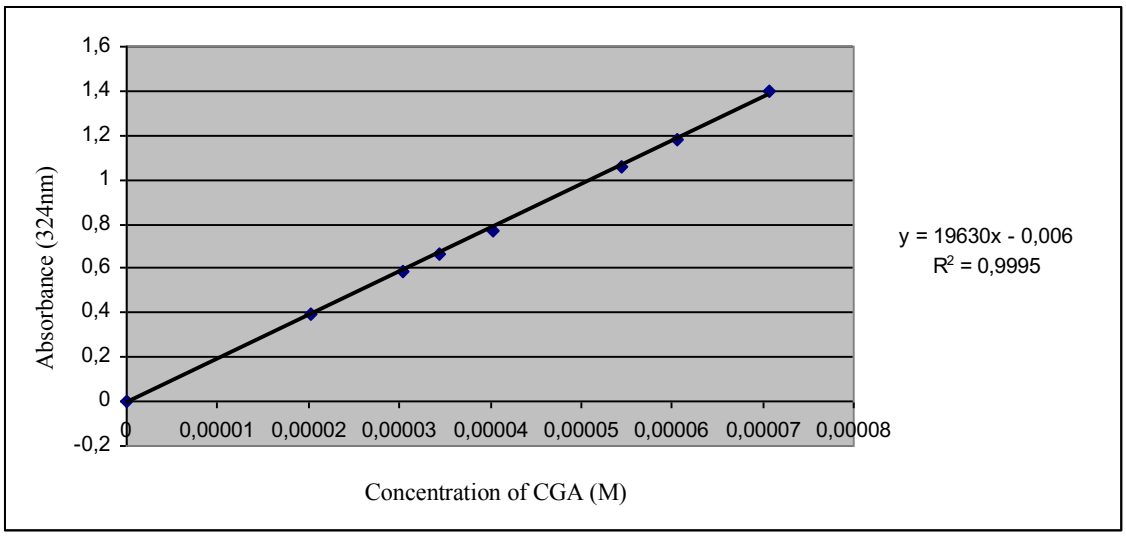

Figure 9. Standard curve of chlorogenic acid measured by UV-spectrophotometry at 324nm.

The results regarding the inclusion of chlorogenic acid into β-cyclodextrin polymer are summed up in the following table (Table 1), in terms of the mass of CGA loaded per gram of dry β-CD polymer.

Table 1. Results of the inclusion of chlorogenic acid into β-CD polymer.

β-CD polymer initial mass (g) before inclusion	CGA mass loaded (mg)	mass of CGA loaded (mg/g of polymer)
0.5024	32.28	64.24
0.5068	32.89	64.88
0.5014	24.90	49.66
0.5056	26.27	51.95
0.5032	25.42	50.52
0.5027	30.15	59.98
0.5018	32.33	64.44
0.5037	26.66	52.93

As it can be observed, the mass of CGA loaded per gram of polymer is different from one sample to another. This is normal as the hydrogel seems to be very heterogenic itself and in comparison with the rest. These samples are the ones that were used for the following studies of stability and scavenging activity of the complex and for the study of the release of chlorogenic acid from the polymer.

4.4. Release of chlorogenic acid from the βCDP

It has already been noted from previous studies carried out in the laboratory that the release of chlorogenic acid from the complex is rapid [27], this was reassured by the manual sampling assay which showed a total liberation in the first 5 minutes. This excessively rapid release may be owed to the fact that most of CGA is found adsorbed in the interstices of the polymeric network and not included in the cavities of this one; however, it is surely because of the high speed (rpm) used in the test, which forces the compound to release more rapidly.

Therefore, for the Sotax dissolution test a time space of 160 minutes was stablished for the release of the bioactive compound. In these trials, the total release of the chlorogenic acid did not happen in 5 minutes, thus, the stirring speed was of 50rpm as indicated by Pharmacopeia, and it was performed in a basket as it has been explained in section 3.2.4; this results in a slower release of the bioactive prolonged in time.

This test was performed in duplicate, and they consisted of 49.7mg CGA per gram of polymer and 52.0mg CGA per gram of polymer; however, the mass of the polymer changed after inclusion and so did the loaded chlorogenic acid. Thus, the first test showed a resulting mass of 21.6mg of CGA, with a percentage of liberation of 74.0% in relation to the inclusion; and the second test showed a mass of 21.9mg of CGA, with a percentage of liberation of 77.1%. Both tests showed reliable results as the mass of chlorogenic acid released increased throughout the 160 minutes, one with a resulting mass of 16.0mg and the other of 16.9mg, respectively, experiencing a release of 69.6% and 68.2% in the first 20 minutes.

As for the mathematical modelling of the controlled drug release, the curve fit for the Korsmeyer-Peppas of only one dissolution test is attached (Fig. 10) as the results are very similar, where it is depicted the percentage of chlorogenic acid released at each time.

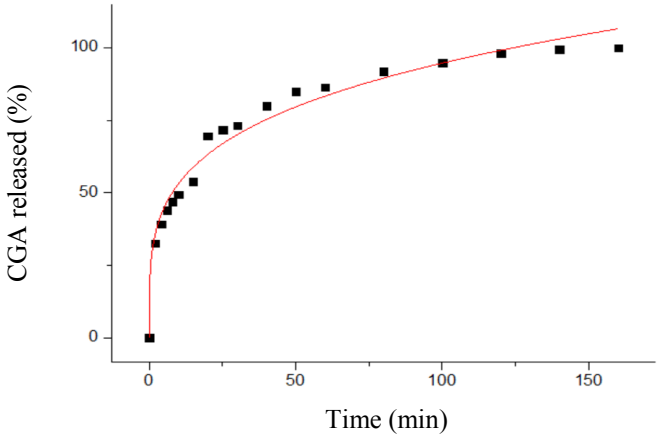

Figure 10. Released profile of chlorogenic acid from β-CD polymer (adjusted by K-P model).

In addition, the following table (Table 2) summarizes the records of the Korsmeyer-Peppas and Higuchi model.

Table 2. Model parameters of two Sotax dissolution tests.

Model	Test	k (min^{-n})	n	R^2
Korsmeyer - Peppas model	1	29.84 ± 1.58	0.25 ± 0.01	0.978
	2	25.81 ± 2.48	0.28 ± 0.02	0.946
Higuchi model	1	10.18 ± 0.59	0.5	0.612
	2	10.12 ± 0.54	0.5	0.717

The fitting for the Higuchi model is not adequate because the value of *n* given in both tests by Korsmeyer-Peppas model is less than 0.5. Zero and first order kinetics were not

included in this table because the data given by these two mathematical models did also not fit well in the equation, due to the same reason stated above. However, the release profile presenting a good fit to the zero order kinetics model are the ones that have a value of *n* greater than 1 (case II transport), and to the first order kinetics model are those that present value of *n* between 0.5 and 1 (anomalous transport) [18]. Consequently, this result does not allow the clarification of a specific release mechanism, although it was expected that the data would fit correctly in the Higuchi model, like the ones showed by previous studies on drug release with this type of polymers [18].

4.5. Determination of the stability of the chlorogenic acid included in the polymer

On one hand, the results for CGA measured in its solid form were compared to those of the chlorogenic acid in the polymer, also in its solid form, both exposed to sun light and in the dark over time (Fig. 11).

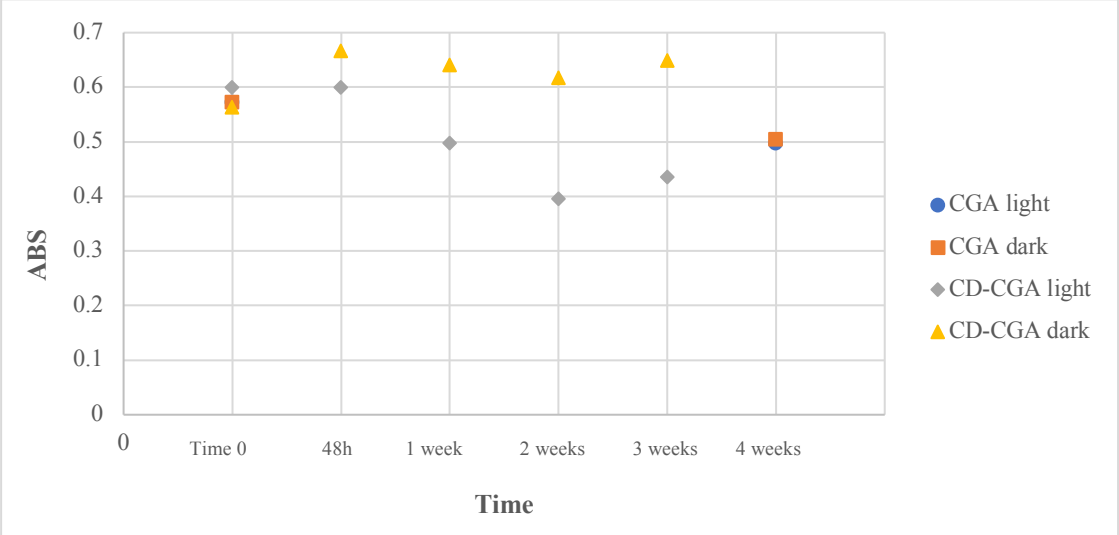

Figure 11. Determination of the stability of CGA and CGA from βCDP in powder (light/dark), in terms of absorbance over time (at 324nm).

As it can be observed, when is not in solution CGA is still stable even though it is exposed to the light (it only decreases in 10%); and therefore, the inclusion complex of this bioactive compound and β-cyclodextrin is also stable and maintains its protective action.

On the other hand, the results of the solutions of CGA 2.8 x 10^{-5}M, were compared to those of the solution of chlorogenic acid released from the βCDP, both at light and in the dark over time (Fig. 12).

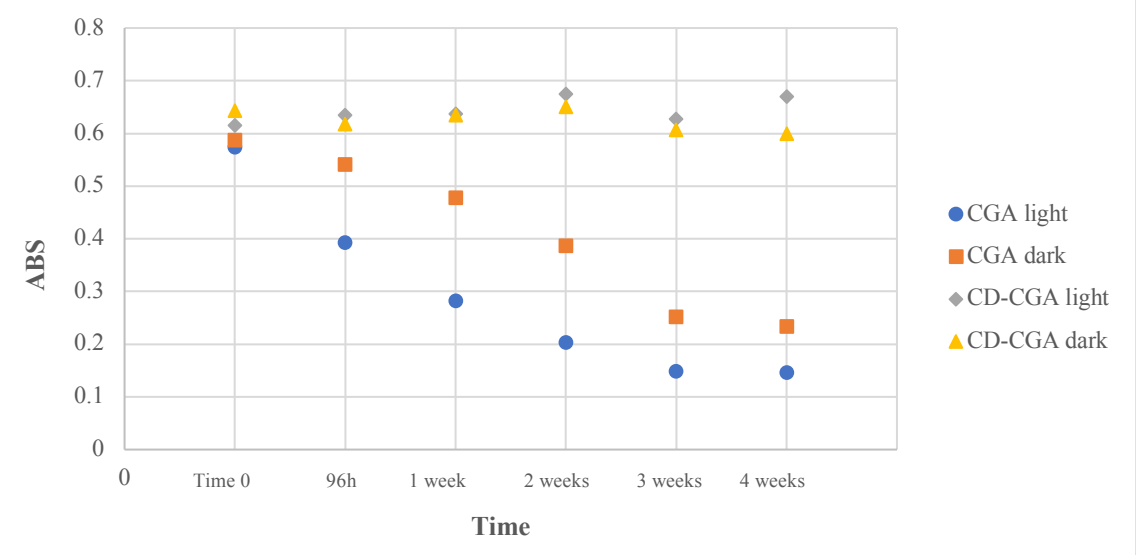

Figure 12. Determination of the stability of CGA and from βCDP in solution, in terms absorbance over time (at 324nm).

These results do show the difference of CGA being inside a protective polymeric system as the physical state of CGA in solution weakens over time and more significantly when exposed to light. However, when the chlorogenic acid is inside the hydrogels of β-cyclodextrin, the protective action takes part and prevents the acid from damaging and so conserving its stability.

In addition to this, the thermal analysis performed on chlorogenic acid showed an already known fact, thus, that the fusion point of chlorogenic acid is 210ºC (Fig. 13). However, when this bioactive compound is inside the β-cyclodextrin polymer, no change in temperature or mass is observed (Fig. 14), proving the protective effect of the polymer. However, the complex suffers a dehydration process at 90-100ºC, due to the H_2O present, as the β-CD polymer did on its own, until it reaches 290-300ºC, when the weight decreases dramatically increasing the temperature, and so suffering a combustion process.

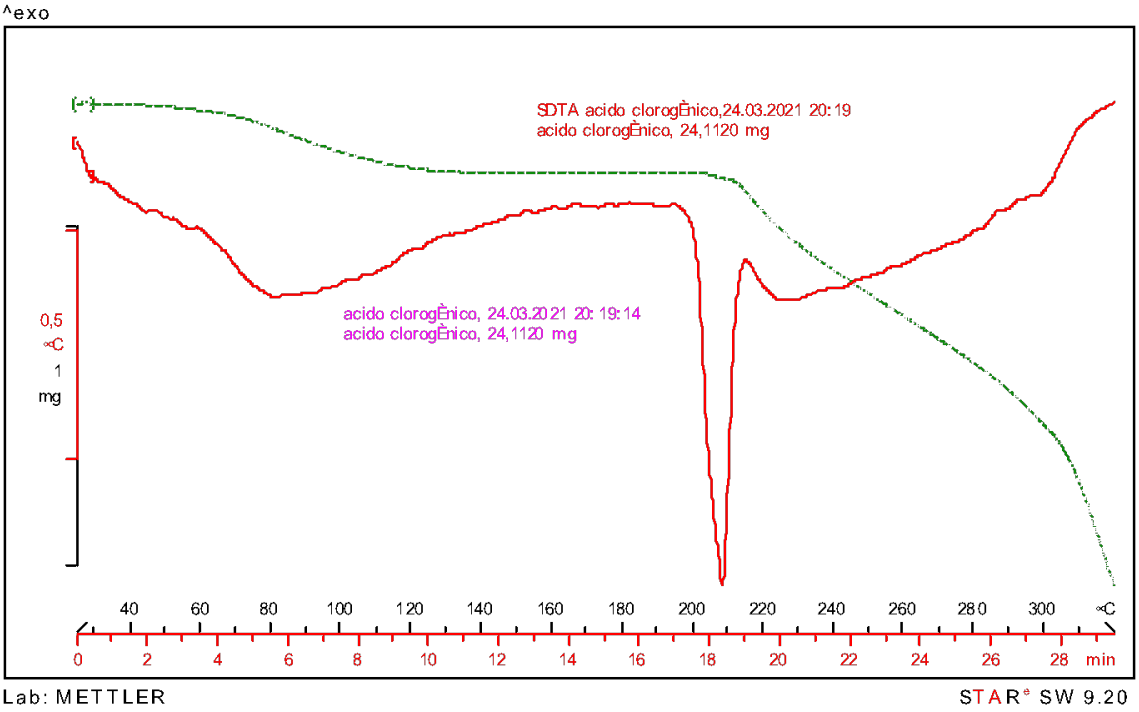

Figure 13. The relationship between TGA and DTA of chlorogenic acid measured by thermal analysis.

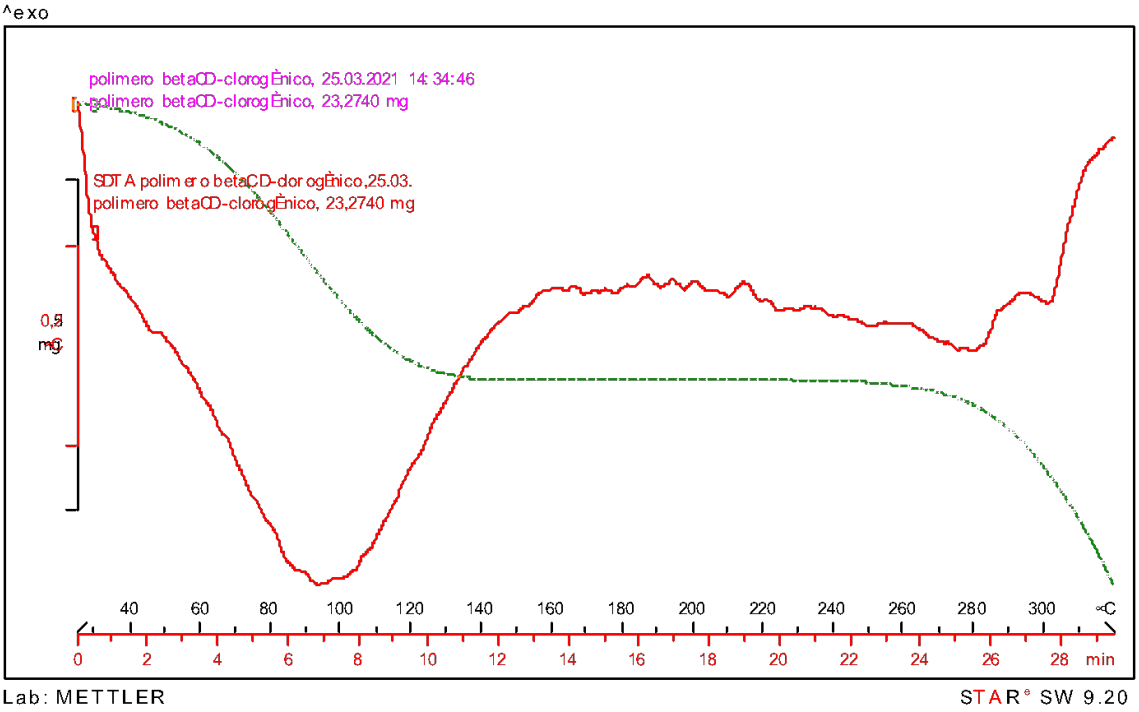

Figure 14. The relationship between TGA and DTA of chlorogenic acid included in βCDP measured by thermal analysis.

Finally, the infrared spectrum of both free and included chlorogenic acid were obtained. In Fig. 15, the spectra of CGA showed what was already known from other studies [32], focusing on the region between 1600 and 1800cm^{-1}, there are 3 peaks: a first one at 1685cm^{-1}, following one at 1636cm^{-1} and the last one at 1600cm^{-1}, these peaks refer to

the carbonyl group (C=O), the ethylene group (C=C) and the phenyl ring [33], respectively; which can be appreciated in the chemical structure of CGA (Fig. 1).

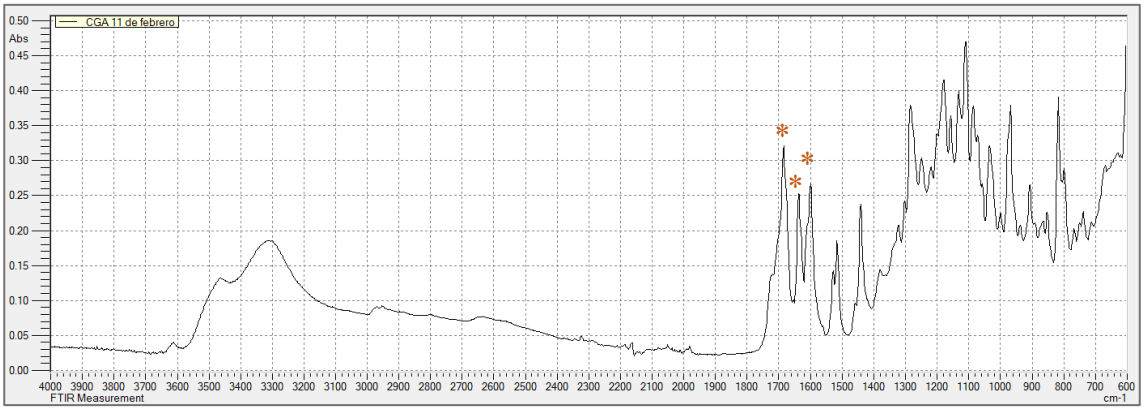

Figure 15. FTIR spectra of chlorogenic acid.

The spectra for the included chlorogenic acid is depicted in Fig. 16; and as it can be observed, no change whatsoever is observed between the spectra of the β-CD polymer itself, and when the chlorogenic acid is inside its cavities. This may be used to corroborate the previous findings suggesting that the polymer does in fact protect the chlorogenic acid.

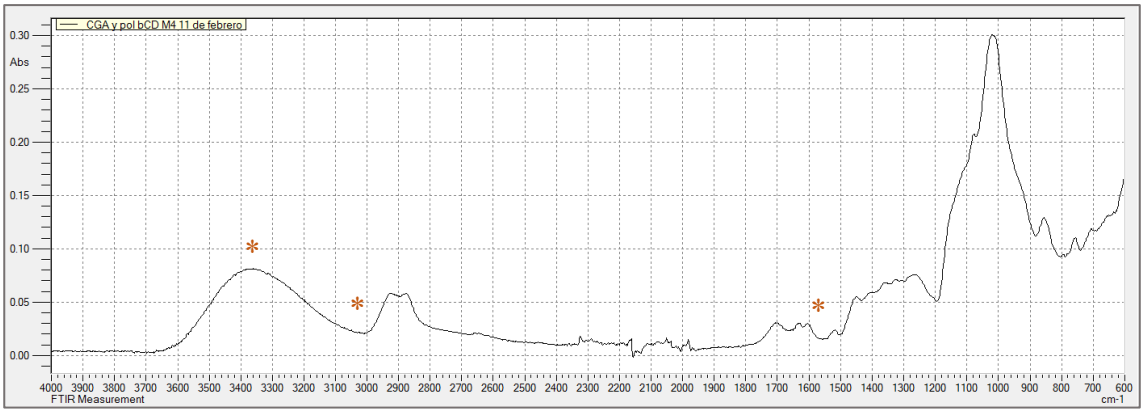

Figure 16. FTIR spectra of chlorogenic acid-βCDP system

4.6. Scavenging activity

As for the chlorogenic acid itself, the resulting scavenging activity at different concentrations was represented in Fig. 17 where the absorbance of each concentration is represented. The data depicted adjusts to a polynomial regression curve as the increase in the concentration of chlorogenic acid decreases the absorbance, indicating a higher scavenging activity.

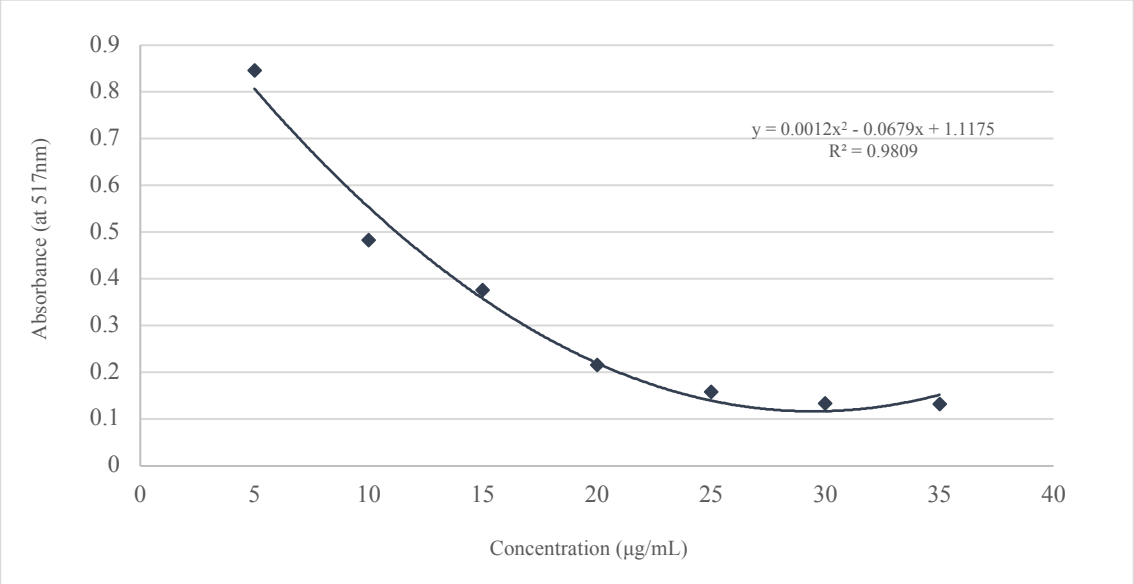

Figure 17. Representation of the absorbance of reduced DPPH at different concentrations at 517nm.

Following, Fig. 18 represents the scavenging activity of samples of chlorogenic acid based on its concentrations which also fits like a polynomial regression curve, however, in this case the slope of the curve increases with the increase in the concentration of CGA.

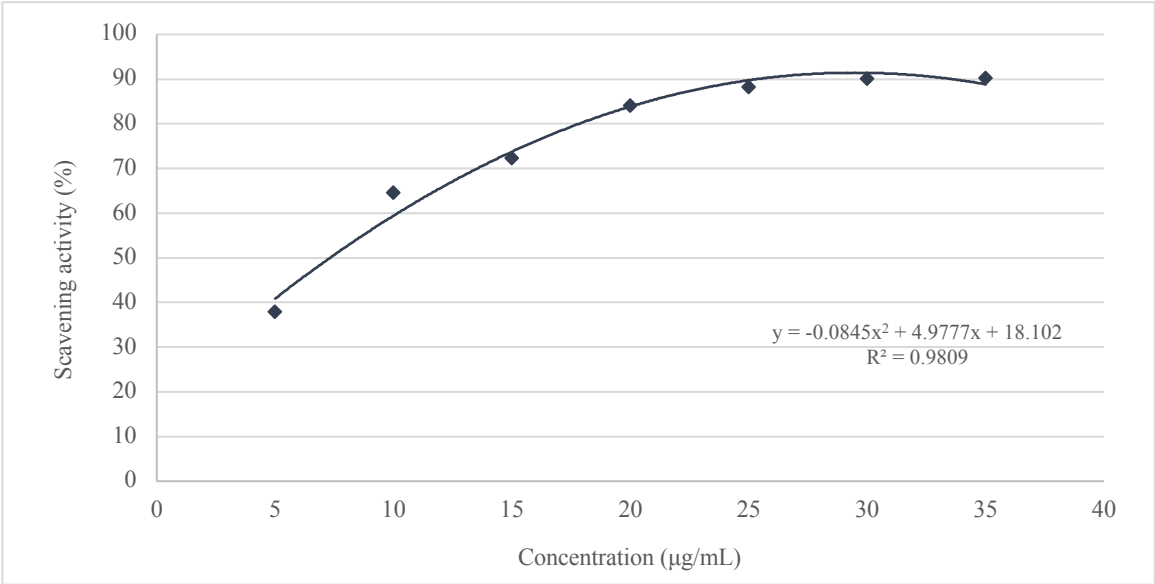

Figure 18. Representation of the scavenging activity of chlorogenic acid at different concentrations.

When the chlorogenic acid was released from the polymer and measured its absorbance in order to settle the time 0, after the replacement of this value in the equation the concentration of the given product was of 19.5µg/mL, resulting in a scavenging activity of 81.7% at first. This was compared to the data obtained above, which gave a scavenging activity of 84.2% when the concentration of chlorogenic acid was of 20µg/mL. As the

concentration now is smaller, the scavenging activity also has to be accordingly; therefore, the data is accurate; and so, the CGA solution made in each measurement was prepared following the concentration of 19.5µg/mL.

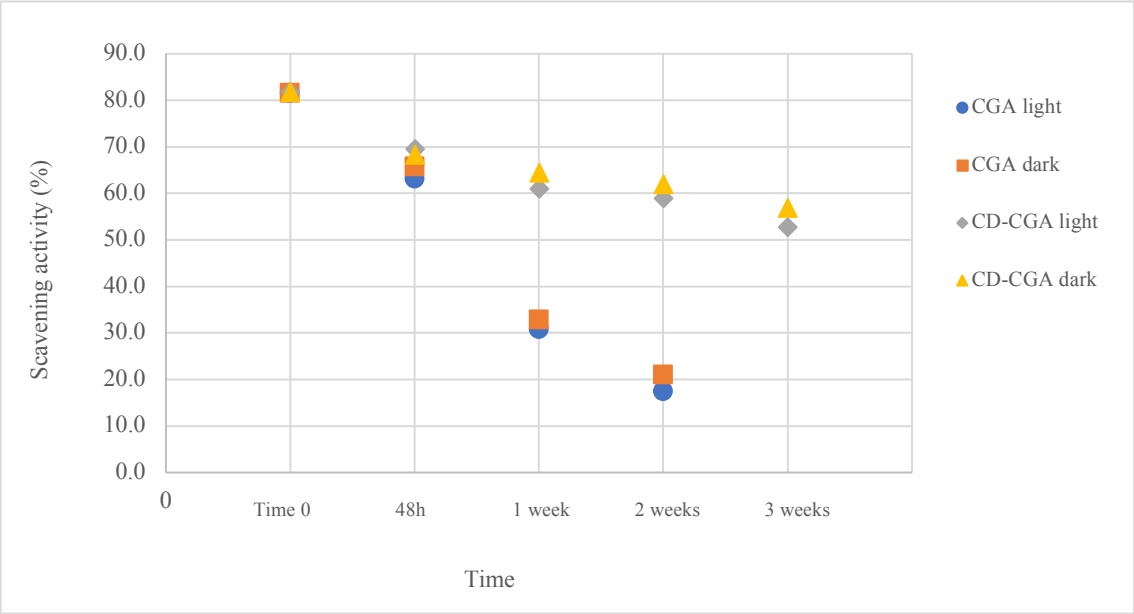

Figure 19. Representation of the scavenging activity of the free and loaded chlorogenic acid (19.50μg/mL) over time.

Even though in the section prior to this one it has been stated that CGA in powder does not experience any damage over time, it does experience a significant decrease when measuring its scavenging activity as it went from 81.5% to 17.4% in a space of just 2 weeks when exposed to light, and to 21.0% when it was kept in the dark; so, it depicted a decrease of more than half of its activity (Fig. 19). This decrease was also noted in the included chlorogenic acid but is not as emphasized as it is when the polymer is not protecting the CGA; experiencing a decrease in the scavenging activity of 29.1% in 3 weeks when exposed to light, and of 25.0% when stored in the dark (Fig. 19).

5. Conclusions

Once the resulting data has been obtained and analyzed, the following conclusions can be drawn:

1. The size of the β-cyclodextrin is adequate enough to hold the chlorogenic acid in its cavities, and the inclusion complex presents a 1:1 stoichiometry.

2. After including the substance in the structure of the synthesized β-CD polymer, with a percentage of cyclodextrin of 80%, it is deduced that it presents certain heterogenicity due to the differences in the chlorogenic acid loaded.

3. The release of the chlorogenic acid from the polymer by manual sampling went as expected, with a rapid release over time. However, when the test was performed with a lower stirring speed and in basket through Sotax dissolution testing, the release was slower and more controlled.

4. When studying the stability of the chlorogenic acid included in βCDP, it was proven through UV-spectrophotometry and thermal analysis, that the polymer helps to maintain the integrity of CGA over time, especially when found in solution and exposed to light.

5. Regarding the study of the scavenging activity, this statement is once again verified as the antioxidant activity is better maintained, to a great degree, when the chlorogenic acid is inside the polymeric matrix.

6. The main objective of this experimental project being the demonstration of the ability of the polymer to protect the chlorogenic acid when it's inside its cavity has been accomplished.

7. This experimental project reinforces previous studies done in this subject and paves the way for further research.

6. Future perspective

As it has been stated, this study was focused on chlorogenic acid as it is one of the phenolic compounds found in coffee, which are what makes this drink to be a source of antioxidants. The perspective that can be applied with further research in this topic is to introduce those antioxidants in our organism without the need to drink coffee, using a polymer of cyclodextrins as the transport system as CDs are well known for their capacity to form inclusion complexes allowing the transport and storage of different compounds, including chlorogenic acid.

Therefore, as antioxidants are known to have a protective effect in a number of diseases especially those implicating oxidative stress; maybe some of these diseases or at least the early manifestation of symptoms may be prevented if we introduce this bioactive compound in our diet, like a powder to add to a drink or beverage.

From this hypothesis, and in order to continue with this work, studies of release from the loaded polymer in bottles of water can be carried out, stabilizing the suitable quantity of the system. On top of that, the antioxidant efficacy in vivo could be verified, through control studies administrating placebo, free antioxidant and the system studied in this project.

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