MICROENCAPSULATION OF BIOACTIVE MOLECULES FROM SPIRULINA PLATENSIS AND HAEMATOCOCCUS PLUVIALIS

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На майка ми, за онези 12 часа посветени изцяло на мен и поради факта, че винаги е от другата страна на линията.

На баща ми, защото е дарен със състрадание и добро сърце, който ми служат за пример винаги, когато злото се опитва да надделее над доброто.

На баба ми, заради всички грижи и защото е гласа на истината в главата ми, когато не знам как да постъпя.

На дядо ми, защото като един истински Артур, събира всички на кръглата маса и защото без корема му нямаше да порасна голяма.

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CHAPTER 1 MICROALGAE

1. Functional ingredients from algae for foods and nutraceuticals

Currently, there is a trend towards a healthier way of living, which includes a growing awareness by consumers of what they eat and what benefits certain ingredients have in maintaining good health (Das et al. 2014). For these reasons in the last decades great effort have been done by science and industries to develop products like functional foods. . A food is considered to be functional when it is fortified, enriched or enhanced, possessing health benefits beyond the provision of essential nutrients (e.g., vitamins and minerals), when they it is consumed at efficacious levels as part of a varied diet on a regular basis (Hasler 2002). Some functional foods are generated around a particular functional ingredient or nutraceutical like for example probiotics, prebiotics, omega-3 fatty acids and others (Williamson 2009).

The development of these products is somewhat challenging since it requires technologies for incorporating health promoting ingredients into food without reducing their bioavailability or functionality (Dominguez 2013).

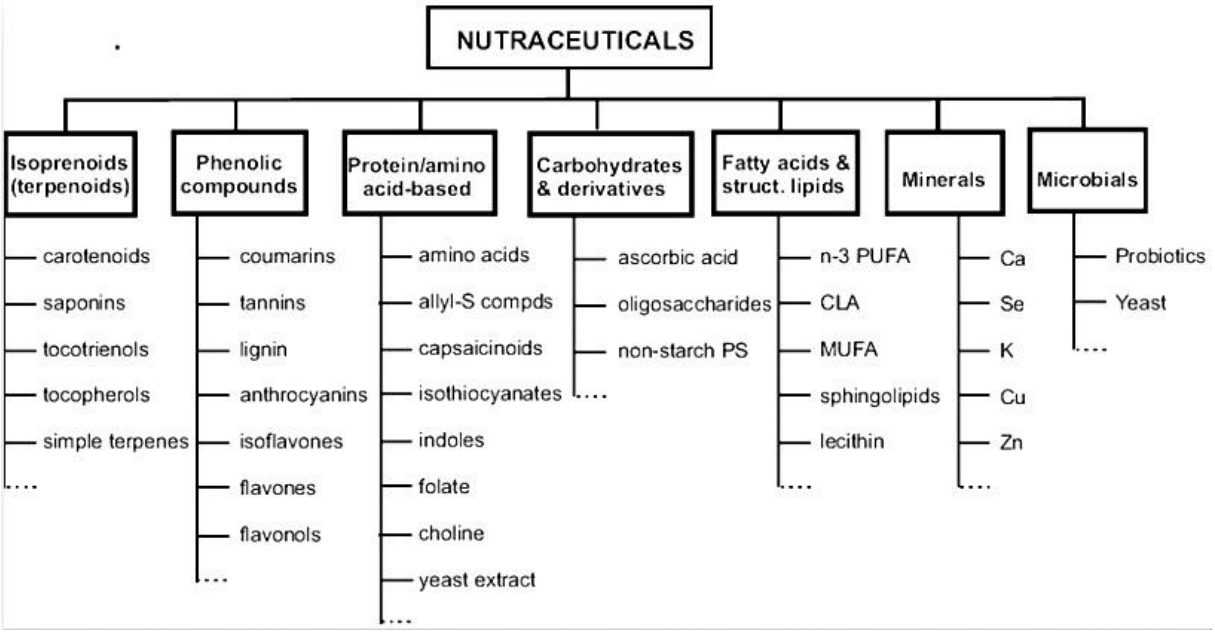
2. Nutraceuticals

The term functional became to gain popularity after the Japanese government in 1980s created a class of "functional foods"-conventional and modified foods that included additional health benefits beyond basic nutrition. In the U.S., the Food and Drug Administration (FDA) does not have a regulatory category of functional foods (Lupton 2009). However, the Academy of Nutrition and Dietetics defines a functional food as: a food that provides additional health benefits that may reduce disease risk and/or promote good health (Hasler et al. 2004).

Nutraceuticals are often defined synonymously with functional foods in the literature (Cencic & Chingwaru 2010). In fact, the term nutraceutical, as used by Stephen DeFelice, M.D., founder of the Foundation for Innovation in Medicine in Cranford, covers dietary supplements, those fortified foods that are enriched with nutrients not natural to the food such as orange juice with added calcium (Das et al. 2012). Thus, nutraceuticals are more correctly

defined as parts of a food or a whole food that have a medical or health benefit, including the prevention and treatment of disease.

Figure 1. Different examples of nutraceuticals



(Nasri et al. 2014). (Gupta 2016).

3.Microalgae

Nowadays there is a growing interest when it comes to the incorporation of nutraceuticals coming from microalgae sources. Microalgae, microscopic photosynthetic organisms that are found in both marine and freshwater environments, could become an important resource for addressing the increasing global demand for food due to the fact that it represent a vast renewable stock with enormous potential when it comes to bioactive compounds (Priyadarshani & Rath 2012;Bagwell et al. 2016). Microalgae is a rich source of proteins, carbohydrates, enzymes, vitamins and minerals (Table 1) and for this reason they are a major source of food, especially in some Asian countries (Priyadarshani & Rath 2012).

Table 1. Some of the most useful substances in microalgae

Vitamins	**B1, B2, B6, B9, B12, C, E, A,**
Antioxidants	Polyphenols, tocopherols
Polyunsaturated fatty acids	DHA(C22:6), EPA(C20:5), ARA(C20:4), GAL(C18:
Carotenoids and pigments	B-carotene, astaxanthin, , zeaxanthin, canthaxanthin, phycocyanin, lutein phycoerythrin, fucoxanthin
Other	Antimicobial, antifungal, antiviral agents, aminoacids, proteins, sterols

Adapted from (Priyadarshani & Rath 2012).

Around 30,000 known species of microalgae exist and only a small number of them are currently on the market and have a commercial significance which gives microalgae a great potential.

Table 2. Nutritional composition of some types of microalgae expressed as g per 100g of dry weight

Component	Spirulina	Heamatococcus	Chlorella	Dunaliella	Aphanizomenon
Thiamin	0.001	0.00047	0.0023	0.0009	0.004
Riboflavin	0.045	0.0017	0.005	0.0009	0.006
Niacin	0.0149	0.0066	0.025	0.001	0.013
Pantothetic acid	0.0013	0.0014	0.0019	0.0005	0.0008
Pyridoxine	0.00096	0.00036	0.0025	0.0004	0.0013
Folic acid	0.000027	0.00029	0.0006	0.00004	0.0001
Cobalamine	0.00016	0.00012	0.000008	0.000004	0.0006

Magnesium	0.319	1.14	0.264	4.59	0.2
β-carotene	0.12	0.054	0.086	1.6	0.42
Chlorophyll	1.15	0.4 red; 1.1 green	5	2.2	1.8
Carbohydrates	17.8	38	15	29.7	23
Fat	4.3	13.8	10	7	3
Protein	63	26.6	64.5	7.4	1

Adapted from (Bishop et al. 2012)

For instance, (Borowitzka 1995) a number of strains of cyanobacteria are known to produce intracellular and extracellular biologically active metabolites with wide antibacterial, antifungal and antiviral activity (Harzevili & Chen 2014). Some of the most important biological activities of different species of red and brown algae may be attributed to the presence of volatile compounds, some phenols, free fatty acids and their oxidized derivatives (Kamenarska et al. 2009). Several microalgae are found to possess therapeutic potential for combating neurodegenerative diseases associated with neuroinflammation (Kim & Chojnacka 2015). In this chapter the main types of microalgae are mentioned because of their well-known nutritional composition (Table 2) and biological activity which provides them with specific health attributes and a perfect example of innovative nutraceuticals to be incorporated in foods.

A special attention will be given in the next chapters to *Spirulina Platensis* and *Haematococcus pluvialis* as they represent an essential part of this manuscript and they have been chosen, due to their specific health benefits, to be studied deeper in order to be therefore prepared for their possible future incorporation in functional foods.

In order to protect and to allow their main biologically active compounds to be more bioavailable for the consumer the microalgae extracts will be stabilized by encapsulation in polysaccharide carriers. The next chapters will then provide an overview of microencapsulation and the application of this technology to microalgae as an object of this thesis.

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CHAPTER 2 MICROENCAPSULATION

1. Fundamental principles of microencapsulation

The main idea behind microencapsulation is the fact that many food components including but not limited to ω-3 fatty acids, vitamins, probiotic bacteria, minerals cannot be directly incorporated in foods before first being encapsulated in some kind of a delivery system (Garti & McClements 2012). The reason for this is that these active compounds are very fragile towards the surrounding environment and factors such as light, oxygen, extreme pH, high temperature but on the other hand their preservation is desirable as they have supposed promoting health benefits.

When we are speaking about oils for example, it is necessary to note that they are difficult to manage in the food industry as are immiscible with water (McClements 2012). If we take as an example ω-3 fatty acids, the reasons for encapsulation can be more complex. Not only the fatty acids are labile to light and oxygen, not mixable with water and therefore difficult to manipulate, but also they might have an unpleasant taste and odour for the consumer. Microencapsulation is a versatile technique which depends on the expectations towards the quality of the final product and of the bioactive compound being encapsulated. Below some examples of bioactive compounds and nutraceuticals and their potential health benefits have been listed (Table 1.).

Table 1. Selection of bioactive compounds and their supposed health benefits

Bioactive compound	Examples	Potential health benefits
Carotenoids	Astaxanthin, beta-carotene, lycopene, lutein	Reducing risk of eye diseases and some types of cancer (Krinsky & Johnson 2005)
Long chain omega-3 fatty acids	DHA, EPA	Promoting cardiovascular health (Abeywardena & Head 2001)
Herbs and species	Essential oils, herbal preparations	Range of health benefits (Baghurst et al. 2006)
Prebiotics	Inulin, oligosaccharides	Promoting gut health, modulation of the gut

		microflora (Schrezenmeir & de Vrese 2001)
Probiotics	Lactobacilli, Bifidobacterium	Improving gut health, immune modulation (Schrezenmeir & de Vrese 2001)

Adapted from (Augustin & Sanguansri 2012)

1.1. Terminology

There are some basic definitions that need to be mentioned when speaking about microencapsulation. First of all we must explain the meaning of an **bioactive compound**. A bioactive compound is a nutraceutical of interested that is going to be encapsulated, and it represents the extra nutritional constituent that typically occur in small quantities in foods (Kris-Etherton et al. 2002). Examples of bioactive compounds can be different kinds of lipids, proteins, minerals, probiotics.

One of the main aims of encapsulation is to make the active compound to be delivered exactly at the right time and in the right place (Agnihotri et al. 2012). The engineering of this delivery is called **controlled release** and there are four main types of controlled release: triggered release, sustained release, burst release and targeted release (McClements 2012). The triggered release is when we use a specific stress applied on the capsule in order to make it break and discharge the active ingredient. The type of stress being used might be mechanical, change in pH, temperature or an enzymatic activity (digestion for example) (Mishra 2015). The targeted release occurs when the microcapsule opens in a specific part (month, stomach, small intestine) as the active ingredient must “survive” until this place. The sustained release is mainly used in the pharmaceutical industry for the encapsulation of drugs as in this type of release we have a prolonged liberation of the active compound in a relatively constant rate (Soppimath et al. 2001). The quickest release type is the burst one because the capsule breaks in very short time.

1.2. Type of microencapsulates

In general, there are two main types of microencapsulates: **reservoir type** and **matrix type** (Zuidam & Shimoni 2010). In reservoir type, the active agents is inside a core entrapped by a barrier or carrier material. It is also called single-core or mono-core or core-shell type. In matrix type, the active agent is dispersed or dissolved throughout the polymer (Zuidam & Shimoni 2010). The coated matrix type is a combination of first two (Figure 1.).

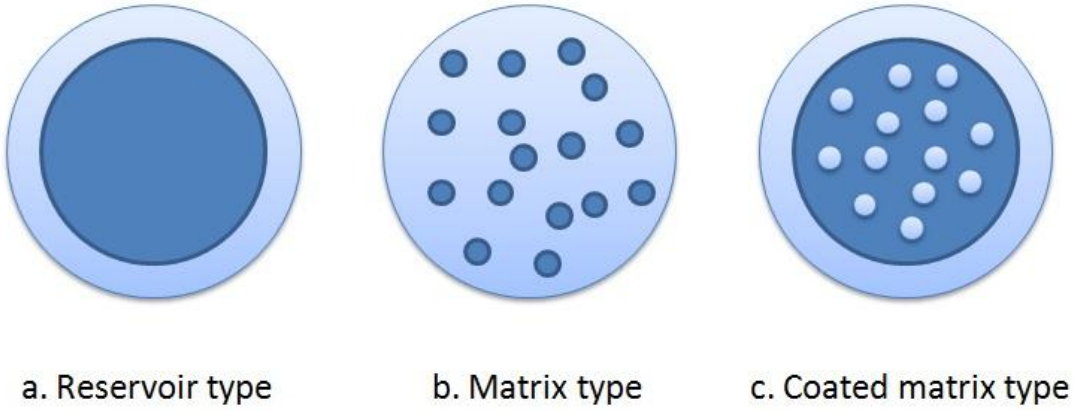

Figure 1. Types of microencapsulates

The main difference between these two type of capsules is the **payload**. The payload in microencapsulation is the amount of core material or bioactive compound being encapsulated in the polymer shell or matrix (Gaonkar et al. 2014). The payload of course depends also on other factors, such as which polymer is being used in the encapsulation process. This is why it is important to chose a suitable carrier depending on the future application of the capsules.

3. Encapsulation materials for food applications

Choosing a suitable carrier might be a challenging mission as there is a limited number of food grade formulations that can be used. For example some of the compounds that are accepted when using encapsulation technologies in the pharmaceutical industry have not been approved in the food one as they have not been certified as "generally recognized as safe" (GRAS) materials (Nedovic et al. 2011). The complete food process must be designed in order to meet the safety requirements of governmental authorities such as the European Food

Safety Authority (EFSA) or Food and Drug Administration (FDA) in the USA (Desai & Jin Park 2005).

Table 2. Examples of encapsulation materials for food applications

Material class	Examples
Cellulose	Methyl cellulose, etyl cellulose, acetylcellulose, carbonmethyl cellulose
Lipids	Fats, beewax, edible oils
Proteins	Gelatin, gluten, peptides, soy protein, pea protein, whey protein, caseinates
Emulsifiers	Liposomes, lecithin, food-grade surfactants
Simple sugars	Fructose, glucose, galactose, maltose, sucrose
Carbohydrates	Chitosan, corn syrup, cyclodextrin, maltodextrin, alginates, pectines, carrageeenan, gum arabic, starch

Adapter from (Garti & McClements 2012;Augustin & Sanguansri 2012)

The main challenge when working with food-grade materials as they are natural materials is that their quality and composition might vary depending on the extraction. However working with natural and biodegradable materials is one of the main trends nowadays in microencapsulation and it has been successfully applied not only in the food sector. Environmental awareness and shift into a healthier way of living as well as preserving the natural balance on the planet has pushed the producers in the different fields where microencapsulation is applied to pay closer attention to using safer materials.

4. Microencapsulation techniques

A wide variety of foods component is encapsulated- antioxidants flavouring agents, acids, oils, artificial sweeteners, colorants, preservatives, agents with undesirable flavours or taste, odours and nutrients, among others (Gibbs 1999). After choosing a suitable type of capsule for the active ingredient and determining the convenient carrier, a technique must be carefully chosen. Since the process is not linear as both bottom to top and top to bottom approaches can

be used, depending on the nutraceutical that needs to be encapsulated we can first appoint a technique and then choose the carriers applicable to this technique or vice-versa. Microencapsulation is a complex science and there are many variables that must be taken into account. Not all of the techniques are appropriate for all active ingredients as not all of the carriers can be utilized for certain nutraceuticals. Only after acquiring a profound knowledge on all the basic principles of microencapsulation it is possible to predict a convenient method to be applied.

Numerous techniques have been developed for encapsulation of bioactive ingredients including spray drying, spray cooling/chilling, freeze drying, inverse gelation, extrusion, co-extrusion, complex coacervation, fluidized bed coating, liposome entrapment and many others (Desai & Jin Park 2005;Madene et al. 2006;Augustin & Hemar 2009).

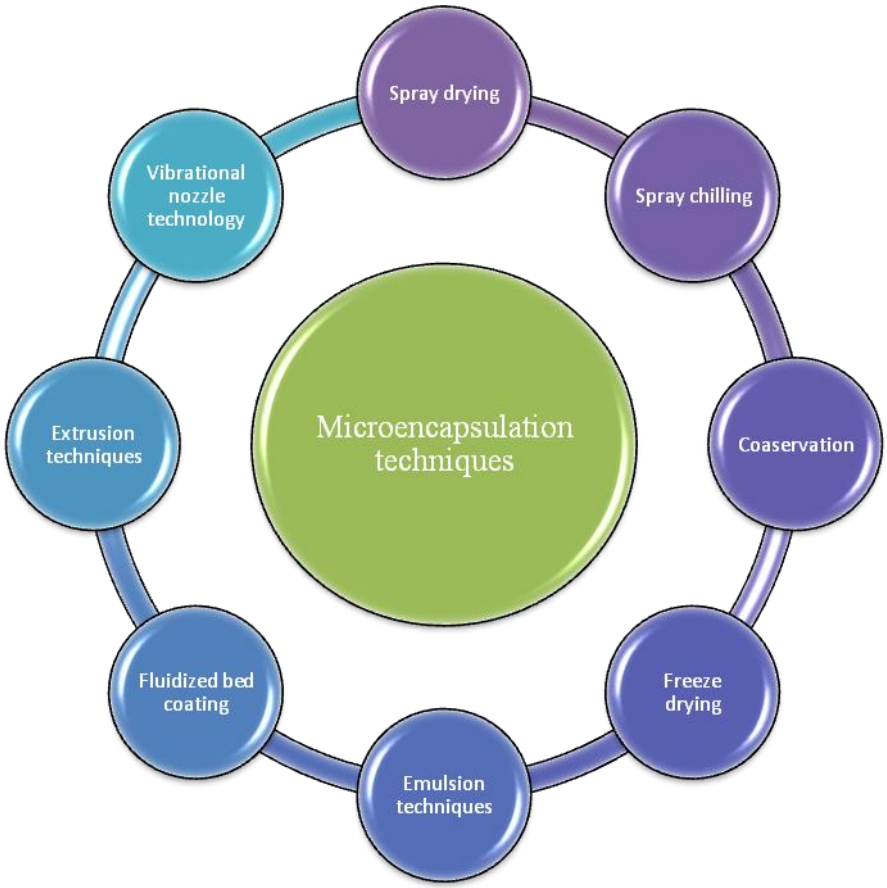

Figure 2. Some of the most common microencapsulation techniques

The selection of the most suitable technique for the process is based on parameters such as targeted applications of the microencapsulated material, desired release mechanism, scale-up

potential, process costs, particle size required, physical/chemical properties of both the bioactive compound and the coating material (Ré 1998). Since every individual food component possesses specific characteristics such as solubility, polarity, stability, different encapsulation approaches have to be applied to meet the specific physicochemical requirements for each particular food ingredient and it should be pointed out that none of the above mentioned techniques is considered as universally applicable (Augustin & Hemar 2009). However in this chapter, the most well-known techniques will be discussed along with some in-depth technical details regarding their operating principles.

4.1. Drying techniques

Among the above mentioned techniques, the drying techniques including spray drying and spray chilling, have often been applied in the food industry because of the simplicity of the process, wide variability of the equipment and ease of handling but also significant merits when it comes to the reduction of the product volume (Masters 1985). Because the final product is in the form of a powder, it can be easily incorporated in foods with other ingredients or subjected to reconstitution by dissolution (Koç et al. 2014). In general, most encapsulation techniques use the drying process to transform the product into a more stable dried food (Fang & Bhandari 2012).

4.1.1. Spray drying

Spray drying is considered the oldest encapsulation technique as it was first applied in the early 1930s for the encapsulation of flavours (Shahidi & Han 1993). It is the most used encapsulation method in the food industry nowadays since it is a well established and studied technology, it is economical (speaking about the process only and not the equipment), flexible and continuous but above all the created particles have a high powder recovery (Desai & Park 2005). A very strong point of this technique is that the materials stay relatively cool until the dried state is reached, as the evaporation takes place on the surface of the capsule(Fogler & Kleninschmidt 1938). For this reason, spray drying is widely used when it comes to heat sensitive bioactive compounds such as probiotics, flavours, oils and enzymes (Jafari et al. 2008).

The process of spray drying starts with feeding the chosen solution that includes the molecule of interested mixed with the carrier material into the spray dryer. An atomization is applied

with the help of an atomizing nozzle (Figure 3). The solution is being atomized inside a hot chamber supplied with hot air at a controlled temperature. When the droplets come into contact with the air due to the temperature, the water is being evaporated and the formation of the dried particles takes place. Then they are being separated by a cyclone from the humid air and collected in the form of a powder inside the product container (Zuidam & Shimoni 2010). Spray drying technique gives also the possibility for solvents to be used where the active compounds are not soluble in water, however this practices have been slowly abandoned due to environmental safety reasons and also when we speak about food applications the above mentioned solvents are not by any means recommended. The prepared encapsulates vary in their shape and size. Normally with this technique they form a matrix structure with a spherical shape that can vary from 10-50 μm to larger particles with a size of 2-3 mm (Gharsallaoui et al. 2007).

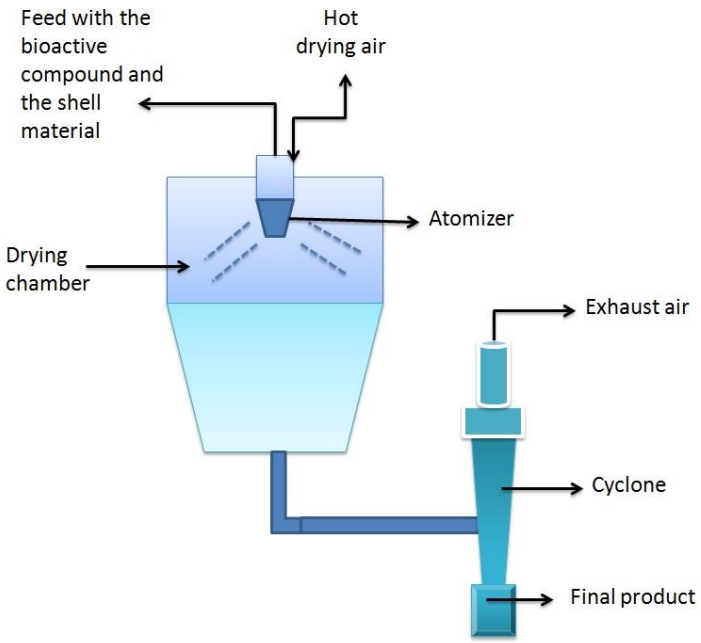

Figure 3. Schematic diagram of spray drying encapsulation

Although the drying techniques are one of the most used ones in present days they do have certain limitations. The first one of them is that the carrier materials available can be quite limiting since the formulations are carried out of aqueous feeds, the wall material must have a good solubility in water (Gouin 2004). Also since some of the powders created using this technology might have a very small particle size, further techniques might need to be applied in order for the particles to agglomerate so easier handling can be achieved. Then, spray

drying might not be the most suitable technique for ingredients that are very volatile as because of the hot temperature in the drying chamber, considerable loses might occur during the process (Reineccius 2001).

4.1.2. Freeze drying

Freeze drying, also known under the name lyophilisation, is a drying process which includes a freezing of the suspension medium before being sublimed from a solid state directly into gas phase (Oetjen et al. 2000). Before the drying processes begins, the active compound is dissolved in water and cooled to very low temperatures (between -20°C and -40°C) because in this manner water is maximally crystallized along with some lipids and soluble solid compounds (Fang & Bhandari 2012).

Encapsulation using freeze drying requires the dissolving or emulsifying the molecule of interest or the so-called core material in the wall material system and then co-lyophilizing them. This process normally results in capsules with a very uncertain structure that are very porous (Fang & Bhandari 2010). This process can be successfully applied in cases where the final appearance of the product is not one of its most valuable characteristics but also when we have molecules that are susceptible to high temperature or are volatile.

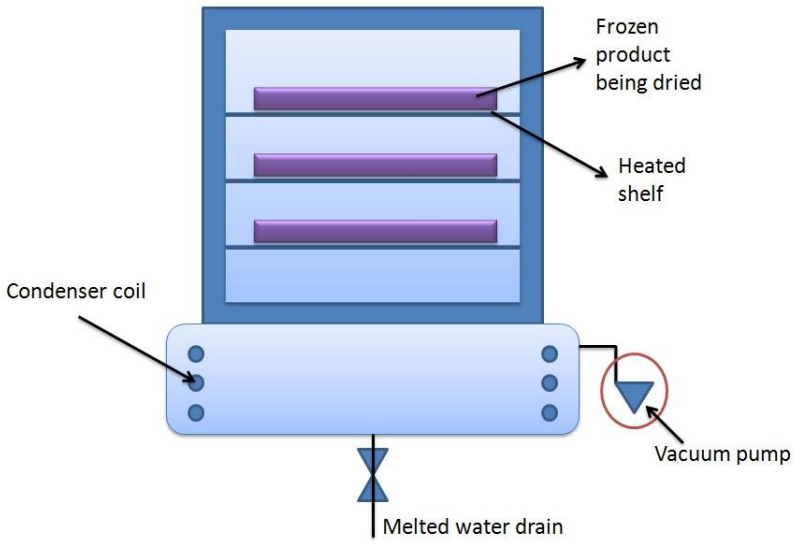

Figure 4. Schematic representation of a laboratory scale freeze dryer

Freeze drying is one of the least harmful methods when it comes to microencapsulation. However there are some limitation when we speak about this technology and they are mainly connected to expenses for the equipment, high energy consumption and long processing time. Also it is important that the final product is packed under vacuum as freeze drying creates a porous matrix which may lead to easier oxidation if in direct contact with air. Freeze drying encapsulated do not offer a great protection into the gastro-intestinal tract as the high porosity offers poor protection when speaking about prolonged release (Manojloviç et al. 2010).

4.1.3. Spray chilling

Spray-chilling is another well known technique in the fields of microencapsulation. It is based on the principle of solidifying atomized spray into particles (Oxley 2012). We might say that spray chilling works on the same principle as spray drying with one main difference- in the chamber where the particles are actually forming, instead of hot air, the particles are being cooled and therefore hardened.

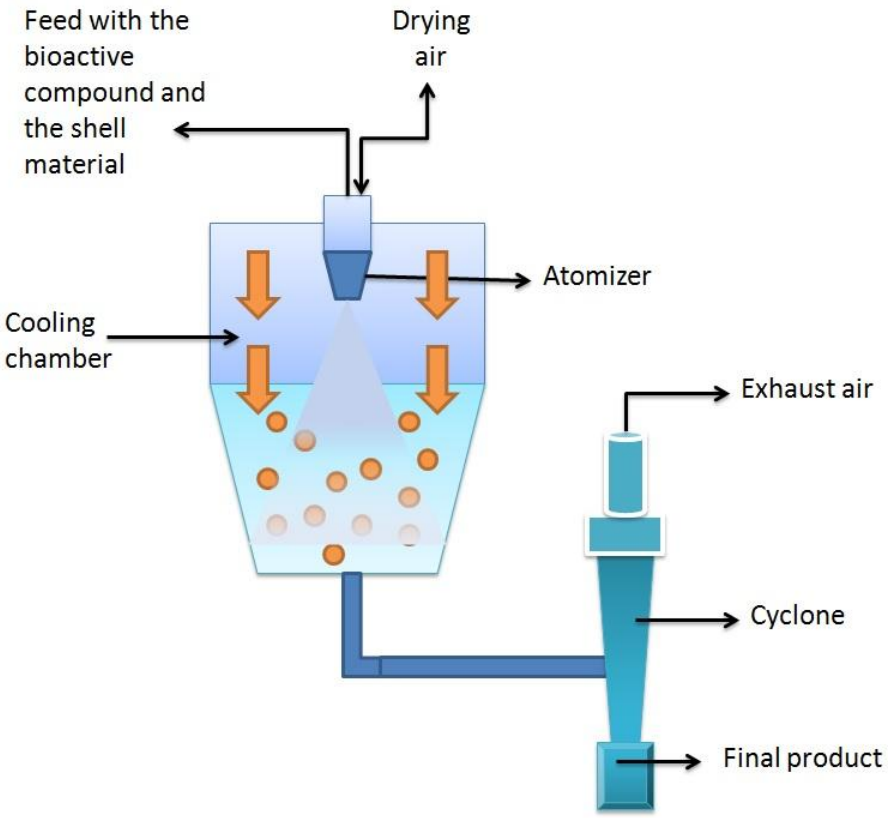

Figure 5. Spray chilling process diagram

Before the atomization, the active compound must be again dispersed into a liquid matrix material and after the cooling the matrix solidifies around the nutraceutical, forming a microcapsule or multi-core microencapsulates (Oxley 2012). It is important that the cooling chamber has a temperature that is below the temperature of the melting point of the carrier material throughout the whole process. This is important as the capsules should be formed before reaching the collection point of the final product.

Spray chilling is characterized with low cost and high production rates. With this technique many temperature sensitive molecules might be encapsulated and spray-chilling can successfully substitute spray drying. However this technique also have its disadvantages some of which are a low payload of the capsules, possibility of expulsion of the active ingredient by the matrix during the storage and the degradation of lipid carrier which may affect the shelf-life of the active ingredient material (Okuro et al. 2013).

5. Coacervation

The phenomenon of coacervation is the process of forming a liquid-rich in polymer-rich phase (coacervate) in equilibrium with another liquid phase. Coacervation involves the separation of a liquid phase of a coating material from a polymeric solution and the wrapping of that phase as a uniforn layer around suspended core particles (Wilson & Shah 2007). There are two types of coacervation- simple coacervation and complex coacervation.

The simple coacervation involves only one polymer and the separation of phases that result in desolvation/dehydratation of the polymer phase (Ansel et al. 1995). These conditions include addition of a water-miscible non-solvent or an inorganic salt. The complex coacervation included two hydrophilic polymers with two opposite charges. When one of the charges gets neutralized by the other one, the polymer is being separated and deposited in the droplet. Once the coacervates are formed, the polymers are stabilized by cross-linking normally using glutaraldehyde (Singh et al. 2011). Since the principles are quite complex and depend on the material being encapsulated a suitable diagram that covers coacervation cannot be given.

6. Fluidized bed coating

Fluidized bed technology is based on the separation of particles in a gas stream (Yang & Keairns 1982). This technology is mainly applied after the particles are already created. It is used for two main purposes- to dry the already created capsules or to coat them with another coating material, providing a second or more barriers. Fluidized bed technology is used when the capsule needs a prolonged release for example or when the core is very volatile and need to be covered with more than one shell material.

The particles are individually accessible for the atomized droplets of the shell material and either by evaporation or melting this shell material covers again the capsules. This is accomplished with the help of a gas steam, serving both for the fluidization of the particles and for spraying of the shell material onto them (Meiners 2012). There are three different types of fluidized bed coating- top spray, bottom spray and tangential spray (Figure 6).

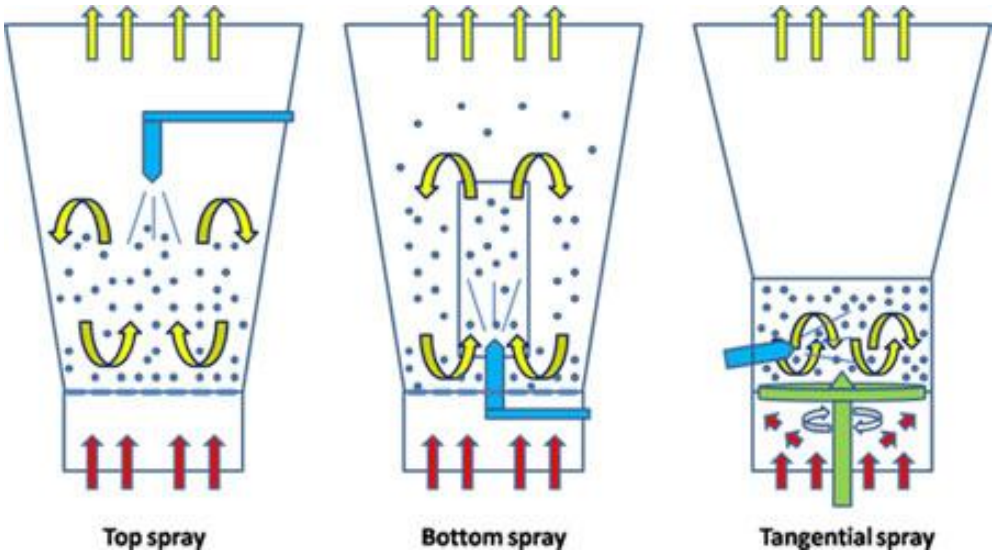

Figure 6. Machine design of bottom spray, top spray and tangential spray Image available at: http://www.colorcon.com

Fluid bed machines require a large volume of air in order to maintain the particles in the air. This technology is often combined with another one such as spray drying (Depypere et al. 2003). The main advantage of this technology is the wide variety of coating materials that can be chosen and they can be either hydrophobic or hydrophilic in nature. The wall thickness can also be controlled in order to obtain the desired wall structure (Gaonkar et al. 2014). The main

disadvantage of this technology is that it requires a lot of energy when applied in industry and can be quite expensive.

7.Nozzle vibration technology

The technique for the production of microencapsulates that is going to be of main interest in this manuscript is called vibrational nozzle technology. This is one of the most widely used methods of production of microencapsulates and it is based on the principle of laminar liquid jet that breaks into droplets by applying a superimposed mechanical vibration onto it. The selected polymer is extruded through a nozzle and in order for the jet to be broken into droplets a controlled, vibrational frequency at a defined amplitude is imposed. To prevent that the droplets stick together during the breakage of the jet, an electrical charge is induced onto the surface of the droplets using an electrostatic voltage system. The system that we have chosen in our laboratory is BUCHI B-390 Encapsulator, Switzerland - one of the leading brands in microencapsulation on the market nowadays.

There can be two types of vibrational nozzle technology- simple extrusion and co-extrusion. In the first case the active compound is mixed with the polymer and a single nozzle is used. In this manner we obtain a matrix type of capsule meaning that the bioactive is dispersed throughout the microencapsulate. In the case of the co-extrusion technique two concentric nozzles are being used as the polymer and the substance of interest are simultaneously extruded.

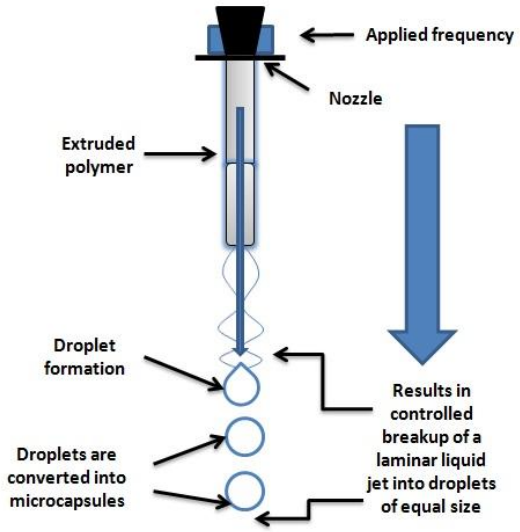

Figure 7. Principle of vibration nozzle technology

When liquid is extruded through a nozzle at certain flow rates, it produces a laminar jet which can break into short lengths by natural irregular disturbances. These segments then form spherical droplets due to the force of surface tension. The droplets then fall into a bathing solution which turns them into solid microcapsules. This system allows mono-dispersed microcapsules to be formed with a standard size deviation of ≤ 5% when using alginate solution.

The size of the microcapsules being produced depends on several parameters. Firstable the nozzle diameter is strongly affecting the size of the forming droplets.. Also, the flow rate must be considered in order to set up the devise in a dripping mode or creating a jet that can be broken by the frequency. The frequency that should be used is strongly dependent on the chosen nozzle size and normally is inversely proportional to the diameter of the nozzle. The viscosity of the extruded solution plays a crucial role in the process. This is why it is very important to pay close attention to the choice of a suitable carrier for the bioactive compound of interest before considering the nozzle size and setting up the parameters of the instrument.

7. 1. Possible biopolymer materials

In food grade microcapsules, proteins, polysaccharides and the combination of these with hydrolyzed starches are some of the most common candidates for shell materials (Garti & McClements 2012). What also should be taken into account is the allergen properties that certain materials might posses and other restrictions depending on cultures and religions.

When it comes to anionic polysaccharides such as alginate, pectin, carageenan and others they are characterized by the fact that in aqueous solutions they are extremely flexible and capable of absorbing large amounts of water.. What is the most important- they easily form the hydrogel structure by the addition of counter-ions (Wang et al. 2012).

7.1.1. Food-grade polysaccharides for hydrogel formation

Among the polymers used as a solid membrane surrounding the capsules, alginates and pectins are widely used in oil encapsulation because of their high gelling capacity, their biocompatibility and low toxicity. In the presence of calcium ions (Ca^{2+}), alginates macromolecules cross-link to form a three-dimensional network called egg-box structure.

7.1.1.1.Alginate

Alginate is one of the most suitable carriers in microencapsulation due to its high capacity to form gels at low concentrations, its relatively cheap price and its biocompatibility and low toxicity. This natural anionic polysaccharide is isolated from the walls of various species of brown algae and it consists of a linear chain of (1-4)-linked residues of β-D mannuronic acid and α-L-guluronic acid in different proportions (Draget et al. 1997).

Alginate hydrogels can be prepared because of the fact that the chains of alginate are cross-linked in three-dimentional networks with high water content in the present of divalent ions (Lee & Mooney 2012). Therefore an "egg-box" is created, a model proposed first by Grant (Grant et al. 1973) in order to explain how the divalent ions bound to the inter-chain cavities also called ionic cross-linking. According to this model, the divalent ions interact simultaneously with the hydroxyl and carboxyl groups of the guluronic acid, forming a stable egg-box structure (Figure 8).

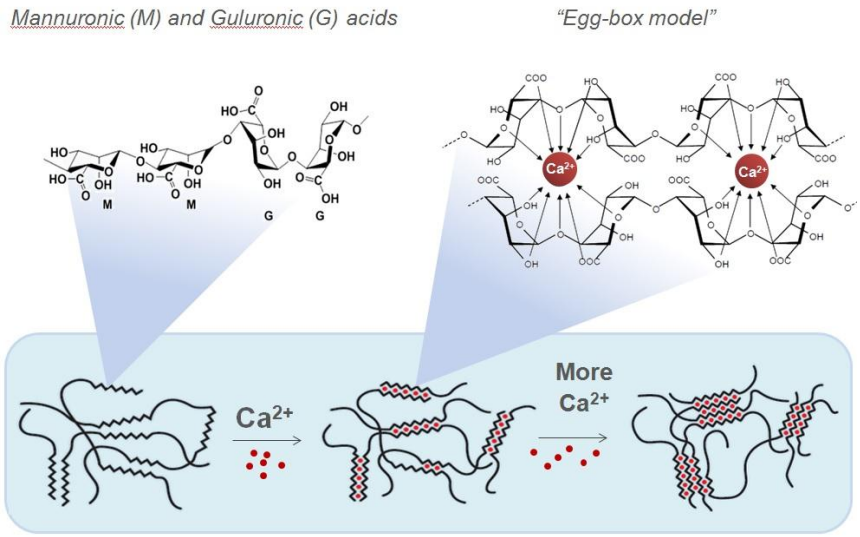

Figure 8. Mechanism of alginate gelation in the presence of calcium ions

In the case where the calcium ions are not enough, more than two carboxyl groups chelate a single ion and thus creating a formation of non stable structures also knowns as imperfect egg-box (Fu et al. 2014). These motifs can influence the hydrogel properties such as pore size, mechanical resistance and release of the active ingredients. The pore size and the mechanical resistance of the alginate hydrogels depends on several other factors including the exposure

time of alginate chains to divalent ions, the divalent ion concentration, the type of cross-linker agent and the presence of organic solvent in the cross-linker solution , (Smidsrod & Skja 1990;Goh et al. 2012;Liu et al. 2016b).

It was also observed that in the presence of calcium divalent ions, alginate chains associated into fibrils and their diameter and network density increased with the increase of calcium divalent ions concentration into the bathing solution (Liu et al. 2016a). It was shown that Ca-alginate matrix have small pores according electron microscopy studies and the diameter of the pores is between 5 and 20 nm (Klein et al. 1983).

The properties of the alginate matrix such as mechanical strength and release of active compounds might also be influenced by the addition of other polymers into the alginate solution (Nussinovitch & Hershko 1996). According to the authors, the addition of polylysine or chitosan in the alginate solution can change the permeability of the alginate membrane and thus slowing down the release of the active compounds. When other cationic polymers are added this can influence the strength of the membrane and affect the fragility of the capsules.

7.1.1.2. Pectin

Pectin is a natural gelling polysaccharide that have received more and more attention in the last years because as a biodegradable polymer (Baracat et al. 2012). Discovered 200 years ago, the chemical structure of pectin and its properties are not completely defined though significant progress is made in the recent years. The gelation of pectin is influenced by number of factors - molecular size, pH, presence of other polysaccharides, degree of methoxylation, number and arrangement of side chains. (Thakur et al. 1997).

Some well defined polysaccharides have been discovered and characterised when it comes to pectin. The first of them is homogalacturonans that are linear chains of α-(1–4)-linked D-galacturonic acid (Hellin et al. 2005). Rhamnogalacturonan I (RG I) is mainly composed of arabinose, galactose, galacturonic acid and rhamnose, suggesting the presence of arabinan and/or (arabino) galactan side chains (Yapo et al. 2007). There are two main regions in pectins- one is the so-called "hairy" regions and the other the "smooth" regions. The smooth regions are characterized by the lack of L-rhamnose residues. The hairy regions have both D-galacturonic acid and L-rhamnose residues and are also characterisez by the fact that they carry side chains of neutral sugars including mainly D-galactose, L-arabinose and D-xylose,

with the types and proportions of neutral sugars varying with the origin of pectin (Campbell-Platt 2011).

Pectins are mainly applied in food for their gelling properties but any sample of pectin, parameters such as the molecular weight or the content of particular subunits will differ from molecule to molecule (Sriamornsak 2003). One of the main advantages of pectin are their ability to protect unstable molecules, their resistance to enzyme hydrolysis and differential solubilities, depending on the pH. (Liu 2014). Pectins can be used independently as a polymer for the production of microcapsules as well as in combination with other polysaccharides in order to form a hyrdogel.

8. Extrusion and co-extrusion techniques

The extrusion technique is a physical method and is the one of the first techniques when it comes to microencapsulation.. What is important to be mentioned is that the technique uses only one nozzle in order to extrude the desired liquid. This means that the bioactive compound should be mixed with the carrier in order to form a homogeneous dispersion. The main goal of this process is to produce hard and dense capsule that will protect the active ingredient (Gaonkar et al. 2014).

The main disadvantage of the technique is the fact that theoretically the bioactive compound and the shell material must be compatible or in other words-mixable. In the case of simple extrusion, matrix type of encapsulates are being formed.

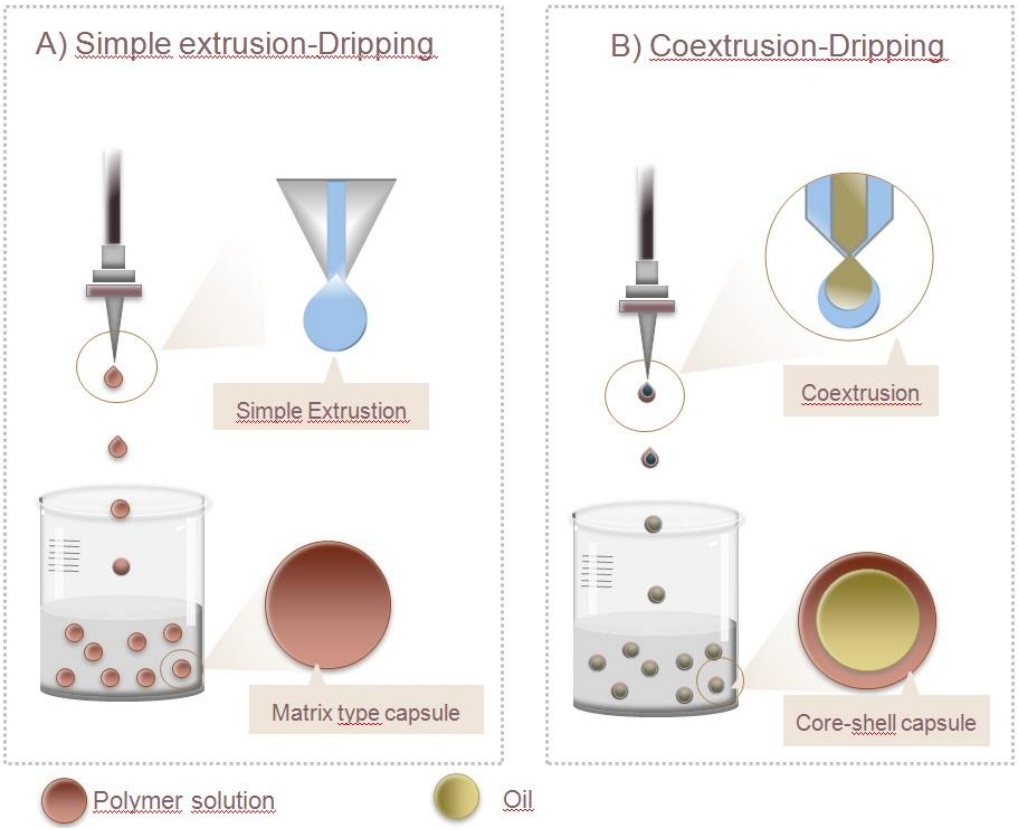

Figure 9. Schematic representation of simple extrusion and co-extrusion methods

On the other hand, though it sounds relatively simple co-extrusion is a lot more complicated and sophisticated technique. Liquid core materials that consist of the bioactive compound and an appropriate shell material are simultaneously pumped through concentric nozzles at a specific flow rate so both of the solutions fall with the same speed in the bathing solution. When reaching the hardening solution, a capsule forms which is consisted of fluid core encased by a layer of shell material. The shell is then harden by the appropriate means-in this case as in the case of simple extrusion, by chemical cross-linking as we are speaking about the extrusion of polymers. As in the case of the simple extrusion, the co-extrusion again depends on many factors such as viscosity of the liquid, flow rate, geometry of the nozzle, amplitude, frequency. The main disadvantage of this technique is the fact that the variables are so many that it is difficult to vary one without affecting the others. This is why the co-extrusion process is always very particular and the setting of the parameters varies from case to case.

Depending on the flow rate given by the operator the capsules might be formed in two main ways- by dripping technique or by forming a jet. In the first case the liquid containing the bioactive compound and the shell solution flow in a very low rate and the capsule begins to

form in the tip of the nozzle. In the case of jet formation, the parameters need to be set a lot more precisely as the capsule is forming in the air due to the breakage of the droplets because of the applied frequency. As long as the flow rate and temperature is constant, this technique can produce really uniformed capsules with small size distribution, however quite large in dimensions. In the case of co-extrusion, core-shell encapsulates are being formed.

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CHAPTER 3 ASTAXANTHIN

Production of stable food-grade microencapsulated astaxanthin by vibrating nozzle technology

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Abstract

Astaxanthin is a carotenoid known for its strong antioxidant and health-promoting characteristics, but it is also highly degradable and thus unsuited for several applications. A sustainable method was developed for the extraction and the production of stable astaxanthin microencapsulates. Nearly 2% astaxanthin was extracted by high-pressure homogenization of dried *Haematococcus pluvialis* cells in soybean oil. Astaxanthin-enriched oil was encapsulated in alginate and low-methoxyl pectin by Ca^{2+}-mediated vibrating-nozzle extrusion technology. The 3% pectin microbeads resulted the best compromise between sphericity and oil retention upon drying. The stability of these astaxanthin beads was monitored under four different conditions of light, temperature and oxygen exposition. After 52 weeks, the microbeads showed a total-astaxanthin retention of 94.1±4.1% (+4 C/−light/+O_2), 83.1±3.2% (RT/−light/−O_2), 38.3±2.2% (RT/−light/+O2), and 57.0±0.4% (RT/+light/+O_2), with different degradation kinetics. Refrigeration, therefore, resulted the optimal storage condition to preserve astaxanthin stability.

1. Introduction

Astaxanthin is a symmetric ketocarotenoid (3,3′-dihydroxy-β-β′-carotene-4,4′-dione) that naturally occurs in a wide variety of marine and aquatic organisms, and it is responsible for the bright red to pink color of Crustacea (shrimp, krill) and Salmonidae (salmon, rainbow trout). Astaxanthin is commonly used as a feed supplement in world-wide fish farming to grow healthy and well-colored fishes.

The importance of this carotenoid rocketed when its protective role against peroxidation of lipids in biological membranes was finally recognized. The activity of astaxanthin, in addition, turned out to be higher than that of other known antioxidants, and this made astaxanthin a candidate molecule to improve human health (Guerin et al. 2003; Mason et al. 2006)

Astaxanthin used for food supplements is usually a mixture of configurational isomers, among which the main active molecule is considered the all-trans isomer. It is produced synthetically or extracted from biological sources, and, in the latter case, it shows a higher chemical stability because it is naturally esterified with fatty acids. Among the biological sources the microalgae *Haematococcus pluvialis* (Hp), a freshwater species of Chlorophyta is acknowledged to contain the highest content i.e. up to 3% on the dry weight (Rao et al. 2007; Johnson & An 2008)

As an antioxidant, astaxanthin scavenges free radicals and other oxidants, and protects the lipid bilayer from peroxidation with its polar ionic rings and non-polar conjugated carbon–carbon bonds. The antioxidant activity of astaxanthin is approximately 10-fold higher than that of other carotenoids, including lutein, canthaxanthin, and β-carotene(Miki 1991; Shimidzu et al. 1996; Kobayashi & Sakamoto 1999; Lorenz & Cysewski 2000; Naguib 2000)

Due to the high level of unsaturation astaxanthin is greatly sensitive to high temperatures, light, and oxidative conditions that catalyze different degradation reactions such as isomerization to cis forms, epoxidation and fragmentation of the polyene chain(Bustos et al. 2003; Kittikaiwan et al. 2007; Bustos-Garza et al. 2013). For instance, the half-life of astaxanthin from Hp dispersed in sunflower oils was 7 days at room temperature (Bustamante et al. 2016). This high instability forces the market to consider new strategies, such as microencapsulation, to stabilize astaxanthin for feed, cosmetic, and food applications.

Spray drying is commonly employed for oil encapsulation and allows to obtain smaller particles (from few to tens micrometers). However, the half-life at 25 C of spray dried astaxanthin is limited to approximately 30 days or even less(Pu et al. 2011; Bustos-Garza et al. 2013; Bustamante et al. 2016).

Differently from spray dry, extrusion process is characterized by milder conditions that minimally affects the integrity of most bioactive compounds(Chew & Nyam 2016). Different approaches to encapsulate astaxanthin by extrusion have been described. (Higuera-Ciapara et al. 2004) discussed the production of chitosan encapsulated beads, but the addition of glutaraldehyde make them not properly compatible with food production.(Kittikaiwan et al. 2007) obtained relatively big (about 0.4 cm) chitosan particles with a procedure which, however, is difficult to automatize. Other attempts to produced chitosan beads allowed to obtain smaller particles (20–100 μm) but with a relatively short half-life (about 30 days) even in dark conditions (Bustos et al. 2003). Alginate has been recently described as a possible encapsulating matrix (Lin et al. 2016). More than 90% of the synthetic astaxanthin encapsulated was measured after 21 days storage. However, alginates have a great limitation, i.e., the loss of nearly 10% of oil upon drying(Taksima et al. 2015). For this reason, different matrices that may ensure longer stability of astaxanthin and minimize the loss of oil during the production ought to be explored.

In the present work we describe the extraction of astaxanthin from *Haematococcus pluvialis*, its microencapsulation by vibrating nozzle extrusion technology and the stability of the encapsulates during one year storage in four different conditions. To make the production process more sustainable we considered two aspects: the avoidance of solvents in carotenoid extraction by the use of high-pressure homogenization in presence of soybean oil, and the utilization of a polymer deriving from plant food-chain side-products, such as pectin, as shell material.

2. Materials and Method

2.1. Algae extraction

Dried Hp cells were provided by AlgainEnergy Srl. The cell aggregates occurring upon drying were powdered by a blender. The powder was mixed with edible soybean oil 1:10

(w:v) at 45°C and kept under gentle agitation with a stirrer for 1 h. The mixture was slowly transferred in a continuous Panther NS3006L homogenizer (GEA Niro Soavi, Milano, Italy) and let recirculate for 150 min at 45 C and 1000 bar of pressure (that represents the maximum pressure obtainable with this system) to increase the extraction yield of the carotenoid. At different time points samples were collected, diluted in ethyl acetate and analyzed by a spectrophotometer at 480 nm as described below to measure the astaxanthin content. The extracted oil was clarified by centrifugation at 3500 g for 15 min to eliminate cell debris and subjected to encapsulation.

2.2. Encapsulation

Food-grade low-methoxyl pectin (PE, Silva Extracts, Bergamo, Italy) and Na-alginate (AL, Sigma-Aldrich) were used for the encapsulation of astaxanthin-enriched oil. Both the polysaccharides are capable of forming gels in presence of divalent cations (e.g. Ca^{2+}) following the ionotropic gelation process. PE is originally extracted from citrus peel and subjected to chemical modification by the manufacturer to reduce the degree of methoxylation (DM) and to increase the degree of amidation (DA) of galacturonic acid residues. The characteristics of the resulting PE provided are: DM: 25–35% and DA: 20–25%.

The polysaccharides were dissolved in water. Two concentrations of PE (2 and 3%) and one of AL (4%) were evaluated. Above these values the solubilization of the matrices was incomplete, while below them the retention of oil was considered unsatisfactory (data not shown). The polysaccharides were mixed with oil to reach a final astaxanthin concentration of 0.03%. The mixture was homogenized with Ultra Turrax (IKA-Werke, Staufen, Germany) at 24,000 rpm for 15 min and degassed in a bath sonicator (Branson Ultrasonic, Danbury, CT, USA) for 20 min at room temperature (RT).

The emulsion obtained was used to feed an Encapsulator B-390 (Büchi, Milano, Italy). The working principle of the instrument is based on the laminar jet break-up by the application of a vibrational frequency with defined amplitude to the extruded jet(Homar et al. 2007; Zhang & Rochefort 2010; Whelehan & Marison 2011; Chew & Nyam 2016). The temperature was set at 40 C in order to diminish the viscosity of the emulsion. After an initial phase of setting, the parameters of the process were fixed as follows: air pressure: 500 mbar, vibrational frequency of the membrane: 600 Hz, electrode potential: 2000 V, amplitude: 3. The diameter

of the nozzle was 750 μm. The gelling bath (0.2 M $CaCl_2$) was positioned 10 cm below the nozzle.

Upon formation, the beads were left in the bath for 30 min to complete the gelling. After that, the beads were collected with a sieve and washed twice (30 min each wash) with distilled water. The beads were finally dried at RT for about 24 h until constant weight was reached. Water activity (Aw) was measured by a HC2AW instrument (Rotronic, Switzerland).

2.3. Morphology

The size and the morphology of the beads were evaluated by a stereomicroscope (Leica Microsystems, Milano, Italy), analyzing 20 beads for each type.

2.4. Sphericity factor (SF)

In order to describe the sphericity of the beads we calculated the SF as previously reported (Chew & Nyam 2016) using the following equation:

$$SF = \frac{(Dmax - Dper)}{(Dmax + Dper)}$$

where Dmax is the maximum diameter passing through the centroid of the bead and Dper is the diameter perpendicular to Dmax. A SF=0 is expected for a perfect spherical shape while SF>0 values indicate higher degrees of shape distortion.

2.5. Evaluation of the oil loss after drying

Ten gram of beads were positioned on a plastic Petri dish whose weight was previously registered and dried as described before. The beads were then accurately removed and the dish, eventually containing traces of oil, was weighted (= weight of dried dish).

The percentage of oil lost during drying was calculated as follows:

$$\%\ \text{oil loss} = \frac{\text{weight of dried dish} - \text{weight of dish}}{\text{weight of the oil}} \times 100$$

where the weight of the oil is the amount of the theoretical encapsulated oil.

The quantification was performed in triplicate.

2.6. Storage stability

The 3%PE beads were divided in 4 groups and stored in transparent plastic vials under different environmental conditions: 1) RT at light with the lid of the vial open (RT/+light/+O_2); 2) RT at dark with the lid open (RT/−light/+O_2); 3) RT under vacuum at dark (RT/−light/−O_2);+4°C at dark, with the lid open (+4°C/−light/+O_2).

At different time points from 0 to 52 weeks an amount of 0.5 g of beads from each group underwent oil extraction as described below to check the astaxanthin content. The experiments were performed in duplicate.

2.7. Degradation kinetics

The kinetics of astaxanthin degradation were analyzed by fitting the data with delayed zero-, first- and second-order kinetic models. The goodness of the fits was evaluated by computing the reduced χ^2 (i.e. χ^2/d.f., where d.f. are the degrees of freedom. A χ^2/d.f.=1 is expected for a perfect fit). Mathematical analyses were carried out using the software Mathematica ver. 10.4.1.0. (Wolfram research Inc., Champaign, Illinois, USA).

2.8. Oil extraction from the beads

The following extraction procedure was developed to calculate the astaxanthin payload of the pectin beads. An amount of 0.5 g of beads was incubated for 2 h with 10 ml of buffer containing 100 mM Na phosphate buffer + 50 mM NaCl, pH 7.4 to destroy the structure of the beads. A volume 0.5 mL of the suspension was placed in a 2 ml tube and 1 ml of ethyl

acetate was added. The tube was vortexed and kept under agitation for 2 h at RT. After centrifugation at 14,000g for 10 min the astaxanthin-containing supernatant was collected.

2.9. Spectrophotometric analysis

Quantification of astaxanthin was carried out by a UV/VIS spectrophotometer (Unicam UV2). The samples were diluted in ethyl acetate and absorbance measured at 480 nm. The concentration of astaxanthin was calculated following the equation:

$$[A] = \frac{10 \times A_{480} \times DF}{E_{(1\%;1\ cm)} \times d}$$

where [A] is the astaxanthin concentration expressed as mg/ml; A_{480}: absorbance at 480 nm;

DF: dilution factor; $E_{(1\%;1\ cm)}$: astaxanthin specific absorbance (2150); d: optical path (cm).

2.10. HPLC

Reverse phase HPLC of astaxanthin-containing samples was performed with a Beckman System Gold (Beckman Coulter) on a C30 column (4.6 × 250 mm, particle size 5 μm) (YMC Europe, Schermbeck, Germany) following a previously described method (Reyes et al. 2014) with minor modifications. The absorbance was monitored at 480 nm by a Beckman 168 diode array detector. The injection volume was 50 μl. The elution was carried out at a flow rate of 1 ml/min using acetone (solvent A) and water (solvent B) as follows: isocratic elution at 84:16 (A:B) for 10 min and a gradient to 97:3 (A:B) for 100 min.

2.11. DPPH

The essay has been performed as described previously (Thaipong et al. 2006) using 1,1-Diphenyl-2- picryl-hydrazyl (DPPH, Sigma–Aldrich) with modifications. The stock solution was prepared by dissolving 24 mg DPPH with 100 mL methanol. The working solution was obtained by mixing 10 mL stock solution with 45 mL methanol to obtain an absorbance of 1.1

units at 515 nm. Beads extracts were dried, suspended in the same solvent used for the assay and allowed to react with 20 volumes of the DPPH solution for 1 h in the dark. The absorbance was read at 515 nm. The results were expressed as percentage of radical scavenging activity (%RSA) using the following equation:

$$\%\mathrm{RSA} = \frac{A_{\mathrm{control}} - A_{\mathrm{sample}}}{A_{\mathrm{control}}} \times 100$$

Each analysis was performed in triplicate.

3. Results and discussion

3.1. Astaxanthin extraction

The extraction profile of astaxanthin from Hp cells is shown in *Fig. 1*. The process is characterized by an exponential kinetics that reaches the plateau approximately in one hour. Notably, nearly 1% (w:w) of astaxanthin is already extracted at time zero (i.e., before the pressure is increased to 1000 bar), indicating that the dried cells released part of the carotenoid during the stirring phase. *Fig. 2* shows microscope images of samples taken at different time points during the extraction process. A decrease of intact number of Hp cells containing astaxanthin (dark-orange color) along the extraction is evident. The yield of the extraction of astaxanthin was approximately 1.9%, not far from the data reported in literature about the carotenoid content in Hp cells extracted with acetone, i.e. 2–3% (Rao et al. 2007). In addition, quantity and extractability of astaxanthin depend on different variables among which the Hp cultivation techniques (Praveenkumar et al. 2015). Since some colored cells are still visible after 150 min the procedure could be probably optimized, e.g. by rising the operating pressure that should help in destroying the cell walls of the microalgae. Anyway, the yield is much higher if compared to previous data which described the extraction of astaxanthin from dried Hp cells by soybean oil (Dong et al. 2014). The reason is probably due to the use of high pressure homogenization that causes the breakage of the cell wall thus allowing a better extraction of the carotenoid.

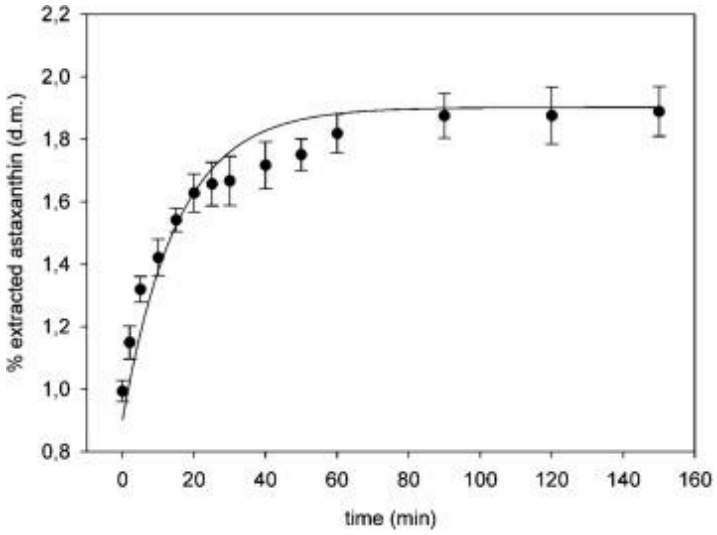

Figure 1. Time-dependent astaxanthin extraction yield in soybean oil. The data are given as mean percentage ± standard deviation of two independent experiments and were fitted (χ^2/d.f.=1.1) with the general exponential function $y(t)=a_1-e^{-a2t,}$ where $a_1=1.90 \pm 0.037$ and $a_2=0.065\pm0.01$ min^{-1}. Using these parameter values it could be calculated that the time to reach 50% and 95% of maximal extraction was 10.6 and 36.2 min, respectively.

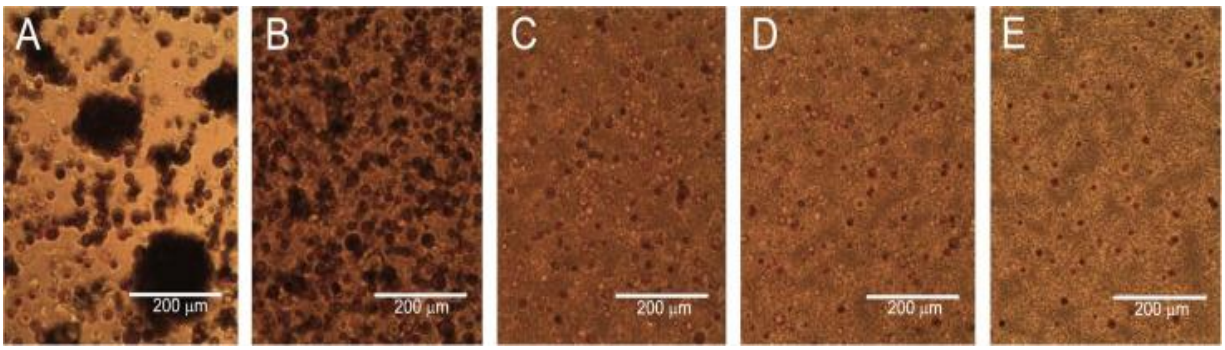

Figure 2. Microscope images of samples taken at different time points of the extraction process showing Hp cells in oil before dispersion (A), or during homogenization at 1000 bar at 0 min (B), 30 min (C), 60 min (D), and 150 min (E).

3.2. Production of matrix-type micro-beads

The astaxanthin-enriched oil was emulsified with the polysaccharides as described in materials and methods section and subjected to encapsulation by the vibrating nozzle encapsulator. Among the different evaluated parameters, the quality of the beads was defined mainly by the loss of encapsulated oil upon drying. Indeed, during the drying process, the evaporation of water leads to a thinning of the polysaccharide matrix that becomes more fragile and that allows drops of emulsified oil to leak. This is of great importance since it represent an economical loss and thus it should be as low as possible. We also considered the sphericity of the beads. Indeed, spherical beads are easier to manipulate than particles with other shapes and, in some cases, more appreciated by the consumers. The SF value of the preparations were 0.24, 0.14 and 0.05 for PE2%, PE3% and AL4% respectively (see *Table 1*). From these data it is clear that the sphericity of alginate beads is definitely higher than PE beads. This different behavior has been reported previously (Pillay & Fassihi 1999; Sandoval-Castilla et al. 2010) and has been associated to differences among the Ca^{2+}-mediated crosslinking properties of the two polysaccharides.

Table1. Characteristics of the microbeads

Type	Size (µm)*	SF*	A_w**	Oil loss (%)**
Pe2%	468 ± 104	0.24	0.350 ± 0.024	0.05 ± 0.04
Pe3%	467 ± 66	0.14	0.335 ± 0.001	0.33 ± 0.05
Al4%	541 ± 81	0.05	0.334 ± 0.013	4.36 ± 0.53

∗ Mean diameter ± standard deviation (n. 20).
** Values expressed as mean ± standard deviation of three independent experiments.

By measuring the oil lost after drying we could identify alginate beads as those more leaking with a loss around 4% of encapsulated oil (*Table 1*). On the contrary, pectin allowed a better retention of the oil with values well below 0.5%. Since PE3% beads represent the better compromise between oil retention and sphericity, we chose this formulation for the

subsequent stability test. The astaxanthin content of the PE3% beads resulted to be 0.95±0.01 mg/g.

3.3. Storage Stability

The stability of astaxanthin was measured for a total duration of 52 weeks. Four conditions were selected to simulate some of the most common storage environments (*Fig. 3*). The data show that the samples stored at+4 C (+4 C/−light/+O_2) and under vacuum (RT/−light/−O_2) retained the higher amounts of the original astaxanthin content with values of 94.1±4.1% and 83.1±3.2% respectively. The percentage of astaxanthin retention was lower for samples kept at RT in contact with standard atmosphere, i.e., 38.3±2.2% at (RT/−light/+O_2) and 57.0±0.4% at light (RT/+light/+O_2).

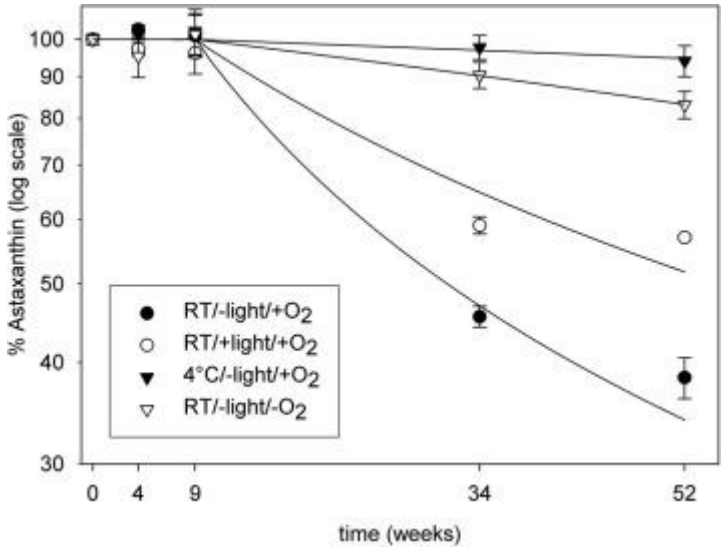

Figure . 3. Stability test of astaxanthin-enriched oil PE3% beads stored at different conditions. The data are expressed as % of the astaxanthin content in respect to time zero.

To analyze the time-dependent astaxanthin degradation we fitted the data with the three standard models from chemical kinetics. The results are summarized in Table 2 where, for each fitting, the decay time constant **k** and the reduced Chi-squared values (χ^2/d.f.) are reported.

Table 2. Kinetics analysis of astaxanthin degradation during the stability test

	order	k (weeks^{-1})	χ^2/d.f.
+4°C/-light/+O_2	0^{th}	0.12 ± 0.05	1.39
	1^{st}	1.2 x 10^{-3} ± 5.6 x 10^{-4}	1.39
	2^{nd}	1.30 x 10^{-5} ± 5.9 x 10^{-6}	1.40
RT/-light/-O_2	0^{th}	0.39 ± 0.03	1.34
	1^{st}	4.2 x 10^{-3} ± 3.4 x 10^{-4}	1.37
	2^{nd}	4.5 x 10^{-5} ± 4.0 x 10^{-6}	1.43
RT/+light/+O_2	0^{th}	1.47 ± 0.14	2.57
	1^{st}	2.0 x 10^{-2} ±1.7 x 10^{-3}	2.47
	2^{nd}	2.2 x 10^{-4} ± 2.9 x 10^{-5}	2.32
RT/-light/+O_2	0^{th}	1.8 ± 0.2	2.60
	1^{st}	2.8 x 10^{-12} ± 2.5 x 10^{-3}	2.41
	2^{nd}	4.5 x 10^{-4} ±3.4 x 10^{-5}	1.97

The reduced χ^2 value provides a statistical justification to prefer one model as the best descriptor of experimental data. From the results given in *Table 2* and *Fig. 3*, there is an objective reason to conclude that the degradation of the samples stored at RT and exposed to the standard atmosphere, independently on whether the samples were kept under light or dark conditions (RT/−light/+O_2 and RT/+light/+O_2), follows 2nd order kinetics. With the present data, however, it is not possible to draw firm conclusions on the degradation kinetics of the other two samples, and both 0th and 1st order kinetic models fitted equally well. Simulations carried out with model equations and parameter values reported in *Table 2* showed indeed that it would be necessary to run stability experiments for at least 200 weeks to unambiguously discriminate between the different degradation kinetics (not shown).

Previous works by other authors described the degradation of astaxanthin as a 1st order reaction (Pu et al. 2011; Takeungwongtrakul & Benjakul 2016). In some cases zero-order

kinetics have been reported although the kinetic order could vary depending on the incubation temperature(Pu et al. 2010; Bustamante et al. 2016). The greater degradation displayed by the samples stored under RT/−light/+O_2 and RT/+light/+O_2 conditions might be explained as the oxidation induced by peroxyl radicals of unsaturated fatty acids, triggered by the presence of oxygen during the 9 weeks induction period. The radicals generated during this period might have started degrading the polyene structure of astaxanthin in the following period. On the contrary, in the other two samples, due to the lack of triggering factors, such as oxygen or a sufficiently high temperature, lipid oxidation might have been partially inhibited.

It is unclear why the beads exposed to light retained a higher content of astaxanthin than those kept in the dark. As described previously, darkness should help preserving the carotenoid integrity (Kittikaiwan et al. 2007). However, similar data were obtained previously (FrancoΓÇÉZavaleta et al. 2010). At room temperature and in standard atmosphere astaxanthin stored in sunflower oil under dark conditions displayed less stability than that exposed to light. The authors described a rather fast degradation of astaxanthin (i.e., 17 days) in all conditions, even when stored in air-free flasks or under refrigeration. The longer stability displayed by our preparation could be explained as the effect of the encapsulation process on molecular diffusion. A limited diffusion would reduce the probability of astaxanthin and radicals to encounter thus preventing their reaction.

Since the spectrophotometer measurements at 480 nm alone do not reflect the actual conversion of the trans-astaxanthin, as many degradation products absorb at a close wavelength (de Bruijn et al. 2016), the extracts of the beads after 52 weeks storage were further analyzed by RP-HPLC (*Fig. 4*, panels B–E) and compared with the extract of the beads at time zero (*Fig.4*A). Free astaxanthin was eluted between 10 and 20 min, whereas astaxanthin monoesters, that constitute the major part of the extract, eluted from 45 to 75 min, while the diesters were eluted from 75 to 100 min. The profile of the chromatogram is similar to those reported previously (Jaime et al. 2010; Reyes et al. 2014)

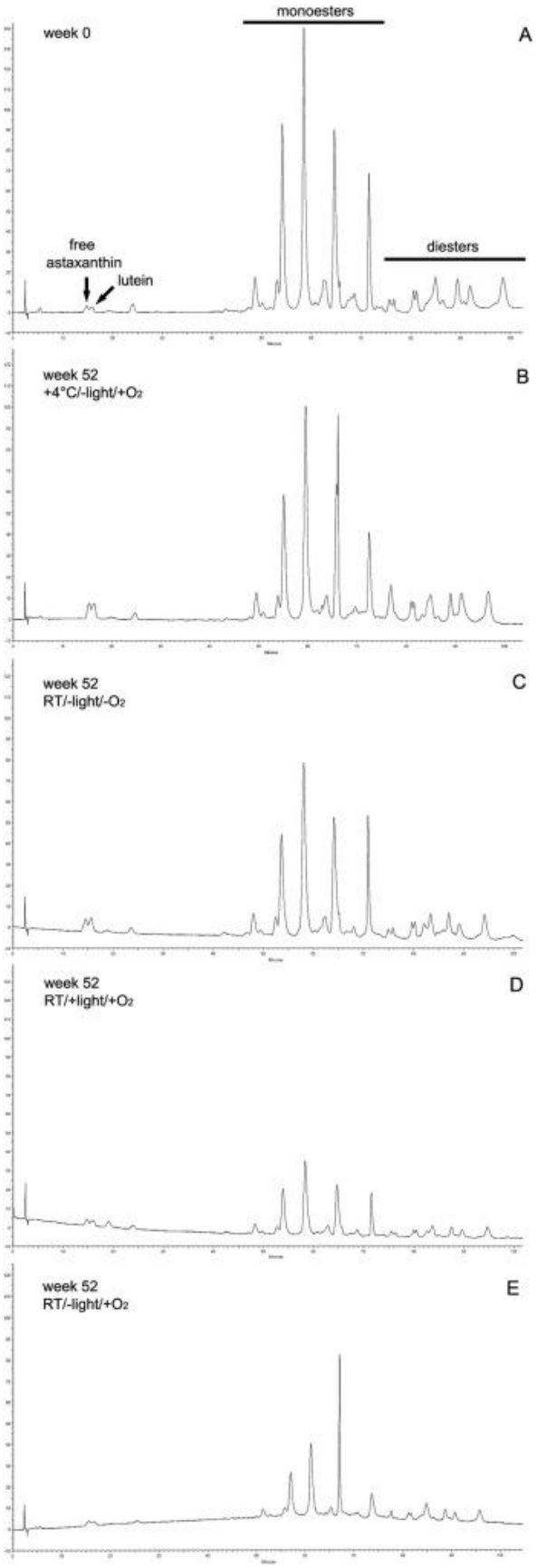

Figure . 4. RP-HPLC chromatograms (480 nm) of the extracts from the beads at the end of the 52 week (B, C, D, E) stability test compared to time zero (A).

From the these data it is possible to observe a strong decrease of the peaks associated to the diesters and monoesters for the samples conserved under light/dark conditions in the presence of oxygen, in comparison to the beads conserved at +4 C and under vacuum. The results shown in *Fig. 4* were further analyzed. The area under the chromatographic traces (AUC) was computed for quantitative comparison purposes, and the results are given in *Fig. 5*.

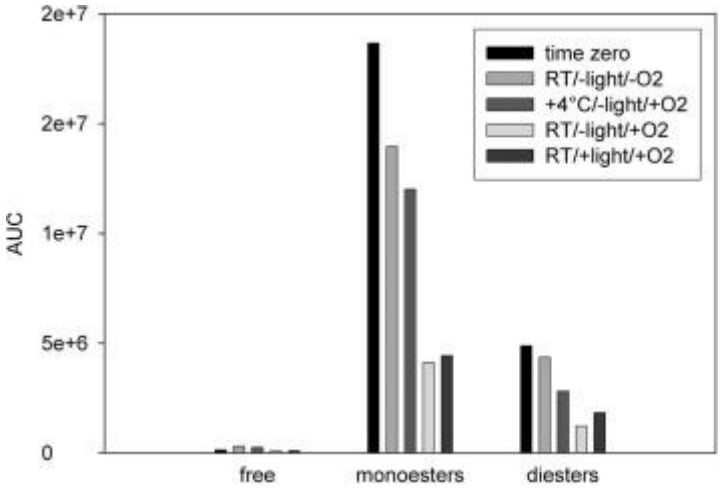

Figure. 5. Free, mono- and di-esterified astaxanthin forms content after 52 weeks storage in the different conditions expressed as integrated peak areas (AUC) of the chromatograms of *Fig. 4*.

These data are in agreement with the astaxanthin retention percentages displayed in *Fig. 3*. Interestingly, the decreasing of the peaks of the esters did not correspond to an equal increase of the peaks associated to the free carotenoid as observed previously (Bustos et al. 2003). This suggests that the storage conditions affects mainly the polyene chain rather than the ester bonds, with a mechanism based on the cleavage of astaxanthin and the production of uncolored fragments. This can occur more rapidly after trans-cis isomerization since 9-cis and 13-cis isomers, the main isomerization products of all-trans astaxanthin, are more susceptible to oxidation (Hernandez-Marin et al. 2013). It is somewhat intriguing that these isomers, and especially the 9-cis isomer, display an anti-oxidant activity higher than the trans-astaxanthin (Liu & Osawa 2007).

To evaluate the antioxidant activity of the encapsulated beads we employed the widely used DPPH assay. In agreement with the spectrophotometric and chromatographic analyses the samples kept at 4 C for 1 year exhibited the highest radical scavenging activity

(%RSA=73.4±1.1) with respect to the initial time of storage (%RSA=84.4±1.2). Interestingly, the samples stored at RT/−light/+O_2 showed a higher %RSA (65.7±1.1) than those at RT/+light/+O_2 (57.5±1.3), even if astaxanthin content was lower in the former samples. The beads stored under vacuum showed a %RSA identical to the samples kept at dark at RT (65.1±1.1). These results indicate that %RSA is not strictly dependent on the astaxanthin content. This is reasonable since other antioxidant molecules present in the formulation, like tocopherols of soybean oil, might degrade with different kinetics depending on the storage conditions. Further studies might elucidate the degradation mechanisms of the major antioxidant compounds present in the encapsulates.

4. Conclusions

High pressure homogenization in soybean oil led to a good extraction of astaxanthin (almost 2%) from ***Haematococcus pluvialis***. Besides being considered more sustainable than other algal polysaccharides, low methoxyl pectin proved to be a suitable material for the encapsulation of astaxanthin-enriched oil by extrusion. The advantage of this approach is the minimal stress that the active molecules undergo. The high stability displayed by the encapsulates in specific conditions during an extremely long storage test makes the whole process feasible for future applications in food, feed, and cosmetic industries.

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CHAPTER 4 SPIRULINA.EXTRACTION OF *SPIRULINA PLATENSIS*

Abstract

Nowadays, there are numerous commercial applications of microalgae in various industrial fields such as nutrition, food and feed, agriculture, cosmetics and many others. The aim of this chapter is to set up a protocol of extraction in order to successfully obtain an aqueous extract of *Spirulina Platensis*. Since there is a growing interest in the incorporation of health promoting bioactive compounds such as Phycocyanin in the food industry, an evaluation of the extract would be undertaken in order to understand better its antioxidant capacity, deferability, its spectrophotometric and fluorescence properties and also its behaviour when subjected to heat treatment that is almost always an inevitable part of the food processing.

Part of the results discussed in this chapter regarding fluorescence analysis were obtained during a collaboration with Professor Nicola Daldosso and his group in Experimental and Applied Physics in the department of Computer Science, University of Verona.

1. Introduction

Algae are simple organisms categorized in two main populations: filamentous and phytoplankton algae (Demirbas & Demirbas 2010). There are four main important classes of algae diatoms (*Bacillariophyceae*), the green algae (*Chlorophyceae*), the golden algae (*Chrysophyceae*) and the blue-green algae (*Cyanobacteria*). The first three types are eukaryotes distinguished by the presence of a nucleus and separate organelles for photosynthesis (chloroplasts) and respiration (mitochondria) and the last one, *Cyanobacteria*, are part of the eubacteria and are prokaryotes lacking a membrane-bounded nucleus (Enzing et al. 2014a; Kim & Lee 2015). Following the increase in the world's population, a special attention should be given to micro-algae as nowadays, they represent a promising source for food and feed. Normally it is sold directly as a dietary supplement without any kind of processing though specific high-value components from micro-algae are also being developed and produced (Enzing et al. 2014a). Micro-algae are used in personal skin products, slimming products, fluorescent protein markers, stable isotope biochemicals and others. However, special attention should be given to food and feed as it represent a large percentage of the market profits of micro-algae products.

Table 1. Market figures of micro-algae based products in food and feed, whole drier micro-algae biomass

Current product based on micro-algae	Production volume (tons/ year dry weight)	Number of producers (key players)	Value of production volume (yearly turnover)	Potential market (synthetic / traditional forms)
Food and feed products: whole dryed micro-algae biomass				
Spirulina	5,000 tonnes/ year (Norsker et al. 2011)	>15 companies (Cyanotech / Earthrise) (Milledge 2012)	US $ 40m (Milledge 2012)	No synthetic alternative
Chlorella	2,000 tonnes/ Year (Norsker et al. 2011)	> 70 companies (Milledge 2012)	US $38m (Spolaore et al. 2006)	No synthetic alternative

Adapted from (Enzing et al. 2014b)

The global marine biotechnology market, with micro-algae as its main component, was estimated in 2011 to be worth €2.4 bn, with an expected yearly growth of 10 % (Guedes et al.

2011). This makes the potential market opportunities enormous and new innovative products from micro-algae- an emerging trend. In this chapter, special attention would be given to *Spirulina platensis* as it represent an indispensable part of the global micro-algae market and it is one of the two bioactive compounds of interest that represent the essential part of this thesis.

2. *Cyanobacteria*

The *Cyanobacteria*, a species of primitive prokaryotes, are among the earliest of inhabitants of Planet Earth, from which is derived the whole biodiversity of the world. Many of these species seem virtually unchanged compared to fossils 3.8 billion years ago and are the oldest forms of life on earth (Kulasooriya 2012). They are photosynthetic organelles in plant cells of plants have evolved, the current chloroplast (Cooper & Hausman 2000; Howe et al. 2008).

The large production of oxygen by *Cyanobacteri*a has subsequently allowed the development of life forms that carry out aerobic respiration as oxygen released by oxygenic photosynthesis gradually changed the original reducing atmosphere to an oxidizing one allowing a dramatic change in biodiversity (Olson 2006).The chloroplasts of eukaryotic algae and higher plants have originated from endosymbiotic relationships with *Cyanobacteria* (Martin & Kowallik 1999; Raven & Allen 2003) stimulating the advent of oxygen tolerant flora and fauna capable of aerobic respiration, a highly efficient mechanism of energy utilization (Kulasooriya 2012).

The *Cyanobacteri*a have evolved resulting in widely diverse morphologies, the cellular organization models ranging from single-celled forms to multicellular forms. They can be found in freshwater, marine and terrestrial ecosystems but also found in extreme habitats including some extreme ecosystems such as hot springs, the desert soils, some hypersaline environments and glaciers (Fogg & Thake 1987; Schirrmeister et al. 2011). In the traditional classification of *Cyanobacteria* the morphological differences are used in order to divide the group into five orders (Rippka et al. 1979; Castenholz et al. 2001): subsection I (*Chroococcales*) and II (*Pleurocapsales*) as unicellular coccoids and subsections III (*Oscillatoriales*),IV (*Nostocales*) and V (*Stigonematales*) that form filaments of varying morphological complexity (Tomitani et al. 2006).

Two species among the vast array of *Cyanobacteria* have been used as food supplements of high nutritive value- *Spirulina platensis* (syn. *Arthrospira platensis*) and *Aphanizomenon flos-*

aquae (Kulasooriya 2012). *Spriulina platensis* is well-known as the source of complete nutrition which no other organism can offer, having a very high protein content (65-71% / dry weight) as well as vitamins, minerals, super antioxidants such as β-carotene, vitamin E and others (Kulshreshtha et al. 2008).

3. *Spirulina platensis (A. platensis)*

Spirulina (*Arthrospira) platensis* is a filamentous non-N_2-fixing *Cyanobacteria* that represents an important source for commercially produced nutraceutical compounds (Estrada et al. 2001) because of the high content of healthy nutritional molecules such as β-carotene, phycocyanin, vitamins, minerals, and polyunsaturated fatty acids (Tietze 1999), as this cyanobacterium utilizes sunlight and CO2 in order to produce chemical compounds that are essential for life (Klanchui et al. 2012). The most recent classification of this *Cyanobacteria* is as it follows (Cheevadhanarak et al. 2012):

• Kingdom: *Bacteria*
• Phylum: *Cyanobacteria*
• Class: *Cyanophyceae*
• Order: *Oscillatoriales*
• Family: *Oscillatoriaceae*
• Genus: *Arthrospira*

The environmental conditions for the growth of *Spirulina platensis* include an aquatic environment with a basic pH between 9.5 and 10.5 (Çelekli et al. 2009), a particularly rich of sodium bicarbonate waters, 10-20 g / l and a daytime temperature of around 35-37 (Danesi et al. 2011). This strongly basic environment combined with the rapid growth of this organism in these conditions prevents the development of other algae like *Chlorella* (Batello et al. 2004; Hamed 2016).

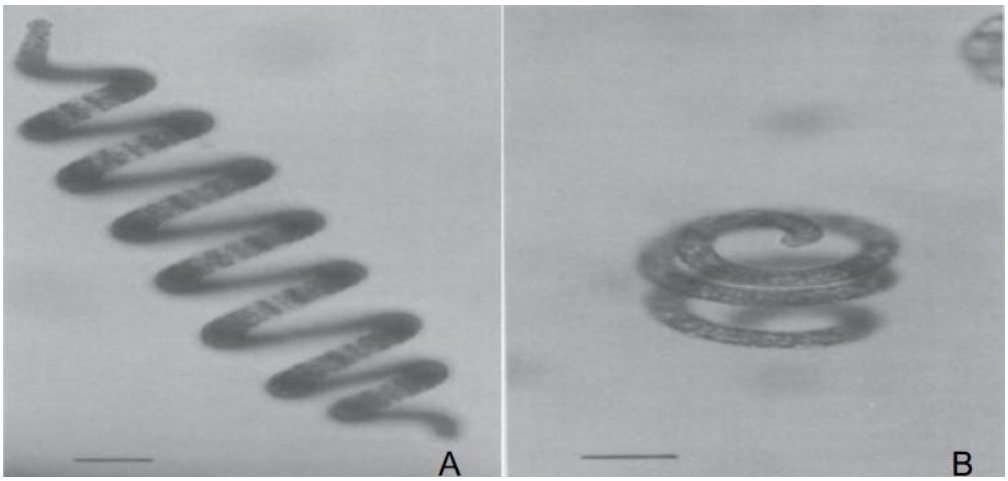


Figure 1. Light micrograph of *Arthrospira maxima*. B. Light micrograph of *Arthrospira platensis*. Bar represents 20μm (Ali & Saleh 2012)

This species reproduces asexually by fission with a typical of the *Cyanobacteria* process, called hormogonia which consists of fragmentation of the filaments into small chains of cells (Mur et al. 1999). The cell wall is Gram (-) and consists of four layers, the outer one (layer IV) consists of lipopolysaccharides, the third layer is formed by fibrillar proteins wrapped helically along the trichome, the second layer is constituted by peptidoglycans and is fused with the inner layer that forms the septum, which delimits the cells. *Spirulina platensis* shows vigorous gliding motility of filamentous cells (trichomes) with rotation along their long axis (Fujisawa et al. 2010) and the cells of the trichomes are broader than long and the width can vary from 3 to 12 μm though it can reach 16μm occasionally with a typical prokaryote with a lack of membrane-bound organelles (Belay et al. 2008).The absence of cellulose in the wall makes *Cyanobacteria* easily digestible by humans (Richmond 2008). The structure is a predominant cytoplasmic thylakoid system (Pessarakli 2016).

3.1. Photosynthetic apparatus and Phycobilisome structure of the *Cyanobacteria*

The photosynthetic apparatus of *Cyanobacteria* is very similar to that of higher plants with the exception of their complex antenna pigments, mainly constituted by three types of macromolecular complexes: the Photosystem I (PS I), Photosystem II (PS II) and the phycobilisome (PBS). Phycobilisomes are complex protein structures located on the cytoplasmic surface of the thylakoid membranes and in *Cyanobacteria* the light harvesting is

mediated by them (Liu 2016). The major components of the PBS are the biliproteins, allophycocyanin (AP), phycocyanin (PC), phycoerythrin, and phycoerythrocyanin, with covalently attached bilin chromophores, composed of two structural domains: an AP core that is in direct contact with the thylakoid membrane and generally six rods of stacked PC and, in some strains, phycoerythrin or phycoerythrocyanin hexamers radiating from the core (Sidler 1994; Miskiewicz et al. 2002).

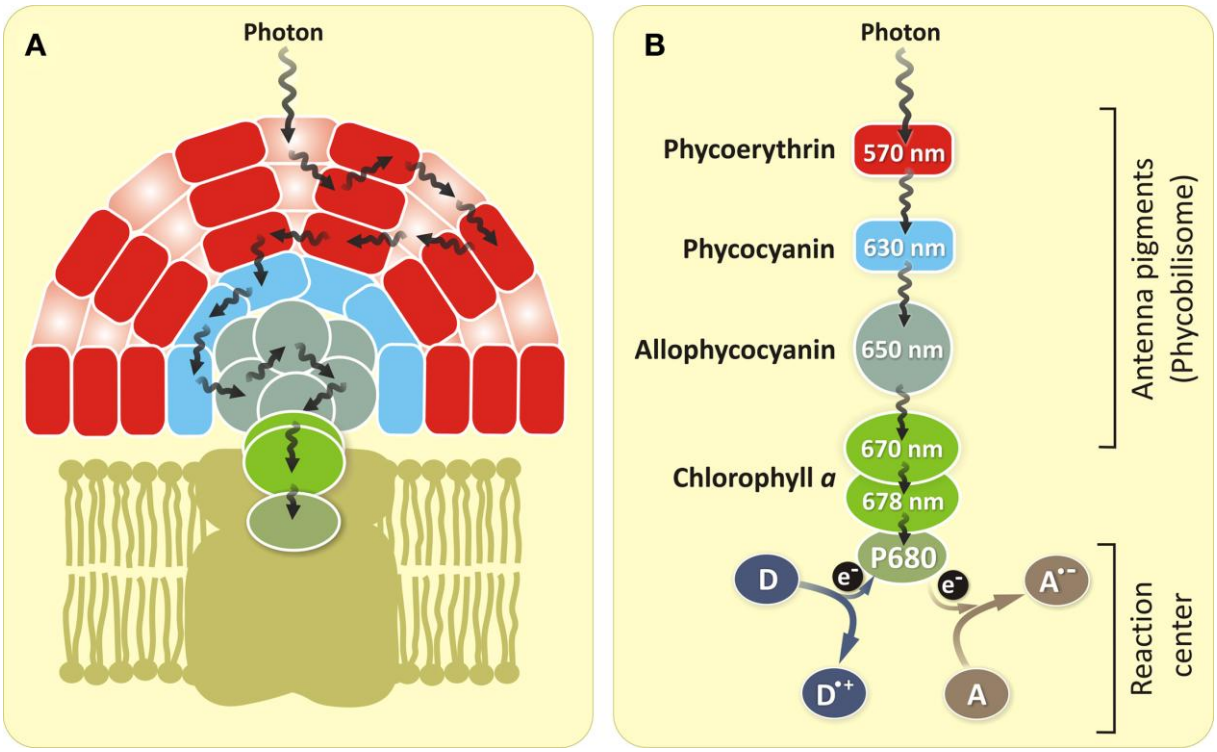


Figure 2. Structural organization of the antenna system of PSII for red algae and cyanobacteria (A) and energy transfer steps including charge separation (photochemical reaction) at the PS II reaction center (B) for cyanobacteria (Shevela 2011)

The main biological role of phycobiliproteins is to capture light for photosynthesis, with Phycoerythrin (PE, $\lambda_{A\ max}$ = 540-570 nm; $\lambda_{F\ max}$ = 575-590 nm), Phycocyanin (C-PC, $\lambda_{A\ max}$ = 610-620 nm; $\lambda_{F\ max}$: 645-653 nm) and Allophycocyanin (APC, $\lambda A_{A\ max}$ = 650-655 nm; $\lambda_{F\ max}$ = 657-660 nm) all of the above mentioned being the regions of the visible spectrum in which chlorophylls have a low absorption coefficient (Sekar & Chandramohan 2008). The absorption maximum is determined mainly by the number of conjugated double bonds in the chromophore (Nollet & Toldra 2012). Phycoerythrobilin contains six conjugated double bonds and absorbs wavelengths below the ones absorbed by Phycocyanobilin that possesses eight conjugated double bonds instead. The structural organization of phycobilisomes allows

the transfer of excitation energy from phycoerythrobilin, which has higher excitation energy, the Phycocyanobilin of Phycocyanin and subsequently to Allophycocyanin. From there, the excitation energy is transferred to the PS II or sometimes to PS I (Eriksen 2008). The C-phycocyanin and Allophycocyanin have the same chromophore, the Phycocyanobilin, but it is present in the α and β subunits in different proportions for the two apoprotein (MacColl 2004; Eriksen 2008; Bermejo et al. 2008).

The aqueous extract of *Spirulina platensis*, when analyzed with the help of a spectrophotometer, reveals the abundant presence of Phycocyanin with an absorption maximum at 615 nm and can also be detected the presence of Phycoerythrin and Allophycocyanin represented as two small shoulders to the left and right respectively of the absorption peak of Phycocyanin. The height of these two peaks is generally less than half compared to that of Phycocyanin and this reflects the richness of these chromophores in *Spirulina platensis* (Ali & Saleh 2012). It is often not possible to quantify the allophycocyanin, this is because the absorption curve of Phycocyanin, which is much more abundant, is covering the one of Allophycocyanin. Moreover, in weakly acidic conditions monomers and trimers of Phycocyanin and of monomers Allophycocyanin all have as the same absorption maximum wavelength of 615 nm, making it impossible to detect the presence of Allophycocyanin in the aqueous extract of the alga (MacColl 2004). To locate the Allophycocyanin by the means of a spectrophotometer in such conditions, you must first purify it by chromatography.

Considering the abundance of Phycocyanin in an aqueous extract of *Spirulina Platensis*, it is used as the main marker. It must be underlined that Phycocyanin maintains its native structure for pH values higher than 5, with a maximum stability between pH 5 and 6, while for values lower that the ones mentioned it denatures gradually (Duangsee et al. 2009; Chaiklahan et al. 2012). When Phycocyanin absorbs wavelengths corresponding to its absorption maximum between 615 and 620 nm, it fluoresces in the red region of the visible spectrum, with a maximum emission between 635 and 640 nm (Pizarro & Sauer 2001; Silveira et al. 2007).

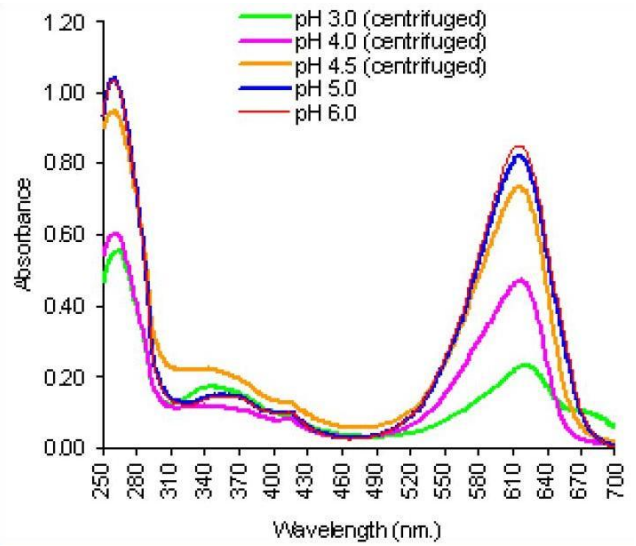


Figure 3. Absorption spectra of Phycocyanin at pH 3.0-6.0 (Duangsee et al. 2009)

Also temperature plays an important role in the stability of the Phycocyanin. The critical temperature, above which C-PC will start to denature is 47 ° C. The degree of denaturation can be determined by comparing the value of the concentration of Phycocyanin before treatment and after treatment, called relative concentration. It is indicated by some authors, that one of the main methods of drying the algae is with the help of an air flow at 55 ° C on seaweed fragments of 3.7 mm in thickness. This system can minimizes the losses of the bioactive compounds as lipids and dyes, compared to other drying technologies, even if the Phycocyanin and the β-carotene are partially degraded. Other authors indicate that the optimum drying temperature should be not more than 45 ° C (Dissa et al. 2010). The instability connected with different changes of pH and temperature might give potential difficulties and make the aqueous extract of *Spirulina Platensis* hard to manage and tricky to incorporate when it comes to food processing as most of the food undergoes some kind of heat treatment and pH changes.

3.2.Nutritional values of *Spirulina Platensis*

Spirulina Plantesis has been used as a food source and medicine since ancient times, nevertheless, only in the last two decades, the true nutritional and pharmacological potential of this alga has been subjected to more profound studies. *Spirulina* has established itself as a nutraceutical food thanks to the discovery and validation of its many beneficial health effects. It poses an antioxidant activity, anti-inflammatory, anti-diabetic, anti-tumor properties, it is immune-stimulating, anti-microbial, cardio protective, and it has the ability to reduce

cholesterol, to induce intestinal growth of lactobacilli, to reduce nephrotoxicity of heavy metals and drugs and it enhances the protection from radiation (Belay et al. 1993; Capelli & Cysewski 2010).

In 2003 the Food and Drug Administration (FDA) has granted the nominative of "General Recognized as Safe" (GRAS) to the dried biomass of *Spirulina platensis*. The biochemical analysis of some of Spirulina strains, have shown that this alga is a rich source of protein, vitamins, essential amino acids, minerals, essential fatty acids including the γ-linoleic acid, glycolipids and sulfolipids (Belay et al. 2008). The biochemistry of *Spirulina* composition may vary according to the species used and the growth conditions, but can nevertheless be summarized for the basic nutrients as follows:

Composition[a]	per 100 g	Composition[a]	per 100 g
1. *Macronutrients*		2. *Vitamins*	
Calories	373	Vitamin A (as 100% β-carotene)[b]	352,000 IU
Total fat	4.3 g	Vitamin K	1090 mcg
Saturated fat	1.95 g	Thiamine HCl (Vitamin B1)	0.5 mg
Polyunsaturated fat	1.93 g	Riboflavin (Vitamin B2)	4.53 mg
Monounsaturated fat	0.26 g	Niacin (Vitamin B3)	14.9 mg
Cholesterol	<0.1 mg	Vitamin B6 (Pyridox. HCl)	0.96 mg
Total carbohydrate	17.8 g	Vitamin B12	162 mcg
Dietary fiber	7.7 g	3. *Minerals*	
Sugars	1.3 g	Calcium	468 mg
Lactose	<0.1 g	Iron	87.4 mg
Protein[b]	63 g	Phosphorus	961 mg
Essential amino acids (mg)		Iodine	142 mcg
Histidine	1000	Magnesium	319 mg
Isoleucine	3500	Zinc	1.45 mg
Leucine	5380	Selenium	25.5 mcg
Lysine	2960	Copper	0.47 mg
Methionine	1170	Manganese	3.26 mg
Phenylalanine	2750	Chromium	<400 mcg
Threonine	2860	Potassium	1,660 mg
Tryptophan	1090	Sodium	641 mg
Valine	3940	4. *Phytonutrients*	
Nonessential amino acids (mg)		Phycocyanin (mean)[b]	17.2%
Alanine	4590	Chlorophyll (mean)[b]	1.2%
Arginine	4310	Superoxide dismutase (SOD)	531,000 IU
Aspartic acid	5990	Gamma linolenic acid (GLA)	1080 mg
Cystine	590	Total carotenoids (mean)[b]	504 mg
Glutamic acid	9130	β-carotene (mean)[b]	211 mg
Glycine	3130	Zeaxanthin	101 mg
Proline	2380		
Serine	2760		
Tyrosine	2500		

Figure 4. Nutritional profile of dried *Spirulina Platensis* **(Belay 2008)**

• **Protein**: *Spirulina* has an unusually high content of protein, between 55 and 70% of its dry weight. Thanks to the absence of cellulose in the cell wall, proteins are more easily accessible to proteolytic enzymes compared to the vegetable ones.

• **Essential fatty acids**: *Spirulina* has a high content of polyunsaturated fatty acids (PUFAs) with around 1.5-2% of the total fat, which is 5-6% of the dry weight of the algae (Marzieh Hosseini et al. 2013). In particular, *Spirulina* is rich in γ-linolenic acid (GLA), which is 36% of total PUFAs, linoleic acid, stearidonic acid (SDA), eicosapentaenoic acid (EPA) , docosahexaenoic acid (DHA) and arachidonic acid (AA).

• **Vitamins**: *Spirulina* contains large amounts of water-soluble B vitamins in particular very concentrated in vitamin B12. There are also vitamins B1, B2, B3, B6, B9. Among the water-soluble is also present in moderate amounts vitamin C. Among the fat-soluble vitamins contained in *Spirulina* is abundant the precursor of vitamin A, β-carotene, but also the content of vitamin E is quite high with a concentration of 19 mg /100g of dry weight. Other fat-soluble vitamins present in *Spirulina* are also vitamins D and K (Kova-ì et al. 2013).

• **Minerals**: *Spirulina* is a rich source of potassium, calcium, copper, iron, magnesium, manganese, phosphorus, selenium, sodium and zinc (Kim 2015).

• **Photosynthetic pigments and enzymes**: *Spirulina* contains many pigments, including chlorophyll A, β-carotene, xanthophylls and moreover, the phycobiliproteins phycocyanin and allophycocyanin.

3.3. Biological effect of *Spirulina*

The recognition that nutrients have the ability to interact and modulate the molecular mechanisms of human physiological functions, has led to a "nutraceutical revolution" in food. There are many foods that contain molecules that can contribute positively to the human health. *Spirulina* with its wide range of bioactive substances it contains, is definitely one of them.

3.3.1. Antioxidant activity

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal cellular metabolism (Rutkowski et al. 2007). The ROS and RNS are known to act as second messengers in the control of various physiological functions of the organism. These free radicals can however also be produced as a result of other metabolic activities, for example when it is required and is produced much energy during intense physical activities, or when a lot of energy must be dissipated for example during exposure to the sun, the X-rays, UV radiation Many free radicals are also produced by the body in response to infection or burns or after the intake of toxic substances. The excessive production of ROS, is often caused by excessive stimulation of NADPH oxidase by cytokine, or the electron transport chain in the mitochondria resulting in partial reduction of some of the oxygen molecules (Turrens 2003).. The free radicals are unstable and highly reactive molecules as characterized by an unpaired electron and all of the above mentioned leads to oxidative stress. To reach a state of equilibrium, they react with other molecules through oxidization. The molecules mainly attacked by free radicals are the nucleic acids DNA and RNA, proteins, and particularly polyunsaturated fatty acids of the lipid membranes (Lobo et al. 2010). The double bonds of the polyunsaturated fatty acids are particularly vulnerable to the attack of radicals structures, due to the presence of double bonds and associated allylic H atoms in their structure (Perluigi et al. 2012). The lipid peroxidation mediated by reactive oxygen species is an important cause of damage and destruction of cell membranes, because a single initial event can lead to the conversion of hundreds of chains of fatty acids in lipid radicals (Belay et al. 2008).

Table 2. Some studies on the antioxidant effects of *Spirulina*

Reference	Type of study	Summary of the study
(Manoj et al. 1992)	In vitro	The alcohol extract of Spirulina inhibited lipid peroxidation more significantly than the chemical antioxidants like alpha-tocopherol, BHA, and beta-carotene. Water extract showed more antioxidant activity than gallic acid and chlorogenic acid.
(Bhat & Madyastha 2000)	In vivo (Rats)	Phycocyanin from Spirulina effectively inhibited CCl_4–induced lipid peroxidation in rat liver in vivo.
(Miranda et al. 1998)	In vivo (Rats)	Plasma antioxidant activity in brain homogenate incubated at 47◦C showed that the antioxidant activity of plasma was 97% and 71% for the experimental group and 74% and 54% for the control group after 2 months and 7 months, respectively.
Vadiradja check	In vivo (Rats)	Carbon tetrachloride (0.6 mL/kg) and R-(+)-pulegone (250 mg/kg) induced hepatotoxicity in rats was reduced significantly when phycocyanin was administered intraperitoneally to rats 1 or 3 h before the challenge
(Romay et al. 2003)	In vivo (Rats)	Oral administration of c-phycocyanin (100 mg/kg) in rats prevented kainic acid induced behavioral and glial reactivity in the rat hippocampus crossing the hematoencepphalic barrier. Authors postulate potential use of phycocyanin in the treatment of neurodegenerative disease such as Alzheimer's and Parkinson's disease induced by oxidative stress-induced neuronal injury.
(Bhat & Madyastha 2000)	In vitro	Phycocyanin showed a potent peroxyl radical scavenger capacity with a rate constant ratio of 1.54 compared to 3.5 for uric acid (a known peroxyl radical scavenger)
(Hirata et al. 2000)	In vitro	The antioxidant activity of phycocyanobilin (a component of phycocyanin) was greater than that of alpha-tocopherol, zeaxanthin, and caffeic acid on the molar basis
(Romay et al. 2003)	In vitro	Phycocyanin inhibited 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH), a free radical generator induced human erythrocyte haemolysis in the same way as trolox and ascorbic acid, two well-known antioxidants. On the basis of the values of IC50 phycocyanin was found to be 16 times more efficient as antioxidant than trolox and about 20 times more efficient than ascorbic acid.

Table adapted from (Belay et al. 2008)

The antioxidant properties of *Spirulina* and its extracts have been analyzed by several research groups. One of these (Manoj et al. 1992) has demonstrated that the alcoholic extract of Spirulina inhibits lipid peroxidation with higher efficiency than that of other antioxidants

such as α-tocopherol, BHA (butylhydroxyanisole) and the β -carotene. Even the aqueous extract of *Spirulina* shows a better antioxidant power of other antioxidants such as gallic acid and chlorogenic acid (Safafar et al. 2015). An interesting aspect of these results is that the aqueous extract showed a significant antioxidant power even after removing polyphenols. The C-PC is the major antioxidant of *Spirulina*. Its ability to neutralize free radicals is well documented in the literature and this property is mainly attributed to its chromophore Phycocyanobilin (Belay et al. 2008).

3.3.2. Spirulina and effects on the immune system

The sulfur-containing polysaccharides, which may be isolated aqueous extract of Spirulina, called calcium spirulan (Ca-SP), possesses immunomodulatory and antiviral activity, stimulates the production of antibodies and white blood cells (Hayashi et al. 1996). Currently the antiviral capacity of Spirulina is attributed to three groups of substances: sulfur polysaccharides, the sulfoglycolipids, and proteins that bind pigments (C-CP and APC) (Karkos et al. 2010).

The calcium spirulan is a characteristic polysaccharide of Spirulina, containing both sulfur that calcium, soluble in water, constituted by: rhamnose, ribose, mannose, fructose, galactose, xylose, glucose and glucuronic acid (El-Baky et al. 2014). Concerning its antiviral activity, it inhibits in vitro the replication of various viruses, such as herpes simplex type I, influenza A, measles, mumps, and Human immunodeficiency-1 (HIV-1) (Hayashi et al. 1996).

Some studies have shown that Spirulina has a positive effect on the immune system, increasing the humoral immunity (Cribb 2004). This property is attributed to Phycocyanin and polysaccharides with high molecular weight. Several polysaccharides were isolated from Spirulina, some of which have molecular weights above 10 million Dalton. It has been shown that these polysaccharides have biological activity and can in fact strengthen the immune system and stimulate hematopoiesis, not only in vitro but also after oral administration.

3.3.3. Anti-inflammatory and anticancer properties of Spirulina

In the last decade many studies have shown that cancer is a multigenic disease caused by the disruption of multiple cell signaling pathways and dysregulation of many gene products. All these phenomena are often linked to chronic inflammatory processes (15% of human tumors are associated with infection and inflammation). It is becoming increasingly clear that many of the lifestyle factors, such as the use of tobacco and alcohol, diet (35% of cancer deaths are attributed to dietary factors), environmental pollution, radiation and infections, can cause chronic inflammation that lead to the development of tumors. Chronic diseases caused by inflammation require continuous and not occasional treatment. Thus, nutraceuticals can be a solution to this problem.

Some components of different foods have a potential use as anticancer drugs. The markers of chronic inflammation include pro-inflammatory cytokines such as TNF (tumor necrosis factor) and certain interleukins, chemokines and pro-inflammatory enzymes such as COX-2 (cyclooxygenase-2), as well as some growth factors such as EGF (epidermal growth factors), and adhesion molecules such as ICAM-1 (intercellular adhesion molecule-1) (Reuter et al. 2010; Landskron et al. 2014; Di Maggio et al. 2015).

Oxidative stress has a deleterious process that can mediate structural damage to the cells and consequently cause various diseases such as cancer and some cardiovascular or neurodegenerative diseases (Uttara et al. 2009). Oxidative stress creates a redox imbalance in the cells, promoting an oncogenesis. In fact, the permanent modification of genetic material caused by oxidative stress is the initial phase of mutagenesis, carcinogenesis and aging (Valko et al. 2007).

4. *Spirulina Platensis* for the production of functional foods

Spirulina possesses a number of bioactive compound. *Spirulina* is different from virtually any other crop for human consumption because it is completely edible, unlike conventional vegetable cultivation of which is often used only the fruit , seeds, leaves or roots. Also, as

mentioned before, the cell wall does not contain cellulose. It is in fact composed of 60% (by dry weight) protein as well as carbohydrates and fats and is easily digested. The protein contained in Spirulina have a good balance in amino acids, which is superior to most of the proteins of vegetable origin, even if lower than the most complete proteins of animal origin. As *Spirulina* is labile to external influences such as pH and high temperature, the aim of this chapter is the characterization of the aqueous extract from *Spirulina platensis* for its future microencapsulation in order to be incorporated in future in functional foods.

4.1. Materials and methods

4.1.1 Extraction from dry *Spirulina*

The dried algae biomass was provided by Algain Energy srl., Italy. It was weighted and finely ground with a mortar and pestle, and the powder obtained was extracted in distilled water by cold maceration (Kamble et al. 2013) with a biomass: water ratio of 1:25 (w /v). After 12 hours stirring at 4 °C in dark conditions the suspension was centrifuged at 10,000 g for 20 min and the supernatant recovered. The extract was then filtered with Whatman paper n ° 4 and stored at -20°C.

4.1.2. Spectrophotometric measurement of *Spirulina* extract

A spectrophotometric method was used to assay the obtained extract using a Thermo scientific Evolution 201 UV-Visible spectrophotometer. The concentration (mg/ml) of phycocyanin (C-PC) was obtained following the equation described by (Silveira et al. 2007):

$$PC = \frac{[OD_{615} - 0.474 \times OD_{652}]}{5.34}$$

where ABS_{615} and ABS_{652} are the absorbance values of the extract measured at 615 and 652 nm respectively.

4.1.3. Heat treatment of *Spirulina* extract

An experiment regarding the loss of C-PC during heat treatment was performed at 65 °C in two different buffer solutions, 20 mM sodium phosphate at pH 6.5 and 20 mM sodium acetate at pH 4.5. The temperature was chosen in order to simulate a possible pasteurization process and the samples were incubated in water baths maintained at the specified temperature for 30 min. Samples we taken at 0, 5, 10, 20 and 30 min and the absorbance was then measured with the help of a spectrophotometer, calculating the amount of C-PC so to understand its possible loss in time under the influence of temperature.

4.1.4. Simulated digestion of *Spirulina* extract

. A two-stage in vitro digestion system (Yi et al. 2015) with slight modifications was adopted. In the first stage, the extract was mixed with simulated gastric fluid (SGF) containing 0.084 M HCl, 30 mM NaCl, pepsin (Sigma) 1:20 (enzyme:protein), pH 1.2 and put at 37°C for 60 min in agitation. Samples were collected at 0, 30 and 60 from the gastric phase. After 1h the remaining SGF was mixed with simulated intestinal fluid (SIF) containing 0.1 M sodium phosphate, + 50 mM NaCl at pH 7.3 and pancreatin (Sigma) 1:20 (enzyme:protein). Simulated intestinal digestion was performed at 37°C for 300 min in agitation. Samples were again collected at different time points.

4.1.5.ABTS analysis of *Spirulina* extract during heat treatment and simulated digestion

Antioxidant capacity analysis was performed using ABTS assay based on the method of den Berg (1999) with slight modifications by Kim (2003). According to this method 2.5 mM of ABTS was mixed with 1 mM of 2.2'-azobis (2-amidinopropane) dihydrochloride in phosphate buffer 0.1 M + 150 mM NaCl at pH 7.4. A water bath was used to heat the solution at 68°C for 1h. The ABTS was cooled to room temperature and filtered with 0.20 μm filter and then diluted with fresh PBS solution in order to reach an absorbance of approximately 0.700 at 734 nm. Afterwards, 950 μL of the ABTS were mixed with 50 μL of the sample and incubated at 37 °C for 10 min. The samples were then centrifuged for 1 min at 14,000 rpm and the absorbance was read using a BIO-TEK Power Wave microplate reader.

4.1.6.Fluorescence analysis of *Spirulina* extract and simulated digestion

The optical properties of the extract, the heat treatment and the simulated digestion of the extract were investigated by using a Horiba Jobin-Yvon Nanolog instrument equipped with a xenon lamp. As for photoluminescent spectra, the setup used was 2 nm slit size, density grating of 1200 g/mm (blazed at 500 nm), integration time of 0.1 second, and cut-off filtration at 370 nm. Photoluminescence excitation at 0.5 second was utilized for the integration time. The measurement of the Spirulina extract was performed at room temperature while the digestion measurement was performed at 37°C as required by the established protocol.

4.1.7. SDS-PAGE

SDS-PAGE analysis was performed in 16% polyacrilamide gel. Samples were diluted with a 2x loading buffer containing 125 mM Tris HCl pH 6.8 with 15% glycerol and 3% SDS, 5% 2-mercaptoethanol and incubated at 95°C for 5 min. Separation was carried out in 25mM Tris Glycine buffer, 192 mM glycine, pH 8.3 The gel was stained using Coomassie Brilliant Blue R250.

5. Results and discussion

The interest in phycocyanin is increased by its nutritional and nutraceutical properties exploited for the preparation of food supplements and healthy foods (Martelli et al. 2014). There is much attention given to it as it is believed to be antioxidant both in *in vitro* and in *in vivo* studies (in experimental animals), and it has suggested anti-inflammatory, anti-viral and anti-cancer properties, and is reported to possess the capacity to stimulate the immune defence system (Shih et al. 2003; Liu & Osawa 2007; Pleonsil et al. 2013; Zheng et al. 2013). Phycocyanin is also used as biochemical tracer in immunoassays due to its fluorescent properties (Silveira et al. 2007; Eriksen 2008).

5.1. Spectrophotometric analysis of *Spirulina* extract

Spectrophotometric measurements of *Spirulina* extract are shown at Figure 5. The peak at 264 nm is due to the nucleic acids that have absorbance maxima normally around 260 and 280 nm and the ratio of absorbance at these wavelengths is as a measure of purity in both nucleic acid and protein extractions (Vonshak 1997). Regarding the absorption peak at 344 nm, many biological assays have as their basis a link to the oxidative status of nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP). Many dehydrogenase enzymes use these coenzymes to transfer hydrogen groups between molecules. Because the reduced forms of these molecules differ from the oxidized forms in their ability to absorb light, it is possible to quantitate reactions based on light absorbance at 340 nm or by the fluorescent emission of light at 445 nm as in this case (Kendrew 2009).

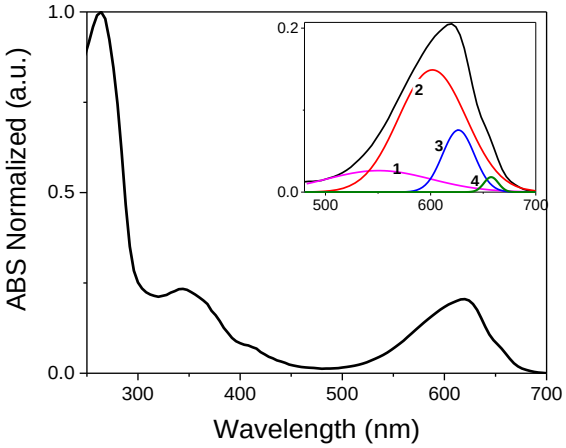


Figure 5. Absorbance (ABS) spectrum of Spirulina extract. Insert: peak separation in the orange range .

As for the third peak at around 600 nm, we can speak about the existence of four spectroscopic classes of phycobiliproteins (PBP) based on peak separation (figure inserted): phycoerythrin PE (500-565 nm, curve #1), phycoerythrocyanin PEC (570-575 nm, curve #2), PC (595-640 nm, curve #3), and allophycocyanin APC (650-655 nm, curve #4). Absorption maximum moves to the red with the increase in the number of conjugated double bonds but differences also occur due to the three dimensional conformation of the bound chromophore and differences in the electrical field from the protein surrounding the chromophore. (Green et al. 2003). Moreover, Phycocyanin, one of the phycobiliproteins present in *Spirulina* is absorbing orange and red light, particularly near 620 nm as it is characterized by absorption

maxima at 650 nm and shoulders at 340 and 620 nm (Patel et al. 2005). A usual extraction of Spirulina platensis would give around 2.63 mg/ml of Phycocyanin depending on the lot.

5.2. Fluorescence analysis of the extract

Energy transfer can be observed by the help of fluorescence analysis. It occurs between the chromophores resulting in the fact that most fluorescence is emitted by the chromophore whose energy level is the lowest in all the complex. In this regard, as it can be seen on solid line of Figure 6, *Spirulina* extract has two emission peaks in the visible regime.

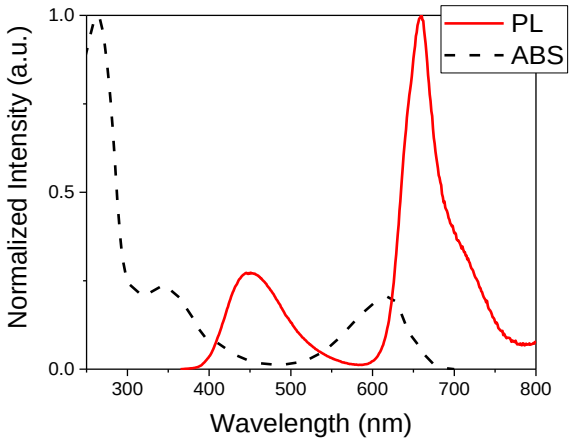


Figure 6. Emission spectrum of *Spirulina* extract by excitation at 350 nm (solid line). Absorption spectrum (dash trace)

By looking at the absorption curve (dash trace), it is clear that absorption peak in ultraviolet (centred at 344 nm) corresponds to an emission in blue (centred at 451 nm). Moreover, absorption in the orange region (centred at 620 nm) leads to emission in red (centred at 660 nm). The presence of PE, APC and PC, based on the absorption spectra, leads to emission in red, as they have PL spectrum at 575, 660 and 640 nm respectively. The red band is characterized by a larger energy band-width with respect to the blue one (the half-height band-width is 15.3 eV for the emission at blue and about 24.8 eV for emission at red). Moreover, there is another absorption band at 260 nm which is impossible with an excitation with a xenon lamp and most probably the emission peak is not in the visible regime.

5.3. Heat treatment of Spirulina extract

5.3.1. Spectrophotometric and fluorimetric measurements

The great commercial interest on C-PC is mainly due to the high protein yield and the relatively easy extraction procedures (Martelli et al. 2014).The effect of temperature on the extract stability was examined at two different pH conditions- pH 4.5 and pH 6.5 at 65°C. In both cases a dramatic loss of C-PC was observed as both samples showed to be highly unstable at the above given temperature which confirms previous results described in literature (Dissa et al. 2010). These pH values were chosen in order to represent common values of food products in food industry. Phycocyanin lost its colour and the spectra taken at time 0 of the heat treatment and the one after showed a great difference as in the second case we could observe the disappearance of the absorption peak (Figure 7,8).

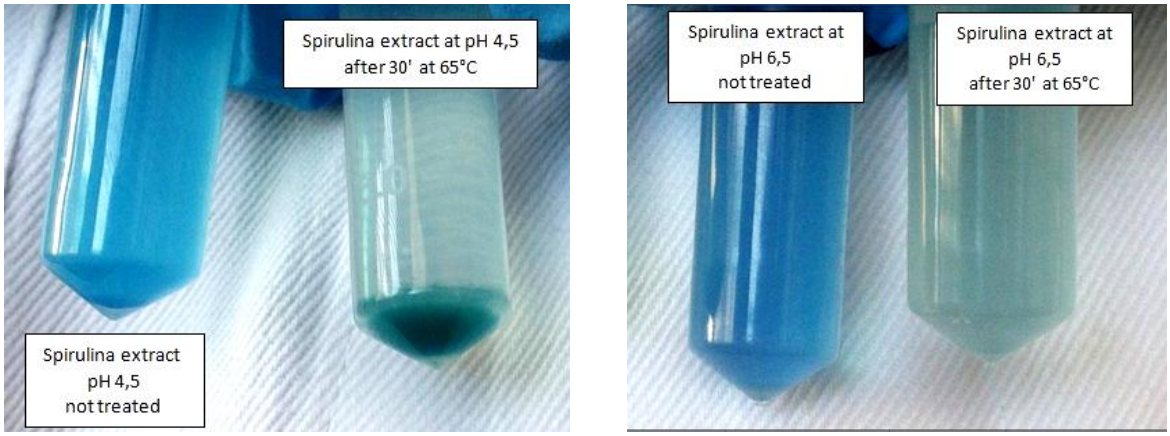


Figure 7. Spirulina extract after 30 min heating at 65 °C at pH 4.5 and pH 6.5

It is interesting to point out that the loss of Phycocyanin was more rapid in pH 4.5 than in pH 6.5. This is more likely to be due to the presence of two suitable conditions- the acidic environment that makes the protein precipitate and the high temperature that accelerates the process of the denaturation. It is also visible that in the case of pH 6.5 the absorbance is slightly shifting left while in the case of pH 4.5 there is a shift in the right.

We should keep in mind the fact that the optical properties of the covalently linked chromophores of biliproteins are deeply influenced by the state of the protein and the absorption.

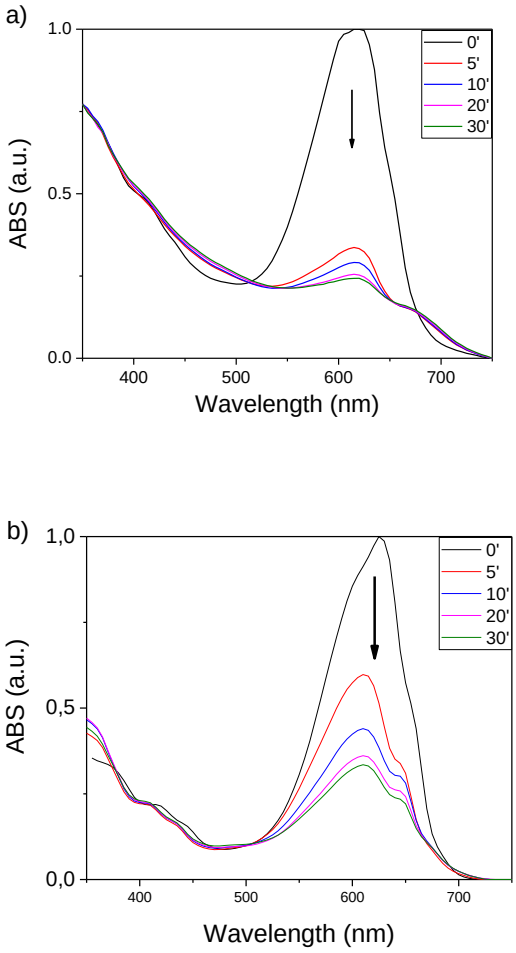


Figure 8. Normalized absorbance (ABS) spectra of the samples of *Spirulina* extract thermally treated at pH 4.5 (a) and at pH 6.5 (b)

We can explain the different direction of shifting in the absorption to the different conformation of the protein caused by the different pH conditions during the heat treatment. Also, as reported earlier by other authors, aggregation of Phycocyanin results in a red shift of the absorption maximum, and a large increase in the extinction coefficient as in the above mentioned experiment under pH conditions of 4.5. As mentioned before, Phycocyanin, under conditions where it is essentially monomeric (phosphate buffer) is characterized by a λ_{max} of approximately 615 nm and the hexameric form (acetate buffer) with a λ_{max} around 621 nm (Glazer et al. 1973). There is a wide variety of studies regarding the possible improvement of the thermal stability of phycocyanin (Mishra et al. 2008; Chaiklahan et al. 2012; Martelli et al. 2014; Braga et al. 2016) but still there are not so many studies concerning microencapsulation as a possible solution to this problem.

5.3.2. ABTS analysis of the heat treatment

The antioxidant capacity of the aqueous extract of *Spirulina platensis* under the influence of heat treatment performed at 65°C was examined in this experiment. The behaviour of the extract in two different buffers was compared (Figure 9)

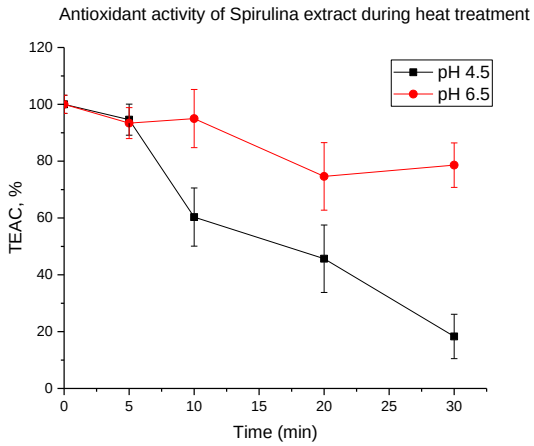


Figure 9. Antioxidant capacity of *Spirulina* extract during heat treatment

As stated in literature *Spirulina* is very labile at high temperatures which makes it difficult to manipulate even as a colorant in the food industry. (Carle & Schweiggert 2016) Therefore it must be added in the very late stages of food processing. In this experiment both high temperature and pH are involved and as we can observe (Figure 9) the extract shows different antioxidant activity in acidic and neutral conditions. In particular in acid conditions the anti-oxidant activity seems to be less retained in respect to the treatment at pH 6.5, in agreement with the major loss of absorbance at 615 nm observed at pH 4.5 in figure 9. TEAC of Spirulina platensis extract is particularly high, i.e. 105±9 umol/g of dry *Spirulina*.

This, as stated before, is probably due to the fact that Phycocyanin maintains its native structure for pH values higher than 5, with a maximum stability between pH 5 and 6, while for lower pH values it denatures gradually (Duangsee et al. 2009; Chaiklahan et al. 2012). Thus, high temperature assists the process of denaturation of Phycocyanin, leading to the very low antioxidant capacity of the *Spirulina* extract at the end of the heat treatment process with a loss of 82% of its activity. On the contrary, when the extract is treated in pH 6.5, the

antioxidant capacity is lost only with 22% from the beginning of the experiment at time 0 maintaining a quite stable behavior.

5.4. Simulated digestion of *Spirulina* extract

5.4.1. Spectrophotometric analysis of *Spirulina* during digestion

A spectrophotometric analysis was performed during the simulated digestion of the *Spirulina* extract. Little information is available about the *in vitro* digestibility of *Spirulina* (De Marco et al. 2014). Simulated digestion was performed onto some types of green, brown and red seaweed. The %digestibility when in the gastric phase under the influence of pepsin has a very wide range- 17-56.7% while % digestibility in the intestinal phase under the influence of pancreatin varies from 56 to 66% (Fleurence 1999).Since Phycocyanin is the main pigment produced by *S. platensis* and may reach 20% in dry weight (Antelo et al. 2008) this was selected as main marker in the digestibility study. We can observe that the absorption spectra at the two fixed wavelengths starts decreasing from the gastric phase under the influence of pepsin which is visible when we consider the concentration of Phycocianin (Figure 10) during the gastric and intestine phase of the simulated digestion. The concentration starts decreasing from the beginning of the simulated digestion, meaning that we are losing valuable parts of the Phycocyanin in the first hour of the experiment due to the fact that it is unstable in acidic conditions.

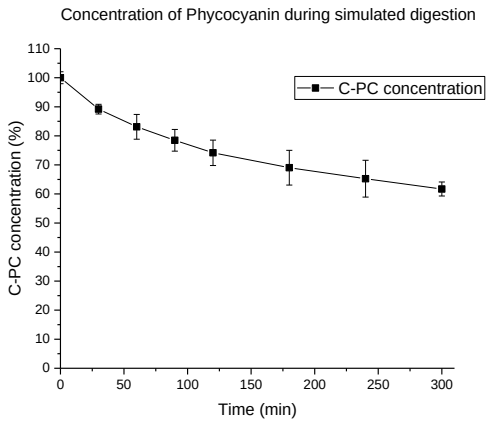


Figure 10. Concentration of Phycocyanin during simulated digestion, gastic and intestinal phase

As stated before, the enzymatic activity during simulated digestion also gives a great impact on the digerability of the proteins in microalgae so the results of the concentraction changes of Phycocyanin (Figure 6) is due not only to the acidic pH but also to the activity of pepsin in the simulated gastric fluid and the incubation at 37°C.

5.4.2. ABTS analysis of simulated digesiton

The antioxidant activity of the extract was observed during simulated digestion (Figure 7). A slight decrease in the antioxidant activity was observed that correlates with the results previously obtained by the spectrophotometric analysis This indicates that the phycocyanin is probably the main responsible of the anti-oxidant activity observed in Spirulina extract.

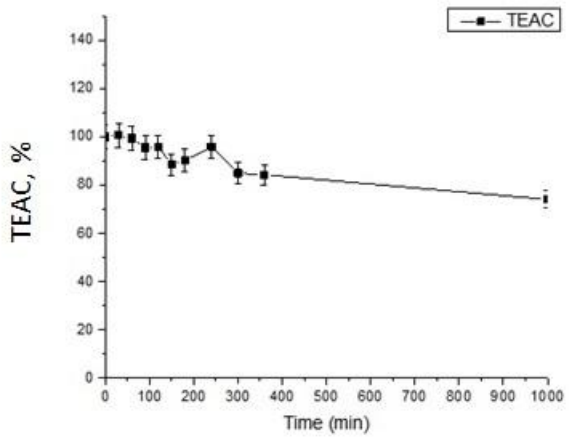


Figure 11. ABTS analysis during full simulated digestion of Spirulina extract. Results are expressed as % Trolox equivalents.

In order to gain more data on these phenomena a fluorescence and SDS-PAGE analysis of the simulated digestion were performed.

5.4.3. Fluorescence analysis of *Spirulina* extract during simulated digestion

During the gastric phase a very low emission signal was measured, if compared with the pure extract analyzed before. It is also important to note that the buffer solution of both simulated gastric and intestine fluids were examined and none of them gave any fluoresce signals

meaning that all fluorescence in the present experiment is due only to the specific properties of *Spirulina* extract. The excitation wavelengths were chosen as for the previous experiments performed. An example for the emission of *Spirulina* extract during gastric digestion is shown in Figure 8 by excitation at 350 nm. As it can be seen it is characterized by two bands in the blue (centred at 455 nm) and in the red range (centred at 705 nm). However the , emission in this phase of simulated digestion did not show time-dependent variations like differently from the emission observed during the intestinal phase of the digestion. This is most probably due again to the fact that Phycocyanin, that is the main fluorescence agent, is insoluble in gastric conditions (i.e. very low pH) and this is why the signal observed was generally low stable. It must be noted that the sharp peaks at 400 and 700 nm are due to the Raman effect and the second harmonic of excitation wavelength, respectively.

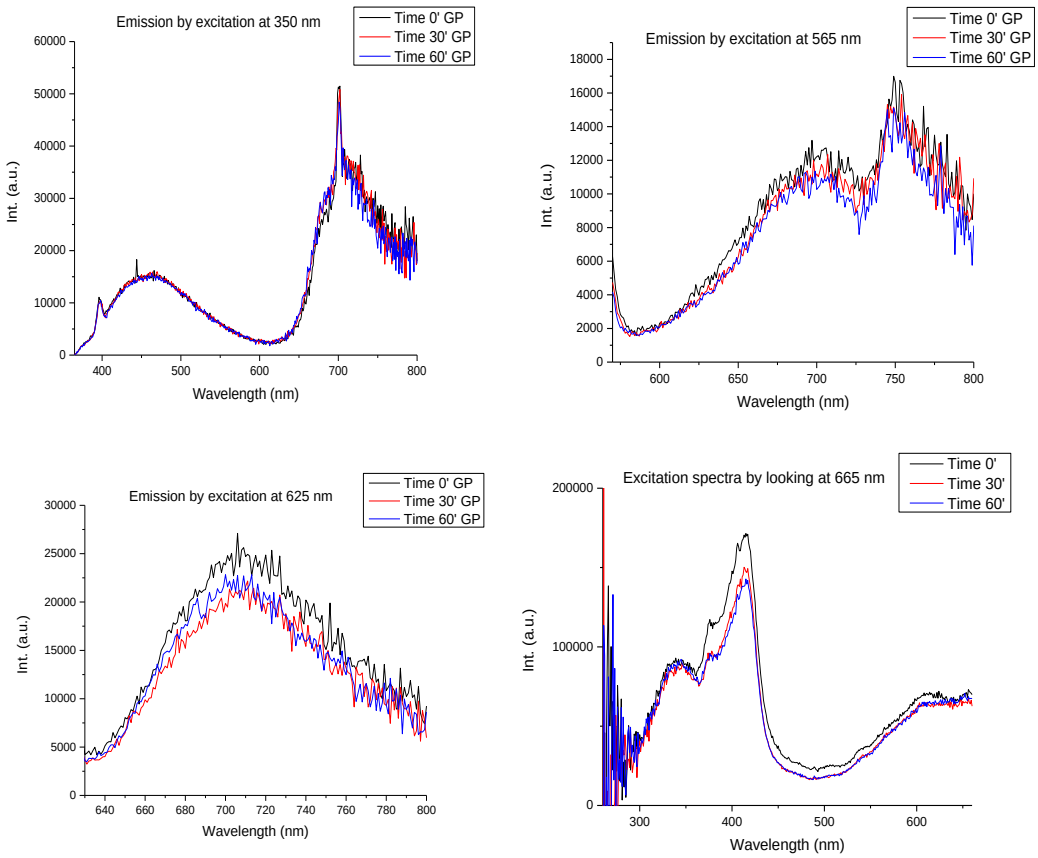
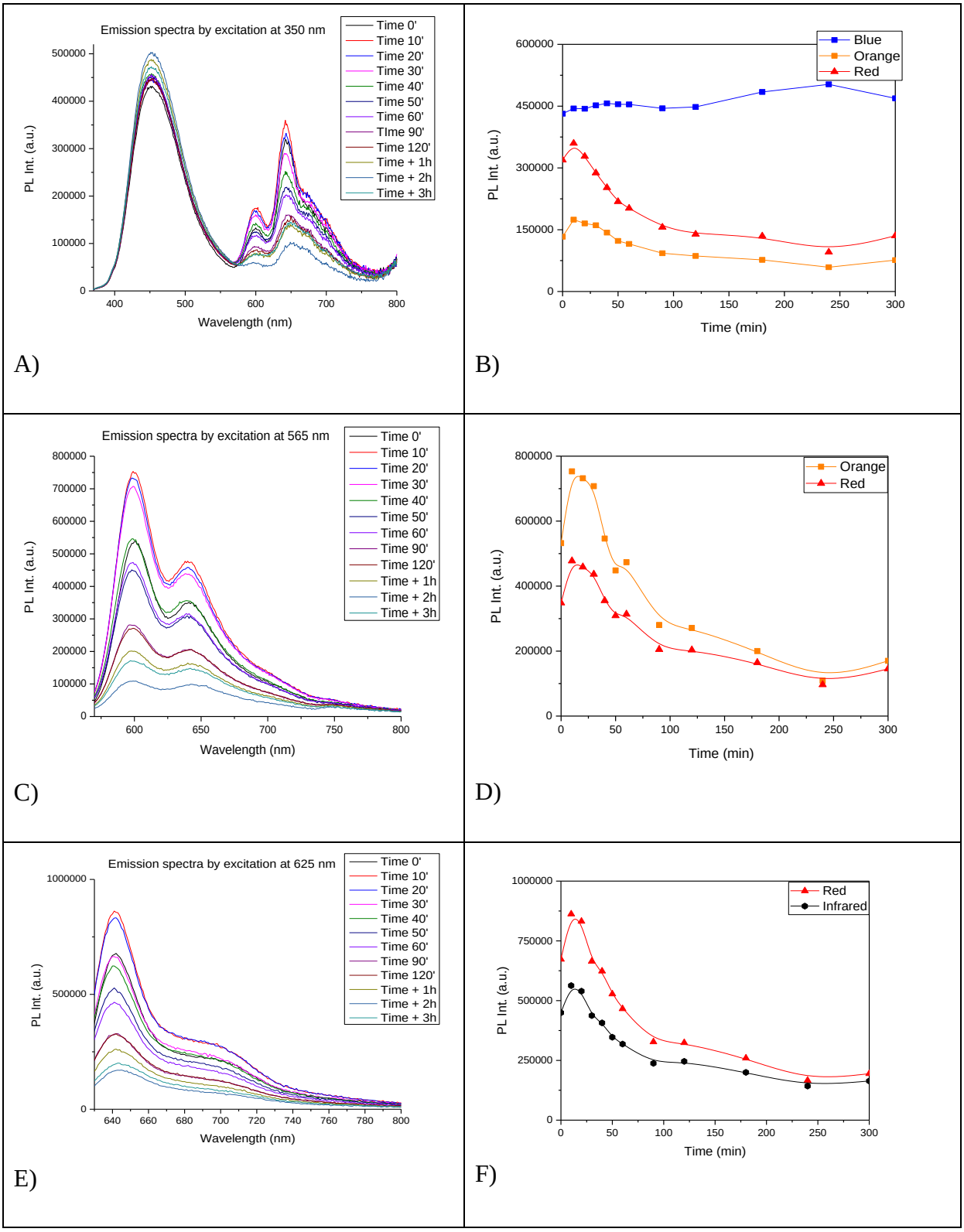


Figure 12. Emission spectra of gastric phase of the simulated digestion phase by excitation at 350 nm as a function of time. The sharp peak at about 400 nm is related to Raman effect of water. Furthermore, the narrow peak at 700 nm is due to second harmonic of excitation.

Optical properties of intestinal phase of the simulated digestion were investigated at different times (Figure 13). Excitation spectra at emission of 665 nm has three main peaks at violet, yellow and orange centred at around 350, 565 and 625 nm respectively.



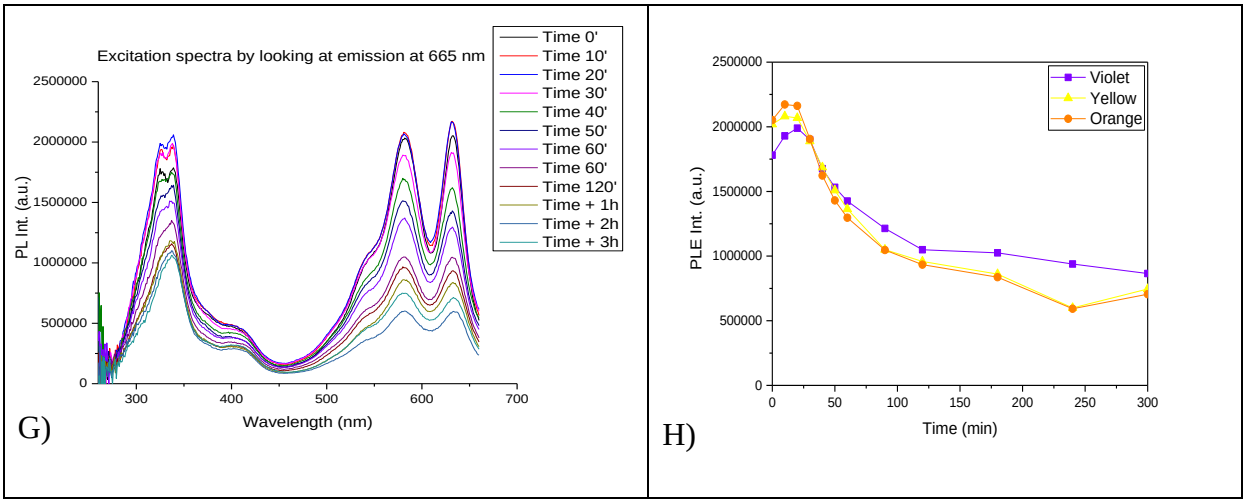


Figure 13. Optical properties of Intestinal phase of the simulated digestion. Right panel (A,C,E,G) excitation spectra at emission of 665 nm, emission by excitation at 350, 565, and 625 nm demonstrated as function of time. Left panel (B,D,F,H) trends of the main peaks also shown as a function of time

The emission of the samples based on the peaks of the photoluminescence excitation (PLE) show that excitation at 350 nm wavelength leads to a bright emission with 3 main bands at blue, orange and red by centre of 453, 600 and 642 nm respectively (Figure 9). The time-dependent intensity of the bands exhibited quenching of orange and red band but stability of the blue band. It must be mentioned that by reduction of PLE at violet which means that the light absorption of the sample is being reduced, Emission spectra by excitation at 565 nm has two main peaks in orange (600 nm) and red (640 nm) region. Moreover, emission by excitation at 625 nm has two peaks at red (641 nm) and infrared (700 nm). Time course of these two excitation peaks intensities is in agreement with the red band by excitation at 350 nm.

These observations show that the emission at the different excitation wavelengths are characterized by a similar trend, i.e. a time-dependent decrease of photoluminescence along the course of intestinal digestion. This could be explained as the effect of pancreatin digestion of fluorescent phycobiliproteins.

5.4.4. SDS-PAGE of *Spirulina* extract during simulated digestion

The SDS- PAGE of the digestion confirmed the results of the fluorescence analysis given above as it shows that the extract is rapidly digested.

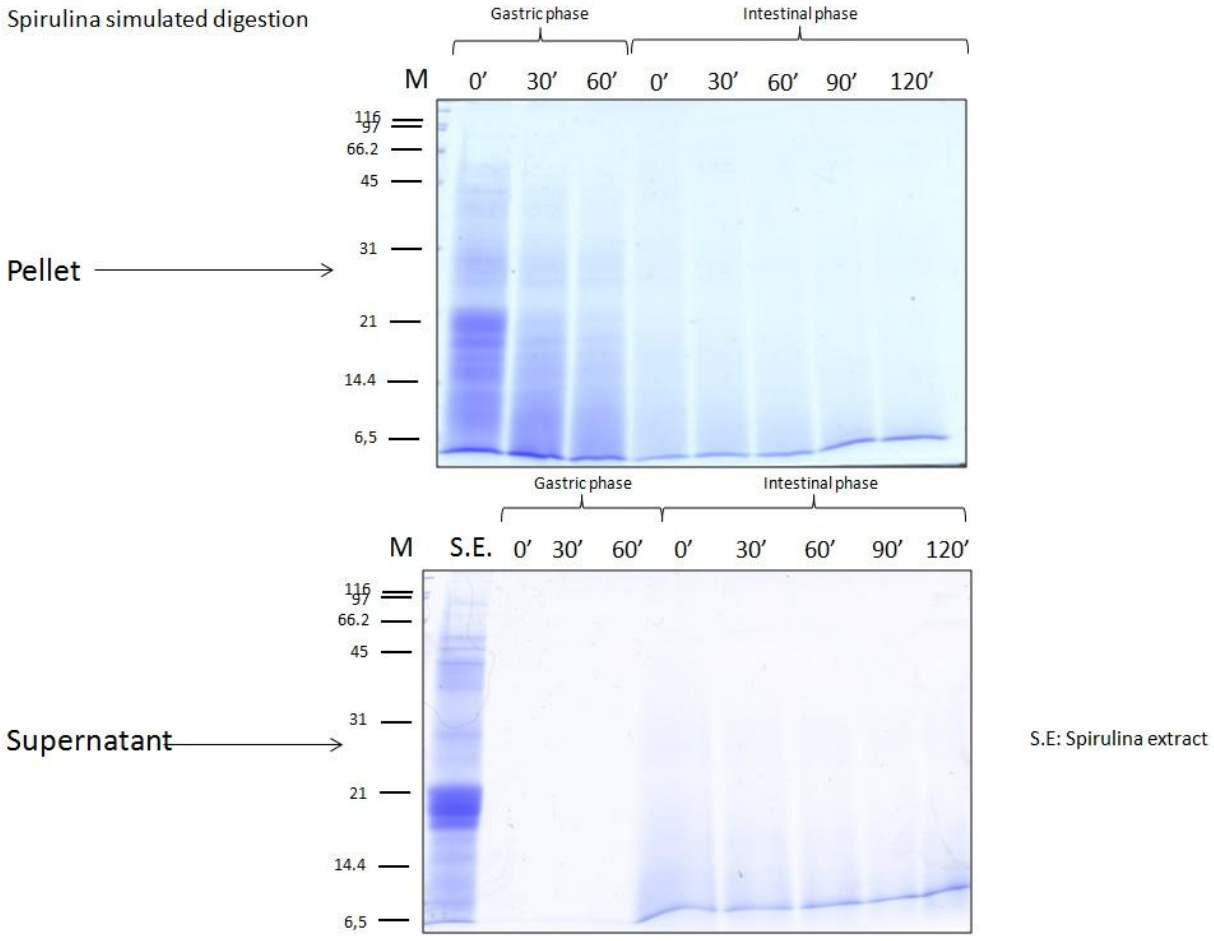


Figure 14. SDS-PAGE analysis of *Spirulina platensis* extract during simulated digestion

In the first stage of the digestion the proteins are accumulated mainly in the pellet, as a consequence of the low pH that induced a generalized protein precipitation. A decreased intensity of the bands was observed along the digestion. No signal was observed in the supernatant, perhaps the peptides generated from the digestion are either not soluble or, too small to be retained in the running gel. In the second stage, the signal of the bands in the pellet are mainly in the range of the low molecular weight as a consequence of the digestion. Also

the bands in the supernatant are very faint suggesting that the proteins are rapidly digested even if not completely.

6.Conclusions

The results obtained shows that the aqueous extract of *Spirulina Platensis* is not stable when treated neither in acidic nor in basic pH loosing much of the characteristic blue colour . Nevertheless, the effect in acid condition led to more dramatic effect due to the denaturing effect induced by the low pH on protein solubility. The anti-oxidant capacity analysis of the extract showed a similar trend indicating that the reactivity towards ABTS is much affected by conformation of the phycobiliprotein. When it comes to simulated digestion, the analysis showed that the digestion starts immediately in the gastric phase, following a rapid and almost full digestion of the proteins in the intestine phase. However the anti-oxidant capacity seems not to be much affected by digestion.

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CHAPTER 5 MICROENCAPSULATION OF *SPIRULINA PLATENSIS* AQUEOUS EXTRACT

Abstract

Taking into account that the aqueous extract of *Spirulina Platensis* alone, could not offer protection to valuable substances such as phycocyanin, various types of microencapsulates were prepared in order to try to protect the extract from the surrounding environment. It was seen in the previous chapter that the extract is labile to heat treatment and that the digestion starts immediately in the gastric phase. It was also observed that when reaching the intestine phase the extract is instantly and almost entirely digested making it impossible for the bioactive substances to survive longer. Microencapsulation was chosen as a possible solution to this problem and the results obtained from encapsulating the extract will be discussed in this chapter.

Part of the results discussed in this chapter were obtained during research activities in L'Ecole Nationale Vétérinaire, Agroalimentaire et de l'Alimentation Nantes-Atlantique ONIRIS, Nantes, under the supervision of Professor Denis Poncelet with the financial support of Cooperint 2015.

1 Introduction

As mentioned above, the extract of *Spirulina Platensis* was easily destroyed under different conditions such as pH change, high temperature, enzymatic activity. For this reason the technologies of encapsulation were applied to the extract in order to protect it. Four different matrix types of capsules were prepared using a vibrational nozzle technology with Encapsulator B-390 This technology was chosen because it is one of the mildest ones in the field of microencapsulation and does not require high temperatures or any additional treatment of the extract.

2. Materials and methods

2.1. Preparation of *Spirulina* microencapsulates

Four main types of encapsulates of *Spirulina* extract were developed (Table 1). The extract was obtained as described in Chapter 4. Alginate Food S60NS was purchased from Satialgine (France), chitosan from Sigma-Aldrich (US), potato starch from Sigma Aldrich (US) and Eudragit L from Evonik.

Table 1. Types of prepared microencapsulates

1,5% Alginate in Spirulina extract (*Spirulina* AL);
1,5% Alginate + 20% Potato Starch in Spirulina extract (*Spirulina* ST);
1,5% Alginate in Spirulina extract coated in 1% Chitosan solution (*Spirulina* CHI);
1,5% Alginate in Spirulina extract + Eudragit L in a ratio 80:20 (*Spirulina* EU).

Each preparation was carried out by adding the polymers directly into the *Spirulina* extract under stirring at 4 °C in dark conditions for 1 to 2 hours until the full dissolution of the powders into the extract. All microencapsulates were prepared using an extrusion method with a BUCHI B-390 Encapsulator with a nozzle size of 750 µm under different conditions of pressure and electrode depending on the type of the solution. The solution was then extruded

in 0.2 M $CaCl_2$ containing 1% acetic acid and stirred for 1 hours. After this, the microencapsulates were washed for 30 min in distilled water. Concerning the preparation of the capsules with 1% Chitosan solution (chitosan was dissolved in 1% acetic acid), the capsules were first extruded and after 30 min in 0.2 M $CaCl_2$ they were transferred into the chitosan solution for another 30 min and then washed in distilled water.

2.3. Morphology studies

The morphology of the produced encapsulates was observed with the use of a Dino Lite Digital Microscope and DinoXcope Software in order to observe the diameter of the produced capsules. Other parameters such as extruded mass, collected mass, loss during extrusion and contraction during gelification (β) were also collected using the following formulas:

$$\text{Extruded mass} = \frac{Container + solution\ before\ extrusion}{Container\ after\ extrusion}, g$$

$$\text{Collected mass} = \frac{Container + CaCl_2\ solution\ before\ extrusion}{Container\ after\ extrusion}, g$$

$$\text{Loss during extrusion} = \frac{Extruded\ mass}{Collected\ mass}, g$$

$$\text{Contraction during gelification } (\beta) = \frac{Extruded\ wet\ capsules}{Collected\ wet\ caplsules}, g$$

2.4. Dry matter

The dry matter of the produced microcapsules was measured after heating at 103 °C in an oven for 24h. Five grams of each sample were measured on an analytical balance and put into previously weighted aluminium cups. After 24 hours the aluminium cups were put for 15 min a desiccator ,weighted and the dry matter was calculated using the following equation:

$$\frac{P3 - P1}{P2 - P1} \text{x } 100 = \% \text{ dry matter}$$

where P1 is the empty aluminium cup, P2 is the cup with the particles inside and P3 is the particles and the aluminium cup after 24h of drying. The experiment must be performed per 5 for each of the samples.

2.5. Drying of *Spirulina* microencapsulates

The capsules were dried using a GLATT GPCG-1 fluid bed. one hundred g of each sample were dried at 35°C using an air flow of 50 m^3/h for the first 1 hour and an air flow of 25 m^3/h for another two hours. All samples were dried until reaching a water activity Aw < 0.3. The capsules were then examined with Dino Lite Digital Microscope and DinoXcope in order to observe their diameter after drying.

2.6. Extraction from dry *Spirulina* microencapsulates

After the drying process a destruction of the microencapsulates with 0.1 M phosphate buffer + 50 mM NaCl and extraction was performed. From each sample a different quantity of dried capsules was destroyed. This was always corresponding to 2 g of wet capsules. Samples were taken at different time points (1, 2, 3, 4, 5 and 7 hours and overnight extraction) and their spectrum and antioxidant activity were examined in order to set up an efficient extraction method from the *Spirulina* capsules.

2.7. Spectrophotometric measurements

C-PC was measured by a spectrophotometer as described in the previous chapter following the equation:

$$PC = \frac{[OD_{615} - 0.474 \times OD_{652}]}{5.34}$$

Where the concentration is expressed as mg/ml and ABS_{615} and ABS_{652} the absorbance values of the extracts measured at 615 and 652 nm respectively (Silveira et al. 2007).

2.8. Heat treatment of *Spirulina* dried microencapsulates

Dried capsules were subjected to heat treatment at 65 °C in two different buffer solutions-20 mM sodium phosphate at pH 6.5 and 20 mM sodium acetate at pH 4.5. An amount of 0.1 g of each sample was weighted and put in 5 ml of each of the two buffer solutions. The temperature was chosen in order to simulate a possible pasteurization. The samples were incubated in water baths maintained at the specified temperature for 30 min. Samples we taken at 0, 1, 5, 10, 20 and 30 min and read with the help of a spectrophotometer. After the supernatant was taken, the pellet was destroyed using 0.1 M phosphate buffer with 50 mM NaCl, centrifuged and the absorbance of the clarified extract was read at the spectrophotometer to measure C-PC presence.

2.9. ABTS Analysis

Antioxidant capacity was tested using ABTS assay as described in Chapter 4.

2.10. Simulated digestion of *Spirulina* dried microencapsulates

A simulated digestion was performed following the protocol of (Yi et al. 2015) with slight modifications. In the first stage, 0.1 g of microcapsules were mixed with simulated gastric fluid (SGF) containing 0.084 M HCl, 30 mM NaCl, pepsin (Sigma) 1:20 (enzyme:protein), pH 1.2 and put at 37°C for 60 min in agitation. Samples were collected at 0, 30 and 60 from the gastric phase. After 1h the remaining SGF was mixed with simulated intestinal fluid (SIF) containing 0.1 M sodium phosphate and 50 mM NaCl at pH 7.3 and pancreatin (Sigma) 1:20 (enzyme:protein). Simulated intestinal digestion was performed at 37°C for 120 min in agitation for another 3 hours. Samples were again collected at different time points.

2.11.SDS-PAGE analysis

SDS-PAGE analysis was performed as described in Chapter 4.

3. Results and discussion

3.1. Preparation of microencapsulates

Depending on the viscosity of the different solutions, parameters such as pressure and electrode had to be adjusted during the process in order to obtain jet and a good separation of the jet into droplets in order for the capsules to be formed (Table 2).

Table 2. Parameters for the production of different types of Spirulina microencapsulates using BUCHI B-390 Encapsulator

Type	Nozzle size, µm	Amplitde	Pressur, bar	Electrod, V	Frequecy, Hz
***Spirulina* AL.**	750	3	677	1600	250
***Spirulina* ST**	750	3	658	1700	250
***Spirulina* CHI**	750	3	650	1600	250
***Spirulina* EU**	750	3	714	1700	250

The frequency and amplitude were chosen as most suitable ones, suggested by BUCHI. The electrode and Pressure were changed depending on the viscosity and behaviour of the solution until a clear-cut jet was not created so to form microcapsules. The parameters had to be changed both for Eudragit and Starch containing capsules as they made the extrusion solution slightly more viscose than the one containing only Alginate.

3.2. Morphology studies

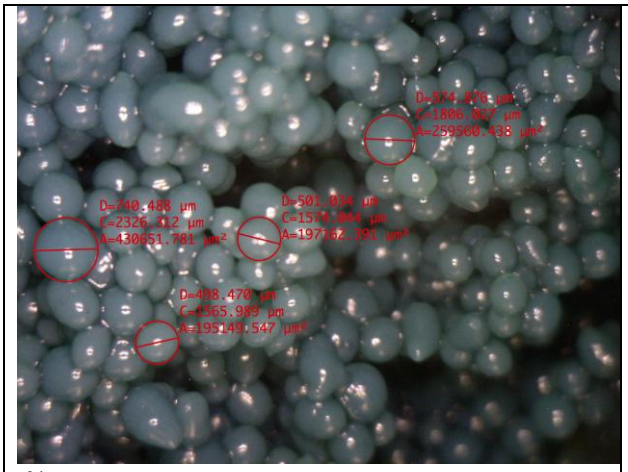
Some of the characteristics of the produced Spirulina microencapsulates were observed such as contraction during gelification (Table 3) in order to better understand the extrusion process for each type of capsule.

Table 3. Characteristics of *Spirulina* microencapsulates

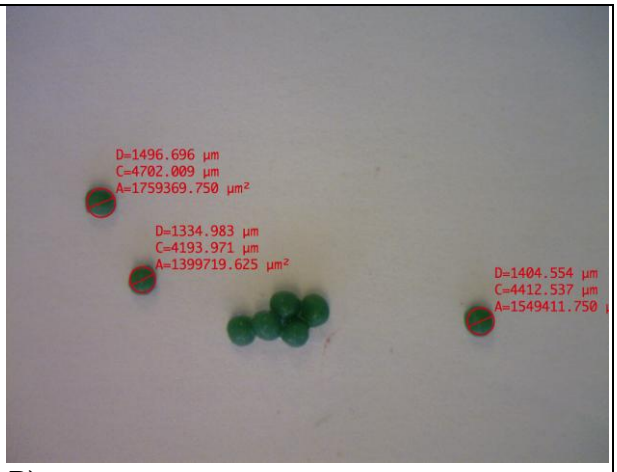
Type	Extruded mass, g	Collected mass, g	Loss during extrusion, g	Contraction during gelification (β)
***Spirulina* AL.**	95,86	90,4	5,46	0,73
***Spirulina* ST**	92,49	90,02	2,47	0,79
***Spirulina* CHI**	95,73	90,73	5	0,49
***Spirulina* EU**	94,41	91,96	2,45	0,75

It can be observed that the encapsulates coated with 1% chitosan solution show a different contraction during gelification in respect with the other samples. This is due to the fact that chitosan has an unique property to change the pore space between the chitosan wall membrane molecules, and achieve the controlled release effect of the content within the microcapsules (Yamamoto et al. 2002; Sriupayo et al. 2005) as previously shown in other studies (Hsieh et al. 2006).

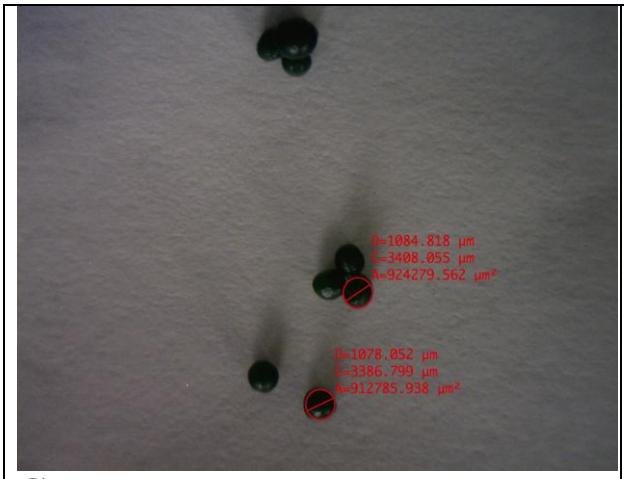
The diameters of the capsules are presented in Figure 1and Table 4.



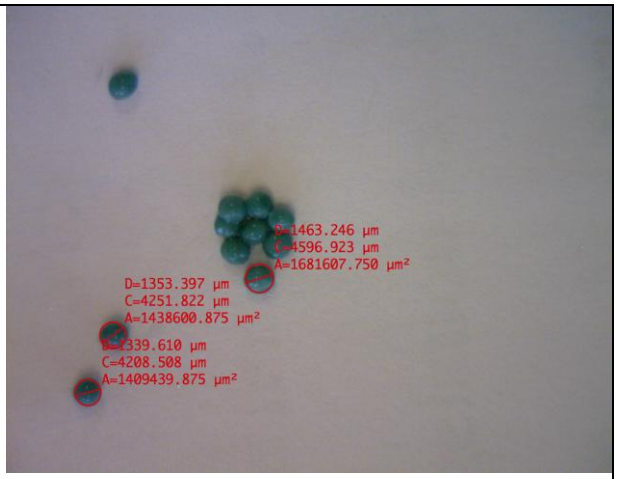
A)



B)



C)



D)

Figure 1. Spirulina microencapsulates after extrusion A) 1 *Spirulina* AL wet B) *Spirulina* ST wet C) *Spirulina* CHI wet D) *Spirulina* EU wet

It can be observed that even after using the same nozzle size during the extrusion of the solution the produced microencapsulates have different diameters and form. The microencapsulates with only alginate show good spherical form thanks to the well known gelling properties of this polymer. In the case of the microencapsulates produced with starch, the samples show very good sphericity and are quite monodisperesed.

Table 4. Average diameter of *Spirulina* encapsulates, wet and dry

Type of microencapsulates	Average diameter, µm
***Spirulina* AL wet**	878.25
***Spirulina* AL dry**	459.78
***Spirulina* ST wet**	1385.42
***Spirulina* ST dry**	689.80
***Spirulina* EU wet**	1081.435
***Spirulina* EU dry**	435.229
***Spirulina* CHI wet**	1412.06
***Spirulina* CHI dry**	1001.47

Different studies show that the addition of starch as a filler in microencapsulation can increase the encapsulation efficiency (Córdoba et al. 2013; Fujiwara et al. 2013) and improve the

microencapsulates morphological characteristics. The % of the starch introduced into the matrix also plays an important role on the size of the particles. The capsules coated with chitosan show a regular and spherical form and the chitosan has also a very obvious contraction influence over the capsules giving them a very good shape.

3.3. Dry matter

The dry matter of the capsules was measured using the protocol mentioned before. The results can be seen in the table below (Table 5).

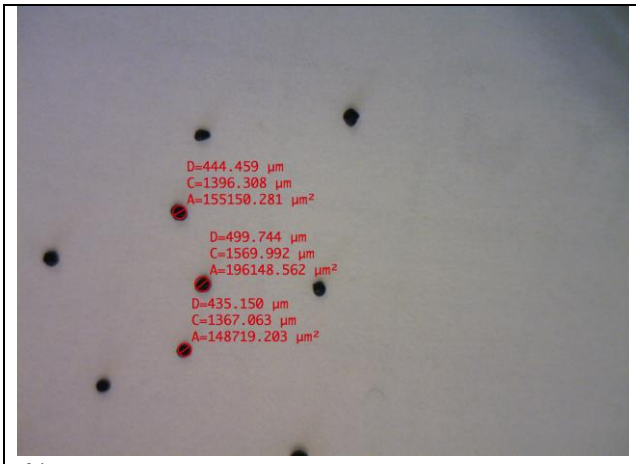
Table 5. Dry matter content in different types of *Spirulina* microencapsulates

Type	Dry matter content, %
1.5% AL Sp.	5,48
1.5%AL Sp.20% Starch	10,48
1.5%AL Sp.1% Chitosan	4,6
1.5%AL Sp.1% Eudragit	12,6

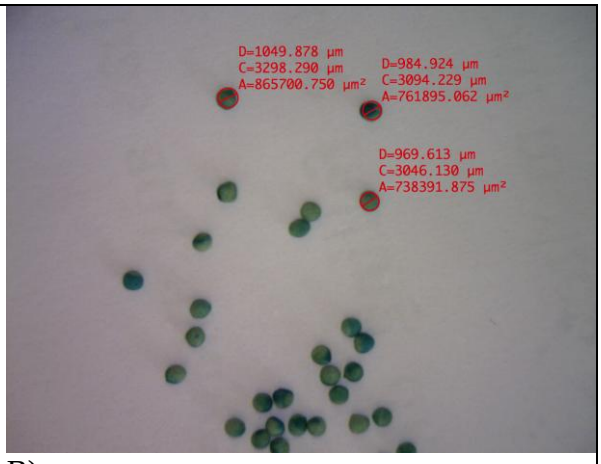
As seen from the table the encapsulates obtained with *Spirulina* AL and *Spirulina* CHI have very similar dry matter content and less than those made with potato starch or Eudragit. This can be explained by the fact that starch and Eudragit were at higher concentrations in respect to alginate and chitosan .

3.4. Drying of Spirulina microencapsulates

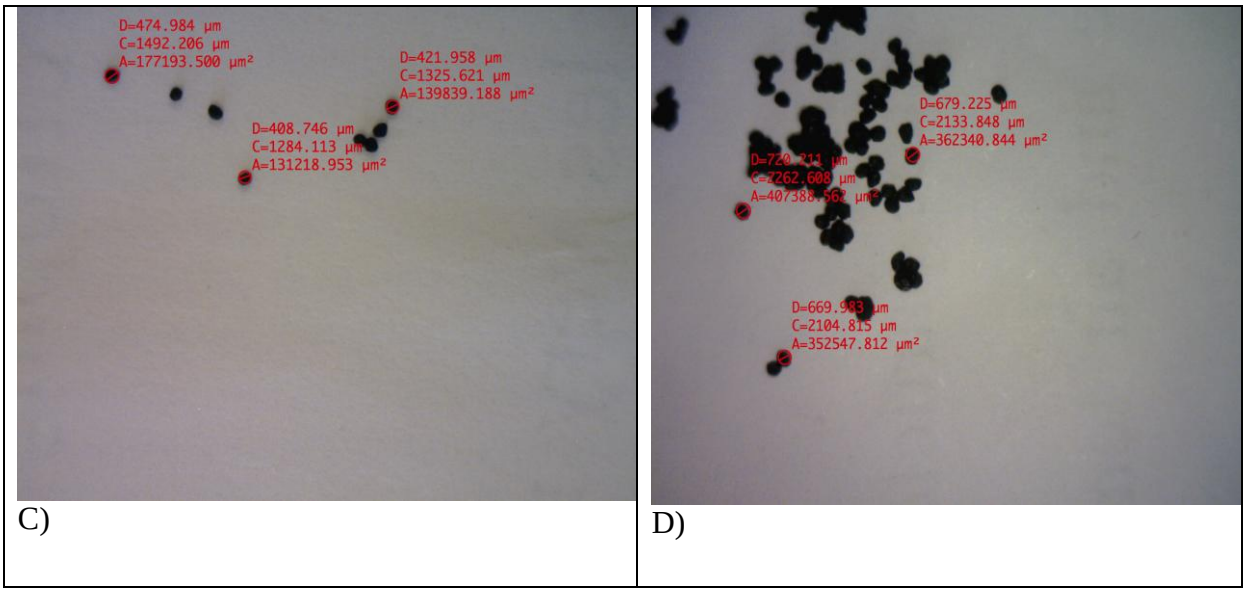
After the capsules have reached a water activity Aw < 0.3, the diameter was observed in order to be compared with the wet capsules (Figure 2).



A)



B)



C) D)

Figure 2. *Spirulina* microencapsulates after drying A) 1 *Spirulina* AL dry B) *Spirulina* ST dry C) *Spirulina* CHI dry D) *Spirulina* EU dry

When the capsules were dried they retained their spherical form. The capsules with Eudragit and starch did not change their dimensions too much after the drying process due to the presence of the fillers that are added exactly to compensate the loss of water during drying thus maintaining the original shape as much as possible. It is noteworthy that the starch microencapsulates do not have a uniform colour. This confirms the date of the dried matter experiment and the fact that starch was not distributed homogenously throughout the matrix.

In the table below there are the correspondences between wet and dry weight of the microencapsulates (Table 6).

Table 6. Ratio between wet weight and dry weight of *Spirulina* microencapsulates

Type	Wet capsules, g	Dried capsules, g
***Spirulina* AL**	100	6.4
***Spirulina* ST**	100	23.67
***Spirulina* CHI**	100	8.02
***Spirulina* EU**	100	13.86

We can observe that the capsules produced with starch and Eudragit have a ratio of dried weight that surpasses the one of the other type of capsules. This is due to the fact that both starch and Eudragit are used as fillers and of course when increasing the % of polymers in the solution the final % of the dried mass will also increase.

3.5. C-PC extraction from dry Spirulina microencapsulates

The extraction of the microencapsulates was run for several hours and stopped when a plateau of extracted C-PC was obtained.

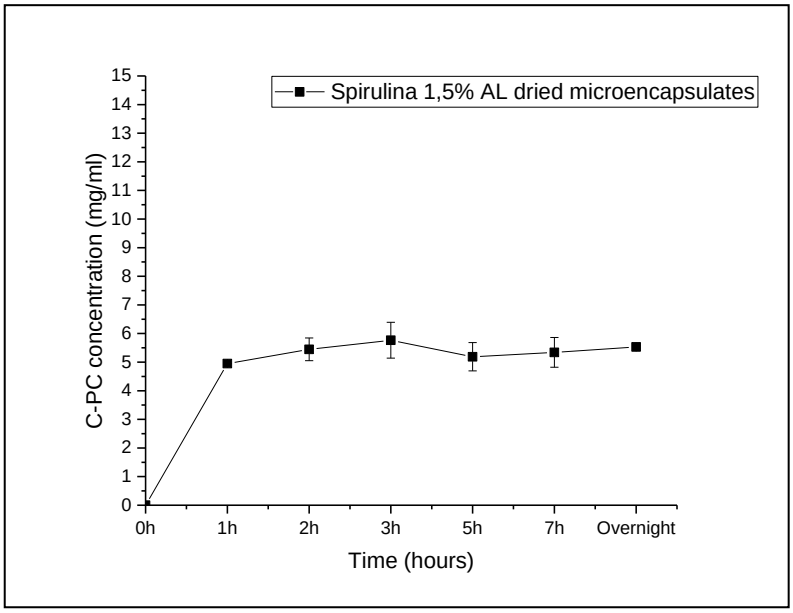


Figure 3. Extraction in time from *Spirulina* AL dry microencapsulates

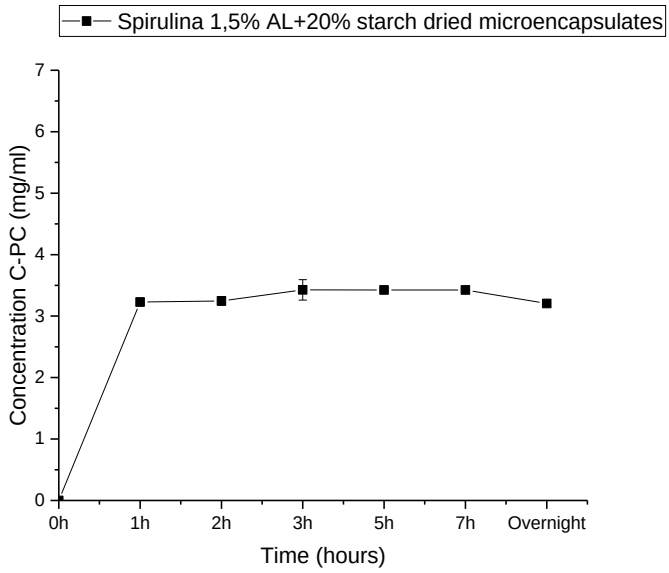


Figure 4. Extraction in time from *Spirulina* ST dry microencapsulates

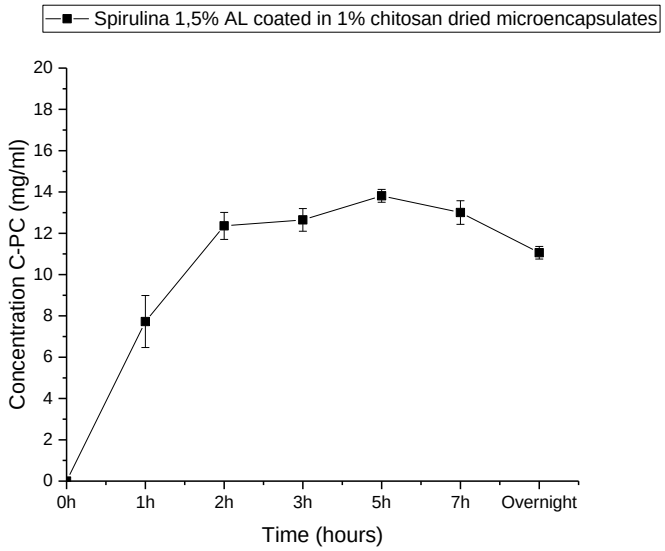


Figure 5. Extraction in time from *Spirulina* CHI dry microencapsulates

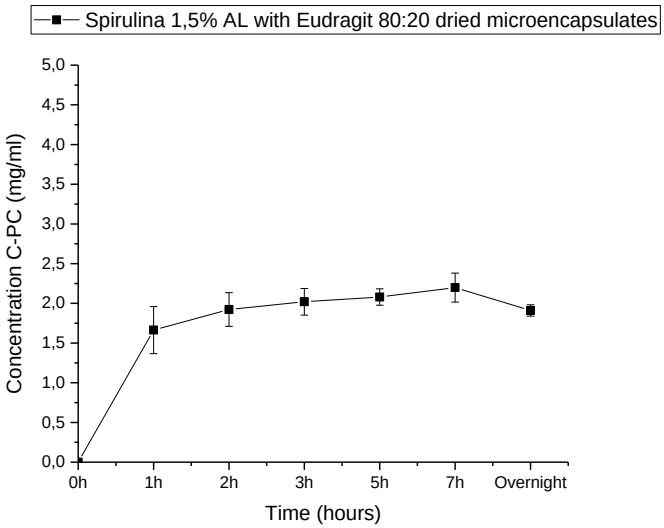


Figure 6. Extraction in time from *Spirulina* EU dry microencapsulates

The graphs show that all samples reach a plateau quite fast after the first hour of extraction. Only Spirulina EU kept increasing the quantity of phycocyanin but with minor changes in the concentration. On the contrary, the capsules coated with chitosan (*Spirulina* Chi) showed a prolonged release, even more than the one performed by Eudragit. However the use of chitosan for the engineering of capsules with controlled release has been also observed in the past and it can be found in literature (Polk et al. 1994; Sezer & Akbuga 1999; Shi & Tan 2002; Agnihotri et al. 2004). Comparing the results it is also evident that the microcapsules contain different concentrations of C-PC due to the different concentrations of polymers and fillers used in the formulations. Hence, the extraction of the same amount (0.1g) from each capsule type highlighted these differences. Experiments changing the amount of materials in order to have the same amount of C-PC in the capsules to be extracted were not taken into account as the purpose of this study was to evaluate the release dynamics of the capsules, so with the same solid-liquid ratio.

3.6. Heat treatment

The heat treatment was performed as in the previous chapter. After each time point the supernatant was removed and the pellet destroyed using 0.1 M phosphate buffer + 50 mM NaCl. From the observation of figures 7-10 it is clear that during the treatment negligible amounts of C-PC were detected in the supernatants of the capsules. Regarding the capsules

much of the C-PC was denatured in the first 5 min of the treatment. Differently from what was observed for the aqueous extract, the samples at pH 4.5 seem to contain surprisingly more C-PC in respect to the samples at pH 6.5 as the low pH protected the phycobiliproteins from thermal denaturation.

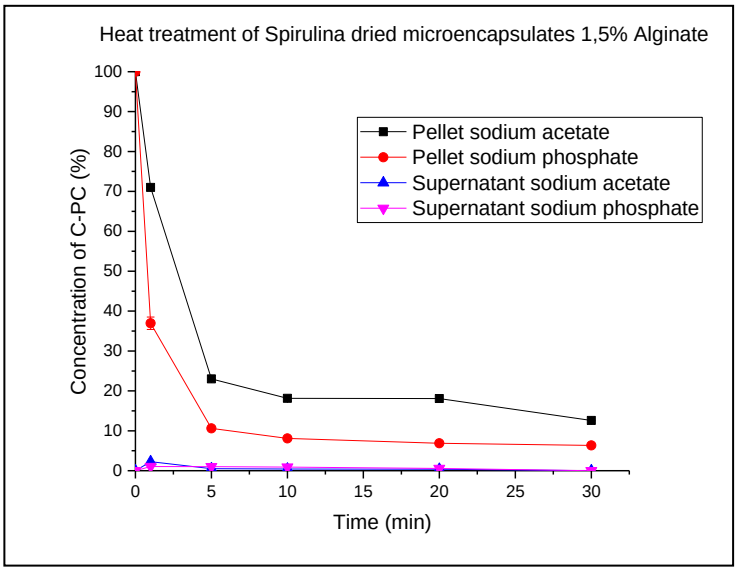


Figure 7. Variation of C-PC concentration determined by spectrophotometry during the heat treatment of *Spirulina* AL capsules at different pH values, max. standard deviation (SD) 0.05.

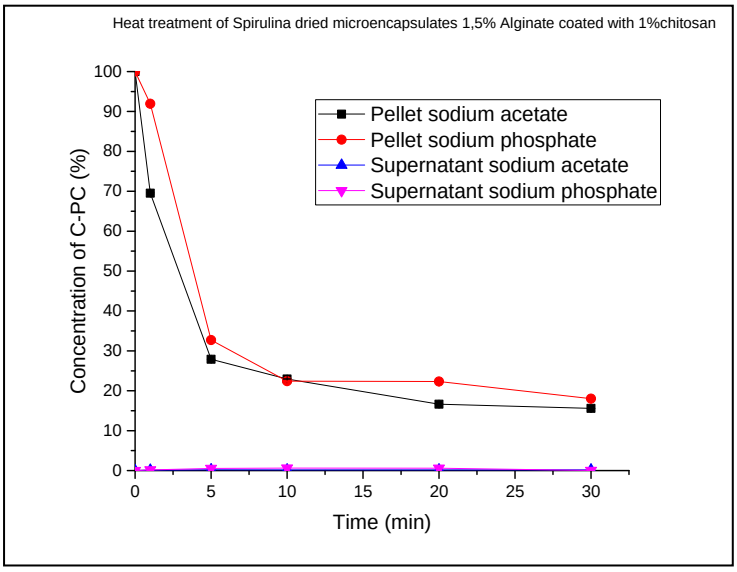


Figure 8. Variation of C-PC concentration determined by spectrophotometry during the heat treatment of *Spirulina* CHI capsules at different pH values, max. SD 0.20.

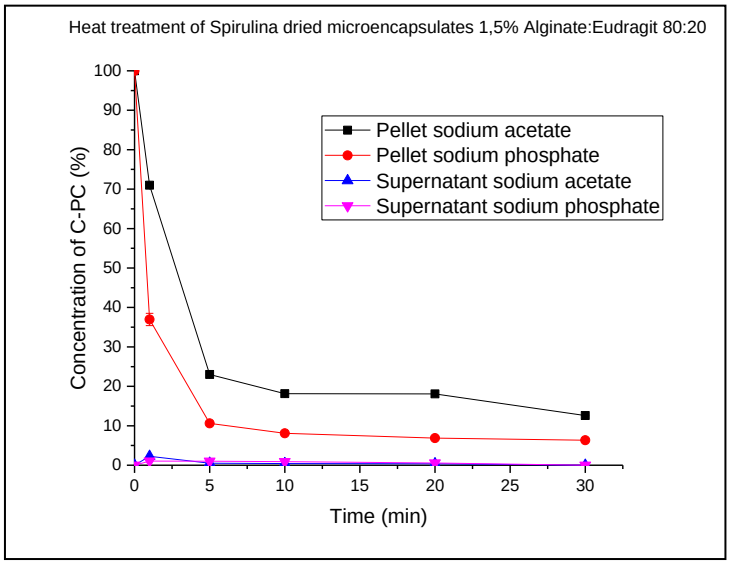


Figure 9. Variation of C-PC concentration determined by spectrophotometry during the heat treatment of *Spirulina* EU capsules at different pH values, max. SD 0.01.

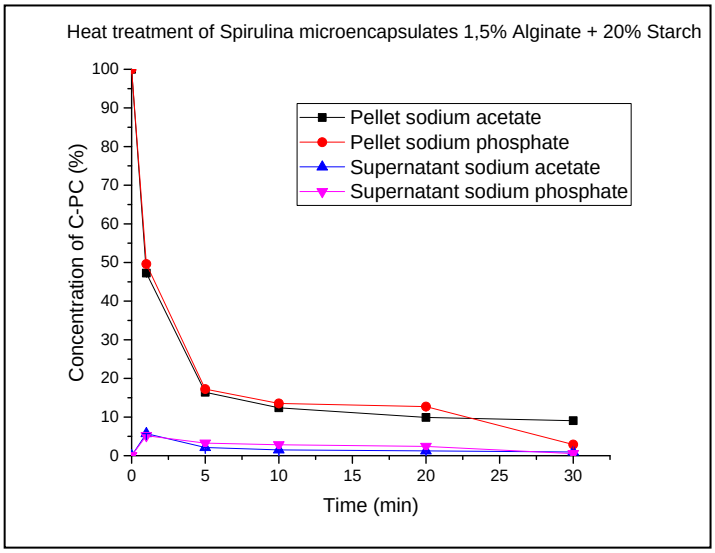


Figure 10. Variation of C-PC concentration determined by spectrophotometry during the heat treatment of *Spirulina* ST capsules at different pH values, max. SD 0.04.

Eudragit L and S polymers are preferred choice of coating polymers as they enable the targeting of specific areas of the intestine. Anionic Eudragit L dissolves at pH > 6 and is used for enteric coating (Thakral et al. 2013) but apparently it does not protect from the influence of temperature as the polymers used in this experiment.

When it comes to the antioxidant capacity (measure by ABTS) upon heat treatment the results agree well with result given by spectrophotometry showing a fast loss of the capacity in the first few minutes of the heat treatment (Figure 11-14)

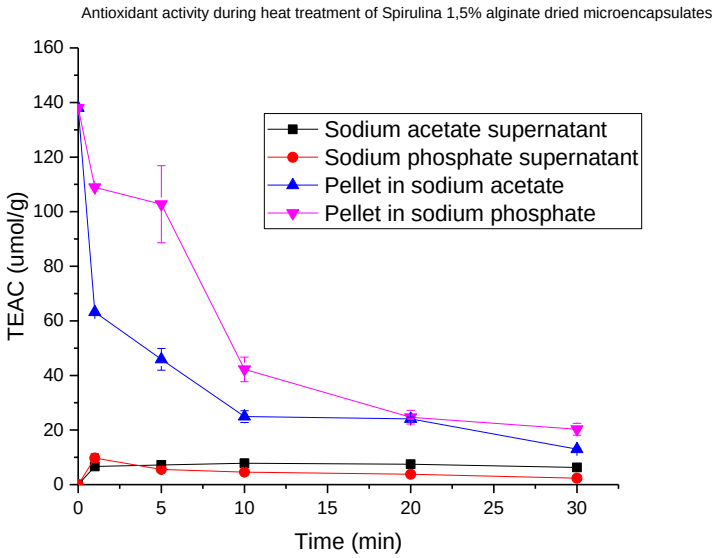


Figure 11. Antioxidadnt activity during heat treatment of *Spirulina* AL microencapsulates

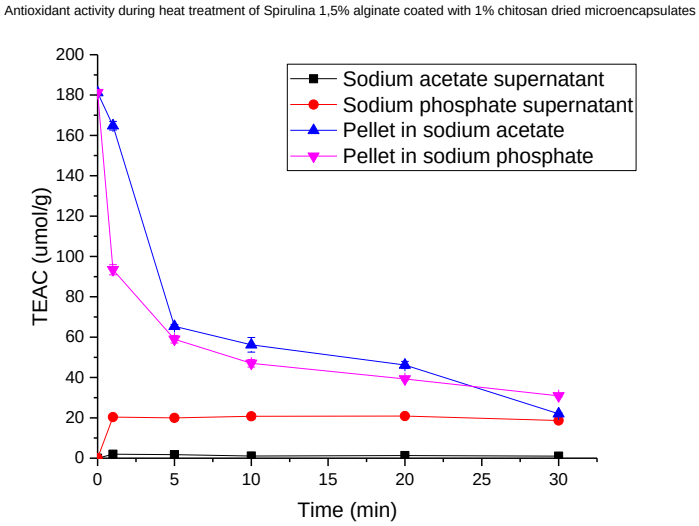


Figure 12. Antioxidadnt activity during heat treatment of *Spirulina* CHI microencapsulates

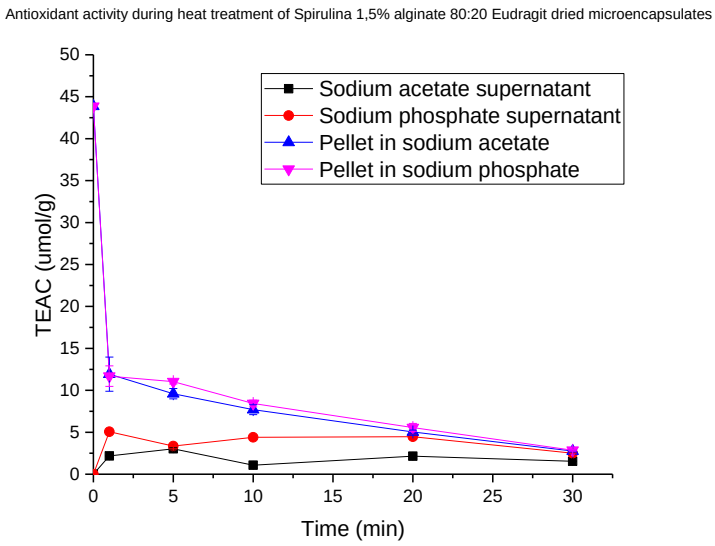


Figure 13. Antioxidadnt activity during heat treatment of *Spirulina* EU microencapsulates

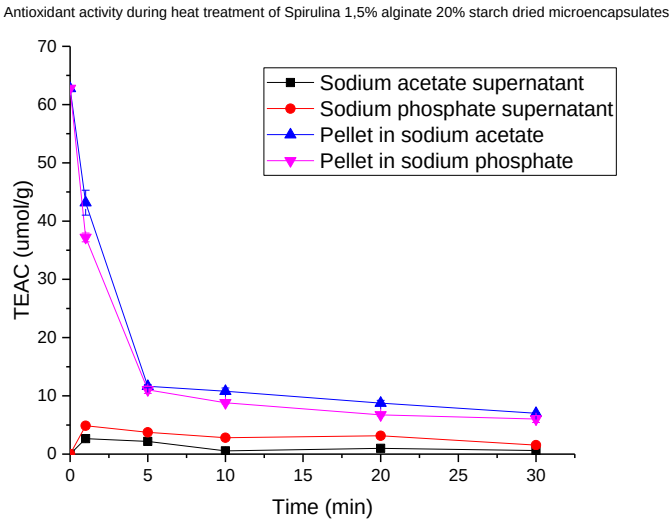


Figure 14. Antioxidadnt activity during heat treatment of *Spirulina* ST microencapsulates

The samples lose their antioxidant capacity approximately following the same trend both in sodium acetate pH 4.5 and sodium phosphate pH 6.5. At the end of the heat treatment, the *Spirulina* AL, *Spirulina* CHI and *Spirulina* ST capsules lost more than 80% of their antioxidant capacity, while *Spirulina* EU lost almost all antioxidant capacity as it is also confirmed by the spectra previously.

3.7. Simulated digestion

The samples collected along the simulated digested were analyzed by spectrophotometry to determine whether C-PC in dried microencapsulates can endure the gastric phase of the digestion without releasing C-PC until entering the intestinal phase.

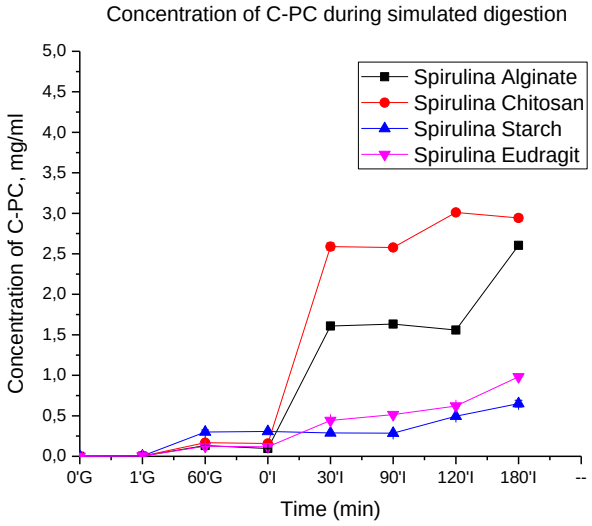


Figure 15. Concentration of Phycocyanin during simulated digestion of *Spirulina* microencapsulates, max. SD 0.04

Each sample consisted of 0.1 g of dried microencapsulates and it can be noticed that in the first stage of digestion they have the same performance- not releasing considerable amount of C-PC. When they enter the intestinal phase, the capsules start breaking and release the encapsulated compound C-PC.

Although the microcapsules are loaded with different concentration of phycocyanin, the experiment could not be performed in another way as each 4 types of capsules consist of different polymers that have diverse characteristics and capacities to absorb water. For example 0.1g of 1,5% alginate microencapsulates coated with 1% chitosan contains more Phycocyanin that the microencapsulates with 20% starch but the ones with chitosan has a water absorption capacity that is a lot lower than the one of starch. Attention was given more to the kinetics of release in this experiment than to the quantity of the released bioactive compound.

All of the encapsulates start releasing the bioactive compound in the intestine phase making it

accessible after the stomach. This result are of great importance since they indicate that it is possible to engineer microcapsules with different release time and targeted delivery.

The ABTS analysis of the digested samples followed nearly the same kinetics. Spirulina ST microencapsulates showed an antioxidant activity of 84% in respect to that]from the maximum obtained during extraction meaning that at 180' min of the digestion the capsules are almost a the maximum of their antioxidant capacity (Figure 16).

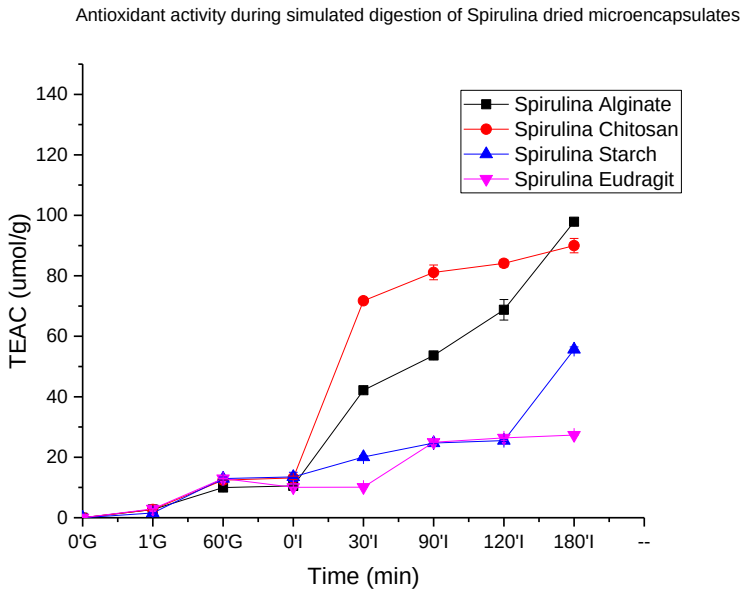


Figure 16. Antioxidant capacity of microencapsulates subjected to simulated digestion, msx. SD 0.02.

Spirulina AL showed a capacity of 70% of their maximum followed by Spirulina EU with 62% and Spirulina CHIcoated microcapsules with 49%. Chitosan and Eudragit are known for its prolonged release profile and are often used in drug delivery because of that.

3.8. SDS-PAGE analysis of simulated digestion

The samples collected after gastric and intestinal digestion were analyzed by SDS-PAGE in order to evaluate the protein integrity.

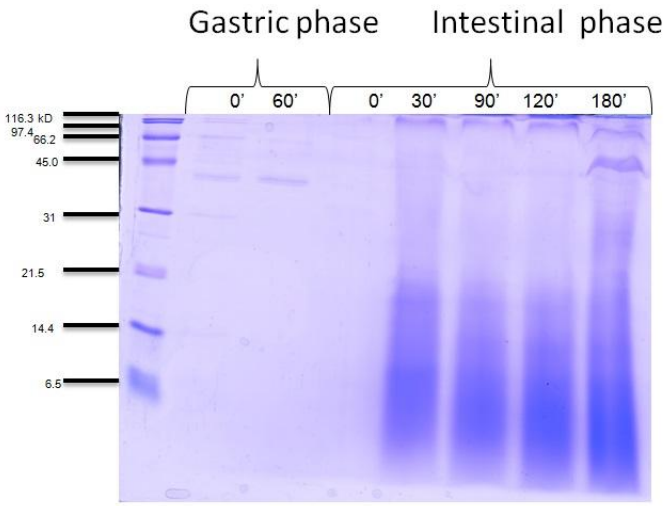


Figure 17. SDS-PAGE of *Spirulina* AL digested microencapsulates.

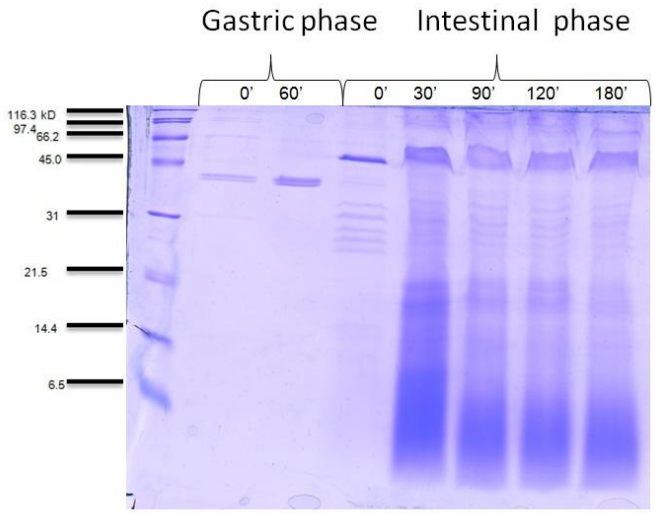


Figure 18. SDS-PAGE of *Spirulina* CHI digested microencapsulates.

Both microencapsulates of *Spirulina* AL and CHI showed very intense bands even at high molecular weight in the samples collected during the intestinal phase of the digestion, meaning that the capsules are broken and the proteins are liberated in the intestine part, but

apparently not affected by proteases. No bands were visible, except those of proteases, in the gastric phase indicating that during this step proteins did not diffuse out from the beads.

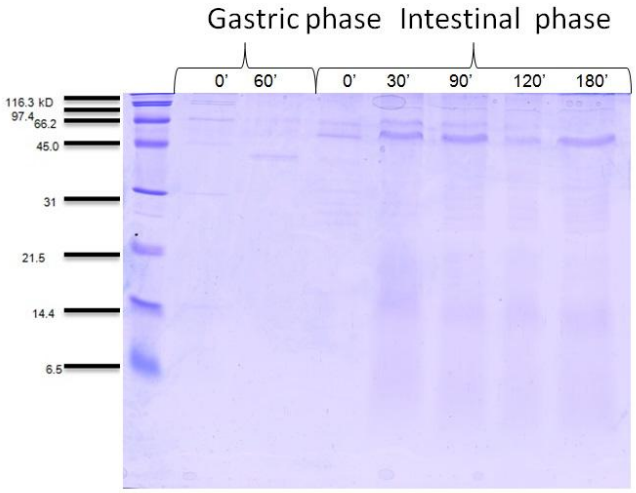


Figure 19. SDS-PAGE of *Spirulina* EU digested microencapsulates

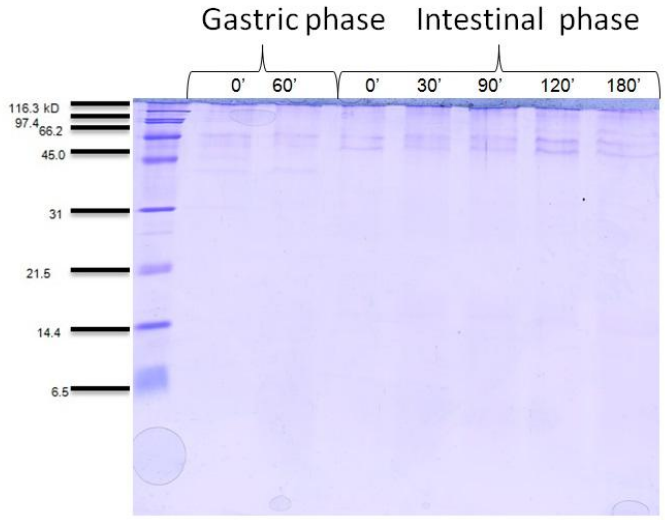


Figure 20. SDS-PAGE of *Spirulina* ST digested microencapsulates

Also *Spirulina* ST and *Spirulina* EU samples did not show signals in the gastric phase, but differently from the previous two samples also the bands in the intestine phase are absent, suggesting that the proteins might have been digested almost completely, in accordance with the ABTS analysis in which the same samples were characterized by the lowest antioxidant capacity. On the other hand, these results do not agree with those obtained for the *Spirulina* aqueous extract in the previous chapter. Indeed this showed a fast digestion in the gastric

phase that was almost completed before the samples could reach the intestinal phase. Taken together these results show that microencapsulation may strongly affect the fate of a given bioactive compound, in this case Spirulina proteins, by changing when and where the bioactive compound ought to be released.

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CONCLUSIONS

There is a trend towards a healthier way of living nowadays, a growing awareness by consumers what benefits for the health certain ingredients have in maintaining it. Preventing diseases through diet is a unique opportunity to use new functional foods. The development and achievement of functional foods requires technologies for incorporating of these investigated health promoting ingredients into food without reducing their bioavailability or functionality.

A very interesting example of new functional foods is microalgae. Our laboratory is investigating the special properties of *Spirulina platensis* and *Haematococcus pluvialis* and its possibilities to be included in functional foods. Experiments and efforts have been made to develop nutraceuticals or functional food in order to prevent or manage different kind of diseases. Spirulina as well as Astaxanthin, with their beneficial health effects, has become one of the nutraceutical foods with a diverse beneficial effect on a big spectrum of disease conditions. Parts of the biologically active compounds in Spirulina and Astaxanthin are quite labile and easily degraded at different conditions such as high temperature, different pH, oxidation and others. This leads to the necessity to find/optimize the ways of how Spirulina and Astaxanthin are preserved in order to be consumed by the human organism.

Spirulina is a spiral shaped microalgae, a *Cyanobacteria* that belongs to a class of bacteria with characteristic photosynthetic capability. Spirulina has very high protein content (up to 70%) but it also contains vitamins (especially B12 and provitamin A), minerals, fatty acids and other nutrients. It is also rich in phenolic acids, tocopherols and γ-linolenic acid. Due to the lack of cellulose walls, Spirulina can easily be digested. Phycocyanin is a phycobiliprotein and one of the major pigment constituents of Spirulina and because of this we chose Phycocyanin as our main marker in the experiments we performed.

Astaxanthin is a symmetric ketocarotenoid (3,3′-dihydroxy-β-β′-carotene-4,4′-dione) that naturally occurs in a wide variety of marine and aquatic organisms, and it is responsible for the bright red to pink color of *Crustacea* (shrimp, krill) and *Salmonidae* (salmon, rainbow trout). Astaxanthin is commonly used as a feed supplement in world-wide fish farming to grow healthy and well-colored fishes. The importance of this carotenoid rocketed when its protective role against peroxidation of lipids in biological membranes was finally recognized.

Since both of these nutraceuticals are labile to external influences such as pH, temperature, oxygen one of the possible solutions to preserve them is microencapsulation. Microcapsules protect sensitive food components, nutraceuticals and labile bioactive compounds, sensitive ingredients can be utilized, and also it can help incorporating unusual or time-release mechanism and ensure protection against nutritional loss or mask and preserve flavours.

The aims of this work was to explore, validate, produce, analyze and improve the production of encapsulates for their future industrial scale-up and successful incorporation in functional foods. A sustainable method was developed for the extraction and the production of stable astaxanthin microencapsulates. Nearly 2% astaxanthin was extracted by high-pressure homogenization of dried *Haematococcus pluvialis* cells in soybean oil. Astaxanthin-enriched oil was encapsulated in alginate and low-methoxyl pectin by Ca^{2+}-mediated vibrating-nozzle extrusion technology. The 3% pectin microbeads resulted the best compromise between sphericity and oil retention upon drying. The stability of these astaxanthin beads was monitored under four different conditions of light, temperature and oxygen exposition. After 52 weeks, the microbeads showed a total-astaxanthin retention of 94.1±4.1% (+4 C/−light/+O_2), 83.1±3.2% (RT/−light/−O_2), 38.3±2.2% (RT/−light/+O2), and 57.0±0.4% (RT/+light/+O_2), with different degradation kinetics. Refrigeration, therefore, resulted the optimal storage condition to preserve astaxanthin stability.

High pressure homogenization in soybean oil led to a good extraction of astaxanthin (almost 2%) from *Haematococcus pluvialis*. Besides being considered more sustainable than other algal polysaccharides, low methoxyl pectin proved to be a suitable material for the encapsulation of astaxanthin-enriched oil by extrusion. The advantage of this approach is the minimal stress that the active molecules undergo. The high stability displayed by the encapsulates in specific conditions during an extremely long storage test makes the whole process feasible for future applications in food, feed, and cosmetic industries.

An analysis of *Spirulina* aqueous extract as well as experiments regarding the loss of Phycocyanin (C-PC) during heat treatment and simulated digestion were performed. The loss of C-PC was examined with a spectrophotometric method, fluorimetric method and an ABTS analysis of the antioxidant activity. The temperature for the heat treatment was chosen in order to simulate a pasteurization process and the samples were incubated in water baths maintained at the specified temperature for 30 min at 65 °C in two different buffer solutions-sodium phosphate at pH 6.5 and sodium acetate at pH 4.5.A two-stage in vitro digestion

system was adopted. In the first stage, the extract was mixed with simulated gastric fluid (SGF), pepsin, pH 1.2 and put at 37°C for 60 min in agitation. After 1h the SGF was mixed with simulated intestinal fluid (SIF) containing 0.1M sodium phosphate + 50mM NaCl at pH 7.3 and pancreatin. Simulated intestinal digestion was performed at 37°c for 120 min in agitation. Samples were collected along the two steps. ABTS is a coloured radical generator that is frequently employed in the evaluation of the antioxidant capacity of a given molecule. The presence of an antioxidant decolours ABTS and this phenomenon is easily measured by a spectrophotometer.

In the case of the heat treatment C-PC lost its colour quicker in pH 4.5 than in pH 6.5 as well as in the second phase of the simulated digestion (intestinal phase). it is known that the optical properties of the covalently linked chromophores of the biliproteins are deeply influenced by the state of the protein and that both absorption and fluorescence monitors different properties of the chromophore. The acid condition combined with the heat treatment has probably catalyzed a faster denaturation of the pigment. Also in the second phase of the simulated digestion (intestinal phase) the colour of the *Spirulina* was almost completely lost. The antioxidant activity and the absorbance decreased in time during the heat treatment –the antioxidant activity dropped with 20% at pH 6.5 and with 80% at pH 4.5 and the absorbance respectively with the same % in the same pH conditions. The antioxidant activity during simulated digestion decreased with 25% at the end of the intestinal phase. This is due to the denaturation of the protein in the case of the heat treatment and the protein being digested in the intestinal phase of the simulated digestion. SDS-PAGE analysis demonstrated that the proteins were completely hydrolysed at the end of the digestion.

Spirulina microencapsulates were produced in order to stabilize the extract. Results showed that microencapsulation does not protect the extract from thermal treatment at 65 °C suggesting that Phycobiliproteins are too labile to such high temperatures. Though it does not protect them from high temperature, microencapsulation showed to be a good method to protect the extract from the influence of the gastric environment. An ABTS and SDS-PAGE analysis showed that microencapsulation of *Spirulina* extract can preserve the capsules in the low pH of the stomach and release the extract in the intestine phase of digestion.

The future perspective of this work is to set up a scale-up method for the production of Astaxanthin and *Spirulina* microencapsulates on an industrial level.