Tutor/s

Dr. Alicia Maestro Garriga
Departament d'Enginyeria Química

Dr. José María Gutierrez Gonzalez
Departament d'Enginyeria Química

Grau en Enginyeria Química

Treball Final de Grau

Cocoa extracts encapsulations for avoiding polyphenols degradation.

Encapsulación de extracto de cacao para evitar la degradación de polifenoles.

Mouna Taifi

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Hi ha una força motriu més poderosa que el vapor,
l'electricitat i l'energia atòmica: la voluntat.

Albert Einstein

Estar aquí no habría sido posible sin los esfuerzos y sacrificios de Papa y Mama. Quiero agradeceros el apoyo, la confianza y el amor incondicional que me brindáis todos los días. A mis hermanos Imad y Youssef, gracias por ser mi pilar.

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REPORT

CONTENTS

1. SUMMARY

Cocoa is highly beneficial for health, because it contains a great deal of polyphenols, which are antioxidants, but also contains arginine, dopamine (neurotransmitter), magnesium, serotonin (neurotransmitter), tryptophan (which is essential to cause the release of the neurotransmitter serotonin), fenilethylamine, and flavones among many others.

The term antioxidant is given to a type of molecules that have the ability to retard or even prevent the oxidation caused by oxygen. Oxidation is the transfer of electrons from one substance to another from an oxidizing agent. In consequence of this transfer of electrons, there is a release of radicals causing cell death.

Polyphenols contained in food have stability problems, because they are sensitive to sun exposure and other environmental factors, such as high temperature, humidity, etc. Therefore it seems adequate to protect them from degradation.

In this work, cocoa extract has been encapsulated into jellified alginate spheres and filaments in order to prevent degradation. However, although this technique has been proposed in the literature as a protection method, there is no published evidence of effective protection of polyphenols against degradation using this type of encapsulation.

Therefore, the main aim of this work is to study the protective effect of encapsulation of cocoa extract into alginate gel against degradation, compared with degradation of free cocoa.

In order to reach this objective, external gelation was used in order to prepare capsules (an external source of gelling agent, Ca^{2+}, is chosen). A solution of alginate + cocoa was dropped into a conventionally stirred $CaCl_2$ solution or, alternatively, introduced in the solution stirred by ultraturrax. In the first case, spheres were formed. In the second one, filament morphologies, due to the high shear, were found. Samples were subsequently lyophilized. Free cocoa extract was lyophilized for comparison.

Once encapsulation was done, the amount of polyphenols contained and their evolution vs. time must be measured in order to study the protective effect. Therefore, an analytical method must be chosen. The Folin & Ciocalteu method was selected as the proper one, and tested first

using polyphenols solutions directly prepared with cocoa extract. It expresses the amount of polyphenols as milligram equivalents of Gallic acid per gram cocoa extract. This method proposes to work at 20ºC. We firstly worked at environment temperature. However, variation of laboratory temperature and a too long analysis time distorted results. Finally, temperature was controlled and raised to 40ºC and reaction time was shortened, finding reproducible results.

On the other hand, before analysis could be done, polyphenols must be extracted from capsules in order to be dissolved in the adequate solution to be measured. Different extraction mediums (water, ethanol, and a mixture ethanol/water) were used, followed by washing with water. It was found that the better solvent was ethanol. Moreover, a basic medium was used in order to partially destroy alginate gel and help extraction process. Under these conditions and with an extraction time of 30 min reproducible results were found.

Several batches of capsules and filaments were prepared and lyophilized, as well as free cocoa extract, and it was found that lyophilization partially degraded polyphenols, although degradation was slighter when they were protected by encapsulation.

These batches were exposed to sunlight, open to the atmosphere, for different periods of time, as well as free cocoa extract, and then polyphenols were extracted and measured. Before that, a big batch was done and several samples of this batch were measured, finding non-reproducible results. It was attributed to segregation of the remnant free $CaCl_2$, which made that different samples had different proportions of cocoa. Therefore, we decided to use the complete batch for each measurement, and reproducible results were found. It was found that encapsulation slightly protected polyphenols from degradation, although this effect probably does not justify all the process.

The release of polyphenols was also studied, in acid, basic and neutral medium. A faster release was found in basic medium, as a result of a partial destruction of gel. There are not significant differences between spherical or filament capsules.

Keywords: Polyphenols, Cocoa, Encapsulation, Alginate, Degradation of Cocoa, Delivering of polyphenols.

2. RESUM

El cacao es un alimento altamente beneficioso para la salud, puesto que contiene una gran dosis de polifenoles, que son antioxidantes, aunque también contiene arginina, dopamina (neurotransmisor), magnesio, serotonina (neurotransmisor), triptofano (esencial para provocar la liberación del neurotransmisor serotonina), feniletalamina y flavones entre muchos otros.

El término de antioxidante, se le otorga a un tipo de moléculas que tiene la capacidad de retardar o incluso impedir, la oxidación provocada por el oxígeno. La oxidación, consiste en la transferencia de electrones de una sustancia a otra a partir de un agente oxidante. Como consecuencia de esta transferencia de electrones, hay una liberación de radicales que ocasiona la muerte celular.

Los polifenoles contenidos en los alimentos presentan problemas de estabilidad, ya que son sensibles a la exposición solar y otros factores ambientales, como pueden ser las altas temperaturas, la humedad, etcétera. Por la cual cosa, se aconseja protegerlos, y así evitar la degradación.

Los polifenoles están situados en un material biológico, como es en este caso, que están ubicados en el extracto de cacao. Para poder analizarlos lo que se ha hecho, es que se ha transportado a los polifenoles presentes en el cacao, al medio de análisis, en unos experimentos ha sido en medio de etanol (cuando se ha querido estudiar la totalidad de los polifenoles presentes en una muestra) y en otros experimentos, ha sido en disoluciones acuosas (variando el pH de estas, con la finalidad de estudiar la liberación que tienen los polifenoles en dichos medios).

El objetivo de este proyecto es el de desarrollar un método analítico para medir la cantidad de polifenoles que contiene una muestra de extracto cacao. Teniendo el valor de la cantidad de polifenoles presentes a tiempo cero en un gramo de extracto de cacao, se ha podido comparar con la cantidad de polifenoles en extracto de cacao, pasado un periodo de tiempo determinado y estando la muestra expuesta a la radiación solar. De esta forma, se ha podido hacer un seguimiento sobre la degradación de los polifenoles dependiendo del tiempo en el que han estado en contacto con la luz solar. Este seguimiento se ha hecho tanto del extracto de cacao encapsulado como del extracto de cacao sin encapsular.

Para analizar la cantidad de polifenoles, por gramo de cacao se ha utilizado el método Folin & Ciocalteu. De esta forma, se ha podido determinar el valor de cantidad de polifenoles, expresados en miligramos equivalentes en Acido Gálico por gramo de extracto de cacao.

Otro objetivo, ha sido el de encapsular el extracto de cacao. La encapsulación se ha realizado añadiendo bajo agitación mezclas de extracto de cacao y alginato, sobre soluciones de cloruro cálcico, para obtener una dispersión de gel conteniendo el extracto de cacao. Esta dispersión se ha secado por liofilización, al no haber aumento de temperatura para eliminar el agua presente en la muestra secar, se conservan las propiedades organolépticas del alimento. Utilizando distintos métodos de agitación, se han obtenido diferentes morfologías del cacao encapsulado, exactamente, el cacao encapsulado en forma de esferas y el cacao encapsulado en forma de filamentos.

Se ha liofilizado cacao sin encapsular, se han medido los polifenoles que conserva después de ser liofilizado y se ha comparado con el extracto de cacao sin liofilizar, de esta forma se ha observado que en la etapa de liofilización el cacao sufre degradación.

Para saber la degradación que sufren los polifenoles, se han dejado muestras expuestas a la luz solar, y a distintos tiempos, se ha medido la cantidad de polifenoles que presentaban dichas muestras. El valor obtenido de polifenoles a tiempo cero y con la muestra protegida, se ha comparado con el valor obtenido de polifenoles a un tiempo t y con la muestra expuesta a iluminación solar. Se han dejado expuestas muestras de cacao encapsuladas y muestras sin encapsular, ambas, sometidas a las mismas condiciones de contacto con aire e iluminación. A partir de los resultados obtenidos, se puede observar que el método y el material utilizado para encapsular, no son del todo efectivos, puesto que el material encapsulado, pierde parte de sus propiedades en el proceso de liofilización, y al exponer la muestra a la luz solar, también sufre degradación a pesar de estar encapsuladas.

En este trabajo también se ha estudiado la velocidad de liberación de los polifenoles en distintos medios. Las muestras encapsuladas y liofilizadas, se han depositado en medio acido, básico y neutro, en continua agitación, y a diferentes tiempos se extrajo una alícuota, la cual se analiza, con la finalidad de determinar la cantidad de polifenoles liberados. Comparando los valores obtenido en distintos medios. Se puede concluir que en medio básico, es donde mejor se liberan los polifenoles del extracto de cacao encapsulado, ya que el hecho de sumergir las capsulas, hace que la basicidad del medio solubilice la capa de alginato encapsulante.

Palabras clave: Polifenoles, Extracto de cacao, Encapsulación, Alginato, Degradación cacao, Liberación polifenoles.

1. INTRODUCTION

Currently, more and more consumers are interested in consuming minimally processed products, able to retain all their properties for as long as possible.

Furthermore, the consumer is very concerned in products that may contain a wider range of beneficial health properties in a single food.

An example of this type of food is cocoa.

1.1. COCOA

Cocoa is a food that comes from the Theobroma cacao tree. The cacao fruit has a crust of about 4 cm thick, and is filled with a viscous and sweet pulp, and cocoa beans are found in the pulp. There are about 30 to 50 grains in each bean, which are long and arranged in a row in the pulp itself.

Figure 1: Cocoa beans.

1.1.1. Chemical process

The chemical process used for obtaining cocoa extract is as follows.

Bacteria and yeast present in the air multiply in the pulp surrounding the beans, and the concentration of sugars that it has. Pulp decomposes forming an acid liquid and alcohol. This process increases the temperature of the whole grains and triggers some internal changes in the cocoa beans. The color of the beans changes to brown and the smell of becomes present.

Fermentation has two objectives, the first one is that the pulp becomes acetic acid evaporated, and the seed size increases to that of an almond. Secondly, it reduces the bitter and astringent flavor of cocoa. [1]

In case of exceeding the fermentation time, it could spoil the cacao, reaching temperatures that override some properties, if there isn't enough time left to carry out fermentation, it could cause the cocoa to taste like raw potatoes and moreover it would be easily attacked by fungi.

Continuing the process the beans are dried. In this last step, the beans lose up to a quarter of their weight.

Cocoa beans contain:

- 54 % cocoa butter
- 11.5 % protein
- 9% cellulose
- 7.5% starch
- 6% tannins
- 5% water
- 2.6% sales
- 1% sugars
- 0.2% caffeine
- 1.2% theobromine
- 2% organic acids and essences

There are three types of polyphenols in cocoa: [2]

- Flavonoids (37%)
- Anthocyanins (4%)
- Proanthocyanidins (58%)

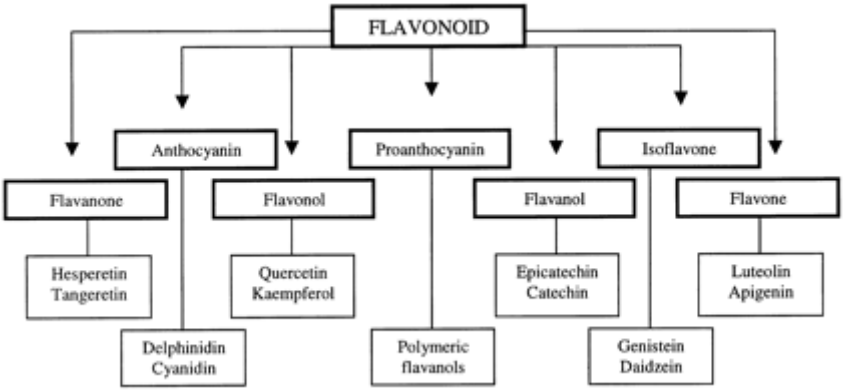

Figure 2: Types of polyphenols

1.1.2. Health benefits of cocoa.

Stabilizes blood pressure: Flavonoids stimulate the production of nitric oxide molecules that promote vasodilation or increased caliber of the blood vessels. Thereby, it increases blood flow and stabilizes blood pressure. [3] [4]

Helps prevent heart disease and strokes: Cocoa consumption is associated with a lower incidence of heart attacks. The flavonoids found in this fruit have the potential to prevent heart disease. Flavonoids are also found in brightly colored fruits, vegetables, tea, red wine and coffee.

Lowers cholesterol: According to scientific studies, consumption of black chocolate with 60-70% cocoa, lowers the levels of low density lipoprotein (LDL).

Influences insulin resistance: The antioxidant effects of cocoa can influence in insulin resistance or disability of the body to respond to insulin, helping to reduce the risk of diabetes.

Improves brain function: The flavonoids in cocoa improve the system that activates thought. When cocoa is consumed for eight weeks, there are improvements in cognitive function and verbal fluency. [14]

Relieves stress: Theobromine, an alkaloid present in cocoa, is a non-addictive stimulant of the central nervous system. It is not as strong as caffeine and its effect is prolonged. It helps the brain in the production of anandamide, a neurotransmitter responsible for the sensation of euphoria and pleasure.

Reduces the risk of cancer: Cocoa helps to reduce the risk of cancer because it is rich in antioxidants that act at the cellular level. These fight against free radicals and prevent the formation of cancer cells.

Eye health improvement: Flavonoids enhance the ability to see in difficult conditions.

Beneficial for the kidneys: Theobromine, like caffeine, but with moderate and long-lasting effects. As coffee, it acts as a diuretic increasing urine output. Besides being a mild stimulant of the nervous system.

Disadvantages of cocoa: As already explained above, cocoa has many health benefits, but also has a major disadvantage, as polyphenols contained have little stability at ambient

conditions. Polyphenols, which are the active agents that provide the benefits discussed previously, are readily degraded at high temperatures and sun exposure.

One solution to this problem is encapsulation.

1.1.3. Encapsulation

In the world of the food industry, encapsulation processes are highly valued, as they contribute to the development and preservation of food products.

The encapsulation process consists in trapping a substance within another which acts as a protective matrix, forming aggregates, particles or capsules from nanometers to several millimeters in size. [5]

Consequently, the life of the active component can be increased, a gradual release can occur and the handling can be facilitated. A capsule, microcapsule, nanocapsule, is formed, depending on their size.

The substances used to encapsulate are starch, starch derivatives, proteins, gums, lipids, alginates and combinations thereof.

1.1.3.1. Benefits of encapsulation

The benefits of encapsulation include:

- Protecting the active components of solar degradation.
- Freezing the active ingredient in processed foods.
- Stabilizing of the final product and during the production process.
- Creating different textures.

1.1.3.2. Encapsulation types

Below is a description of what the best known encapsulation techniques are:

- **Coacervation:** The concept behind coacervation microencapsulation is the phase separation of one or many hydrocolloids from the initial solution and the subsequent deposition of the newly formed coacervate phase around the active ingredient suspended or emulsified in the same reaction media. This is done by thermal, cross-linking, or other techniques.

- **Interfacial polymerization**: It is the reaction of a monomer dissolved in an organic phase with another monomer, this monomer dissolved in an inorganic phase at the interface between the two immiscible phases.

- **Ionic gelation:**
    - **External gelation**: It is the dispersion of one liquid containing alginate solution and component to be encapsulated, in another liquid with a source of calcium which will promote gelation of alginate
    - **Internal gelation**: The alginate solution contains the compound to be encapsulated and a dispersion of an insoluble calcium salt. It is dispersed in another liquid which contains an acid that travels to the disperse phase and enters inside the droplets, decreasing pH and, therefore, solubilizing the calcium, making it available to act as the gelling agent.

- **Liposome entrapment**: they are colloidal particles consisting of a membranous system formed by lipid bilayers encapsulating aqueous space(s). Owing to the possession of both lipid and aqueous phases, liposomes can be utilized in the entrapment, delivery, and release of water soluble, lipid-soluble, and amphiphilic materials. The underlying mechanism for the formation of liposomes and nanoliposomes is basically a hydrophilic-hydrophobic interaction between phospholipids and water molecules. A major advantage of their use is the ability to control the release rate of the incorporated materials and deliver them to the right place at the right time. Bioactive agents encapsulated into liposomes can be protected from digestion in the stomach, and show significant levels of absorption in the gastrointestinal tract, leading to the enhancement of bioactivity and bioavailability.

- **Molecular inclusion:** an inclusion compound is a complex where one chemical compound forms a cavity in which molecules of a second compound are located. Molecular inclusion is generally achieved by using cyclodextrins (CDs) as the encapsulating materials. CDs are a group of naturally occurring cyclic oligosaccharides derived from starch. The external part of the cyclodextrin molecules is hydrophilic, whereas the internal part is hydrophobic. CDs are a satisfactory medium for encapsulation of less polar molecules (such as essential oils) into the apolar internal cavity through a hydrophobic interaction.

- **Spray drying**: it is a method of producing a dry powder from a liquid or suspension by rapidly drying with hot gas. This is the best method for thermally sensitive materials such as food and pharmaceuticals.

1.1.4. Alginate

Alginate is a compound found in the cell wall of brown algae (Phaeophycae). Properties of alginate vary according to the type of algae from which it derives. [6]

Alginate in the form of sodium, potassium or magnesium salt, is soluble in aqueous solutions at a pH above 3.5.

Also, it is soluble in mixtures of water and miscible organic solvents, such as alcohol, but is insoluble in solutions containing calcium.

The viscosity of alginate solutions depends on the concentration, rising significantly from 2%, and temperature. The viscosity decreases with increasing concentration or temperature.

Alginates have the capacity to retain water, and because of that, have the characteristics of gelling, stabilizers, thickeners. It has a flexible structure and mechanical strength.

Alginates are hydrocolloids considered because of their affinity with water and their rheological properties.

In the presence of calcium, alginate can form a structure known as an "egg crate". In this structure, the calcium ions are situated as bridges between the negatively charged groups of glucuronic acid.

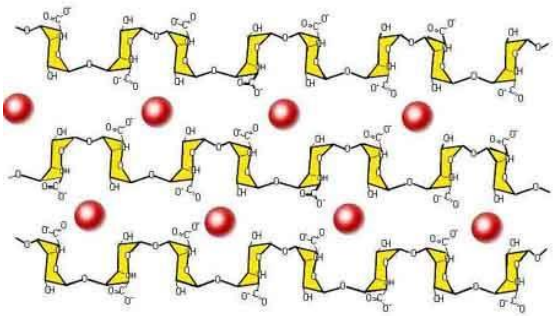

Figure 3: Alginate structure in the presence of calcium

Alginate behaviour is affected by the following factors:

- **Temperature**: the solubility of alginates decreases by 2.5% per degree of temperature increase.

- **pH**: Between the range of 5 to 10 in the pH scale there aren't not much influence on the behaviour of alginate. In the ranges of 3 to 3.5 on the pH scale it begins to observe changes in the behaviour of alginate. Alginate solutions are stable at an ambient temperature, at pH 3-4, but below pH 3.5 the alginate is insoluble in aqueous solutions because, under pH 2 and above pH 6 alginates lose viscosity.

- **Ionic strength**: the viscosity of alginate solutions decreases slightly in the presence of salts with monovalent cations, as the polymer tends to shrink with increasing solution ionic strength.

- **Concentration and molecular weight**: viscosity of alginate solutions increases with concentration. Moreover, at higher molecular weights the thickening power is greater.

2. OBJECTIVES

The general objectives of this work are to study the degradation of polyphenols contained in cocoa extract, and to study how encapsulation protect them from their degradation. In order to achieve these general objectives, the following studies will be carried out:

- Encapsulation of cocoa extracts. Encapsulation will be carried out by gelling of aqueous dispersions of cocoa extract containing sodium alginate with addition of calcium ions. The dispersion of encapsulated cocoa extract is lyophilized to obtain a dry product containing the encapsulated cocoa extract. Influence of stirring method on encapsulation will be studied.

- Analysis and extraction of polyphenols. Concentration of polyphenols in aqueous solutions will be analyzed by Folin & Ciocalteu method.The extraction of polyphenols contained in cocoa extract will be studied by using water or hydro alcoholic mixtures, and determining the extraction time required, amounts of solvent and pH needed for a complete extraction.

- Degradation of polyphenols. The degradation of polyphenols in encapsulated and non-encapsulated cocoa extract will be studied. To test the effectiveness of encapsulation, lyophilized capsules will be exposed to environmental conditions and sun exposure in order to determine if there are degradation of polyphenols and how much degrade over a period of time. It will be compared with the degradation of non-encapsulated cocoa extract.

- Release of polyphenols from encapsulated cocoa extract. The release of polyphenols in different mediums, acid, basic and neutral, will be studied by analyzing the polyphenols concentration in the medium as a function of contact time.

3. EXPERIMENTAL

3.1. MATERIAL AND EQUIPMENT

3.1. Material

3.1.1. Solids

- Sodium Carbonate anhydrous– Sigma-Aldrich (nº CAS 497-19-8, nº CE 207-838-8)
- Calcium Chloride anhydrous– Sigma-Aldrich (nº CAS 10043-52-4, nº CE 233-140-8)
- Sodium Alginate - Sigma-Aldrich (nº CAS 9005-38-3)
- Sodium Hydroxide - Sigma-Aldrich (nº CAS 1310-73-2, nº CE 215-185-5)
- Gallic Acid - Sigma-Aldrich (nº CAS 149-91-7, nº CE 205-749-9)
- Calcium Carbonate– Sigma-Aldrich (nº CAS 471-34-1, nº CE 207-439-9)
- Cocoa Extract – (Theobroma Cacao L.)- Powder supplied by the Faculty of Pharmacy of the University of Barcelona.

3.1.2. Liquids

- Sulfuric Acid– Sigma-Aldrich (nº CAS 7664-93-9, nº CE 231-639-5)
- Folin & Ciocalteu's phenol reagent (2N).
- Ethanol– Sigma-Aldrich (nº CAS 64-17-5, nº CE 200-578-6)
- Deionized water.

3.2. Equipment

3..2.1. Lyophilizer of LyoQuest-50 serie.

Lyophilization is a process in which the product is frozen and thereafter introduced into a vacuum chamber for removing the water by sublimation. In this way, the water is removed from the solid state to a gaseous environment without going through the liquid state. This process is used for the dehydration of foods, biological material and other heat-sensitive products.

Figure 4: Lyophilizer of LyoQuest-50 serie

3.2.2. Ultraturrax UTC.

The ultraturrax is a machine wich serves to disperse one solution into another by stirring. It creates different types of suspensions in batch processes. It is used in emulsions that are difficult to be mixed with normal agitation devices, such as alginate mixed with water. To mix alginate and water, a high speed stirring is required to get a homogeneous emulsion.

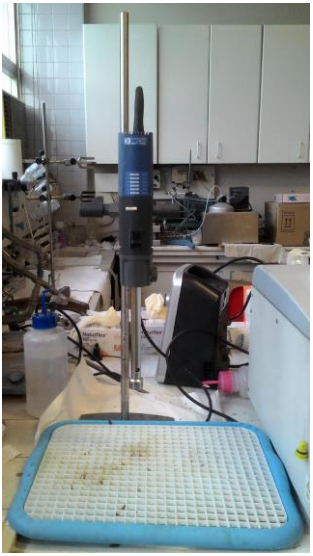

Figure 5: **Ultraturrax UTC**

3.2.3. Sonicator.

An electric current transmits its energy to a mechanical system that will make it vibrate to generate high intensity ultrasound waves. The ultrasonic vibrations generated in the sample.

Figure 6: Sonicator

3.2.4. Magnetic shuffler.

A magnetic stirrer, is an electronic device that uses a magnetic field to mix a solvent and one or more solutes. This device consists of a small bar magnet and a plate below, which has a rotating magnet to create a rotating magnetic field.

Figure 7: Magnetic shuffler

3.2.5. Spectrophotometer.

This is an instrument used in chemical analysis that measures, depending on the wavelength, the ratio between values of the same size and concentration photometric or chemical reactions that are measured in a sample.

Figure 8: Spectrophotometer

3.2.6. Optical Microscopy.

The microscopes are used to examine very small or thin sliced samples. It is used to increase or enlarge images of objects and organisms invisible to the naked eye.

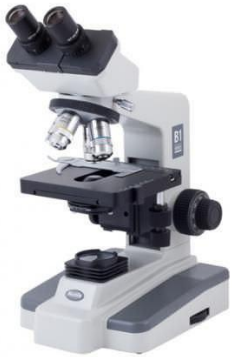

Figure 9: Optical Microscopy

3.3. Techniques

3.3.1. Calibration Curve

The calibration curve is extremely useful because it relates the analytical signal (in this case the absorbance) vs concentration (polyphenols present in the sample). The calibration curve was done in order to quantify the amount of polyphenols in different samples of different concentrations. [7]

For this, the Folin-Ciocalteu method was used. Two solutions were prepared, one containing a known amount of polyphenols, the gallic acid solution, and the second solution is one of the reactants, sodium carbonate.

To prepare the solution of gallic acid, 0.5 g of gallic acid were weighed, dissolved in 10 ml of Ethanol, and finally made up to a volume of 100 ml of water. The Gallic acid solution will be the solution A.

For the solution of sodium carbonate, 200 g of sodium carbonate were weighed, and 800 ml of water were added. The solution boiled, and once it reached 100 °C, was allowed to cool for 24 hours. After 24 hours, the solution crystallized, then the crystals were filtered and added to water to a volume of 1 liter. The sodium carbonate solution will be the solution B.

Of the solution A 0 ml were taken (blank), 5 ml, 10 ml, 13 ml, 15 ml and 17 ml, put into separate flasks, and water was added to 100 ml respectively.

In a test tube with a stopper, they are taken for the calibration curve 20 µl separate flasks (those containing 0, 5, 10, 13, 15, and 17 ml of solution A), 1580 µl of water are added, 100 µl of Folin reagent, and stirred vigorously to ensure homogenization, finally add 300 µl of solution B.

These tubes are allowed to react in the reactor, which is at 20 ° C for a period of 2 hours. After this period, the spectrophotometer is used to read absorbance.

3.3.2. Extraction and analysis of polyphenols from the cocoa powder

Before being able to analyze the amount of polyphenols, they must be extracted from biological material in which they are, and must be transported to the test medium. In the first

part the test medium is ethanol. And finally, when it wants to study the release of polyphenols in different medium, different aqueous solutions of different pH were carried. [8]

To carry cocoa polyphenols, into the midium of analysis, which have proceeded to do is the following:

Has been measured a sample of cocoa, of which polyphenols are extracted. This amount of cocoa, has been deposited in a beaker, protected and covered of the sun light, and added a volume of ethanol. It has been left stirring for a period of time mixture.

To 1 gram cocoa extract a volume of 10 ml of ethanol is used, and with a stirring time of 10 min.

Also, if he has studied different extracting mediums, they could extracted more effectively polyphenols. [9]

To analyze the polyphenols present in pure cocoa extract, after the polyphenols have been extracted in ethanol, the mixture was filtered and was washed the remaining solid in the filter with a volume of 5 ml of water three times.

The process of washing the filtered solid, is intended to drag polyphenols that may have become trapped in the filter, thereby ensuring that filtered liquid contains the majority of polyphenols possible. Then water is added to a total volume of 100 ml.

To analyze, they are taken for the analysis 20 µl of the solution containing the extracted cocoa polyphenol, 1580 µl of water are added, 100 µl of Folin reagent, and stirred vigorously to ensure homogenization, finally add 300 µl of sodium carbonate.

The next step is allowed to react tubes for a period of 2 hours at a constant temperature of 20 °C.

Finally, the samples are carried to the spectrophotometer, and the obtained values, corresponding to the concentration of polyphenol in the sample, expressed in milligrams gallic acid equivalents per gram cocoa extract.

3.3.3. Encapsulation.

Keep in mind that cocoa extract, during the entire process has been protected in an amber colored bottle and stored in the closet. This is because as will be shown below, it is sensitive to sun exposure. And therefore it is necessary to protect the cocoa from sun exposure

by encapsulation, in order to prevent or reduce the degradation suffered. To do this, it was encapsulated in alginate, and added to a calcium solution. [10] [11]

Capsules with sphere and filament shape were done. To make spheres and filaments, two solutions were prepared, the first containing water, sodium alginate and cocoa. The second solution is a solution of calcium chloride, which serve to surround the encapsulated material and provide the source of calcium.

For the preparation of the first solution 2 g of alginate were weighed, and were added by stirring provided by the ultraturrax to 97 ml of water. The mixture was left to stand for 24 hours in the refrigerator to degas thoroughly. The next morning 1 g of the above cocoa extract alginate solution was added, and mixed thoroughly by ultraturrax, and let to rest for an hour.

The calcium chloride solution was prepared by adding 0.5 g of calcium chloride in 99.5 ml of water. It was homogenized for 3 min with a magnetic stirrer.

Spheres: to create spheres, a syringe was used, without an mouthpiece and dripped with an amount of 25 ml of the alginate-cocoa-water mixture over 75 ml of the solution of calcium chloride, while stirring with a magnetic stirrer.

Figure 10: Lyophilizes spheres

Filaments: *t*o create the filaments instead, a syringe was, without any mouthpiece and dripped with an amount of 25 ml of the alginate-cocoa-water mixture over 75 ml of the solution of calcium chloride while stirring with ultraturrax, thus using shear force. The morphology of structures obtained is in the form of filaments.

Figure 11: Lyophilized filaments.

After having created such spheres as the filaments, these are frozen by liquid nitrogen to thereby, lyophilize. Lyophilisation was used in order to remove water containing solutions, without raising the temperature which could damage the properties of cocoa extract.

Once encapsulated cocoa samples lyophilized, lyophilizer trays are removed, and proceed to the extraction of polyphenols.

The first thing that is done, is that on the same tray, the ethanol content is poured to start the extraction process. The tray is washed with ethanol and deposited mainly in a beaker and allowed to stir for a time period of 30 minutes. During this time the beaker is protected and covered of the sun light.

After the extraction time, the content of the beaker is filtered and stored in a 100 ml flask volume. The solid remaining on the filter with 5 ml of water is washed three times, thus is to be ensured that all the polyphenols have been extracted into the liquid volume and not retained in the filter.

Once the filtration and washing process is finished, water is added to the aqueous solution until a volume of 100 ml.

They are taken for the analysis 20 µl of the solution containing the extracted cocoa encapsulated, 1580 µl of water are added, 100 µl of Folin reagent, and stirred vigorously to ensure homogenization, finally add 300 µl of sodium carbonate.

The next step is allowed to react tubs for a period of 30 minutes at a constant temperature of 40 ° C.

Finally, the samples are carried to the spectrophotometer, and the obtained values, corresponding to the concentration of polyphenol in the sample, expressed in milligrams gallic acid equivalents per gram cocoa extract are read.

3.3.4. Degradation of Polyphenols.

It is known that cocoa, when exposed to sunlight and environmental conditions, tends to degrade and thus lose polyphenols. It is therefore, both pure extract and encapsulated cocoa, is left exposed to sunlight, in order to be able to analyze the degradation rate at different time.

In petri dishes, has therein a known quantity of spheres and filaments, separately, were lyophilized according to the same procedure as in the previous section, and once finished the lyophilization step, they were left exposed to sunlight. These samples have been analyzed on different days, being able to observe the deterioration in the encapsulated cocoa, with each passing day.

3.3.5. Release of encapsulated Polyphenols.

Depending on the applications of the capsules, it should be able to free itself, more or less power depending on the medium in which it is going to liberate.

To do this, the polyphenols are extracted spheres and filaments encapsulated in various mediums, precisely in the basic medium, acid medium and in neutral medium.

The encapsulated cocoa was released in basic pH, acid pH and neutral pH, simulating the pH of the intestine, pH 4, stomach, pH 10 and an intermediate pH, pH 7.

For a basic medium, it has been achieved, adding to a volume of 100 mL of water, a volume of 80 µL of a solution of sodium hydroxide (4 g sodium hydroxide in 100 ml water). The acid medium instead was achieved by adding 400 µL of sulfuric acid to 100 ml water. In case, for the neutral medium, deionized water was used.

4. RESULTS AND DISCUSSION

4.1. CALIBRATION CURVE

The first step was to determine the calibration curve, since it is the tool to know the concentration of polyphenols present in a sample from the absorbance value.

The method followed is the Folin-Ciocalteu, which consists in mixing the sample with a volume of water, Folin reagent and sodium carbonate, after mixing allowed to react for 2 hours at 20 ° C. Initially, it was thought that the laboratory temperature, about 25 ° C approximately, was allowed to react for appropriate samples and if the laboratory temperature varied some degree, would not affect the results.

When samples which are allowed to react for 2 hours at laboratory temperature are analyzed, it is observed, that the analytical method was very sensitive to temperature, and when the ambient temperature fluctuated analysis also obtained. For that reason, the temperature was controlled at 20 °C.

To solve this problem, what was done, samples was allowed to react in a reactor that kept the temperature constant at 20 ° C. In this case the results were expected, for the calibration curve.

The results obtained in the calibration curve when the samples were allowed to react for 2 hours at 20 °C are:

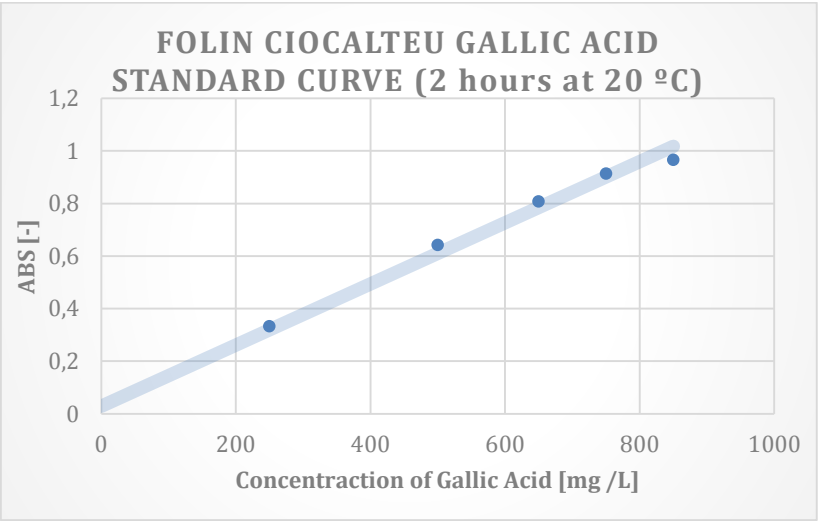

Figure 12: Folin Ciocalteu Gallic acid standard curve (2 hours at 20 ºC)

The calibration curve in this case is: y = 0.0292 + 0,0012x.

Then, with the purpose of saving time, it was tested, the same procedure but in this instance allowing the samples to react at a temperature of 40 °C for 30 minutes.

The goal here was, to check that the result would be similar to the previous case and if it did react in this way reactant would save time in the experimental phase.

The results of the calibration curve obtained for a reaction temperature of 40 ° C and a reaction time of 30 minutes are:

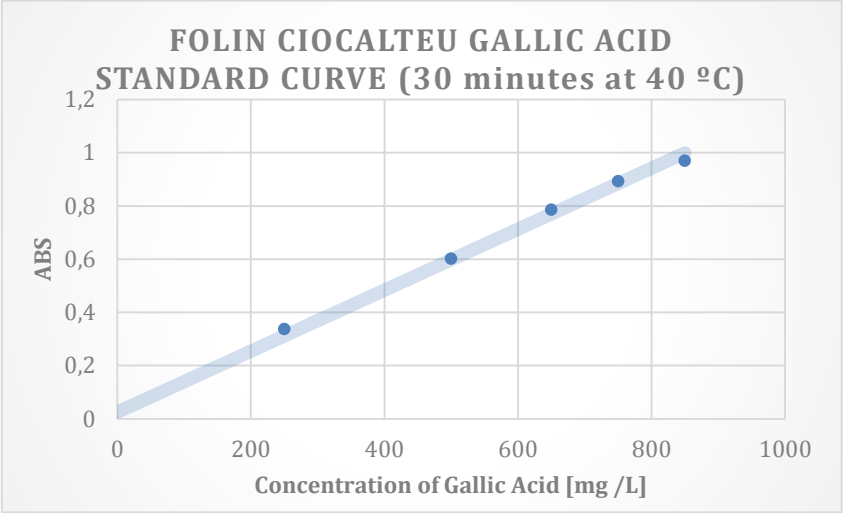

Figure 13: Folin Ciocalteu gallic acid standard curve (30 minutes at 40 ºC).

In which the calibration curve is: y = 0.0248 + 0,0011x.

Independently, if the sample is allowed to react for 2 hours in 20 ºC or for 30 minutes in 40 °C, the results are the same.

4.2. Extraction and Analysis Method

As has already been mentioned above, the polyphenols found in cocoa extract and to analyze them should be removed in an ethanol or aqueous medium. [12] [13]

In order to analyze the amount of polyphenols present in a sample of extract cocoa, first it had to decide which would be the best diluent to extract polyphenols. Initially, ethanol was chosen to extract the polyphenols in cocoa extract, but has also been proven, if other extractors could extracted better.

In this case, it have been studied, extraction with a volume of ethanol, ethanol-water mixture (50% water and 50% ethanol) and water. The total volume was 10 ml.

Having a mass of cocoa extract was added one volume of A, B or C, and allowed to stir for 30 minutes. Next, all of it was filtered and water was added to a volume of 100 ml. Using the

Folin & Cicolteu method, the polyphenols in the sample was analyzed with the spectrophotometer.

By making extractions, the solid filtrate was then washed with 5 ml of water.

According to the obtained values, it could be seen that the best option to extract polyphenols from the cocoa extract is ethanol.

Extracted Volume	Ethanol	Water - Ethanol	Water
Quantity of extracted polyphenols [mg EGA/g Cocoa]	382,01	299,10	161,37

Figure 14: Quantity of extracted polyphenols, using different types of extractors.

So, the polyphenols were extracted with one volume of ethanol, and the filtered solid washed with water.

The amount of polyphenols per gram cocoa are**:** 382.01 mg EGA/ g Cocoa.

Analyzing cocoa extract, both encapsulated and unencapsulated cocoa, it has been observed that adding some improvements, most of the polyphenols could be extracted more effectively.

The first modification was the volume of ethanol used in the extraction. Above 10 ml of ethanol was used to extract 1 g cocoa extract. In the case of encapsulated cocoa, the same amount of ethanol was also used.

However, it is observed that increasing the volume of ethanol in the extraction step, increases the inertia of the polyphenols to be extracted by the concentration difference. That was, in the solid there were a higher concentration of polyphenols, while in the ethanol solution no. Therefore, polyphenols tended to move toward ethanol solution more quickly.

The second improvement that was used was the extraction time. In this case, it sets an extraction time within 30 minutes, because in this way could ensure that cocoa extract, or in the case of encapsulated cocoa had enough time to be extracted.

Volume of ethanol [ml]	10 ml Etanol	40 ml Etanol
Quantity of extracted polyphenols [mg EGA/g Cocoa]	371,19	382,01

Figure 15: Quantity of extracted polyphenols, using different volumes of ethanol.

The third improvement, was to add a basic medium, in the case, for the extraction of spheres and filaments, as being coated with alginate, it was more difficult to be extracted at the same time, and therefore a basic medium was added to solubilize the alginate coating.

	Spheres	Filaments
Quantity of Polyphenols [mg EGA/g Cocoa]	252,34	256,17

Figure 16: Quantity of polyphenols in spheres and filaments

It is observed that in the filaments and spheres, less polyphenols are obtained than are obtained in pure extract.

This is because, during the encapsulation and lyophilization process, cocoa powder suffers a minor degradation. Therefore the difference in values is obtained.

One of the analyzes that has been done in this work, is the lyophilization of unencapsulated cocoa, and see if the lyophilization fact, managed to degrade polyphenols, and the result is that, the lyophilization process causes a deterioration in the powder cocoa.

	Non Lyophilized cocoa	Lyophilized cocoa
Quantity of extracted polyphenols [mg EGA/g Cocoa]	382,01	124,25

Figure 17: Quantity of polyphenols in encapsulated and non-encapsulated cocoa

In conclusion, it has been developed a method to encapsulate cocoa polyphenols. The final product is dried by lyophilization, and depending on the stirring used can be obtained capsules as spheres or filaments.

It was developed an analytical method to quantify the amount of polyphenols that has a cocoa extract sample. With ethanol and basic medium, it has been achieved solubilize alginate capsules, and thus able to extract containing cocoa inside. The concentration of polyphenols in aqueous solution was determined by the Folin & Ciocalteu method with some modifications.

The lyophilization process causes a degradation of cocoa polyphenols.

4.3. POWDER COCOA EXPOSED

In this section, cocoa extract was exposed to sun-light and environmental conditions, in order to see how long it takes to degrade and on what proportion it is degraded.

What has been done is that different capsules, has been cocoa extract and has been left exposed, periodically, has been analyzing the samples, to track the deterioration in the cocoa extract of step the days.

Figure 18: Powder Cocoa Exposed

As seen in the pictures, cocoa, by sun exposure and contact with oxygen, becomes dark in color. The appearance of this obscure pigmentation, causes a negative effect, because it produces changes in taste, and nutritional food value.

Considering that the samples have been analyzed on different days, the results obtained are shown in the following graph:

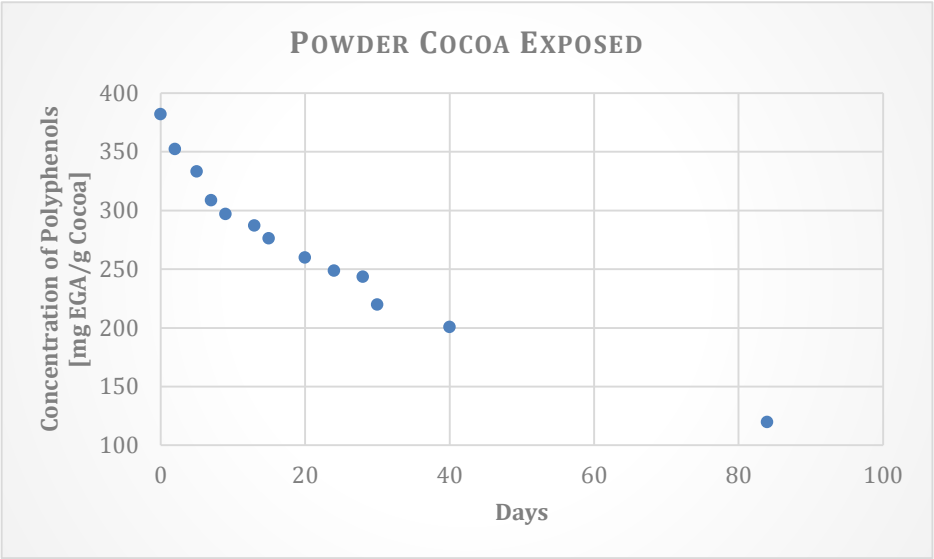

Figure 19: Cocoa powder degradation with time.

4.4. ENCAPSULATED COCOA EXPOSED

The spheres and filaments are exposed to the same conditions of temperature and sun exposure.

The spheres and filaments degrade over time. However, because it is coated by the alginate, they degrade more slowly. In the 24 days of study, pure cocoa unencapsulated degrades at 30%, whereas the encapsulated cocoa degrades at 11%.

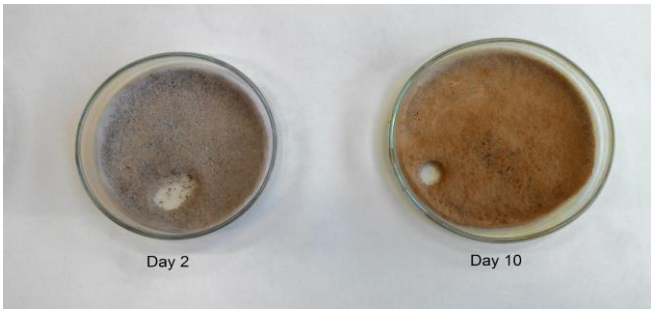

Figure 20: Exposed filaments

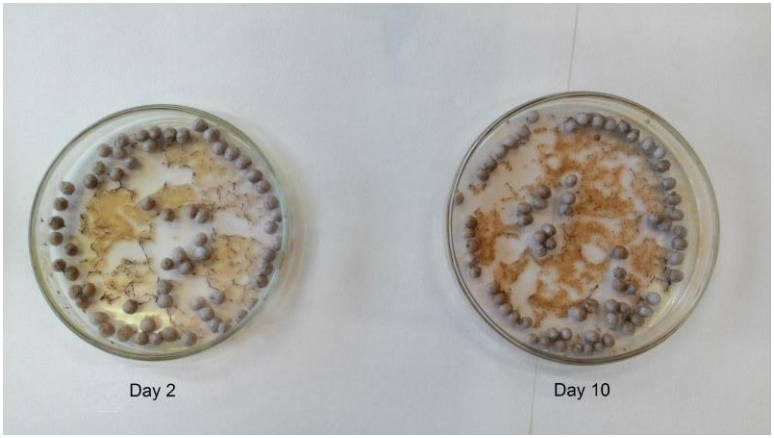

Figure 21: Exposed spheres

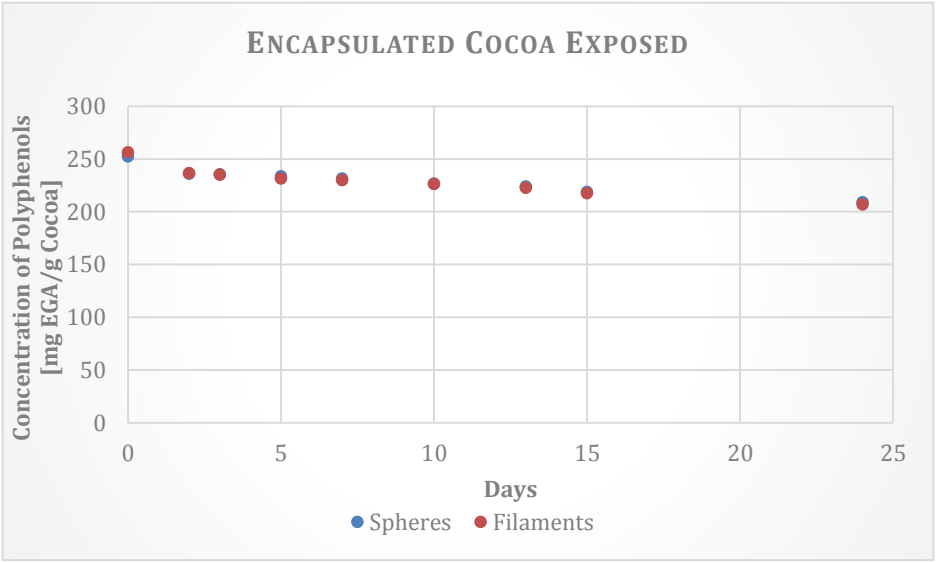

Figure 22: Encapsulated Cocoa degradation with time.

In the unencapsulated cocoa, the initial quantity of polyphenols is 382.01 mg EAG/ g Cocoa, and after 15 days, the amount of polyphenols presents in the sample is 276.30 mg EAG/ g Cocoa, 26.67% is the percentage of degradation of polyphenols.

In the encapsulated cocoa, the initial quantity of polyphenols in spheres is 252.34 mg EAG/ g Cocoa, and after 15 days, the amount of polyphenols presents in the sample is 218.71 mg EAG/ g Cocoa, 13.32 % is the percentage of degradation of polyphenols.

In the encapsulated cocoa, the initial quantity of polyphenols in filaments is 256.17 mg EAG/ g Cocoa, and after 15 days, the amount of polyphenols presents in the sample is 217.57 mg EAG/ g Cocoa, 15.07 % is the percentage of degradation of polyphenols.

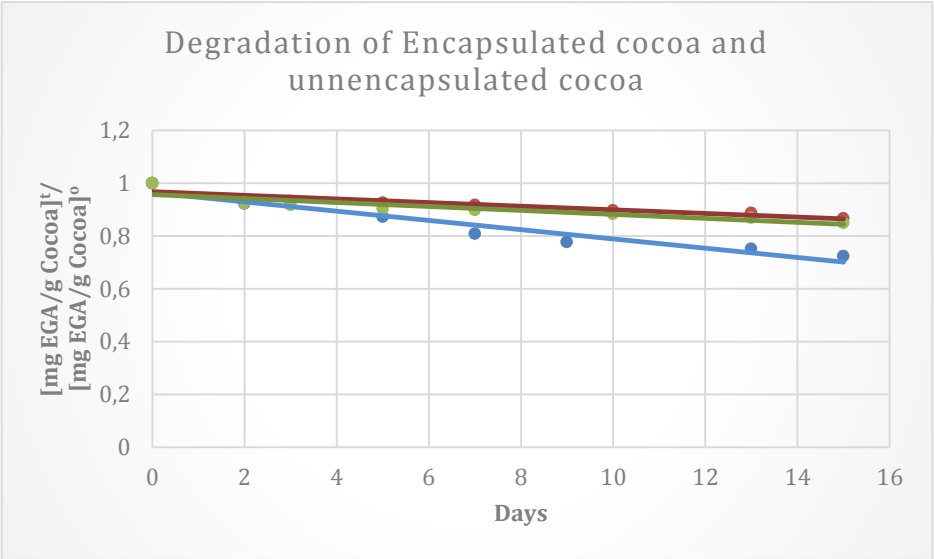

Figure 23: Degradation of encapsulated and non-encapsulated cocoa

In the graph, it is shown in the axis x, there polyphenols concentration at time t divided by the initial concentration of polyphenols. In this way it can be compared the trend of degradation which cocoa and capsules for a while.

In case of spheres and filaments they degrade similarly. And the case of cocoa extract, it is observed that for the same period, cocoa degrades faster. Polyphenols suffer degradation when exposed to sunlight and air contact. This causes a significant decrease in the amount of polyphenols.

The encapsulated cocoa, being in contact with solar lighting and air, has also degradation of polyphenols. Certain is, it is that the encapsulated cocoa degraded less than unencapsulated cocoa. Still, the degradation caused by the encapsulation process, it may lose many polyphenols, and therefore this method is not appropriate to encapsulate.

4.5. RELEASE RATE IN DIFFERENT MEDIUM

The release rate of polyphenols, was studied, supposing that polyphenols should be removed in different media, depending on the final application for which it is intended.

To proceed to determine the release rate of polyphenols, in various mediums, which has been done, it has taken a sample extract cocoa encapsulated and lyophilized in the form of spheres and filaments.

After being lyophilized, capsules were placed in a beaker, and the three extraction mediums were added. The three mediums consisted of: the basic medium, an aqueous solution with 80 µl of sodium hydroxide dissolution; the acidic medium, an aqueous solution, with 400 µl of concentrated sulfuric acid, was added; and neutral medium consisting of deionized water. Thus we obtain the different media at different pH.

It was constantly stirred, covered and protected from the sun. At each time interval an aliquot was extracted, which was filtered and washed with small volumes of water (5 ml Water 3 times), and analyzed by the Folin method, discussed above.

Thus, it was possible to prepare the following graphs, in which, the tendencies that have polyphenols when released in different mediums can be observed.

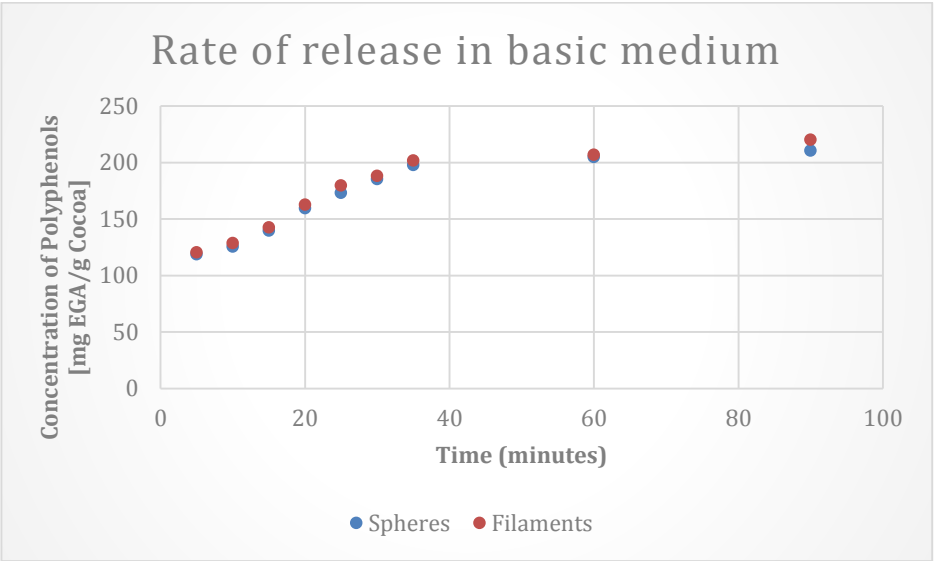

Figure 24: Rate of release of polyphenols in basic medium

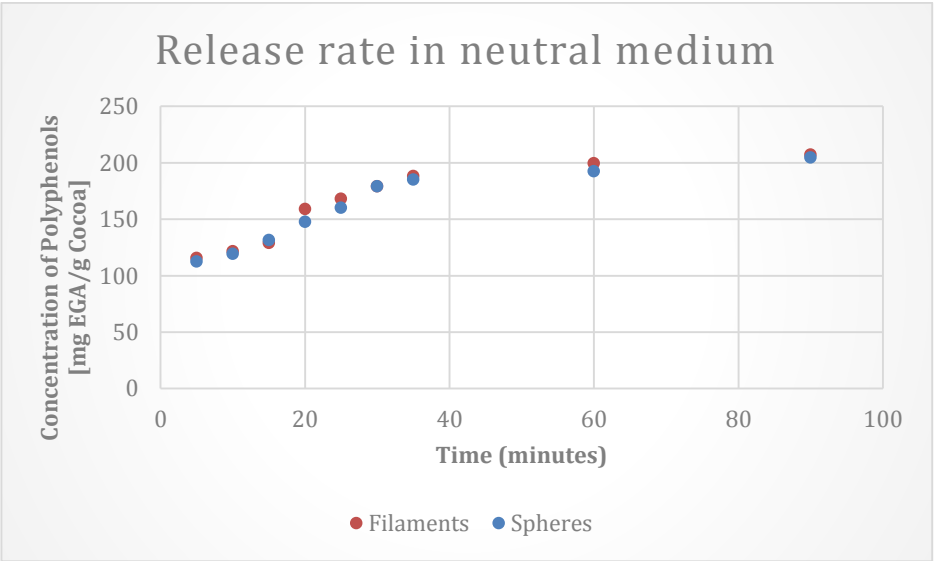

Figure 25: Rate of release of polyphenols in neutral medium

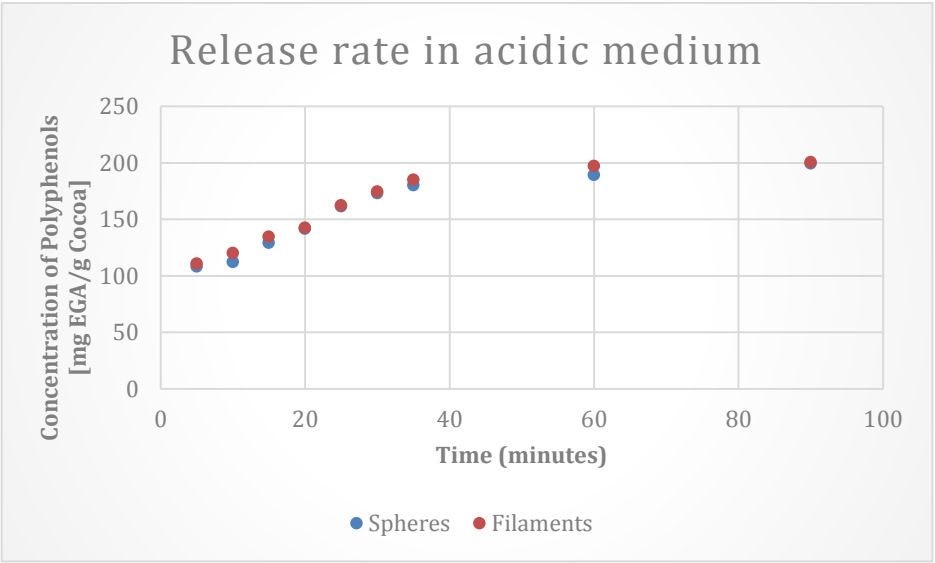

Figure 26: Rate of release of polyphenols in acidic medium

In conclusion, the basic medium, is where better polyphenols are released. Because, in that medium the alginate solubilized. In neutral medium, the release is complicated, basically for the coating, and therefore takes longer to release the active component.

Finally, the acid medium is the medium where the process of release takes more time to be completed.

There are not significant differences between spherical or filament capsules.

5. CONCLUSIONS

This experimental study has permitted to reach the following conclusions:

- A method to effectively encapsulate polyphenols contained in cocoa extract has been developed. The final product is a dry encapsulated cocoa extract obtained by lyophilization. Depending on addition and stirring method, capsules in spherical form or filament form can be obtained.

- A method for analyzing content of polyphenols in encapsulated cocoa extracts has been developed. Extraction with ethanol and basic medium permit a complete solution of polyphenols contained in encapsulated cocoa extract. The concentration of polyphenols in the aqueous solution obtained is determined by a slightly modified Folin & Ciocalteu method.

- The encapsulation and lyophilization processes cause a degradation of polyphenols, as determined by a significant diminution of its content in the cocoa extract.

- Cocoa extract exposed to ambient air and light present a significant diminution with time in polyphenols content.

- Encapsulated cocoa extracts exposed to ambient air and light present less diminution with time in polyphenols content than non-encapsulated ones. However, the degradation caused by the encapsulation process makes might make no convenient the encapsulation.

- Encapsulated cocoa extracts release the contained polyphenols slightly faster in basic medium than in acid or neutral mediums. There are not significant differences between spherical or filament capsules.

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ACRONYMS

LDL: Low density lipoprotein

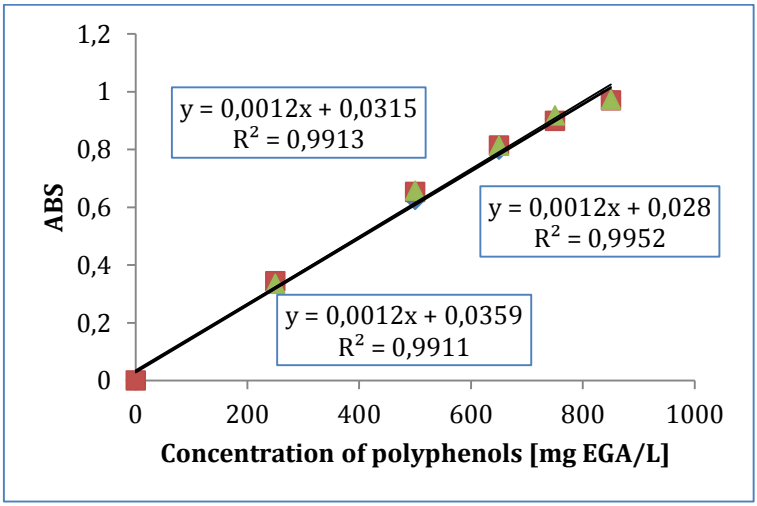
CDS: Ccyclodextrin

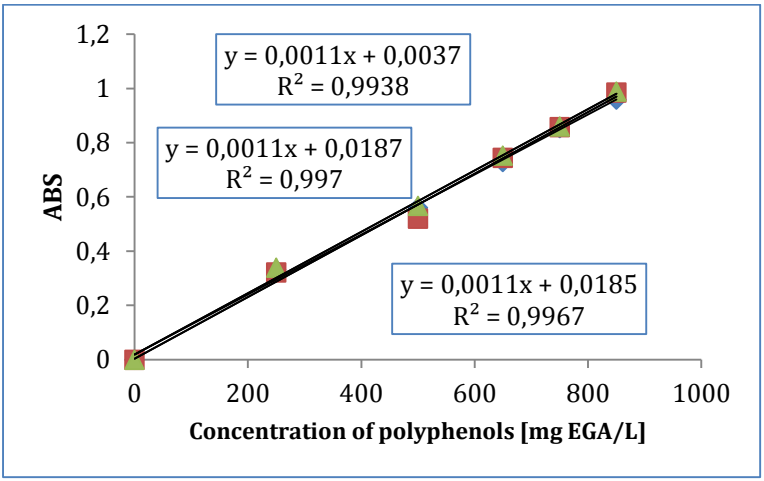
EGA: Equivalent in Gallic Acid

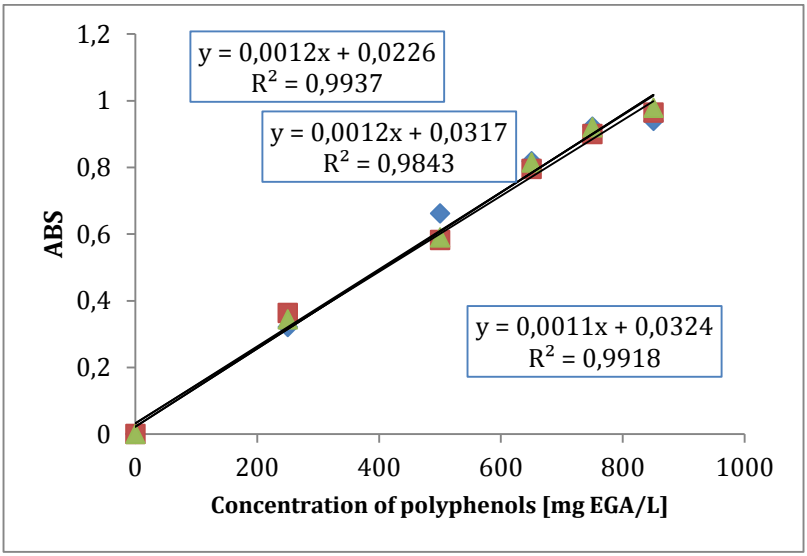
APPENDICES

APPENDIX 1: CALIBRATION CURVE

The results obtained in the calibration curve when the samples were allowed to react for 2 hours at 20 °C are:






y = 0,0012x + 0,0226
R² = 0,9937
y = 0,0012x + 0,0317
R² = 0,9843
y = 0,0011x + 0,0324
R² = 0,9918
ABS
1,2
1
0,8
0,6
0,4
0,2
0
0
200
400
600
800
1000
Concentration of polyphenols [mg EGA/L]

APPENDIX 2: EXTRACTION METHOD

The extraction step values:

Experiment	Ethanol	Ethanol + water	water	
1	*238.31*	*288.666*	*78.80*	*washing with 10 ml of water, and stirring during 5 min*
2	*307.58*	*294.890*	*173.95*	*washing with 5 ml of water, and stirring during 5 min*
3	*334.01*	*313.75*	*231.36*	*washing with 5 ml of water, and stirring during 30 min*

Experiment	Spheres	Filaments	Unencapsulated Cocoa	
1	*223,84*	*157,71*	*122,79*	*washing with 10 ml of water, and stirring during 5 min*
4	**262,06**	**222,20**	**125,15**	*washing with 5 ml of water, and stirring during 5 min, basic medium*
5	**252,34**	**256,17**	**124,25**	washing with 5 ml of water, and stirring during 30 min, in basic medium