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PRODUCTION AND CHARACTERIZATION OF ASTAXANTHIN NANOPARTICLES

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LIST OF ABBREVIATIONS

AOC: Antioxidant capacity

ASX: astaxanthin

CAA: cellular antioxidant activity

CLSM: confocal laser scanning microscopy

DCFH-DA: 2',7'-Dichlorofluorescin di-acetate

DLS: Dynamic Light Scattering

DMSO: Dimethyl sulfoxide

EE: encapsulation efficiency

EFSA: European food safety authority

FDA: food and drug administration

FITC: fluorescein isothyocianate

GRAS: generally recognized as safe

GS: gastric stage

H.p: Haematococcus pluvialis

IP: isoelectric point

IS: intestinal stage

NEs: nanoemulsions

NNI: national nanotechnology initiative

NPs: nanoparticles

PDI: polydispersity index

PPI: pea protein isolate

PPS: potassium persulfate

ROS/RNS: reactive oxygen/nitrogen species

RPI: rice protein isolate

sASX: superficial astaxanthin

SD: simulated digestion

SEM: scanning electron microscope

SGF: simulated gastric fluids

SIF: simulated intestinal fluids

SLN: solid lipid nanoparticles

SPI: soy protein isolate

TAG: triacylglycerol

TEAC: Trolox equivalent antioxidant capacity

WPC: whey protein concentrate

Abstract

There is increasing interest on behalf of consumers and the food industry for the enrichment of common food with health-promoting bioactive molecules. Clinical studies have demonstrated that tangible health benefits may derive from the intake of bioactive compounds, in the prevention of dietary related pathologies such as diabetes, cancer, obesity and cardiovascular diseases. The beneficial effect is usually given by the presence in food of peculiar molecules such us carotenoids, polyphenols, polyunsaturated fatty acids and bioactive peptides, to cite a few. Unfortunately, these compounds display high susceptibility to environmental conditions such as light, extreme pH and temperature, and to standard food manufacturing processes. They can also account for undesirable flavors, colors or affect final product stability and appearance, thus rendering their presence in the product an issue rather than a useful addition. The addition of nutrients in small quantities into a food system may not significantly affect its proprieties, but the high amounts, often required to meet certain health claims and benefits, might bring to a product with a poorly acceptable sensory profile and a scarce stability. In particular, lipophilic bioactive ingredients display a major challenge. Besides their limited solubility in most of the foods and beverages, they are characterized by high susceptibility to oxidation and by a lower adsorption through the gastrointestinal tract in comparison to more hydrophilic substances, meaning a scanty bioavailability. Hence, there is a pressing need for the production of edible delivery systems or carriers that could efficiently encapsulate, protect and improve the handling of lipophilic molecules.

The objective of this thesis was to develop a system suitable for the encapsulation of lipophilic molecules, capable of: a) protecting the ingredient from the surrounding environment (extreme pH, heat, UV light, oxygen); b) preserving its functionality (e.g. antioxidant activity); c) reducing the impact on the organoleptic level; d) improving the bioavailability of the encapsulated molecules. This last point in particular could be achieved by using sub-cellular delivery systems referred to as nanoparticles or nanocarriers, which may potentially enhance the transport across the intestinal wall.

To this purpose, astaxanthin was chosen as a model bioactive compound. Astaxanthin is a keto-carotenoid that displays several biological activities, such as high antioxidant capacity, that may contribute to the prevention of degenerative pathologies like diabetes, cancer, cardiovascular diseases and chronic bowel disease. However, like all carotenoids it is characterized by a strong

lipophilic character that makes its inclusion in many types of aqueous-based foods and beverages rather a challenge. This aspect is the main cause of its poor absorption through the human intestinal mucosae. Moreover, astaxanthin is labile to common processing conditions such as the presence of light and oxygen, extreme pH and high temperatures. For these reasons a lot of efforts have been put in these past years to find suitable solutions for its protection and manipulation.

In order to develop the suitable encapsulate, in **chapter 2** an emulsification and solvent-evaporation technique was used as encapsulation approach; whey proteins were selected as the matrix to envelope the core constituted by an astaxanthin-enriched oleoresin derived from *Haematococcus pluvialis*, a microalgae representing the main natural source of the carotenoid. The process was optimized by varying crucial parameters and the stability of the nanoparticles was tested in different conditions. This analysis highlighted a better performance of the encapsulated molecule in comparison to the starting oleoresin. Good release properties during *in-vitro* simulated digestion and the increase of the solubility in water were observed. In **chapter 3**, the study was focused on the research for plant alternative proteins as encapsulating matrices in order to satisfy the increasing interest of the consumers for substitutes of animal-deriving ingredients. This allowed to identify pea protein isolate as a valid candidate for the development of a vegetarian/vegan-allergen free nanocarrier. Finally, **Chapter 4** dealt in depth with the antioxidant properties displayed by the astaxanthin nanoparticles through *in vitro* colorimetric assay and by the development of a cell-based assay. The encapsulates showed higher antioxidant capacity in comparison to the oleoresin. The uptake of the nanoparticles was studied in cell model systems through confocal laser microscopy and flow cytometry that indicated a probable energy-dependent mechanism.

Chapter 1. Introduction

1. Interest of the study

In the past years the market of functional foods and nutraceuticals have faced an exponential growth, due to the increasing demand of the Western society for natural products, together with the crescent awareness of consumers that an intimate link exists between food consumption and its health-beneficial effects (*Link, Balaguer, & Goel, 2010; Scalbert et al., 2011; Liu, 2013*). New products are emerging on the market, with the aim to couple the nutritive and healthy effects of food consumed every day. Consequently, incorporation in foods and in nutraceutical formulations of bioactive compounds to improve some traits such as shelf-life, aspect or taste, avoiding the use of synthetic additives, as well as to give to the final products new features (e.g. antioxidant or anti-inflammatory activity, etc.) is highly desirable for the food and food supplements industries. In particular, more attention is now being payed to the capacity of the products in guaranteeing the delivery and the bioavailability of the bioactive, limiting its impact on the sensory level. Encapsulation, intended as surrounding a molecule with a barrier, could be a solution to all these issues. In particular, nano-encapsulation, referred to the production of nanometric delivery encapsulated systems, is rapidly emerging as the best choice to this purpose. Currently, the market of nanotechnology products in the food industry is worth about US$ 1 billion, and is based mainly on nanometric delivery systems for packaging coatings (*Chau, Wu, & Yen, 2007*). In this chapter, the most employed encapsulation techniques and the ways to design an encapsulate will be presented.

1.2 The concept of encapsulation

Encapsulation is a rapidly expanding technology with numerous potential applications in different areas, including food, feed, cosmetics and pharmaceuticals. Encapsulation can be defined as a process that leads to the entrapment of one or more substances (active agents) within a matrix (wall material). The encapsulated substance can be called in different manners: core, fill, payload or internal phase. The matrix that constitutes the wall is often called coating, membrane, shell, capsule, carrier material or external phase (*Wandrey, Bartkowiak, & Harding, 2010; Champagne & Fustier,*

2007; Bernard F. Gibbs Selim Kermasha, 1999; Shahidi & Han, 1993). The process of encapsulation could also be defined as a technology of packaging solids, liquids, or gaseous materials in small capsules that release their contents at controlled rates over prolonged periods of time and under specific environmental conditions (*Desai & Park, 2005*). Produced particles usually have diameters of a few nm to a few mm (*Wandrey et al., 2010*).

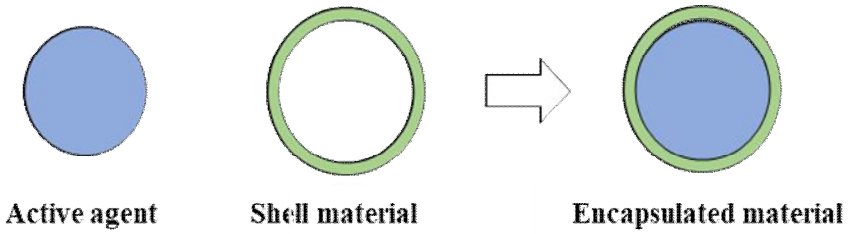


Fig.1.1. Schematic representation of the encapsulation mechanism.

The terminology of encapsulation is based mainly on few terms, the knowledge of which could help in having a better understanding of the process:

Active agent: The component, ingredient or nutraceutical that needs to be encapsulated within the delivery system. The active component can vary in chemical and/or molecular characteristics (charge, molecular weight, polarity and physical state) and in its functionality. Bioactive components that can be encapsulated include lipids, vitamins, peptides, fatty acid, antioxidants, minerals but also living cells such as probiotics (*Burey, Bhandari, Howes, & Gidley, 2008; McClements, Decker, & Park, 2008*).

Delivery: The process of carrying the encapsulate material to the site of action. This may be the small intestine, stomach or colon if we are speaking about the human body.

Controlled release: Refers to the process of releasing the encapsulated component with a specific concentration-time profile at the site of action. The release could follow different patterns:

- *Burst release*: release of most of the entirety of the active agent in a short period of time.
- *Prolonged released*: the active agent is released over a long period of time at a constant rate. An example could be the long release of sugar in a chewing gum.
- *Triggered release*: the release is activated in response to a particular environmental component like: pH, ionic strength or the high temperature required for the release of encapsulated baking soda when baking a cake.

1.3 The need for encapsulation

Sixty years ago, the main sector in which encapsulation was employed was that of biotechnology, primarily for the separation of cells from the produced metabolites in bioreactor tanks. But nowadays, one of the major players along with the pharmaceutical sector, is the food industry (*Bernard F. Gibbs Selim Kermasha, 1999*). This sector is responding fast to the requirement by the consumers to have healthier food on the market, without losses on the sensory profile. To change the nutritional value and add improved features of a specific product, phytochemicals, vitamins, antioxidants and fatty acids need to be included into the final product. These compounds are usually highly susceptible to degradation by environmental factors, processing and gastrointestinal conditions, and therefore, encapsulation represents an approach for their effective protection (*Nedovic, Kalusevic, Manojlovic, Levic, & Bugarski, 2011*; *McClements, Decker, Park, & Weiss, 2009)*.

Apart from that, the incorporation of nutraceuticals, aromas and proteins could ameliorate the palatability, appearance and texture properties of the final product.
The physical separation from the food matrix of the active compound through an inert barrier could avoid undesirable interactions between them (e.g. the precipitation of protein given by the presence of high concentration of polyphenols).
Encapsulation could be used also to mask the taste or odor associated to the presence of the functional molecule (e.g. the odor of fish oil or the bitterness of polyphenol and amino acids mixes).
The dilution of the bioactive inside the encapsulant matrix could be advantageous when it is needed in very small amounts. In this way the delivery system could be guaranteed in a pre-dosed form, avoiding eventual toxic effects (*Desai & Park, 2005*).

The encapsulation of lipidic substances in small delivery systems soluble in water could ameliorate their bioavailability. As stated by the Food and Drug Administration (FDA), the term bioavailability refers to the rate and extent to which the active drug ingredient or therapeutic moiety is absorbed from a drug product and becomes available at the site of drug action (*Allam, El Gamal, & Naggar, 2011; Fathi, Martín, & McClements, 2014*). In addition to the above, encapsulation can be applied for modification of physical characteristics of the original material in order to allow easier handling and to provide an adequate concentration and uniform dispersion of the active agent.
In particular, nanotechnology, referred to the production of encapsulate of diameter in the range of 1-100 nm, has become one of the most promising technologies to revolutionize conventional food science and food industry. Nanoparticles proved their efficacy in enhancing food security,

extending storage life, improving flavor and nutrient delivery, allowing pathogen/toxin/pesticide detection, and serving as functional ingredients (*Mihindukulasuriya & Lim, 2014; Rashidi & Khosravi-Darani, 2011*).

1.4 Capsule forms

The forms of the encapsulates generally fall in two main categories: reservoir and matrix type. The reservoir-type system (Fig.1.2 A) is characterized by the presence of a thick shell, an inert membrane that physically separates the active compound that resides in the interior part of the capsule from the surrounding environment (*McClements, 2015*).

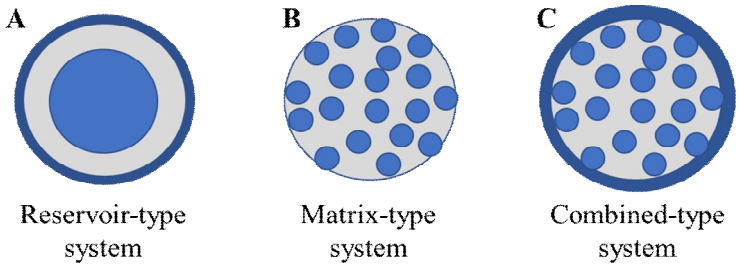


Fig.1.2. Schematic representation of the encapsulate system: A) Reservoir; B) Matrix-type and C) Combined-type system.

In the matrix type (Fig.1.2 B) the active agent is uniformly distributed over the formed net, constituted by the encapsulant material, in this way the active agent can be found also on the surface of the carrier that is normally called bead (*Soukoulis & Bohn, 2018*). Additionally, there is also a combination of the two forms, the reservoir-matrix system (Fig.1.2 C), where the matrix encapsulate is embedded by a thin membrane. This type of system is used mainly for long lasting release. In addition to the above, the microencapsulates can also be mononuclear with multiple shells/walls, and the capsules/beads may have irregular shape.

1.5 Desired encapsulate characteristics

Since food is more easily accessible to everyone in comparison to pharmaceuticals, the regulation about encapsulation in the food sector is much stricter (*Nedovic et al., 2011*). The material used for encapsulation needs to meet two important attributes: it has to be Generally Recognized As Safe (GRAS) and safe for the major governmental agencies: the European Food Safety Authority (EFSA) for Europe, and Food and Drug administration (FDA) for the United States. For example,

many of the synthetic chemical polymer coatings that are used for the delivery of pharmaceutical products and drugs cannot be employed in designing delivery systems for food (*McClements, 2015*; *Acosta, 2009*).

Other important factors that need to be considered are that the selected materials need to be:

- *Food grade*: the delivery system needs to be composed of food ingredients and through a process that meets the legislation or cultural belief approved by the country in which the final product is sold (e.g. lipids, proteins, carbohydrates and water).
- *Economic feasibility*: the encapsulate should be capable of being economically manufactured. From this requirement arises the need to search for new inexpensive encapsulating materials (*McClements, 2015*). A simple example could be the use of pectin (extracted from fruits peels) to produce hydrogel beads containing enriched oil of astaxanthin (*Vakarelova et al., 2017*) and flax seed oil (*Menin et al., 2018)*.
- *Compatibility with the food matrix*: the presence of the particle system should not adversely affect the sensory, rheological and appearance characteristics of the final product. The chosen material must be inert (*Bernard F. Gibbs Selim Kermasha, 1999; Donsì, Sessa, Mediouni, Mgaidi, & Ferrari, 2011*).
- *Protection against degradation:* the chosen material should produce a stable and strong barrier against the condition that could negatively affect the functionality of the bioactive agent when introduced in the final product (e.g. high temperature, pH, oxygen presence etc.) (*McClements, Decker, & Weiss, 2007)*.
- *Loading capacity and retention:* normally the higher the amount that can be encapsulated for unit mass of encapsulant agent the better is. This is mainly for two reasons: 1) from the economic point of view it is very advantageous and 2) for the consumer the amount of functional food needed to reach the daily intake is reasonable.
- *Bioavailability/bioactivity:* the encapsulated bioactive ingredient must be delivered and released at the site of action. The system should preferably enhance the absorption of the active, resulting in an amplification of its bioactivity (*McClements et al., 2007*).

Since no single coating material can meet all of the criteria listed above, they are often employed in combination or modified to obtain peculiar characteristics (e.g. OSA-starch).

1.6 Nanoparticles versus microparticles

As already mentioned, nanotechnology is defined as the production, utilization and manipulation of materials, devices or systems in the nanometer scale (smaller than 100 nm) (*Aguilera, 2008; Fathi et al., 2014*). Furthermore, according to the definition of the National Nanotechnology Initiative (NNI), it is the "understanding and control of matter at the nanoscale, at dimensions between roughly 1 and 100 nm, where unique phenomena enable novel applications" (*Tamjidi, Shahedi, Varshosaz, & Nasirpour, 2013*). The properties such as color, solubility, viscosity, and biological properties are substantially modified at a nano-scale, owing to the interactions of individual atoms and molecules (*Neethirajan & Jayas, 2011*). The major applications in food industry regards the packaging materials, new nutrient delivery systems and formulations with improved bioavailability characteristics (*Weiss, Gaysinsky, Davidson, & McClements, 2009*). Nanoencapsulates show long-term kinetic stability and increased surface-to-area ratio with respect to microencapsulates, an aspect that potentially may enhance the solubility and the bioavailability of the active ingredient. For example, at sizes below 100 nm, it was shown that the particles appear to be readily adsorbed at the intestinal mucosa (*Desai, Labhasetwar, Amidon, & Levy, 1996*).

It is important to underline that often in the scientific literature mainly dealing with food, several reports describe nanoparticles with sizes higher than 100 nm, which is in contrast with the definition given by the NNI (*Sanguansri & Augustin, 2006*). This is not surprising since in some cases particles with a diameter larger than 100 nm display features similar to those having a diameter below this value. Size is anyway important as it affects the optical clarity, physiochemical stability, encapsulation and release characteristics (*Ezhilarasi, Karthik, Chhanwal, & Anandharamakrishnan, 2013*). For example, numerous studies have pointed out the importance of the size of the particle in relation to the transport across the mucus layer, the absorption by the epithelial cells and overall the fate of the particle within the human body. In general, the smaller the particle size, the higher the absorption of these particles through the gastro-intestinal tract (GIT). Particles larger than 300 nm are not directly transported into the systemic circulation (*Jani, Halbert, Langridge, & Florence, 1990)*, although they have been detected in the lymph and blood as they can 'leak' through loosened tight junctions (*Hillyer & Albrecht, 2001*).

Although nanoencapsulation is a promising technology with many advantages compared to microencapsulation, the number of products based on nanotechnology present on the market at the moment is still very limited when compared to microtechnology, mainly for three reasons: 1) the potential toxicity due to the small size of the delivery system, which is still elusive to both regulatory agencies and consumers (*Bieberstein, Roosen, Marette, Blanchemanche, &*

Vandermoere, 2013); 2) difficulty in scaling up the processes and 3) use of organic solvents, which are toxic, and may be present as residues in particle formulations (*Müller, Mäder, & Gohla, 2000*).

1.7 Materials for encapsulation

Typical ingredients for encapsulation include amorphous sugar (sucrose, lactose and trehalose), polyols (sorbitol and mannitol), and non-crystalline polymeric materials such as chemically modified starch, maltodextrin, polysaccharides (arabic, mesquite or gellan gum, alginates and pectins), and proteins (gelatins, whey proteins, sodium caseinate, vegetable proteins) (*Wan, Bankston, Bechtel, & Sathivel, 2011; Soukoulis & Bohn, 2018*). Common ingredients used for encapsulation are listed in Tab.1.

Category	Materials	Methods
Carbohydrate	Starch, maltodextrins, corn syrup solids, dextran, modified starch (N-lok®, Capsul®, HICAP®), cyclodextrin	Spray- and freeze-drying, extrusion, coacervation, inclusion complexation
Cellulose	Carboxymethylcellulose, methyl cellulose, ethylcellulose, celluloseacetate-phthalate	Coacervation, spray-drying, edible films, fluid bed
Gum	Gum acacia, agar, sodium alginate, carrageenan	Spray-drying, ionic gelation, fluid bed
Lipids	Wax, paraffin, beeswax, diacylglyerols, oils, fats, phospholipids	Emulsion, liposomes, film formation, spray chilling, fluid bed
Protein	Gluten, casein, zein, gelatin, albumin, peptides, whey protein, vegetable proteins (rice, pea, sunflower, chickpea etc.)	Emulsion, spray-drying, ionic gelation, fluid bed
Emulsifiers	Mono- and di-glycerides, lecithin, liposomes, food-grade surfactans	Emulsion, spray dry, ionic gelation, Fluid bed

Tab.1. List of encapsulating materials for food applications.

For encapsulation purposes the ideal wall material should have: good emulsifying properties, be a good film former, have low viscosity at high solid concentrations, low hygroscopicity, good property of release, low in cost, bland in taste and odor, stable in supply and able to protect the active ingredient (*Desai & Park, 2005; Bangs & Reineccius, 1990*). From the moment that none of the materials listed above could have all these characteristics, frequently the wall materials are used in combination with each other to obtain mixture of material with enhanced performance. The problem of working with natural ingredients or processed food ingredients is mainly the variation in composition and quality of the original source from which they are isolated.

1.8 Encapsulation techniques

There is a number of techniques available for encapsulation of food compounds, in which the processes are divided into two main categories: the mechanical/physical and the chemical ones. The mechanical processes involve the controlled precipitation of a polymeric solutions where a physical change occurs, meanwhile the chemical processes involve polymerization reactions (*Anandharamakrishnan, 2015*). The most employed technologies include spray drying, spray cooling/chilling, freeze drying, extrusion, fluidized bed coating, coacervation, liposome entrapment, inclusion complexation and emulsion (*Bernard F. Gibbs Selim Kermasha, 1999; Augustin & Hemar, 2009; Desai & Park, 2005*). Tab.1.2 lists a series of technologies and the type of process they belong to. The choice of the technique to be used depends on different factors that need to be accurately analyzed, such as: the particle size, the appearance, physical/chemical properties of the active agent, target application of the encapsulate, etc.

Mechanical process	Chemical process
Spray drying	Simple coacervation
Spray cooling	Complex coacervation
Spray chilling	Ionotropic gelation
Lyophilization	Interfacial polymerization
Centrifugal suspension preparation	Solvent evaporation
Co-crystallization	Liposome entrapment
Emulsification	Inclusion complexation
Fluidized bed coating	Solvent exchange method
Centrifugal extrusion	
Spinning disk	
Pressure extrusion	
Hot-melt extrusion	
Electrospraying or electrohydrodynamic techniques	

Tab.1.2. Encapsulation techniques

Among all these techniques only a few allow to obtain particles in the nano-range. In the following paragraphs, these technologies will be discussed. In particular, for the aim of this thesis, only the

techniques indicated for the encapsulation of lipophilic ingredient will be discussed, which are a combination of chemical and mechanical processes.

1.9 Nano delivery systems

1.9.1Nanoemulsions

Nanoemulsions (NEs) are nanoscale droplets of a multiphase colloidal dispersion formed by dispersing two immiscible liquids through physical rupturing (*Solans, Izquierdo, Nolla, Azemar, & Garcia-Celma, 2005*; *Fryd & Mason, 2012*). Different size ranges of nanoemulsions are reported in literature: from less than 100 nm (*Rao & McClements, 2011*; *Ghosh, Mukherjee, & Chandrasekaran, 2013*) to 100-600 nm (*Sakulku et al., 2009*). Due to the size, nanoemulsions display interesting physical properties, that microemulsion lack. For instance, the droplet size in a nanoemulsion is much smaller than visible wavelength, in this way most of them appear optically transparent, a very favorable feature when applied to beverages. Additionally, the reduced dimension of NEs enhances stability against gravity, due to the Brownian motion of the nano-sized droplets (*Fryd & Mason, 2012*), avoiding deposition phenomena of the particles. NEs are also metastable, i.e. they can be diluted with water without changes in droplet size distribution. The methods used to produce NEs can be divided into mechanical and non-mechanical processes. For the former the most used techniques are high-pressure homogenization, microfluidization and ultrasonication (*Villalobos-Castillejos et al., 2018*; *Anton & Vandamme, 2011*).

With high-pressure-homogenization, the solution is passed through a small inlet orifice at pressures in the range of 500-5000 psi. Microfluidization uses higher pressures, up to 20000 psi, to pass the liquid through microchannels (the collision chamber) where the nano-droplets are produced. The driving forces in the production of the droplets by these two techniques are cavitation and mechanical shear. The pressure applied and the number of passing cycles have a strong effect on particles size.

Ultrasonication uses bubble cavitation as a mechanism of droplet formation. The ultrasound waves (usually in the range of 20 kHz) result in the sequential formation, growth and collapse of microscopic vapor bubbles in the liquid; the collapse of these cavities provides sufficient energy to increase the surface area of the droplets (*Patil & Pandit, 2007*). In this case, increasing sonication time is often useless because the droplet size decreases until a certain limit after which coalescence phenomena can be observed due to the collision of existing particles (*Fathi, Mozafari, & Mohebbi, 2012*).

Nanoemulsions can also be obtained using a non-mechanical method by which organic solvents are used to dissolve the molecules of interest and the component is emulsified with a stabilizer dissolved in aqueous solution. A stabilizer is a molecule that thanks to its amphiphilic characteristics is able to absorb to a lipidic surface stabilizing it. The evaporation of the solvent leads to the stabilization of the emulsion.

NEs are good candidates for the delivery of lipophilic or poorly water-soluble ingredients, such as edible oil, vitamins and carotenoids. The materials used as stabilizers are for example polysorbate, lecithin carbohydrate and proteins, to cite a few.

1.9.2Nanoliposomes

Nanoliposomes (the nanometric version of a liposome), are colloidal structures formed by the input of energy to a right combination of constituent molecules in aqueous solution. A liposome, in particular, could be define as a closed, continuous, vesicular structure composed mainly by a phospholipids bilayer(s) in an aqueous environment (*Mozafari et al., 2006*; *Mozafari, Khosravi-Darani, et al., 2008*). The materials used for the formation of liposomes are mainly phospholipids, that are also the major component of the cell membrane bilayer. The key characteristic that drives the process of liposome formation is the amphiphilicity, due to the presence of polar and non-polar regions in their structure (*Mozafari, Johnson, Hatziantoniou, & Demetzos, 2008*). Due to their biocompatibility, biodegradability and low toxicity they are widely used in the food, pharmaceutical and cosmetic industries.

An important characteristic of liposomes is that they are 'targetable', i.e. the ability to reach a designated target site, and in this way deliver the molecule to a defined region (*Mozafari et al., 2006*).

In contrast to NEs, nanoliposomes can be employed not only for the encapsulation of lipophilic bioactive, but also for also water-soluble ones (*Torchilin, 2005*). The preparation procedure is simple: when amphiphilic molecules (dissolved in methanol, ether or chloroform) are placed in an aqueous system, they tend to form aggregated complexes in an attempt to cover their hydrophobic section, exposing their hydrophilic groups. If a sufficient amount of energy is provided to the aggregated structure, they can arrange themselves to form organized, closed bilayer vesicles (*Lasic, 1988; Mozafari et al., 2006*). During the rearrangement process, liposomes can entrap hydrophilic molecules present in the aqueous solution. The energy required for liposome formation can be delivered through sonication, homogenization or heating. Another way to obtain nanoliposomes is by passing pre-formed liposomes of micrometric size through a filter-membrane with small nanometric pores, employing a system of extruders (*Olson, Hunt, Szoka, Vail, & Papahadjopoulos,*

1979).

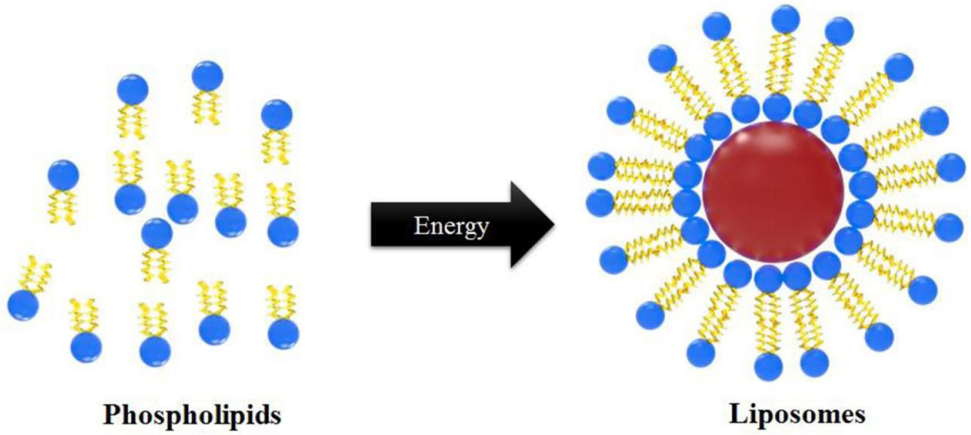


Fig. 1.3. Encapsulation of bioactive molecules through liposome technique. Source (*Shukla et al., 2017*)

A problem related to the use of liposomes is the scarce stability. During storage they can undergo aggregation, fusion and precipitation phenomena, this because lipid vesicles are thermodynamically unstable.

1.9.3Solid lipid nanoparticles

Solid lipid nanoparticles (SLN) could be considered as an evolution of the nanoemulsion system (Fig.1.4). Indeed, it is possible to produce lipidic nanoparticles from nano-/micro-emulsions by using a lipid phase that is able to crystallize at the final temperature of application (*Müller, Radtke, & Wissing, 2002*). SLNs contain lipid droplets that are fully crystallized with the bioactive components being a part of the lipid matrix (*Müller, Petersen, Hommoss, & Pardeike, 2007*; *Weiss et al., 2008*). SLNs consist of a core of solid lipids with the bioactive being a part of the lipid matrix. Triacylglycerols (TAGs) are commonly used as carrier lipids. They exhibit polymorphism upon cooling, thus leading to different stacking of TAG molecules in different assemblies, with different stability. The surfactants are more or less the same used in nanoemulsion production. Two are the methods used for large scale production of SLNs: 1) hot homogenization and 2) cold homogenization (*Müller et al., 2000*). In the first one, the lipid mixture is melted 5-10°C above its melting point, the bioactive compound is added to the lipidic solution and then dispersed in aqueous solution containing a surfactant, at the same temperature. The obtained emulsion is then homogenized at controlled temperature. The result of the process is an Oil/Water emulsion. Cooling of the emulsion leads to the recrystallization of the lipid and the formation of solid lipid nanoparticles. In the second technique, the bioactive is incorporated to a melted lipid, then the lipid microparticles are dispersed at room temperature in a cold aqueous solution that contain a

surfactant. The produced lipid suspension is then homogenized (*Fathi et al., 2012*).

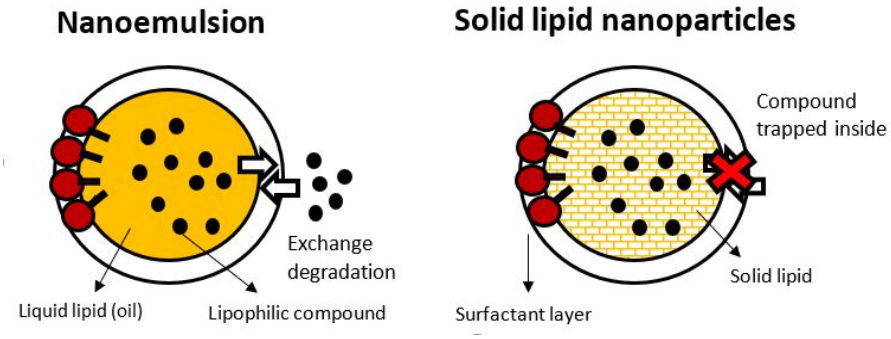


Fig.1.4. Differences between nanoemulsion and solid-lipid nanoparticles system.

1.10 Post processing for industrial use

While the development and production of nanoparticles at lab-scale is well described and characterized, limited research integrated concurrently the post-treatment need to have a product scalable at the industrial level. One of the aspects is for example the concentration of nanoparticles that often are obtained at very low solid concentration, limiting their use in commercial applications and leading to high costs of transportation and storage. For example, most nanoparticle preparations are in the liquid form, and storage of a product in the form of a suspension or concentrate is detrimental for the shelf life of the product that could undergo chemical and physical instability phenomena. Transforming the nano-system into a powder is a suitable way to increase the stability of the product, the easy handling for costumers, expand the versatility of the product and reduce costs of storage and transportation. The chosen technique must allow the conservation of the nano-structure and the stability of the encapsulated bioactive. In the case of dried forms of product, it is also necessary to have an appropriate dispersibility of the powder into native particles, without altering its physicochemical and biological behavior.

In the following chapters, different techniques belonging to the microencapsulation sector will be discussed. These techniques due to their versatility are often use to stabilize and drying particles produced with other encapsulation techniques.

1.11 Spray dry

Spray-drying encapsulation has been used in the food industry since the late 1920s in the field of milk powder production, and it is one of the oldest processes for encapsulation of food ingredients (*Ray, Raychaudhuri, & Chakraborty, 2016*). The utilization of spray dry technology became very important during the 2nd World War, when the necessity for the transport of huge amounts of food

emerged, causing a search for new methods to reduce food volume and weight (*Cal & Sollohub, 2009*).

Nowadays, spray-drying is the most widely used microencapsulation technique in the food industry, typically used for the preparation of dry stable food additives. It is recognized as the cheapest technique to produce microencapsulated food materials (*Desai & Park, 2005*), due to the fact that the equipment is readily available and production costs are lower than most other methods (*Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007*).

Although most often considered as a dehydration process, spray-drying can be used to encapsulate active material within a protective matrix formed from a polymer. From a practical point of view, spray-drying is a unit operation by which a liquid product is atomized in a hot gas current to instantaneously obtain a powder. The gas generally used is air or occasionally an inert gas. The liquid feeding the sprayer can be a solution, an emulsion or a slurry.

Spray-drying, depending on the starting feed material and operating conditions, produces a very fine powder (10–50 μm) or large size particles (2–3 mm) (*Gharsallaoui et al., 2007*). In most cases, particles produced via spray dry have a core dispersed throughout the wall material and the central area is occupied by an empty space resulting from expansion of particles during the drying stages (*Reineccius, 2004)*. Capsules with this structure might have a core loading of 20-30% of total capsule weight (*Thies, 2004*).

The preparation of a solution to be sprayed requires the production of a stable emulsion of the encapsulant and the core materials. The mixture is prepared by dispersing the active agent (often hydrophobic) in the solution of the polymer dissolved in water, producing an immiscible phase. The dispersion is then homogenized. Stability of the produced emulsion must be evaluated, to avoid phase separation during the atomizing step (*Gharsallaoui et al., 2007*; *Jafari, Assadpoor, He, & Bhandari, 2008*). Emulsion viscosity and particles size distribution of the emulsion have great influence on the process itself and on the final product.

Fig. 1.5 shows the basic structure of a spray dryer. The process comprises three main phases: 1) atomization, 2) drying and 3) powder collection.

1) *Atomization* refers to the process of simultaneous reduction of particle size. This is of great importance, because the formation of small drops exposes a very large surface area to the drying gas, therefore the heat transfer is facilitated, and the solvent evaporates within seconds. As a result, the drying materials never reach the inlet air temperature of the drying gas. This allows the employment of spray dry technology also for the encapsulation of heat sensitive bioactive (e.g probiotics, flavors and polyunsaturated fatty acids) (*Cal & Sollohub, 2009*).

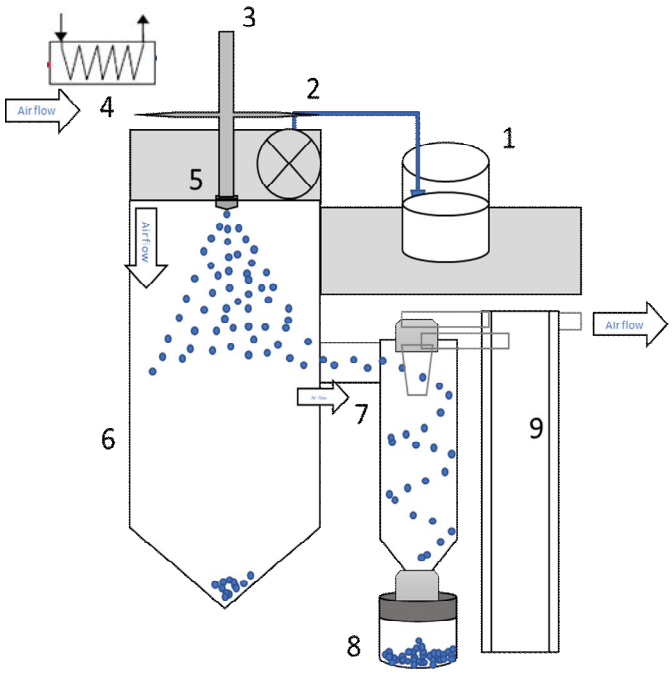
Commonly used atomizer types are pressure, centrifugal and pneumatic atomizer (*Gharsallaoui et al., 2007*). The important characteristic for a spray dry atomizer is to obtain uniform and homogenous drops (*Crosby, 1989*). This has great incidence on the quality and stability of the final powder.

2) *Drying chamber*: After atomization, the droplets are exposed to the action of a drying gas, usually atmospheric air. Air is directly drawn from the atmosphere, passed through a filter system and pre-heated (*Mujumdar & Law, 2006*).

In the chamber, the dispersion droplets move in various air streams, due to non-laminar gas flow.

Usually, drying times are of the order of 5–100 seconds (*Corrigan, 1995*). However, in a well-designed system 15–30 seconds is a fair time for the passage of the sprayed particle through the drying zone (*Fogler & Kleinschmidt, 1938*). The most frequently used chambers are the vertical ones (Fig.1.3), which are characterized by a cylindrical shape that convert to an inverted cone at the bottom.

Typically, the hot air inlet temperature may range from 150-220°C and evaporation occurs instantaneously. Consequently, the product is exposed to moderate temperatures (50-80°C), which mitigate the thermal degradation of the products (*Cal & Sollohub, 2009*; *Fang & Bhandari, 2012*).



Due to its assets, the machine is able to select particles with the right dimensions. Small droplets < 5µm are immediately dried upon contact with the drying air and are transported with the airflow. The medium-sized droplets have time to move out from the air inlet zone falling into the reversing whirl of the air occurring below the air inlet. The largest droplets escape the air whirl, and due to the large dimensions do not have the time to dry completely, thus depositing on the chamber wall (*Southwell, Langrish, & Fletcher, 2001*).

Fig.1.5. Typical schematic diagram of a spray dryer: 1 Feed solution, 2 peristaltic pump, 3 nozzle cleaning system (in the case of pneumatic nozzle), 4 hot air system, 5 atomizer, 6 drying chamber, 7 cyclone, 8 powder collector, 9 filter systems.

Particles with diameter < than 1 μm move with the exhaust air and are collected in special filter bags, positioned at the end of the operations unit.

New assets of spray dry comprise multi-stage spray dryers, that allow to increase the resident time of the product in the air-dry flow and to decrease the drying temperature, limiting in this way thermal denaturation of the product and rising process effectiveness (*Bimbenet, Jean-Jacques, Schuck, Pierre, Roignant, Michel, Brulé, Gérard, & Méjean, Serge, 2002*). Moreover, coupling physically a spray dryer with a fluidized bed on the drier outlet side, allows to better control particle size and moisture content (*Turchiuli et al., 2005; Cal & Sollohub, 2009*).

3) *Powder collection*: After the drying process, the particles descend towards the bottom of the chamber. The particles may settle on the bottom and be collected, or they may leave the chamber together with the outgoing air. In this case the separation takes place in cyclones or bag filters. Cyclones are the most used separating device (*Cal & Sollohub, 2009*). They consist of an upper cylindrical part, referred to as the barrel, and a lower conical part, referred to as the cone. The gas stream, loaded with solid particles, leaving the spray dryer enters tangentially at the top of the barrel and travels downward into the cone, forming an outer vortex. The increasing air velocity in the outer vortex exerts a centrifugal force on the particles, separating them from the gas stream. When the gas stream reaches the bottom of the cone, an inner vortex is created, thus reversing its direction and exiting out at the top as clean gas. The particulates fall into the collection chamber attached to the bottom of the cyclone (*Anandharamakrishnan, 2015*).

Particle shape is an index of many parameters such as particle size distribution, flowability, friability, bulk and particle density.

The interaction between variables such as inlet and outlet temperature, flow rate of drying gas, the feed properties (such as concentration, solute diffusion coefficient and solvent latent heat), govern the final particle morphology, which can be that of a hollow particle or of a solid spherical particle. Fig 1.6 shows different scanning electron microscope (SEM) pictures of particles obtained by spray dry technology representing different morphologies.

In spray dry process, significant parameters that need to be carefully evaluated are: inlet temperature and feed rate which coupled give control over the drying rate of the particles and final water content of the product (*Anandharamakrishnan, 2015*). The feed rate depends on the viscosity and surface tension of the feeding solution: solutions with low viscosity are atomized with a higher efficiency giving a more homogeneous final product.

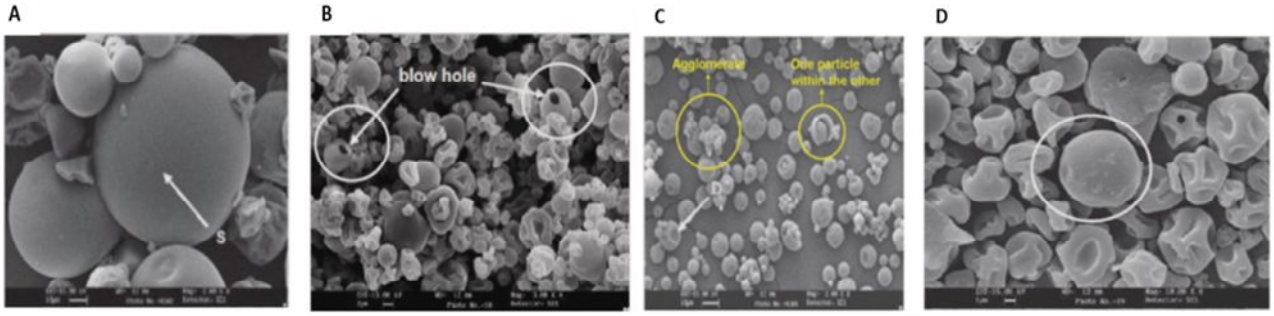


Fig. 1.6 Different morphology patterns of spray dried particles. (A) Smooth surface (B) Blow-hole formation (C) Agglomerate (D) Dented surface. From: (B and D) *Rajam et al., 2012*. (A and C) *Ezhilarasi et al., 2013*.

The outlet temperature is a parameter that cannot be directly controlled, because it depends on the drying characteristic of the material used. It is the highest temperature to which the product may be heated (*Cal & Sollohub, 2009*). Ideal temperatures of the final product have been identified to be in the range of 50-80°C in order to avoid thermal degradation of thermo-degradable products. The drying air flow, if possible need to be adjusted to the maximum permitted in order to maximize operation efficiency of the cyclone (*Maury, Murphy, Kumar, Shi, & Lee, 2005*).

Because of the complexity of the various spray-drying steps, spray-drier procedures have been traditionally based on experience, trial-and-error, and pilot scale work. The advantage of this technique is that it is economical, flexible and continuous. It can produce particles of good quality with diameters of less than 40 μm, a dimension that could leave the final product unaltered on a textural and sensorial perspective (*Crosby, 1989*; *Anandharamakrishnan, 2015*).

The main limitation of the spray-drying technique in microencapsulation is the limited number of wall materials available, which must have a good solubility in water. Another disadvantage of spray-drying that should be considered is that it produces a fine powder which needs further processing, such as agglomeration (*Gharsallaoui et al., 2007*).

1.12 Spray chilling/cooling

This is a technology in which the opposite principle of the spray drying is applied, i.e. the process of solidifying an atomized solution in particles. As in spray drying, the three main phases consist in: 1) atomization, 2) particles formation and 3) collection of the particles. The primary difference is given by the particles formation zone, where the particles are formed from the cooling and hardening, and not as a consequence of solvent evaporation (*McClements, 2015*). The second one, is the nature of the carrier material used primarily constituted by lipids deriving from vegetable oil with melting point in the range of 45-122°C (fats and stearins) and 45-67°C (mono and di-glycerides) (*Anandharamakrishnan, 2015*).

The feed preparation starts with the dispersion of the active material in the matrix solution. The basic process consists in the feed of a slurry or a homogenous mixture of the active ingredient and the matrix encapsulant into a nozzle system. The nozzle atomizes the mixture into a chamber where the droplets are cooled or gelled into particles. The formed particles are then collected through a cyclone, electrostatic collector, liquid hardening bath, powdered beds or filter bag system depending on the machine design. Particles solidification takes place in the cooling chamber, this process is dependent from the cooling chamber characteristics and material properties. The cooling chamber needs to maintain a temperature below the melting point of the solid during the entire atomization process. Different methods of droplet formations are available, but the most common is the spray-atomization one. This system (showed in Fig.1.7) ranges in capacity from less than 1kg/hr to tons/hr. The particle size of the final products could go from 40-100µm, depending on the type of atomization unit used.

Spray chilling requires to use ingredients for encapsulation that will solidify when atomized in the presence of low temperatures (*Desai & Park, 2005)*. The two most common categories of material used with spray chilling are melts and gels. Melts are composed of a molten matrix material, such as wax or fat (e.g polyethylene glycol, bees wax, palmitic and stearic acid, carnauba and candellia wax, shellac). An advantage derived from the use of these materials, is that since there is no need for evaporation, the production rate is equivalent to the feed, assuming 100% recovery of the atomized material. Gels are composed of matrix material dissolved in water (e.g gelatin, carrageenan, agar, gellan gum, pectin and alginate). Differently from melts, gels often need to be dried after collection to remove water.

The main disadvantage of this techniques is represented from the fact that most of the microparticles produced are insoluble in water due to their lipidic coating.

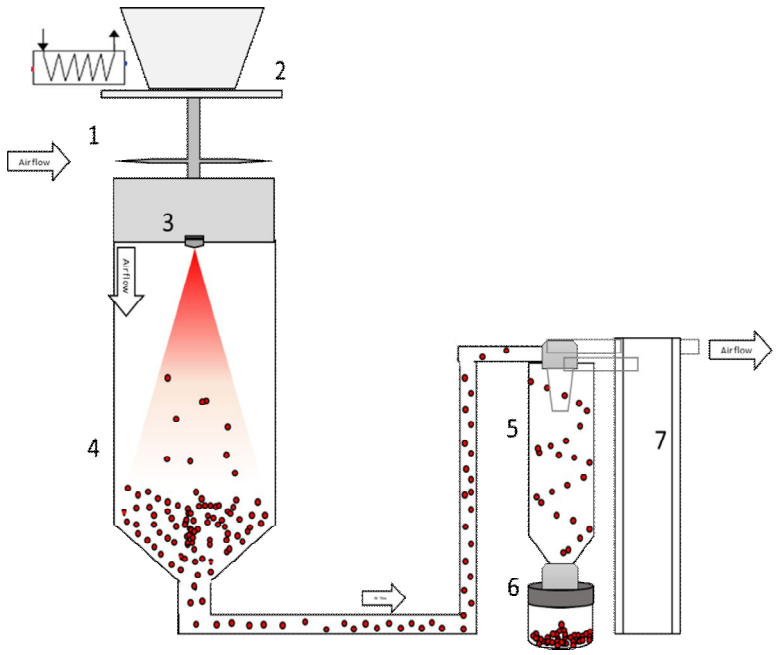


Fig. 1.7 Spray chilling schematic diagram: 1) Drying air; 2) hot/warm feed system; 3) nozzle system; 4) drying chamber; 5) cyclone; 6) powder collector; 7) filter bag system.

1.13 Fluid bed

Fluid bed was originally developed as a pharmaceutical technique, but it is now increasingly applied in the food industry (*Desai & Park, 2005; Shilton, & Niranjan, 1993*).

Fluidized bed was first developed in the 1950 by Dale E. Wuster, to encapsulate tablet with sugar for medical purpose (*Dixit & Puthli, 2009*). Today, this method is widely employed because thanks to the different assets of the machine it can be employed for freezing and cooling, drying, puffing, freeze-drying, spray-drying, coating, agglomeration and granulation.

The machine is basically composed by four major components showed in Fig.1.8: 1) an air handling system; 2) a product container; 3) an expansion chamber and 4) an exhaust system to retain particles within the processing chamber (*Burggraeve, Monteyne, Vervaet, Remon, & Beer, 2013*).

Fluidized bed technology is based on the separation of particles in a gas stream. The operating principle of fluidized systems is based on the fact that if a gas is allowed to flow through a bed of particulate solids at velocities greater than the settling velocity of the particles, the solids become suspended in the stream of upward moving gas (*Dixit & Puthli, 2009*). The gas stream negates the gravitational pull due to the weight of particles enabling the suspended state of the solid. The definition “fluid bed” describes the behavior of the solid particles that act like a fluid (*McClements, 2015*).

The machine design is classified according to the spray direction of the feeding solution from the nozzle (Fig.1.8) as:

A) Bottom/Wuster spray (Fig.1.8 A): the nozzle is positioned at the base of the chamber, in the middle of the distributor plate, and liquid is sprayed in the same direction of the fluidizing air.
B) Top spray (Fig.1.8 B): the binder liquid is sprayed from the top down onto the fluidized bed, counter-currently to the fluidizing air.
C) Tangential spray (Fig 1.8 C): the nozzle is introduced at the side of the chamber, embedded in the powder bed during granulation.

The nozzle is used to facilitate the dispersion of a liquid into spray of small droplets. The most popular energy source for the nozzle is compressed gas. Of fundamental importance is the choice of the right amount of liquid and the volume of atomizing air used to produce the droplets, in order to obtain a final product with the desired characteristics. As a consequence of the modification of these two parameters the final product can be classified as a raspberry structure, an agglomerate or granules.

As a simple rule, larger particles will be better coated with a relatively low atomization air pressure, giving slightly larger particles as a final product. Larger droplet size can cause agglomeration between the particles, this could be an unwanted characteristic due to the risk of separation of the agglomerate in a later stage in the process (*Schaafsma, Kossen, Mos, Blauw, & Hoffmann, 1999*).

Fluid bed machines require a large volume of air to maintain the particles suspended in the air. In order to avoid the blow out of the particles, filters are an unavoidable part of the equipment. They are made of textile and the pores are large enough to permit the passage of the air, but small enough to retain the particles. The filter can also be composed of stainless steel and Teflon.

From the encapsulation point of view, in this technology the bioactive food, in the form of powder, is suspended in an air flow and the matrix components are sprayed onto the bioactive components. This forms an encapsulate (*Champagne & Fustier, 2007*).

In particular, granulation process via fluid bed, applied to enhance wettability and flowability properties of powder is the most important application of fluid bed. It requires the suspension of particles in a conical shaped container by the use of a (heated) air stream. The applied air velocity should allow proper particle movement in the container but keep the material out of the filter bags (Fig. 1.8. Good fluidization can be visually monitored by the free downward flow of the granules at the windows of the container.

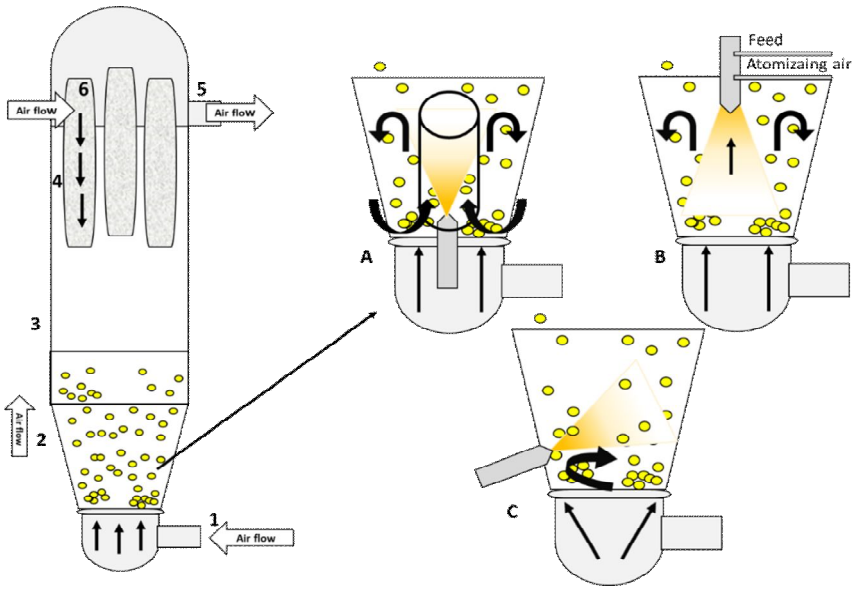


Fig. 1.8. Fluid bed schematic diagram: 1) Process air inlet; 2) coating chamber; 3) expansion chamber; 4) filters system; 5) air outlet; 6) pulse air; in the right part is represented the machine design referred to the nozzle position: A) bottom/Wuster spray, B) top spray and C) tangential spray.

As particles move up and down in the container, a binder solution is sprayed. Binder liquid droplets are deposited onto the fluidized particles in the spray granulation zone. After spraying the required amount of binder liquid, further fluidization enables rapid drying of the particles. Drying reduces the residual moisture of the granules to a level that ensures the stability of the final product. A good granulation is achieved when particles are uniformly mixed and liquid bridges between the particles are strong and easy to dry.

In food industry this technology gives the opportunity to deposit a multi or single layer of encapsulant matrix on powdered ingredients. The deposition of the layers can present different features: protection, target release, avoid the stickiness of the product during storage, taste and odor masking. This technology gives also the opportunity to increase the particle size of fine powder through agglomeration, a process in which primary particles are joined together so that bigger porous secondary particles (conglomerates) are formed (*Palzer, 2005*).

Nowadays, this process is widely used to obtain instant powder, which that are quickly dispersible with minimum stirring (*Freudig, Hogekamp, & Schubert, 1999*). The materials used for coating are mainly water-soluble biopolymers, lipids, proteins, gums, carboxymethylcellulose, sodium alginate, pectin, kappa-carrageenan, gelatin and starch hydrolysates (*Dewettinck & Huyghebaert, 1999*; *Meiners, 2012*) .

The main advantage of this technique is the possibility to increase the size of fine powder, with an improvement in the handling, wettability, flowability and compressibility. The disadvantages are the low yield and scarce uniformity of the final product (*Liske, & Mobus, 1968*).

1.14 Freeze drying

This technology involves the crystallization of a solution or suspension at low temperature, followed by its sublimation from the solid state directly into the vapor phase (*Liu, Zhao, & Feng, 2008*). This technique comprises three main stages: 1) freezing, 2) primary drying and 3) secondary drying. In the first stage the sample is crystallized to ice at sub-zero temperatures. The primary drying involves the sublimation of the crystal ice formed during the first step. Sublimation is an endothermic process that takes place at low values of pressure and temperature. This process leaves in the lyophilized structures pores, that gives to the product excellent rehydration ability. The secondary drying is related to the desorption of unfrozen water at a comparatively higher temperature than that of primary drying.

As an encapsulation technique, the core and wall are dissolved and then dispersed in water. The solution is then frozen and dried under low pressure conditions. The result is a slab of dried product which is flaky in shape.

The particle size of the encapsulates after freeze drying is in the range of 20–5000 μm, and the structure is of the matrix type (*Zuidam & Shimoni, 2010*).

This technique is used also in order to stabilize and dry particles obtained with other techniques (e.g. emulsion, coacervation and extrusion) that for example suffer of high heat instability.

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Chapter 2.

Preparation and stability evaluation of astaxanthin-containing protein-based nanoparticles

Abstract

Astaxanthin (3,3' dihydroxy-β,β-carotene-4,4'dione) (ASX) is a carotenoid of industrial and commercial interest due to its potential health benefits but its use in the food, feed and pharmaceutical fields is limited due to low bioavailability, scarce stability to thermo-chemical treatments, susceptibility to oxidation, and poor organoleptic characteristics. The aim of this work was to develop a method to stabilize astaxanthin from *Haematococcus pluvialis* oleoresin and to improve its bioavailability through nano-encapsulation. Nano-particles (NPs) were produced by emulsion solvent-evaporation technique using whey proteins concentrate (WPC) as stabilizer. The efficacy of encapsulation was 96%. The particle size was found to be in the range of 80-130 nm. Superficial charge of the capsules was negative. The shelf-life of the NPs was investigated by an accelerated system. Encapsulated ASX was found to be more stable to chemical and light stresses in comparison to the starting oleoresin. A simulated gastro-enteric digestion was conducted to study the release in physiological conditions. This showed that the bioaccessibility of astaxanthin was 93%. The data collected suggest that WPC ASX NPs might have possible future applications as supplements for human and animal diets.

Keywords: *Encapsulation*, *astaxanthin, Haematococcus pluvialis, stability, nanoparticles, bioaccessibility.*

1. Introduction

Free radicals and oxidative stress are acknowledged to cause damages to important biomolecules such DNA, lipids and proteins and for this reason they are correlated to cancer, cardiovascular disease, diabetes, autoimmune and neurological disorders (*Surh, 2003*). There is an increasing knowledge given by scientific, clinical and epidemiological data of a direct link between the daily assumption of antioxidants, such as carotenoids and polyphenols, and a lowering effect of such diseases (*Nishino et al., 2002*). Astaxanthin (ASX) (3,3'-dihydroxy-β-β'-carotene-4,4'-dione) is a symmetric ketocarotenoid widely distributed in nature, responsible for the bright red to pink color of some crustaceans, salmons and birds. The major natural source of ASX is *Haematococcus pluvialis* (*H.p.)* a freshwater algae which under stress condition can accumulate 2-5.3% of ASX on the dry weight (*Ambati, Siew Moi, Ravi, & Aswathanarayana, 2014*; *Poonkum, Powtongsook, & Pavasant, 2015*). This molecule attracted the interest of the scientific community thanks to its well-known antioxidant capacity, estimated as 10 times greater than other carotenoids, including lutein and β-carotene, and as 100 times higher than alpha tocopherol (*Higuera-Ciapara, Félix-Valenzuela, & Goycoolea, 2006*; *Naguib, 2000*; *Kobayashi & Sakamoto, 1999*). It has been proved to be effective against diabetes, cardio-vascular disease, some type of cancers, gastric ulcers and in skin protection against UV rays (*Guerra & Otton, 2011; Pashkow, Watumull, & Campbell, 2008*; *Nakao et al., 2010*).

The enrichment of food with ASX is limited due to the high instability of the carotenoid to light, high temperatures and extreme pH due to its conjugated structure (*Martínez-Delgado, Khandual, & Villanueva–Rodríguez, 2017*; *Kittikaiwan, Powthongsook, Pavasant, & Shotipruk, 2007*). In addition, the strong lipophilic nature of ASX underlies its scarce bioavailability (*Anarjan, Tan, Nehdi, & Ling, 2012*).

ASX is commercially available mainly in the forms of ASX oleoresin and *H.p.* aplanospores. *H.p.* oleoresin, is a dense oil, very difficult to manipulate due to the high insolubility in water. Furthermore, it does not meet the consumer perception due to the algae-related taste and smell. Aplanospores represent the *H.p.* growth stage in which ASX accumulation reaches the maximum yield. These cells are collected, dried, physically broken and used directly as a supplement. It is important to underline that aplanospores are characterized by three walls made of sporopollenin and algenan (*Hong et al., 2018;Huang, Liu, Sun, Xue, & Mao, 2018*) that act as a natural barrier against

oxidation and chemical attack (*Mendes-Pinto, Raposo, Bowen, Young, & Morais, 2001*) but also as an obstacle for human digestive enzymes (*Krichnavaruk, Shotipruk, Goto, & Pavasant, 2008*).

Different technologies have been employed to reduce ASX degradation and to increase its bioavailability. These include the encapsulation by different approaches using a number of shell materials (chitosan, alginate, pectins, maltodextrin etc. (*Vakarelova et al., 2017*; *Kittikaiwan et al., 2007*) but these structures are sometimes difficult to be digested due to the lack of specific enzymes. In some other cases the micrometer range of the obtained capsules require a mechanical disruption to release ASX. Another approach employed is represented by solid lipid nanoparticles (SLN) (*Tamjidi, Shahedi, Varshosaz, & Nasirpour, 2017*; *Li, Zahi, Yuan, Tian, & Liang, 2016*) but this system sufferes thermal instability due to the relatively low melting point of the fats employed, that does not allow the inclusion of SLNs in cooked or pasteurized products. In another work chitosan was used to protect ASX against oxidation with great success (*Higuera-Ciapara, Felix-Valenzuela, Goycoolea, & Argüelles-Monal, 2004*; *Kittikaiwan et al., 2007*) , but the insolubility in water and at neutral pH of chitosan, and its scarce release properties render this solution not suitable for many food applications.

Besides improving the stability of active molecules, nanoencapsulation represents a strategy to improve also their bioavailability. Indeed, the incorporation of ASX and other carotenoids in colloidal systems with dimensions close to 100 nm could improve the cell uptake of compounds with a bioavailability inversely proportional to the diameter of the nanostructure (*Anarjan & Tan, 2013; Anarjan et al., 2012*; *Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013*).

In the present work whey proteins, a generally recognize as safe (GRAS) material with high nutritional value, have been employed as a carrier to produce a water-dispersible ASX formulation particularly suitable for human consumption. The incorporation of carotenoids in a nanocarrier poses relevant stability issues. Indeed, in comparison to a microencapsulated form, the higher surface/volume ratio typical of nanocarriers can promote oxidation (*Britton, 1995*). For this reason, NPs stability was assessed in different conditions of pH, in presence of oxidant species, and upon UV light and heat treatment. The release of ASX in al conditions and the bioaccessibility were studied by simulated digestion.

2. Materials and methods

2.1. Chemical and reagents

Whey protein concentrate (WPC) 80 was gently provided by I.T.ALI. (Reggio-Emilia, Italy). The protein content was 80% (w/w). *Haematococcus pluvialis* powder was obtained from a local supplier. Ethyl acetate, acetone HPLC-grade, pepsin, pancreatin, trypsin, sodium cholate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Astaxanthin extraction from *H.p.*

H.p. oleoresin was obtained using the protocol proposed by Bustos-Garza and co-workers with minor modifications (*Bustos-Garza, Yáñez-Fernández, & Barragán-Huerta, 2013*). Briefly, the algae powder were pretreated by mixing 5 g of algae and 1 ml of 3 M HCl and treating the sample in a microwave oven for 1 min at 100 W (*Sarada, Vidhyavathi, Usha, & Ravishankar, 2006*). The pretreated algae were extracted with 25 ml of ethyl acetate in a tube with a screw cap, for 60 min under agitation at 50 °C in a thermal bath. The solid portion was separated by centrifugation at 3000 g for 10 min to eliminate the biomass. The oleoresin was kept in the dark at 4 °C until use.

2.3. Spectrophotometric analysis

2.3.1. ASX quantification

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

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

Where [A] is the concentration of ASX expressed as mg/ml; A_{480} the sample's absorbance at 480 nm; DF: dilution factor; $E_{(1\%;1cm)}$: ASX percent solution extinction coefficient [$(g/100\ ml)^{-1}\ cm^{-1}$] in ethyl acetate (2150); d: the optical path (cm).

2.3.2. Turbidity analysis

Turbidimetric analysis was performed by monitoring the absorbance at 660 nm (*Tang, Wang, Yang & Li, 2009*).

2.4. HPLC analysis

Reverse phase HPLC of astaxanthin-containing samples was performed with a Beckman System Gold (Beckman Coulter) on a C30 column (4.6 x 250 mm, particle size 5 µm) (YMC Europe, Schermbeck, Germany) following a previously described method (*Reyes, Mendiola, Ibanez, & del Valle, 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.

2.5. Astaxanthin nanoparticles preparation

Astaxanthin nanoparticles (ASX NPs) were produced following the method described previously by (*Yi, Lam, Yokoyama, Cheng, & Zhong, 2015*) with some modifications. WPC was dissolved in distilled water in a concentration range between 1 and 10%. The solution was stirred for 30 minutes at room temperature. *H.p.* extract was differently diluted in ethyl acetate and combined with the protein solution at a ratio of 9:1 (protein solution:oleoresin). A fine emulsion was produced using an ultra-sonicator for 10 minutes at a potency of 10 W (Microson ultrasonic cell distruptor XL). At the end of the process ethyl acetate was removed using a nitrogen flow in the dark. The WPC ASX NPs were kept in the dark at 4 °C until use. (Different methods, e.g use of high shear homogenizer, sonicator and combination of them, intensity and times were evaluated as well for the production of ASX NPs).

2.6. Characterization of astaxanthin nanoparticles

2.6.1. Surface charge and average diameter

Zeta-potential, mean diameter and polydispersity index (PDI) of WPC-ASX nanoparticles were analyzed by dynamic light scattering principles using a Malvern Zetasizer (Nano-ZS; Malvern

Instruments, Worcestershire, U.K). Prior the analysis the sample were diluted 80 times to avoid multiple scattering effect. PDI value ranging from 0 to 1, indicated the distributions of the particle sizes, value close to 0 indicated a uniform population of particles and a value close to 1 indicated a wide variety of dimensions among particles size distribution. Zeta potential give an important information about particles stability, for values closer to 0 the system is considered not stable, due to the absence of a net charge that can contrast the aggregation process of NPs.

2.6.2. Encapsulation efficiency

WPC ASX NPs were treated enzymatically with 2 mg/ml of trypsin for 4 h at 37°C in a thermo-shaker (Biosan, Riga, Latvia). The enzymatically digested solution was mixed with a double volume of ethyl acetate and placed on a rotating shaker for 60 minutes. The solution was centrifuged at 12,000 g for 5 minutes to allow the separation of the two immiscible phases. ASX was recovered from the upper phase, diluted opportunely and quantified by spectrophotometry as described above. The efficiency of encapsulation was estimated by the subsequent formula:

$$\text{Encapsulation Efficiency \%} = (ASXf/ASXi)*100$$

Where ASXi represents the initial amount of ASX loaded in the NPs, whereas ASXf refers to the amount of ASX recovered from NPs after the breakage of the protein shell via enzymatic extraction.

2.6.3. Chemical stability

The chemical stability of ASX NPs was tested in solutions with different pH values (1-10) adjusted using NaOH or HCl solutions. The stability was evaluated using a UV/VIS spectrophotometer (Unicam UV2) reading the absorbance at 650 nm as a turbidimetry index.

2.6.4. Oxidation stability

Stability of WPC ASX NPs and ASX from *H.p.* extract against chemical oxidation was analyzed according to the method proposed by Pan and co-workers (*Pan, Yao, & Jiang, 2007*) with some modifications. Briefly, the *H.p.* oleoresin was solubilized in Dimethyl sulfoxide (DMSO) and diluted in water to reach a final concentration of 7.5 µg/ml. The samples (980 µl) were mixed with 10 µl of a $FeCl_3$ solution (350 µg/ml) and incubated at room temperature for 24 hours. At different

time points: 20, 60, 180 and 1440 min. , an aliquot of the solution was analyzed and the % of ASX retained evaluated. The % of residual ASX from NPs was analyzed through enzymatic digestion with trypsin as previously described. The sample containing *H.p.* oleoresin was directly extracted in a double volume of ethyl acetate. Prior the extraction a solution of ascorbic acid (10 µl from a 1.3 mg/ml stock solution) was added to the sample in order to stop the oxidative reaction.

The % of ASX retained was evaluated using the following equation:

$$ASX\ \% = (ASXr/ASXi) * 100$$

where ASXr corresponds to the amount of ASX retained after the exposure to the oxidation condition and ASXi is the initial amount of ASX determined through enzymatic extraction after encapsulation process.

2.6.5. UV stability

The stability against UV-B light of WPC ASX NPs and *H.p.* oleoresin (solubilized in DMSO as described above) was studied using a trans-illuminator (Bio-Rad, Hercules, CA, USA). During the exposure aliquots of the samples were taken at different time points: 5, 30, 60 and 120 min. . The % of residual ASX was determined by spectrophotometry as previously described.

2.6.6. Storage stability

Storage stability of WPC ASX NPs and *H.p.* oleoresin was evaluated at 65°C. The samples were kept in a static oven (Memmert, Germany) in a closed tube. To study the stability of the *H.p.* oleoresin, ethyl acetate was removed to obtain only the oleoresin from *H.p.* At different time points the samples were analyzed for the content of residual ASX by spectrophotometry and an HPLC analyses were performed.

2.6.7. Simulated *in vitro* digestion

In vitro digestion was performed following Infogest protocol (*Minekus, Alminger, Alvito, Ballance, Bohn, Bourlieu & Dufour, 2014*). Simulated gastric fluids (SGF) and Simulated Intestinal Fluid (SIF) were prepared following the protocol. The duration of the two phases were 1 h for the gastric

and 4 h for the intestinal phase. The enzymes used were porcine pepsin and pancreatin for the gastric phase (pH 3) and the intestinal phase (pH 7) respectively. Bile salt in the form of sodium cholate was added to SIF to a final concentration of 10 mM. Briefly, NPs and SGF were mixed in a ratio 1:1, the pH was eventually adjusted to 3 with HCl 1M. After 1 hour, SIF was added in a ratio 1:1 and the pH adjusted to 7 with NaOH 1M. The reaction was conducted at 37°C in a rotating shaker. Every 30 min. samples were extracted with a double volume of ethyl acetate. The amount of ASX released from WPC ASX NPs were evaluated by spectrophotometry. De-esterification degree was evaluated through HPLC analysis.

2.7. Statistical analysis

All measurements were performed in triplicate and the results were reported as mean value ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA).

3. Results and discussion

3.1. Physical evaluation of WPC ASX NPS

ASX was extracted from *H.p.* cells with a maximum yield of 1.1% w/w. HPLC analysis of the oleoresin was performed to understand if the microwave-assisted extraction and the thermal treatment at 50°C could have detrimental effects on ASX integrity. The HPLC profile showed the presence of ASX mainly in the mono-esterified form (80%), followed by the di-esterified form (18%) and a low amount of free form (2%). Traces of other carotenoids were observed but not quantified. This result, reflects with minor differences the ones found in literature (*Rao, Sarada, & Ravishankar, 2007*) regarding *H.p.* oleoresin.

WPC was employed as a biocompatible matrix to improve the bioavailability and dispersibility of ASX in water through emulsification-solvent evaporation technique. The process was optimized considering two parameters. The first parameter considered was WPC concentration. As reported earlier (*Yi, Lam, Yokoyama, Cheng, & Zhong, 2014*) the amount of the matrix material can influence not only the dimension of the NPs, because of the layering effect of the proteins that tend to form growing structures until a stable conformation is reach, but also the time requested to release the bioactive compound during digestion process.

The solution containing ASX NPs was transparent with a red-orange bright color (Fig. 4), but the increasing concentration of protein led to a loss of transparency as demonstrated by the increase of the absorbance registered at 660 nm (Fig.1).

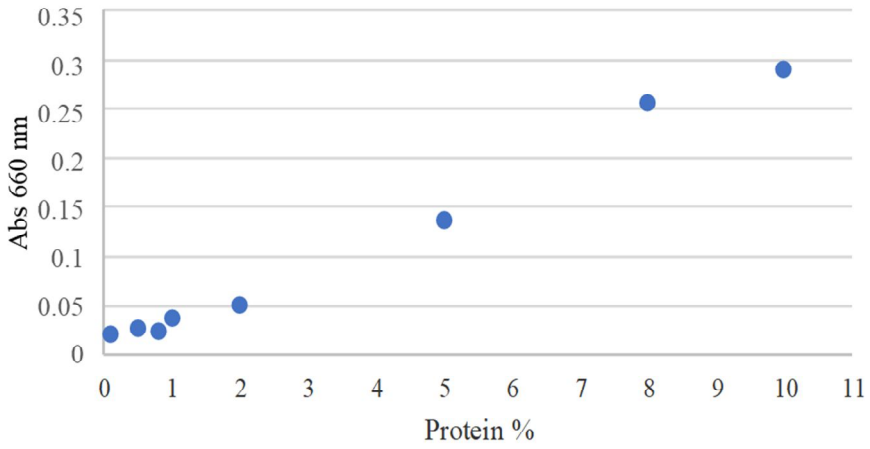
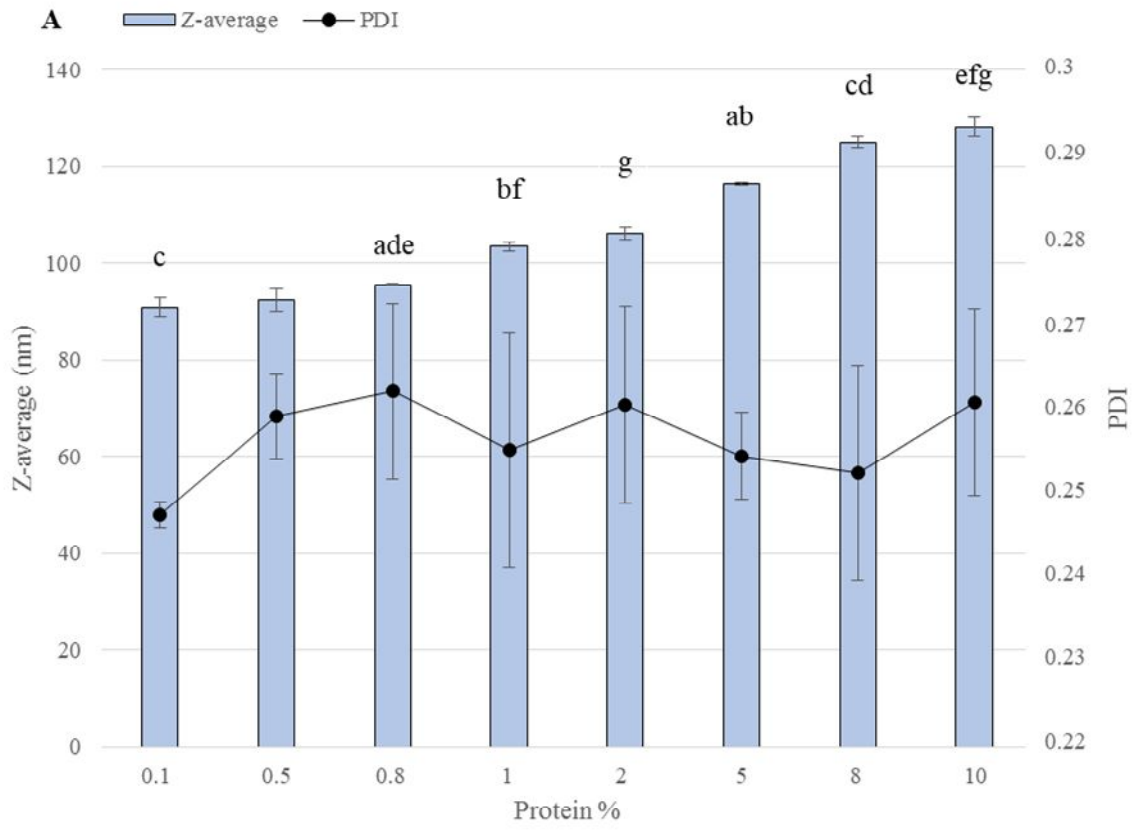


Fig.1. Turbidity of different WPC ASX NPs produced varying the WPC concentration.

The average size, PDI and zeta potential were considered as reference parameters to evaluate the process. As shown in Fig.2 A**,** the increase of proteins amount led to an increase of the dimensions of ASX NPs from an average size of 90 nm, obtained with the lowest concentration, to 128 nm with 10% WPC. The data showed an evident dependency of the dimensions from protein concentration. PDI values were low for all the formulations, indicating a homogeneous size NPs population, but no statistically significant differences were observed among the samples. Zeta potential values were highly negative (Fig.2 B), due to the WPC shell that at neutral pH are negatively charged. Values higher than +20 and less than -20 are normally associated to stable NPs (*DeLuca, Kaszuba, & Mattison, 2006*). This is because of strong repulsive forces that inhibit the aggregation phenomena. What we observed was a decrease of zeta potential value by increasing the % of WPC: the 10% preparation showed a zeta potential of -17.0 ± 4. Although the best values of zeta average, PDI and zeta potential were obtained with WPC concentration ranging from 0.1 to 1%, a rapid degradation of ASX was experienced at WPC concentrations from 0.1% to 0.5%. As a consequence, the subsequent experiments were performed using a WPC concentration of 1%.



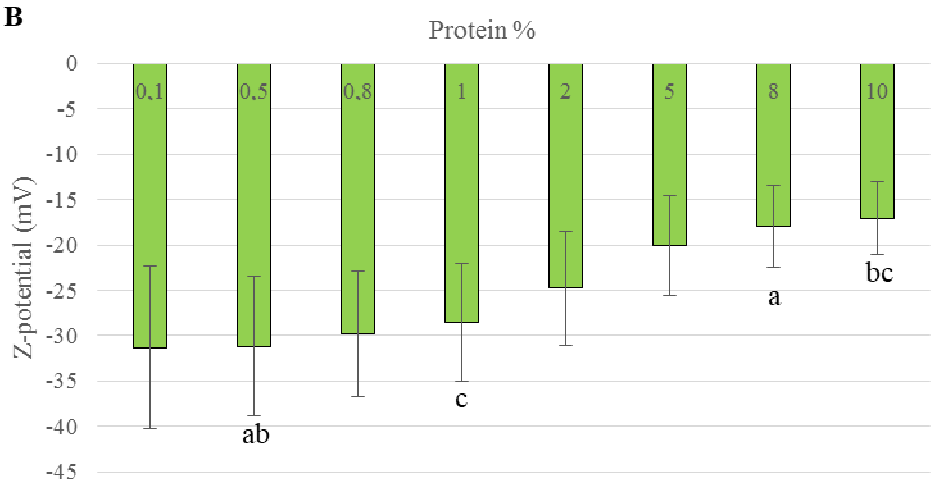


Fig. 2. Variation of size and PDI (A) and z-potential (B) as a consequence of the protein concentration used to produce ASX WPC NPs. Differences between values indicated by the same letter are statistically significant ($P<0.05$)

The second parameter considered was the amount of *H.p.* oleoresin used. As shown in Fig.3 (A), the diameter of ASX NPs decreases with the increase of oleoresin, reaching a minimum of 95 nm. This sample showed a surface charge higher than -20 mV (-17.9 ± 4.56 mV) (Fig.3 (B)), underlining the instability of the structure. This is possibly due to the high amount of oleoresin exceeding the amount of proteins constituting the shell. Also, in this case the PDI value were low and the differences given by the increasing amount of oleoresin were not significant.

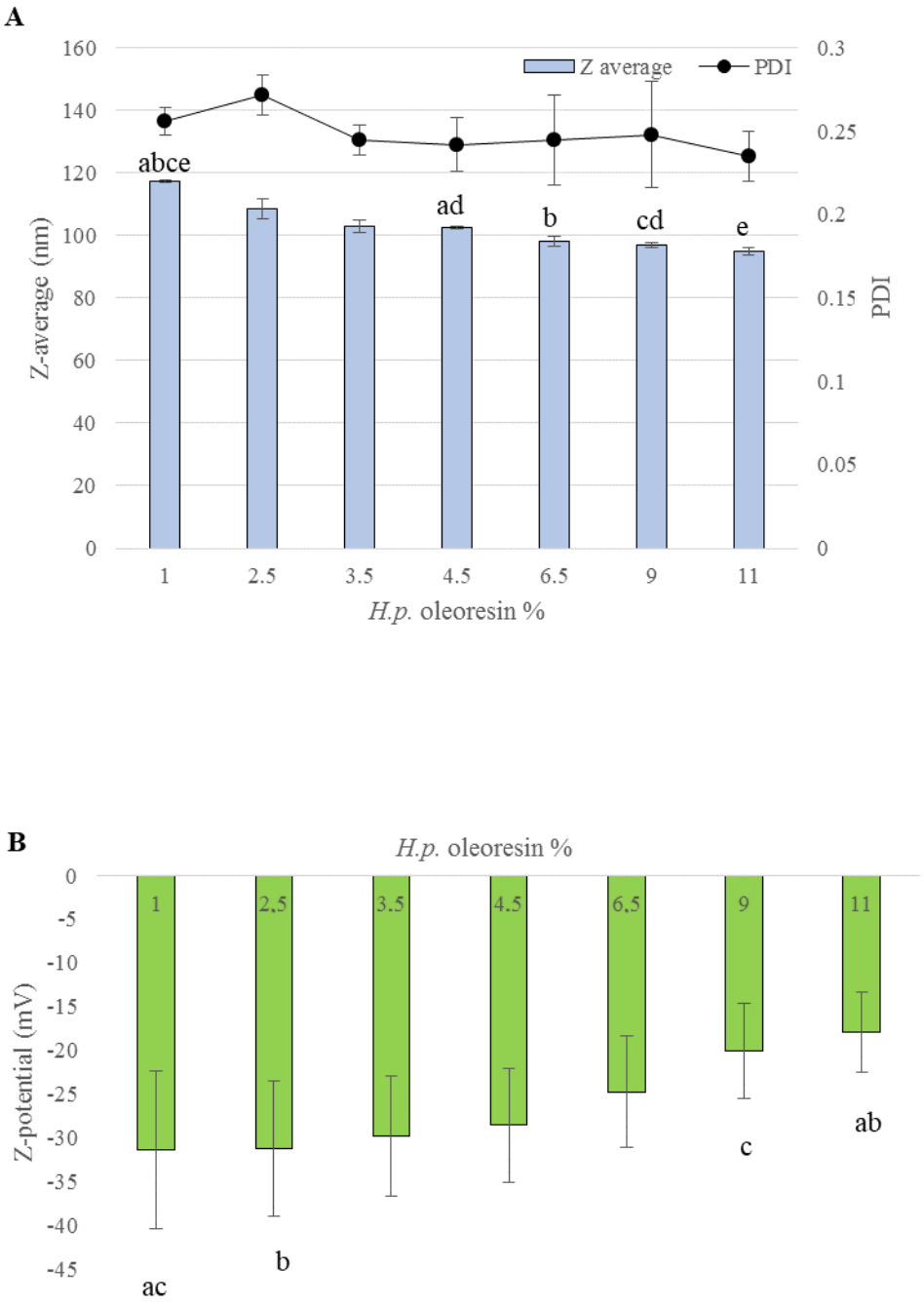


Fig.3. Variation of size and PDI (A) and z-potential (B) as a consequence of the different *H.p.* oleoresin concentrations used to produce ASX WPC NPs. Differences between values indicated by the same letter are statistically significant (P<0.05)

Satisfactory results were given by the NPs made with 4.5% of *H.p.* oleoresin with a Z-average of 102.7 ± 0.36, PDI of 0.242 ± 0.016 and a surface charge of -28.5 ± 6.5.

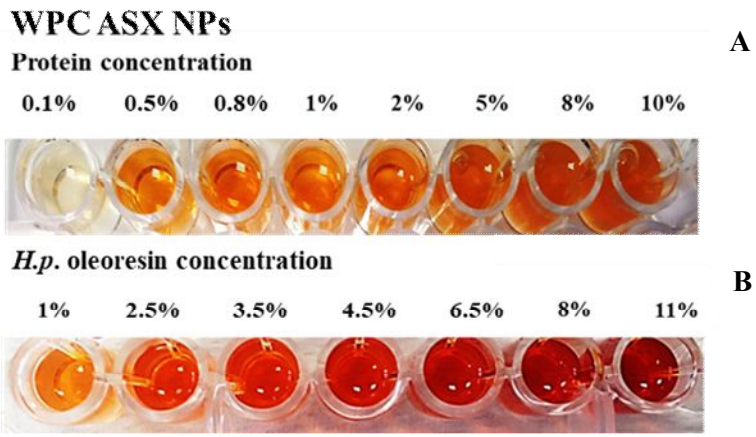


Fig.4. Appearance of WPC ASX NPs as a function of proteins concentration (A) and *H.p.* oleoresin concentration.

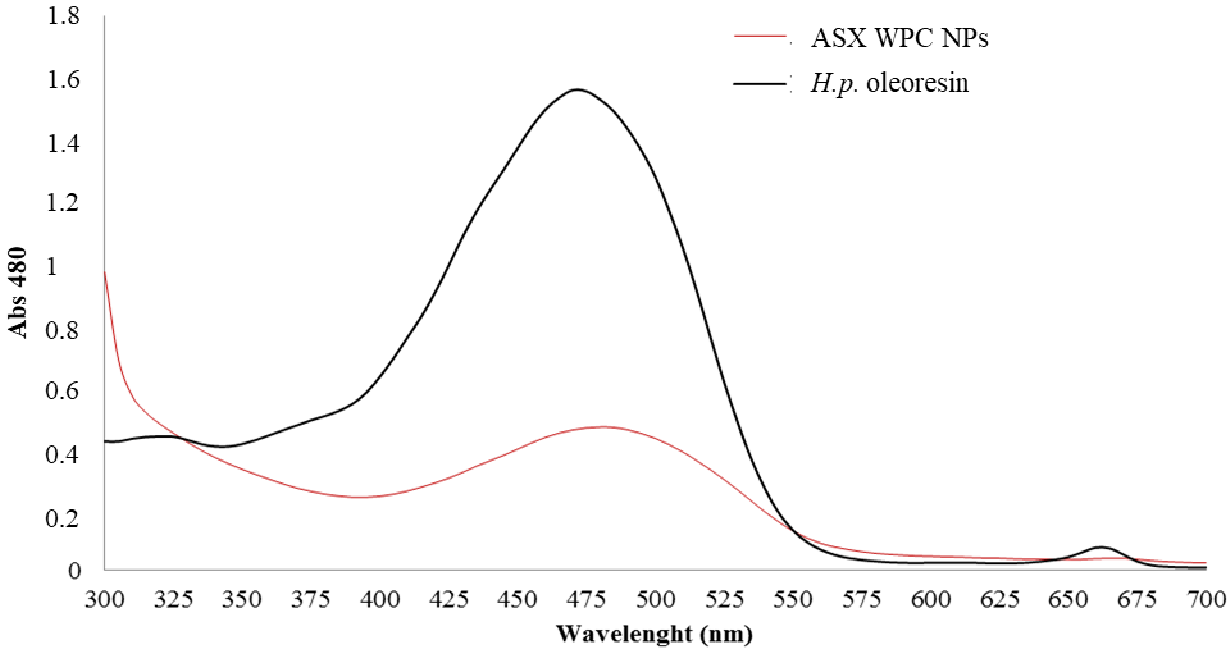


Fig.5. Comparison between the absorption spectra of the *H.p.* oleoresin and WPC ASX NPs.

A comparison between the absorption spectra of *H.p.* oleoresin in ethyl acetate and WPC ASX NPs in water revealed a small shift of the maximum absorption of ASX from 480 nm to 470 nm after encapsulation process. This might be due to the presence of the protein shell together with the fact that the solvent is necessarily different. In the case of WPC ASX NPs, a great absorption at

wavelengths <300 nm given by the presence of proteins was detected. On the whole the absorption characteristics of encapsulated astaxanthin in the visible spectrum are conserved.

The encapsulation efficiency was 96 ± 2.5%. The minor loss of ASX could be caused by the oxidation generated from the sonication process, or from the incomplete degradation of the protein shell during the enzymatic extraction. HPLC analysis of the extract from WPC ASX NPs was performed and compared to the one of the *H.p.* oleoresin before the encapsulation process (Fig.6). No qualitative differences were observed, indicating that encapsulation process did not affect the nature of the esters distribution. It could be interesting to evaluate if the process leads to the formation of *cis* isomers by a different chromatographic approach. The biological activity of these isomers is still matter of debate (*Gòmez-Estaca et al. 2017, Liu & Osawa 2007*).

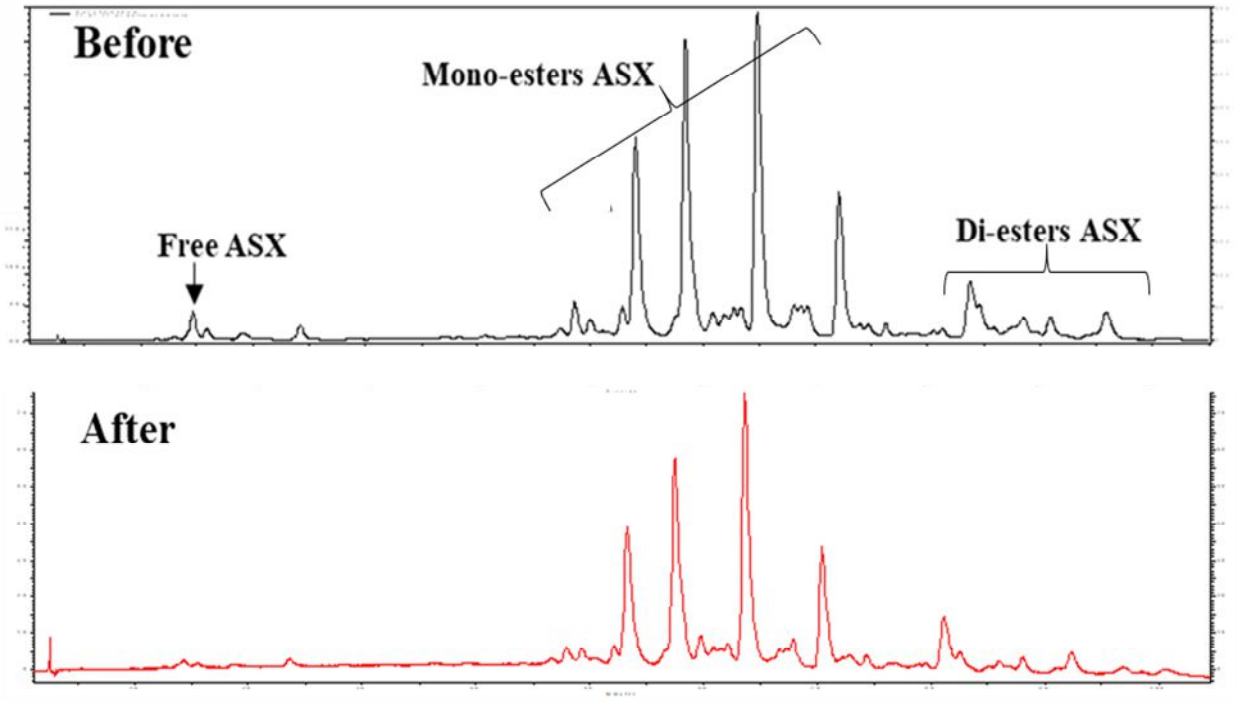


Fig.6. HPLC chromatograms of *H.p.* oleoresin composition showing free, mono-esters and di-esters of ASX before and after encapsulation.

3.2. Characterization of WPC ASX NPs

3.2.1. pH stability

WPC ASX NPs stability was analyzed at different pH values. NPs were found unstable at pH between 3,5 and 5,5, giving the formation of aggregates that tend to precipitate. The pH range corresponds to the average isoelectric point of the shell proteins. Qian and co-workers (2012) reported that the aggregation of protein-stabilized nano-emulsions might originate from the small net surface charge registered at pH close to the pI of proteins, not sufficient to exert electrostatic repulsion among the particles (*Qian, Decker, Xiao, & McClements, 2012*).

3.2.2. UV degradation

As already reported, one of the major factors responsible for the degradation of ASX is light (*Vakarelova et al., 2017*). It is also acknowledged that ASX could function as a protective agent for human skin against UV rays (*Rao et al., 2013*). Given the importance of this aspect we decided to analyze the behavior of WPC NPs ASX formulation in comparison to that of *H.p.* oleoresin exposed to direct UV light. As shown in Fig.7, after two hours of exposure to UVB light the ASX % retained in WPC ASX NPs and in *H.p.* oleoresin was 70.5% and 4.1% respectively.

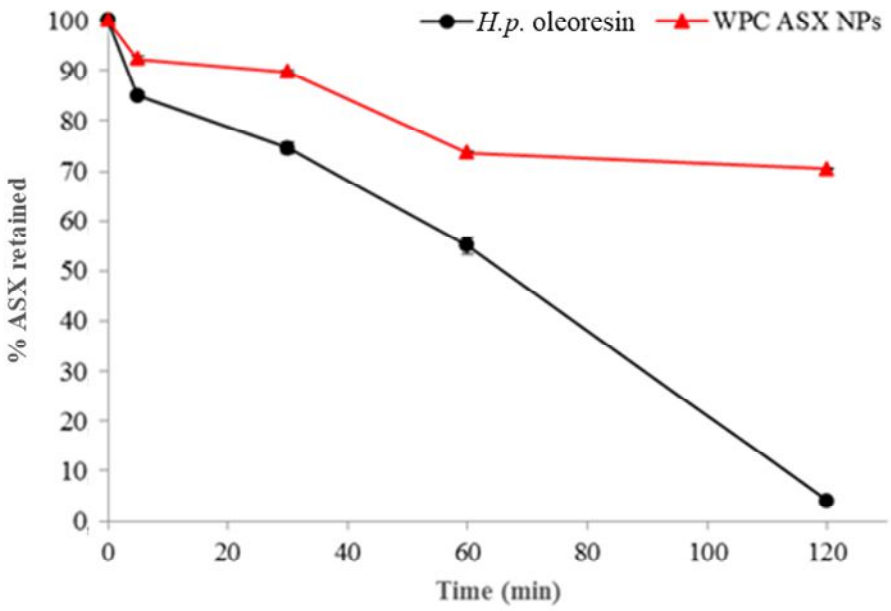


Fig. 7. Comparison between ASX retained in the WPI ASX NPs and *H.p.* oleoresin after exposure to UV rays.

This result shows that the protein shell could exert a protective effect towards the ASX contained in the NPs. The initial degradation of ASX from WPC ASX NPs could derive from the oxidation of the ASX exposed in the outer layer of the NPs, indeed this effect seems to reach a plateau after 1 hr of exposure to UV light.

3.2.3. Chemical degradation

Another factor that could affect the stability of ASX is the presence of oxidant species, i.e. iron, that is ubiquitous in food products and known as a harsh oxidizer. The physical barrier represented by the WPC shell and its intrinsic capacity to chelate metal ions could influence the stability of ASX contained in NPs (*Boon, McClements, Weiss, & Decker, 2010*; *Woodall, Lee, Weesie, Jackson, & Britton, 1997)*. To this purpose ferric chloride ($FeCl_3$) is commonly used as an oxidizing agent for studying carotenoid stability (*Boon et al., 2010*). The results reported in Fig.8 underline a different behavior between the samples: the *H.p.* oleoresin showed a pattern characterized by a rapid degradation kinetics within the first 20 min of exposure with a loss of nearly 40 % of the ASX content, followed by a slower rate of degradation until the end of the experiment with the retainment of only 5.6% ASX after 24 h. The WPC ASX NPs sample showed a slower decrease of ASX compared to the former. Indeed, after 20 minutes the amount of ASX retained was 95.4%. After 24 hours the amount of ASX was 31.1%. The results show a protective effect of the WPC protein shell towards Fe^{3+}-mediated degradation.

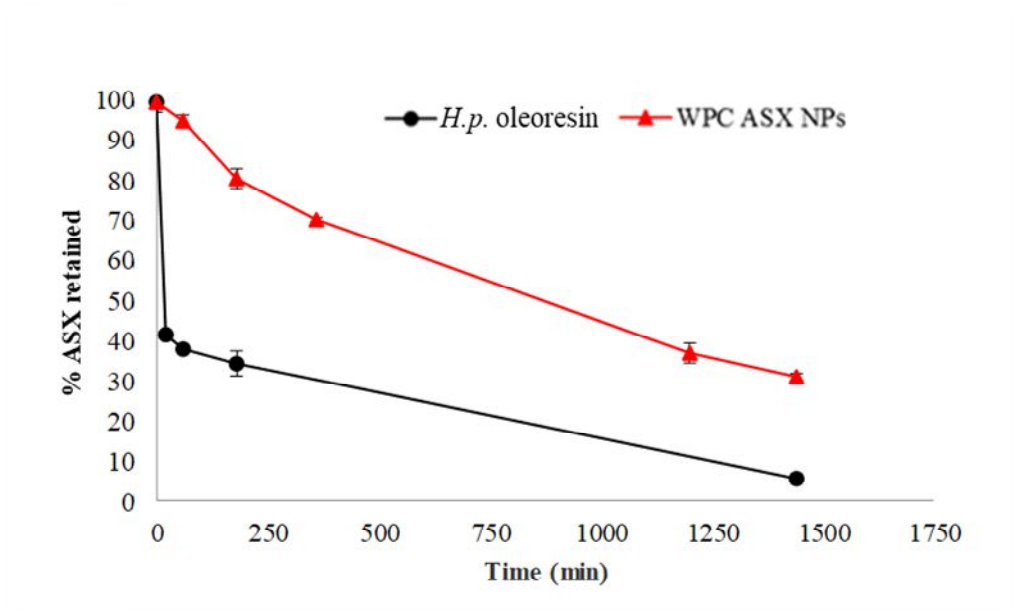


Fig. 8. Comparison between ASX retained in the WPI ASX NPs and *H.p.* oleoresin after exposure to $FeCl_3$.

3.2.4. Thermal stability

Schall oven test (*Slater, 1984*) is regularly applied to study the stability of lipophilic substances such as edible oils. This test was employed to study the thermal stability of WPC ASX NPs and *H.p.* oleoresin. The samples displayed similar ASX degradation rates (Fig. 9), with *H.p.* oleoresin characterized by a slightly higher stability until the 4th day. After this period an inversion of the degradation rates was observed. To further analyze the data obtained, first order kinetics equation was applied to describe the degradation kinetics of ASX (Fig. 10). This fit was chosen because already described for the degradation of ASX and other carotenoids (*Vakarelova et al., 2017*; *Lim, Burdikova, Sheehan, & Roos, 2016;Villalobos-Castillejos, Cerezal-Mezquita, Hernández-De Jesús, & Barragán-Huerta, 2013*) .

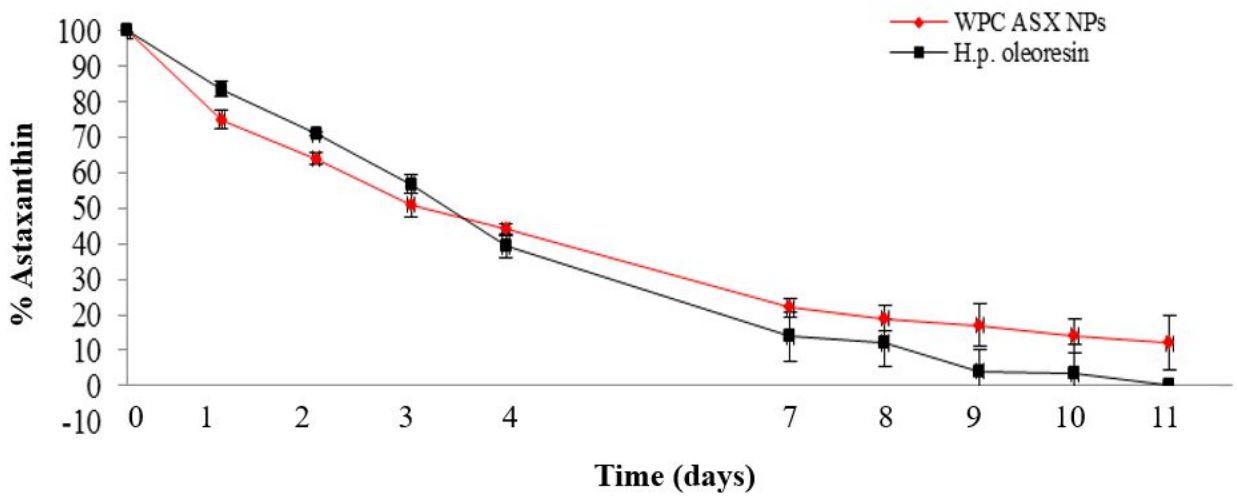


Fig.9. Comparison between WPC ASX NPs and *H.p.* oleoresin stability at 65°C.

A straight line was found from the fitting on the data obtained from WPC ASX NPs describing a first order degradation kinetics. Different was the result obtained with *H.p.* oleoresin: while until the 4th day a straight line was obtained from the plot, after this period an acceleration of the degradation rate was observed.

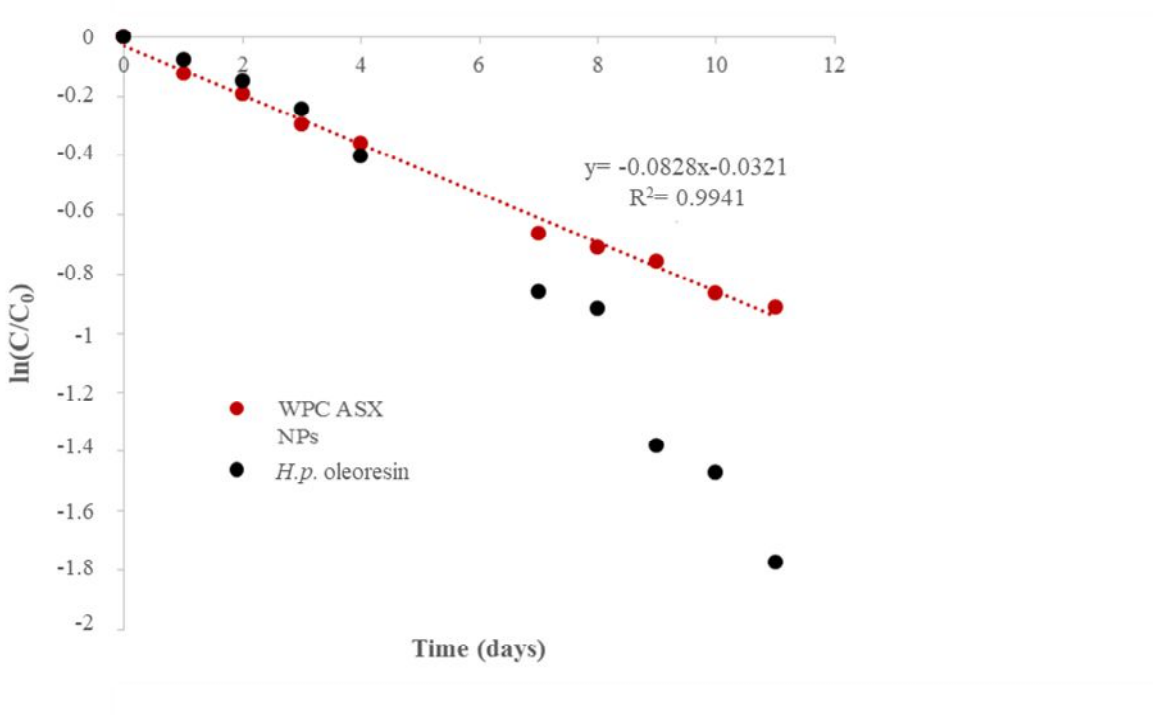


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Fig.10. Kinetics of degradation of WPC ASX NPs and *H.p.* oleoresin at 65°C.

The quicker degradation of *H.p.* oleoresin could derive from the absence of the protective glassy matrix present in the NPs system that allows for a faster accumulation of reactive degradation species (*Boon et al., 2010*; *Takeungwongtrakul & Benjakul, 2016*) originating from the oxidation. When these degradation species reach a certain concentration they could further oxidize the carotenoids present in *H.p.* oleoresin.

A gradual loss of color was observed for both the samples. As reported previously the auto-oxidation products of carotenoids do not present color properties due to the lack of chromophores at the absorption wavelength of visible light (*Wagner & Warthesen, 1995; Villalobos-Castillejos et al., 2013*). HPLC analyses of ASX extracted from WPC ASX NPs and the *H.p.* oleoresin underline the lack of a selective degradation of ASX: indeed, losses were observed for all the compounds present in the encapsulated *H.p.* oleoresin (data not shown). The absence of new peaks detected at 480 nm suggest the conversion of carotenoids in non-carotenoid products. It is reported that the thermal degradation of carotenoids in the presence of oxygen results in the formation of volatile compounds and larger non-volatile compounds (*Bonnie & Choo, 1999*). The scarce protection of WPC shell against temperature could be due to the high surface exposed of the NPs, that could lead to a major exposure of ASX to heat and as a consequence to the degradation of the ASX carbons chain.

3.2.5. Evaluation of bioaccessibility by simulated digestion

The absorption of carotenoids during digestion occurs mainly in the first part of intestine, where the uptake through the intestinal mucosa takes place (*Chitchumroonchokchai & Failla, 2006; Desmarchelier & Borel, 2017*).

The amount of ASX released, and thus bioaccessible, from NPs after the gastric phase was about 45% (Fig. 11). This release can be explained considering that during this phase an evident aggregation of the NPs was observable probably as a consequence of the low pH value close to the pI of the whey proteins.

During the intestinal phase the aggregates disappeared. This is confirmed also by the average size of the particles 165.5 nm and a PDI of 0.290. The release of ASX reached about 65 % after 2 hours of intestinal digestion, that, in this model, represents the small intestine (*Versantvoort, Oomen, de Kamp, Rompelberg, & Sips, 2005; Kopf-Bolanz et al., 2012*). After 4 hours of intestinal digestion the amount of ASX bioaccessible was about 90%.

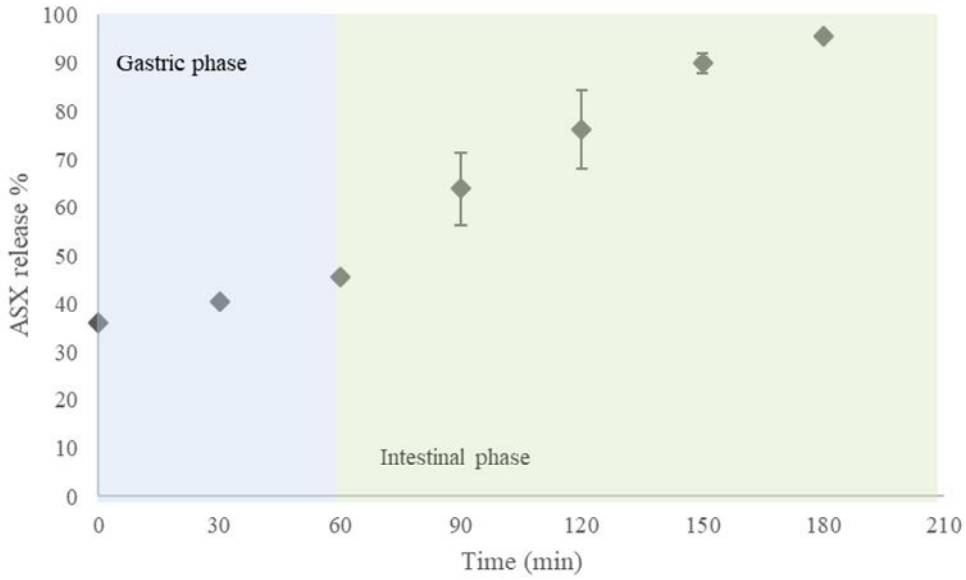

Fig.11. Release of ASX from WPC ASX NPs during *in-vitro* simulated digestion. $p<0.05$.

To evaluate the contribution of the buffer conditions to ASX release observed, a second SD in absence of digestive enzymes was performed. As shown in Fig.11 the incubation of the NPs with the simulant buffers produced a fast release of ASX (35,7%) in the gastric medium, not far from the 45% release shown in Fig. 11 at the same time point. This means that almost 80% of the released ASX was due to the acidic conditions that probably denatured partially the structure of the NPs, causing the release of the carotenoid. Note worthily, no ASX was released during intestinal phase in absence of enzymes, indicating that probably NPs structure was somehow restored (Fig.12). Hence the release of ASX in the intestinal phase was mainly due to the action of pancreatic proteases.

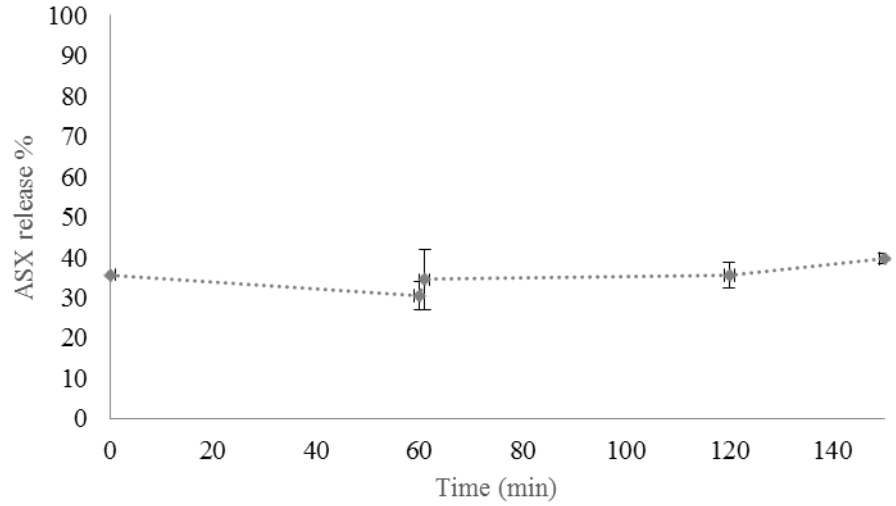

Fig.12. release of ASX from WPC ASX NPs during *in-vitro* simulated digestion in absence of digestive enzymes.

The simulated digestion (SD) was performed not only to address the bioaccessibility of the encapsulated ASX but also to evaluate possible chemical modifications of the carotenoid. HPLC analysis of the oleoresin extracted from NPs collected before and after digestion could give a picture of the variation of the esterification degree of ASX. Fig. 13 shows that the esters composition before digestion was mainly represented by monoesters and diesters, accounting on the whole for the 99% of the ASX present, while after two hours of the intestinal digestion the major form was represented by free ASX (66%). After 4 hr of digestion the conversion to the free form reached 75%. This result is of great importance because the de-esterification of carotenoids is a crucial step required for their uptake through the intestinal mucosa.

Montero and co-workers (2016) observed only 20% of total ASX in the free form upon digestion of a microencapsulated ASX system constituted of a gum arabic shell. The lower de-esterification experienced can be explained considering different factors: 1) the difference in the matrix

composition (whey protein vs gum arabic), 2) the oleoresin: matrix ratio [1:1 in our case, 1:10 in Montero et al. (2016)], and 3) the difference in surface to volume ratio, that in our case is higher being the particles diameter in the nano-range while the particles diameter described by Montero et al. measured was of few micrometers (3.3 to 6.8 µm) (*Montero et al. 2016*).

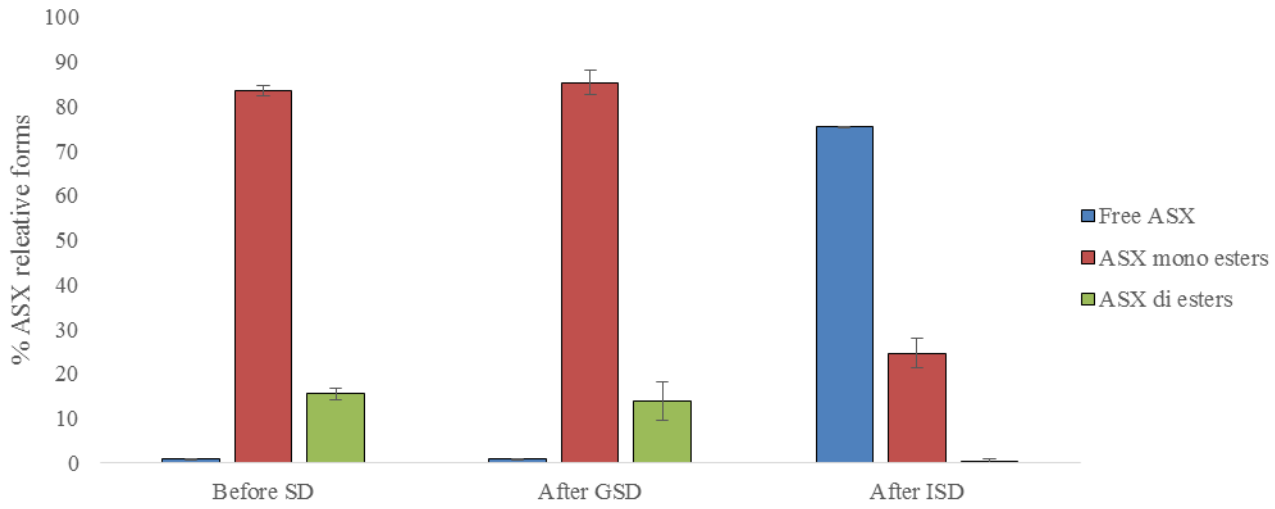

Fig.13. Concentration of the different chemical forms of ASX: free form, monoesters and diesters of ASX before simulated digestion (Before SD), at the end of gastric stage (After GSD) and at the 2nd hour of intestinal stage (After ISD).

4. Conclusion

In the present work ASX oleoresin obtained from *H.p.*, was successfully nano-encapsulated through emulsion solvent-evaporation technique. This allowed to disperse a strongly hydrophobic molecule like astaxanthin in water maintaining the light absorption characteristics of the carotenoid. In this work we described the optimization of different parameters to identify the conditions leading to best compromise between the lowest achievable diameter, the highest stability and the greatest payload. In particular, besides a scarce solubility in the pH range of 3.5-5.5, the NPs obtained showed a higher stability toward UV light, metal-induced oxidation and heat degradation with respect to the oleoresin. Simulated digestion of NPs showed a high ASX bioaccessibility (90%) with 75% conversion to the free form that represents the most bioavailable ASX form. The formulation can be considered a good candidate carrier for the delivery of high amounts of ready-to-absorb astaxanthin for human diet.

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Chapter 3.

Evaluation of different plants proteins for the nano-encapsulation of *H.p.* oleoresin

Abstract

The aim of this study was to research for plant alternative proteins as encapsulating matrices for astaxanthin in order to satisfy the increasing interest of the consumers for substitutes of animal-deriving ingredients. Astaxanthin (ASX), a ketocarotenoid with high antioxidant activity, was nano-encapsulated using soya protein isolate (SPI), pea protein isolate (PPI) and rice protein isolate (RPI) through emulsion-solvent-evaporation method. While by SPI and PPI it was possible to obtain nanoparticles (NPs) with diameters between 80-200 nm on average, and high encapsulation efficiency (EE), RPI did not allow to obtain positive results, since most of the ASX was not encapsulated, probably due to the scarce emulsifying properties of RPI. To further optimize the process, SPI and PPI proteins were subjected to different heat and pH treatments prior encapsulation. The results were analyzed on the basis of mean particle size, polydispersity index (PDI), Z-potential, encapsulation efficiency (EE) and presence/absence of ASX exposed on the surface of the NPs. The pre-treatments did not influence substantially the encapsulation efficacy of the proteins. The release of ASX in simulated digestive fluids from SPI and PPI NPs was compared to that of WPC NPs disclosed in the **chapter 2**.

Keywords: *Astaxanthin, Encapsulation, plant proteins, rice, soya, pea, simulated digestion, nanoparticles, emulsifier.*

1. Introduction

The enrichment of foods with lipophilic bioactive components, such as phytosterols, carotenoids and omega 3 fatty acids, is a growing area in the food industry (*McClements, 2010*). Nano-encapsulation, referred to the production of encapsulates with dimensions in the range of 1-100 nm, seems to be the most suitable approach to increase the scarce bioavailability of lipophilic components and to avoid substantial modification of the sensory and rheological profiles of the enriched products (*Augustin & Hemar, 2009; L. Chen, Remondetto, & Subirade, 2006*). Among the different systems (e.g. solid lipid nanocarriers and liposomes) emulsion-based technologies are particularly suitable for the design and fabrication of delivery systems for bioactive lipids, thanks to their high stability, aqueous solubility, enhanced bioavailability and an ease of processing with high monitoring possibilities of particle size, depending on the technology employed (*MacAdam, Shafi, James, Marriott, & Martin, 1997*; *Kakran & Antipina, 2014*; *Lu, Kelly, & Miao, 2016*). Solvent-evaporation is one of the most popular encapsulating methods. By this technique, nanoparticles (NPs) are produced through the use of emulsifiers such as globular proteins (e.g. whey proteins, sodium caseinate, soy proteins, etc.) that thanks to their structure, flexibility and amphiphilic nature, rapidly adsorb to the emulsion surface, where they self-aggregate and form continuous and homogeneous membranes around oil droplets constituted by the lipophilic molecules dissolved in an organic solvent (*Dickinson, 1994*; *Hu, McClements, & Decker, 2003*; *Lefèvre & Subirade, 2003*). Proteins can also stabilize the formed emulsion due to the superficial charge (negative or positive) that they are able to impart to the droplets surface at pH above and below their isoelectric point (pI). This surface charge reduces the tendency to coalesce and flocculate. Another fundamental parameter is the crystallization of the lipidic molecules in the NPs core, that is caused by the evaporation of the solvent (*Chu, Ichikawa, Kanafusa, & Nakajima, 2007*).

Astaxanthin (ASX) is a ketocarotenoid, belonging to a group of oxygenated carotenoids termed xanthophylls (*Higuera-Ciapara, Félix-Valenzuela, & Goycoolea, 2006*). Due to the high presence of conjugated carbon-carbon double bonds and of hydroxyl and ketonic groups at both the end of the molecule, astaxanthin displays a unique antioxidant activity (*Chen, Lin, & Hsieh, 2007; Ambati, Siew Moi, Ravi, & Aswathanarayana, 2014; Rodríguez-Sáiz, de la Fuente, & Barredo, 2010*; *Rao et al., 2013*). The successful use of ASX was reported in the treatment of cardiovascular diseases, different types of cancer, diabetes and neurodegenerative disorders (*Guerin, Huntley, & Olaizola, 2003; Fassett & Coombes, 2011*; *Pashkow, Watumull, & Campbell, 2008*; *Zhang, Leng, Zhang, &*

Li, 2009; *Palozza et al., 2009*). However, its use in the food industry is limited because of its unsaturated nature, that causes extreme instability when the molecules is exposed to environmental stresses such as light, extreme pH, oxygen and heat (*Vakarelova et al., 2017; Zhao et al., 2006*). Furthermore, its low solubility in aqueous-based systems (such as the one present in the digestive system) causes a low absorption rate and consequently, low bioavailability (*Tan & Nakajima, 2005; Anarjan et al. 2012*).

As previously described in **chapter 2**, ASX can be successfully encapsulated through emulsion-solvent evaporation technique using whey protein as the emulsifier/stabilizer. Protein concentrates and isolates used in food industry are mostly derived from whey, soybean and wheat, but because of dietary restrictions and novel trends, food manufactures and consumers are looking for alternative proteins sources. Many plant proteins have been extensively studied in recent years, because of their biodegradable and renewable character and good functional properties, such as emulsifying, film forming capacity and water solubility (*Euston, Al-Bakkush, & Campbell, 2009*; *Ducel, Richard, Saulnier, Popineau, & Boury, 2004*). Furthermore, in recent years, health-promoting biological activities and regulatory functions have been associated to several proteins of plant origin (e.g. cardiovascular and bone health, inflammation, cancer prevention, weight control, insulin sensitivity and mineral absorption) (*Carbonaro, Maselli, & Nucara, 2015*). Proteins from soybean, pea, wheat, corn and barley have already proved their ability to protect sensitive bioactive substances and flavors thanks to their similar behavior in terms of emulsifying, chemical and gelling properties to those of whey protein. This was achieved through micro-encapsulation, mainly using spray-drying technique (*Fernandez-Avila, Arranz, Guri, Trujillo, & Corredig, 2016; Ducel et al., 2004; Charve & Reineccius, 2009*). However, plant proteins have rarely been reported as nano-encapsulation matrices (*Yi, Lam, Yokoyama, Cheng, & Zhong, 2015; Fernandez-Avila et al., 2016*).

The aim of this study was to evaluate the properties of some commercial proteins isolated from soy, pea and rice, for the nano-encapsulation of ASX. Size, stability, encapsulation efficiency and the presence of ASX on the NPs surface were analyzed. The release of ASX from the different NPs during simulated digestion was evaluated in comparison to that of the NPs developed using whey protein concentrate (WPC) (**chapter 2).**

2. Material and methods

2.1. Chemical and reagents

Soya protein isolate (SPI) was purchased from ACEF (Milano, Italy). The protein composition (w:w) was protein 80%. Pea protein isolate (PPI) and rice protein isolate (RPI) were purchased from Raab Vitalfood (Rohrbach, Germany). PPI protein composition was 80% (w/w). RPI protein composition was 80% (w/w). *Haematococcus pluvialis* powder was purchased from a local supplier (Italy). Ethyl acetate, acetone HPLC-grade, pepsin, pancreatin, trypsin and sodium cholate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Astaxanthin extraction from *H.p.*

H.p. oleoresin was obtained using the protocol proposed by Bustos-Garza and co-workers with minor modifications (*Bustos-Garza, Yáñez-Fernández, & Barragán-Huerta, 2013*). Briefly, the algae powder were pretreated by mixing 5 g of algae and 1 ml of 3 M HCl and treating the sample in a microwave oven for 1 min at 100 W (*Sarada, Vidhyavathi, Usha, & Ravishankar, 2006*). The pretreated algae were extracted with 25 ml of ethyl acetate in a tube with a screw cap, for 60 min under agitation at 50 °C in a thermal bath. The solid portion was separated by centrifugation at 3000 g for 10 min to eliminate the biomass. The oleoresin was kept in the dark at 4 °C until use.

2.3. Spectrophotometric analysis

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

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

Where [A] is the concentration of ASX expressed as mg/ml; A_{480} the sample's absorbance at 480 nm; DF: dilution factor; $E_{(1\%;1cm)}$: ASX percent solution extinction coefficient [$(g/100\ ml)^{-1}\ cm^{-1}$] in ethyl acetate (2150); d: the optical path (cm).

2.4. Astaxanthin nanoparticles preparation

Astaxanthin nanoparticles (ASX NPs) were produced following the method described previously by (*Yi et al., 2015*) with some modifications. SPI and PPI were dissolved in distilled water in a concentration range between 1 and 15%. The solution was stirred for 30 minutes at room temperature. *H.p.* extract was differently diluted in ethyl acetate and combined with the protein solution at a ratio of 9:1 (protein solution:oleoresin). A fine emulsion was produced using an ultra-sonicator for 10 minutes at a potency of 10 W (Microson ultrasonic cell distruptor XL). At the end of the process ethyl acetate was removed using a nitrogen flow in the dark. The WPC ASX NPs were kept in the dark at 4 °C until use. In the case of encapsulation with pre-treated protein: the proteins solution (1.0% w/w) used for encapsulation were prepared in three different ways 1) the pH of the aqueous solution was adjusted at 8 with NaOH 2 M; 2) Heat treatment at 80°C for 1 minute; 3) combination of 1) and 2). As control, proteins dissolved in demineralized water were used. The solution was stirred for 30 minutes at room temperature. *H.p.* oleoresin was diluted in ethyl acetate at a final concentration of 2.5% and combined with the protein solution at a ratio of 9:1 for the production of the emulsion (protein solution:oleoresin).

2.5. Characterization of astaxanthin nanoparticles

2.5.1. Surface charge and average diameter

Zeta-potential, mean diameter and polydispersity index (PDI) of NPs were analyzed by dynamic light scattering (DLS) principles using a Malvern Zetasizer (Nano-ZS; Malvern Instruments, Worcestershire, U.K). Before the analysis the sample were diluted 80 times to avoid multiple scattering effect. PDI value ranging from 0 to 1, indicated the distributions of the particle sizes, value close to 0 indicated a uniform population of particles and a value close to 1 indicated a wide variety of dimensions among particles size distribution. Zeta potential give an important information about particles stability, for values closer to 0 the system is considered not stable, due to the absence of a net charge that can contrast the aggregation process of NPs.

2.5.2. Superficial astaxanthin

A volume of 0.5 ml of NPs was extracted with 1 ml of ethyl acetate by continuous agitation for 10 minutes. The sample was centrifuged at 12.000 rpm for 5 minutes. The supernatant was collected, diluted and analyzed by a spectrophotometer as previously described.

2.5.3. Simulated *in vitro* digestion

In vitro digestion was performed following Infogest protocol (*Minekus, Alminger, Alvito,, Ballance, Bohn, Bourlieu, ... & Dufour, 2014*). Simulated gastric fluids (SGF) and simulated intestinal fluids (SIF) were prepared following the protocol. The duration of the two phases were 1 h for the gastric and 4 h for the intestinal phase. The enzyme used were pepsin for the gastric stage and pancreatin for the intestinal phase. Bile salts in the form of sodium cholate were added to SIF to a final concentration of 10 mM. Briefly. NPs and SGF were mixed in a ratio 1:1, the pH was adjusted to 3 with HCl 1M. After 1 hour, SIF was added in a ratio 1:1 and the pH adjusted to 7 with NaOH 1M. The reaction was conducted at 37°C in a rotating shaker. At different time points samples were extracted with a double volume of ethyl acetate. The amount of ASX released from WPC ASX NPs were evaluated by spectrophotometry.

2.6. Statistical analysis

All measurements were performed at least three times and the results were reported as mean value ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA).

3. Results and discussion

SPI, PPI and RPI were employed as alternative encapsulant matrices to improve the water-dispersibility and bioavailability of ASX through emulsion-solvent evaporation technique. Plant proteins are often better accepted by consumers since they can comply with specific cultural and religious indications, i.e. vegetarian, vegan, lactose-free and kosher. In addition, in compliance with the EU regulation (Annex II of the EU Food Information for Consumers Regulation No.1169/2011 and Commission Delegated Regulation (EU) No. 78/2014 amending Annex II to Regulation (EU) No 1169/2011), pea and rice proteins are not considered as allergenic sources.

3.1. Production of SPI and PPI ASX NPs

Using the same approach described in **chapter 2** the optimization of the encapsulation parameters for SPI and PPI started from the study of the variation of dimensions and z-potential of the NPs as a function of the concentration of protein and *H.p.* oleoresin. The first parameter considered, i.e. the concentration of proteins needed to encapsulate ASX, is crucial since the presence of free unabsorbed protein molecules in the continuous phase may promote depletion and flocculation of oil droplets (*Dickinson & Golding, 1997)* or the formation of unabsorbed proteins aggregates (*Dickinson, 1994*).

3.1.1. ASX SPI NPs

SPI are currently one of the most abundant plant proteins. They exhibit high nutritional value and desirable functional properties as emulsifiers and texturizing agents (*Teng, Luo, & Wang, 2012)*. From the chemical point of view they are composed by a balanced composition of polar, non-polar and charged amino acids, and thus they are able to incorporate molecules with different chemical characteristics (*Riche & Williams, 2010*). The major fraction of SPI is composed by glycinin and β-conglycinin.

In the case of SPI ASX NPs production, differently from WPC, it was impossible to obtain NPs using solutions more concentrated than 5% due to the limited protein solubility (see Fig.1). Average size, PDI and zeta-potential were considered as reference parameters to evaluate the performances of the process. In the first experiment, all the NPs were produced keeping constant the amount of *H.p.* oleoresin (1%) and varying the amount of encapsulant matrix. As presented in Fig.1 A**,** the

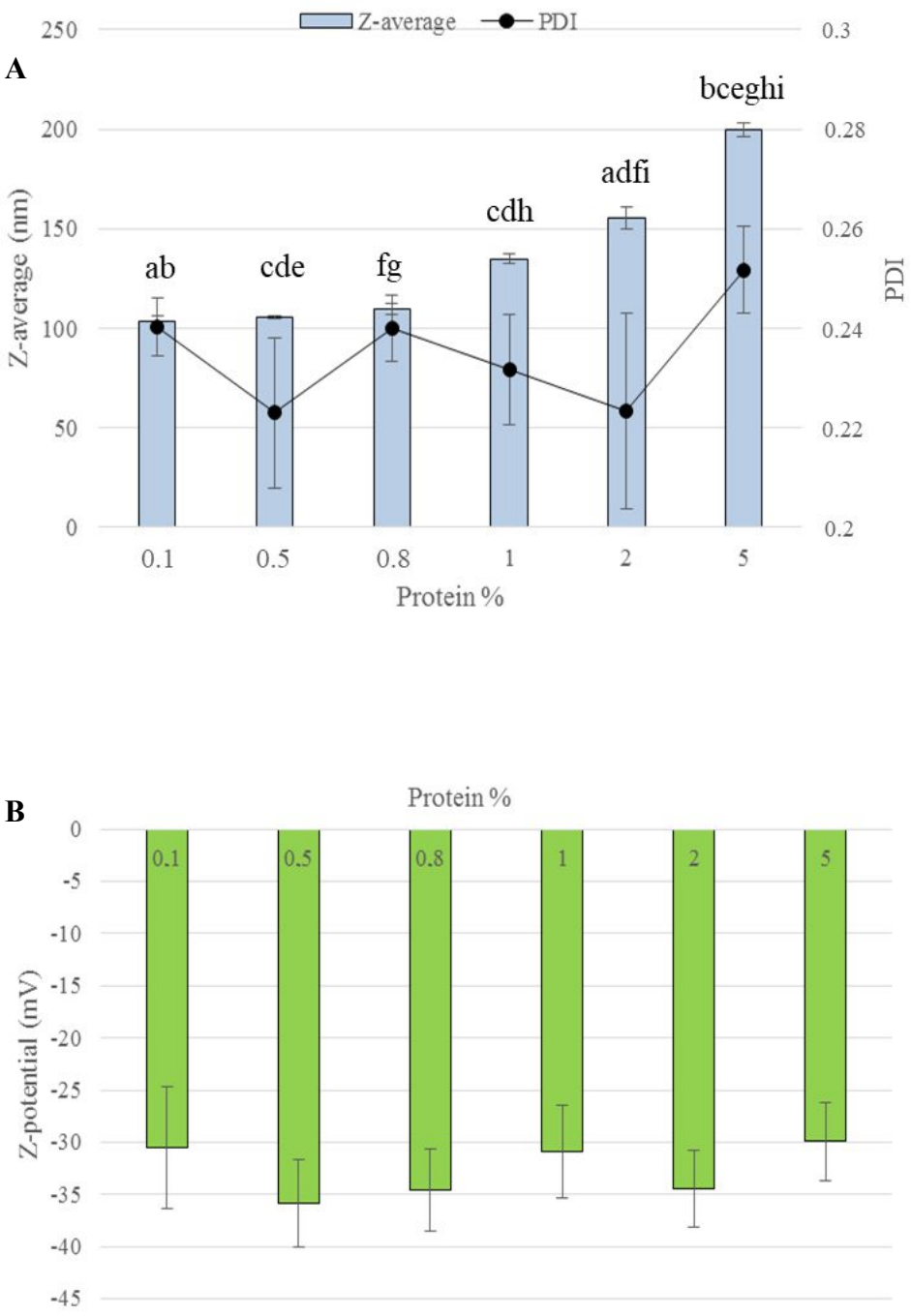


Fig.1.Variation of size and PDI (A) and z-potential (B) as a consequence of the different protein concentrations used to produce ASX SPI NPs. Differences between values indicated by the same letter are statistically significant ($P<0.05$)

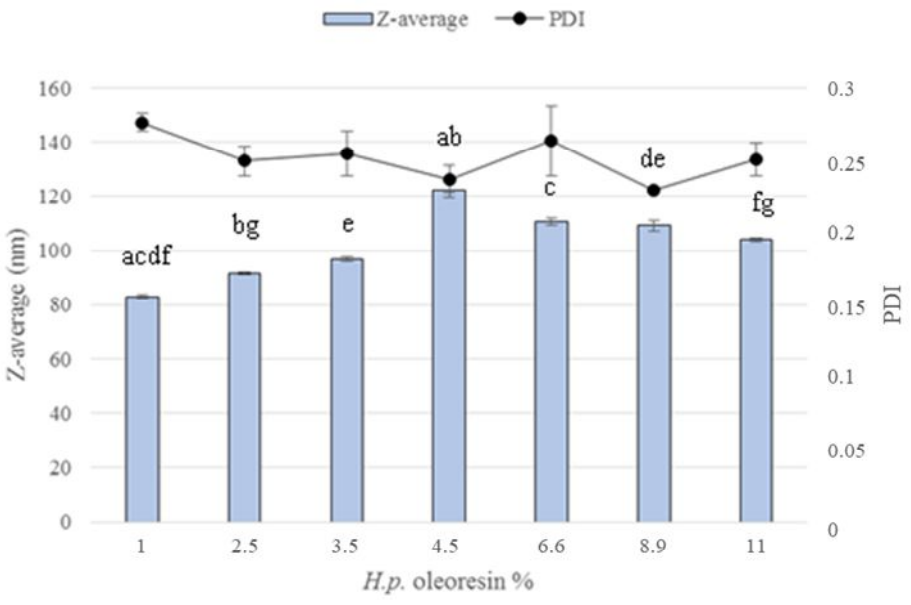
dimensions ranged from 103 to 200 nm, confirming the strong dependence of size on proteins concentration, and the tendency of plant proteins to give bigger NPs probably due to the higher

interfacial tension given by the higher tendency of this proteins to bind water, increasing as a consequence also the viscosity of the solution (*Keerati-u-rai & Corredig, 2010*; *Chu et al., 2007*),

rigid structure (*Yi, Lam, Yokoyama, Cheng, & Zhong, 2014*), higher molecular weights (between 200,000 and 350,000 Da) that renders the diffusion of proteins slower through the oil/ water interface (*O'Sullivan, Murray, Flynn, & Norton, 2016*) and the lower presence of hydrophobic amino acids in comparison to dairy proteins (*Hu et al., 2003*; *Yi et al., 2015)*. PDI values were acceptable for all the formulations ranging from 0.24 (0.1% SPI) to 0.25 (5% SPI), describing moderate polydisperse samples. As already mentioned, zeta potential values higher than +20 and lower than -20 mV are good indicators of NPs stability (*Müller, Jacobs, & Kayser, 2001*), SPI ASX NPs displayed highly negative values for all the samples (**Fig.1B**), from -30 to -35.8 mV. The more negative values compared to those obtained with WPC, are due to the chemical structure of soy proteins and the higher presence of negatively charged groups at neutral pH.

In order to evaluate the effect of *H.p.* oleoresin concentration on NPs production, SPI concentration was set to 1%. Differently from what described in **chapter 2** using WPC, increasing concentrations of oleoresin led to the growth of NPs dimensions (Fig.2 A), from 83 nm using 1% oleoresin to 125 nm with 4.5% oleoresin. Increasing further the concentration of oleoresin resulted in an unexpected decrease of the diameter. PDI values were in the range of 0.23-0.28. Like in the case of WPC, no particular trends could be appreciated, and no correlation of PDI to the diameter was found. The sample at 1% oleoresin gave the less negative Z-potential value (-19.7 mV) (Fig.2 B). This last result could be explained taking into account that a specific ratio between the protein acting as emulsifier and the lipophilic molecules should be respected (*Dickinson, Golding, & Povey, 1997*).

A



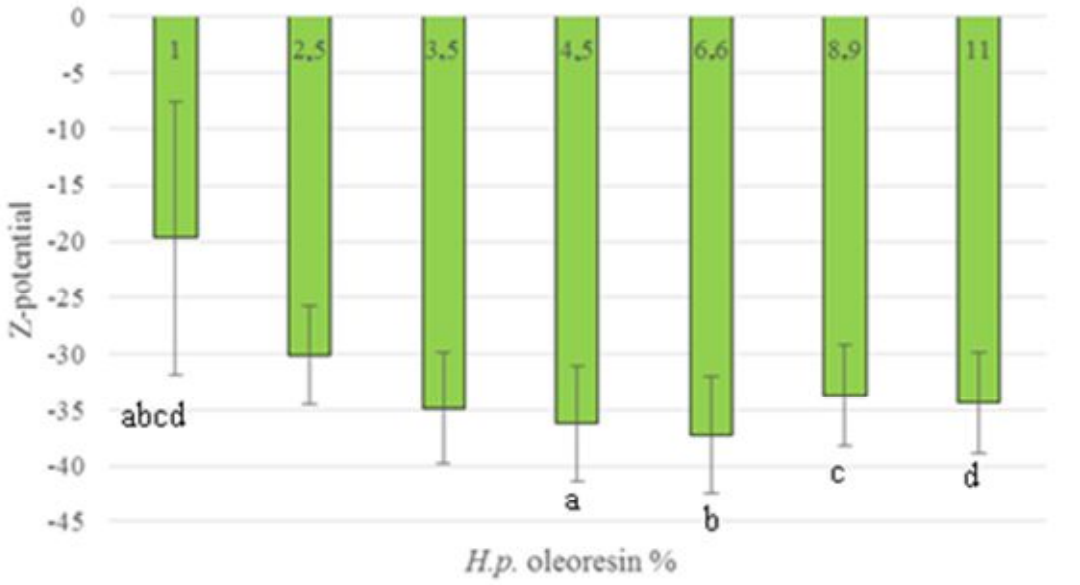


Fig.2 Variation of size and PDI (A) and z-potential (B) as a function of the different *H.p.* oleoresin concentrations used to produce ASX SPI NPs. Differences between values indicated by the same letter are statistically significant ($P<0.05$)

Probably, in the case of the sample composed by 1% proteins and 1% *H.p.* oleoresin, the amount of proteins was too much higher in comparison to the oleoresin.

The interaction of this free protein fraction together with the neo-produced NPs could result in a partition neutralization of the superficial charge, thus explaining the low Z-potential for this sample. Another phenomenon described in literature is that non absorbed stabilizer can also aggregate/flocculate, and probably interact with the NPs surface.

With the increased amount of *H.p.* oleoresin, the decrease of the ratio protein/oleoresin could allow for the migration of the proteins to the oil droplet surfaces, producing the higher charge measured for these samples (*Yerramilli, Longmore, & Ghosh, 2017*).

It must be noted that the results concerning the average size of the NPs here obtained are very different from those obtained by Yi e co-workers (2015), that using the same carrier system obtained β-carotene SPI NPs with an average diameter of 372 nm. This difference can be a consequence of the different molecular structure of the active ingredients encapsulated. Another important difference was that in our case the NPs were produced only with sonication while Yi and co-workers used a high pressure homogenization approach a 700 bar. Probably, the two methods have differently affected SPI characteristics (*O'Sullivan et al., 2016*).

The ASX SPI NPs obtained (**Fig.3**) were characterized by a red-orange color and a slight opalescence, especially at high protein concentration (**Fig. 3 A)**. Opalescence in this type of system can arise from the presence of particles characterized by high particle size (higher than 100 nm) or from the interactions among proteins that tend to form aggregates.

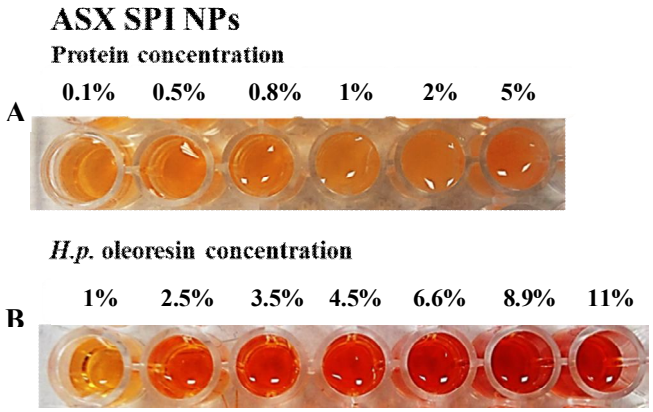


Fig.3. Appearance of ASX SPI NPs as a function of the different protein concentrations (A) and *H.p.* oleoresin concentrations (B).

3.1.2. ASX PPI NPs

The same analyses were conducted also for the nanoencapsulation of ASX using PPI as encapsulant matrix. The main fraction of PPI is composed by legumin (60,000 Da), vicilin (50,000 Da) and convicilin (70,000). As in the case of SPI, the percentage of protein concentration tested was from 0.1 to 5% due to the fact that higher protein concentrations led to the production of a very viscous solutions not suitable for our purpose. As shown in **Fig.4 A,** the average diameter of ASX PPI NPs obtained ranged from 94 to 130 nm, with the NPs characterized by bigger dimensions produced respectively with the 0.1 and 5% protein concentrations. Z-potential ranged from -29.9 to -24.4 mV, underlying that all the NPs produced were characterized by a highly negative charge, and thus stability against flocculation and aggregation phenomena (Fig.4 B). No correlation was found between protein concentration and Z-potential. Keeping protein concentration constant (1%), by increasing the amount of *H.p.* oleoresin the dimensions of the obtained ASX PPI NPs ranged from 100 to 140 nm (**Fig.5 A**), where the dimension of the NPs produced was correlated to the % of oleoresin, confirming the trend already observed in the case of ASX SPI NPs.

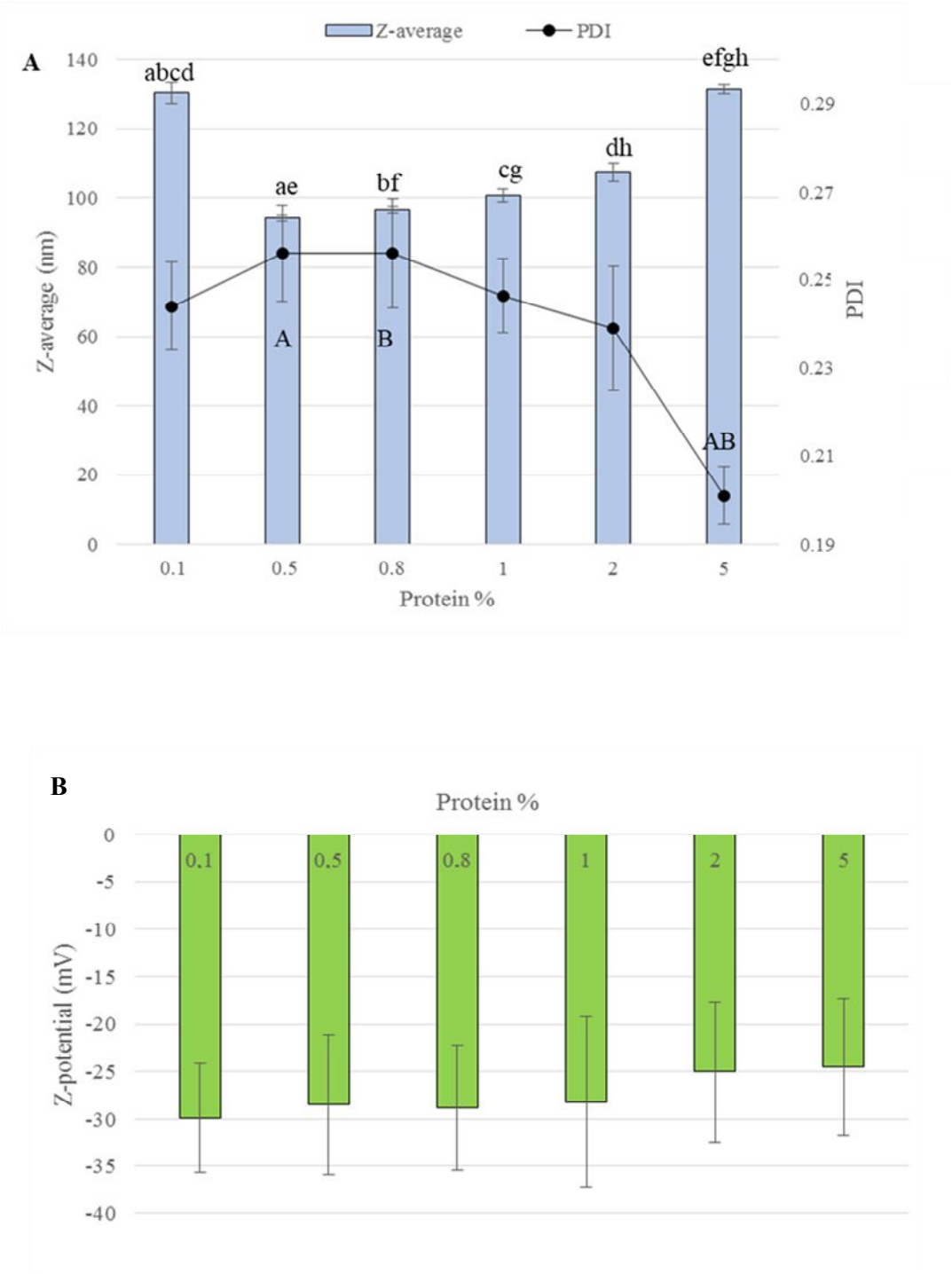


Fig.4. Variation of size and PDI (A) and z-potential (B) as a consequence of the different protein concentrations used to produce ASX PPI NPs. Differences between values indicated by the same letter are statistically significant ($P<0.05$). Capital letter correspond to the significance of PDI.

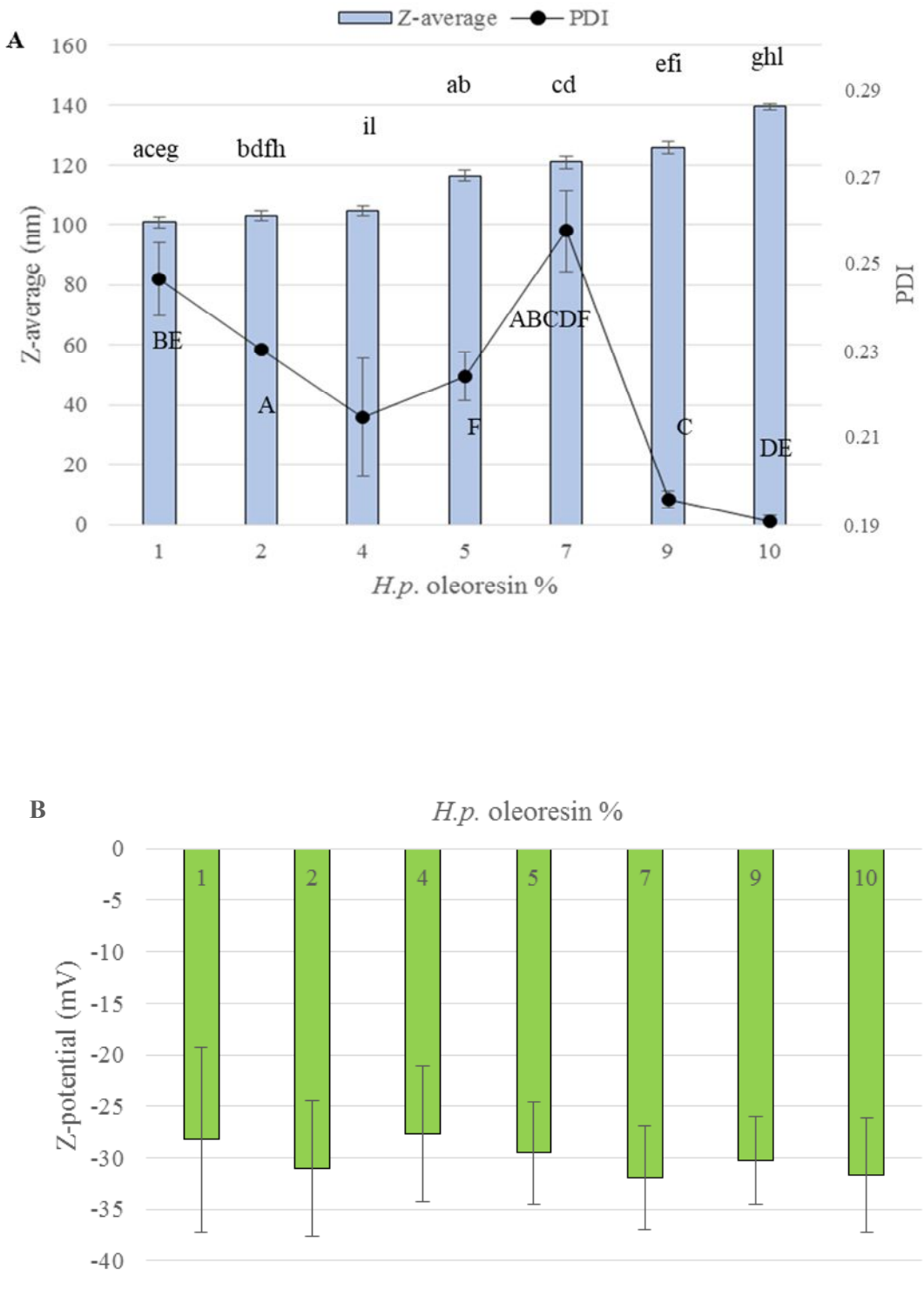


Fig.5. Variation of size and PDI (A) and z-potential (B) as a consequence of the different *H.p.* oleoresin concentrations used to produce ASX PPI NPs. Differences between values indicated by the same letter are statistically significant ($P<0.05$). Capital letter correspond to the significance of PDI.

The PDI was comprised between 0.21 and 0.25 for the preparation containing *H.p.* oleoresin from 1 to 5%, meanwhile the NPs produced with 7% of *H.p.* oleoresin showed a higher PDI, i.e. 0.257, that can be still considered associated to a moderate polydisperse system. For the NPs produced with the highest concentration of *H.p.* oleoresin (9 and 10%) a significantly lower PDI was unsuspectedly observed (i.e. 0.19). The lower protein to oleoresin ratio of these samples produced a more homogeneous NP population with bigger average size. The surface charge was highly negative for all the samples, from -27.7 to -31.9 mV (Fig.5 B), without an evident dependency on the amount of *H.p.* oleoresin.

3.2. Evaluation of plant proteins encapsulation properties

Commercial proteins isolates are commonly used as emulsifier in emulsification-solvent evaporation technique but sometimes they show poor solubility in the aqueous phase compared to the laboratory purified proteins (*Donsì, Senatore, Huang, & Ferrari, 2010*; *Avramenko, Low, & Nickerson, 2013; Liang & Tang, 2013*; *Shi et al.,2015*; *Fernandez-Avila et al., 2016*) thus limiting the possibility to further scale-up the process. A typical problem associated to isolates is the possible incomplete dissolution of the matrix that could generate the production of big particles and agglomerates, hampering the efficiency of the process. For this reason, we decided to study the encapsulation properties of proteins subjected to different pre-treatments: heat treatment, adjustment of pH to values far from isoelectric points of the proteins, and the combination of these two parameters. It is known that globular proteins may denature, dissociate and aggregate under different conditions of pH, ionic strength and temperature (*Chen et al., 2006*). For example, thermal treatment could lead to the unfolding of polypeptide chains followed by re-aggregation with concomitant exposure by the new formed complex of initially buried hydrophobic amino acid residues (*Utsumi & Kisella, 1984*). Such complexes can have different functional properties and digestibility (*Duodu, Taylor, Belton, & Hamaker, 2003*).

3.2.1. SPI

Comparative tests were performed in order to understand if the pre-treatments (heat, pH and a combination of the two) of proteins before encapsulation could have an effect on their solubility, and as a consequence, on the encapsulation efficiency of ASX. Fig.6 shows the obtained ASX SPI NPs: all the solutions were transparent and show orange-red bright color.

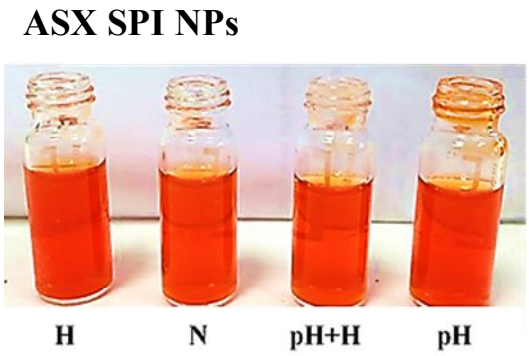
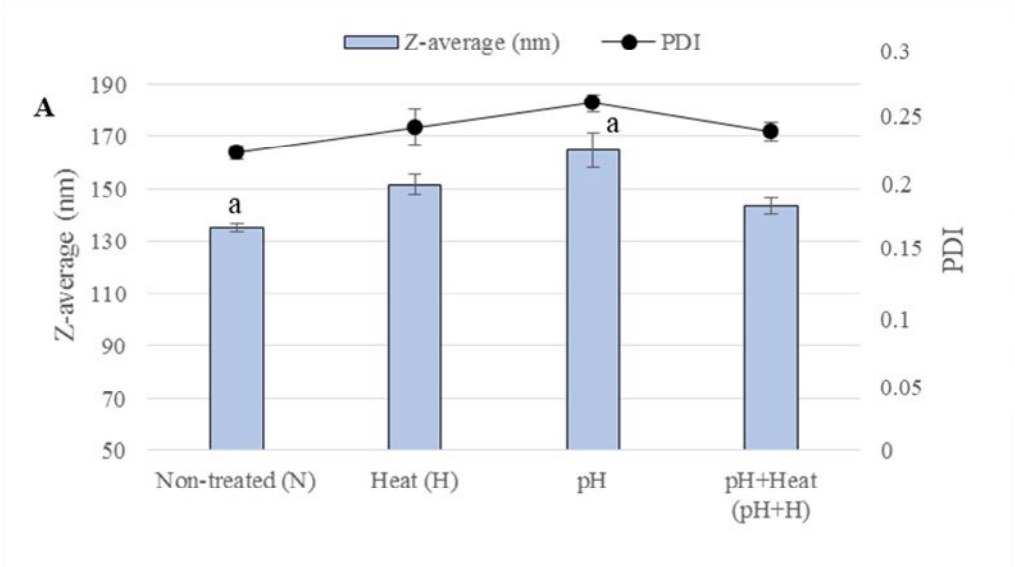


Fig. 6. Appearance of ASX SPI NPs produced with differently treated proteins : H (Heat), N (non-treated), pH and pH+H (pH+heat).

The smallest diameter was obtained with the non-treated proteins (N) i.e., 135 nm, the biggest diameter with the proteins solubilized at pH 8, i.e. 164.6 nm (Fig.7A). PDI values were in the range of 0.22 and 0.26 for all the preparations. Z-potential values (Fig.7B) were highly negative for all the preparations, underling a substantial stability of all the ASX SPI NPs prepared.



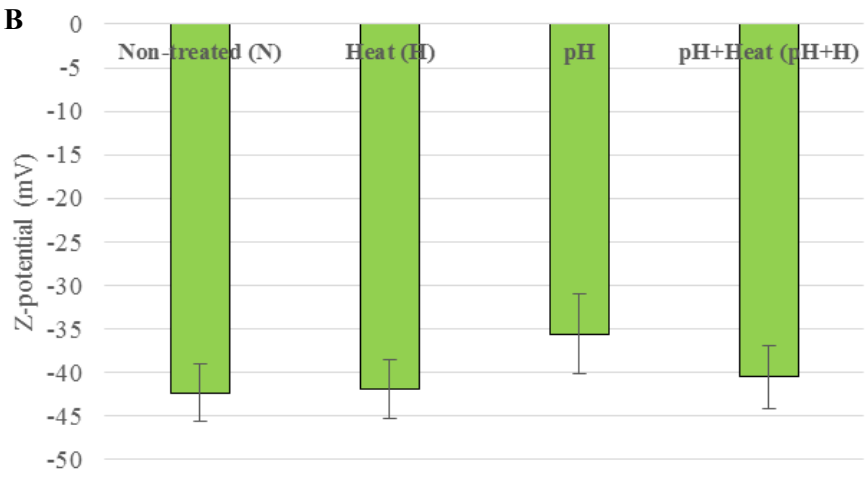


Fig.7 Dependence of ASX SPI NPs size and PDI (A) and Z-potential (B) on the different protein treatments. Differences between values indicated by the same letter are statistically significant (P<0.05).

In Table 1 two significant parameters for the evaluation of the encapsulation process are reported: the encapsulation efficiency (EE), referred to the amount of ASX effectively encapsulated inside the NPs, and the superficial ASX (sASX), that describes the amount of ASX present on the NPs surface. sASX could easily undergo oxidation reactions losing its structural integrity and functionality. For this reason, a very low value of superficial ASX is recommended.

ASX SPI NPs	**EE%**	**sASX %**
Non-treated	93 ± 0.01^{abc}	0.0037 ± 0.0001^{AB}
Heat	99 ± 1.03^{a}	0.0097 ± 0.0004^{C}
pH	99 ± 0.85^{b}	0.0261 ± 0.0023^{AC}
pH + heat	96 ± 0.14^{c}	0.0153 ± 0.0004^{B}

Tab.1. Effects of the pretreatments on encapsulation efficiency (EE%) and superficial ASX % of ASX SPI NPs. Differences between values indicated by the same letter are statistically significant (P<0.05).

The EE was high for all the samples, with the highest value of 99% obtained for the heat-treated sample. The lowest values were obtained for the sample N with an EE of 93%, a value very similar to the one obtained by Yi and co-workers 2015. The higher EE displayed by sample H could be a

consequence of the temperature-induced protein conformational changes that could allow to better allocate the oil phase (*Wang, Li, Jiang, Qi & Zhou, 2014*). The amount of superficial ASX was negligible for all the samples: from 0,026 to 0,0037%. Although the higher EE shown by the H samples the diameter of the particles was too big to be considered as satisfactory for our purpose.

3.2.2. PPI

The appearance of ASX PPI NPs is shown in Fig.6, The solutions were transparent and orange-red in color. No evident differences were observable among the samples.

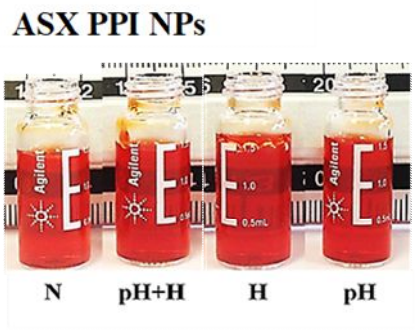
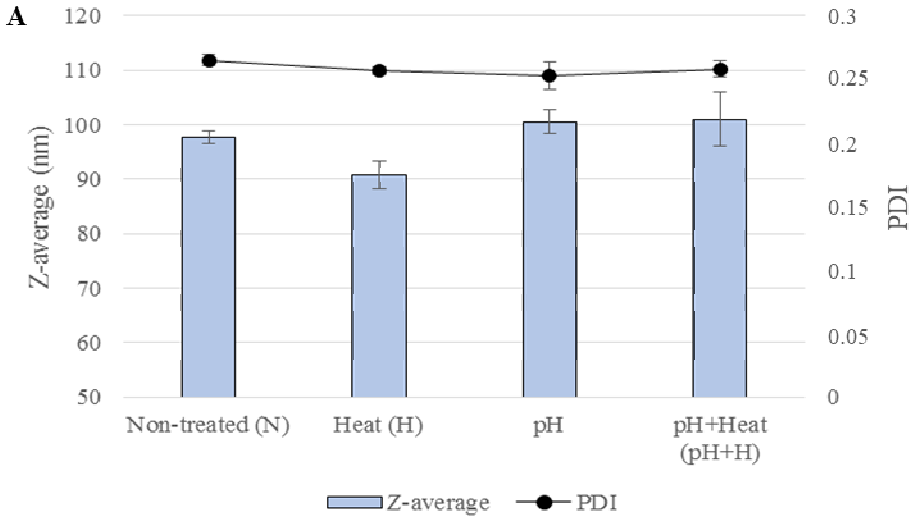


Fig.8 Appearance of ASX PPI NPs produced with the differently treated proteins : H (Heat), N (non-treated), pH and pH+H (pH+heat).

The average sizes of the obtained NPs (Fig.8A) were smaller than those obtained with SPI and much more similar to those obtained in **chapter 3** with WPC. Sample H gave the NPs with the smallest average dimension (91 nm), pH and pH + heat treated samples gave the bigger NPs diameters (around 100 nm).



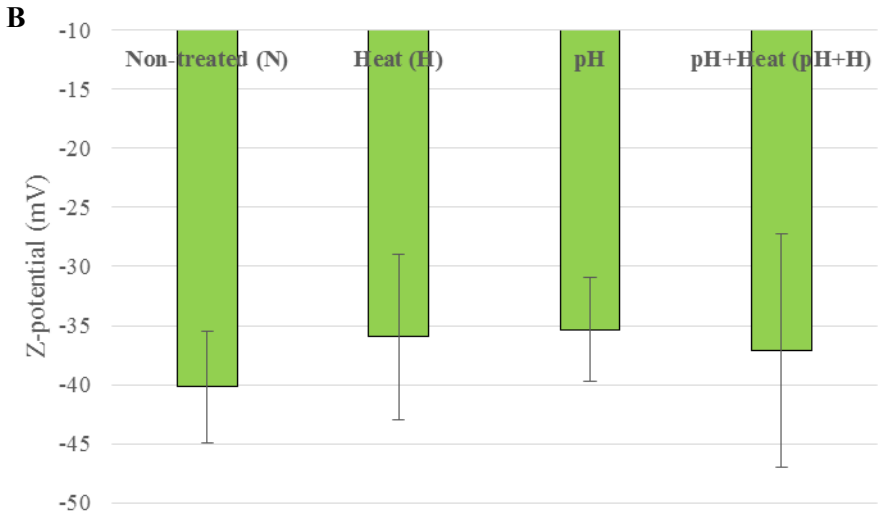


Fig.9 Dependence of ASX PPI NPs size and PDI (A) and Z-potential (B) on the different protein treatments.

PDI values were very similar for all the samples, ranging from 0.25 to 0.265, underling that probably the pre-treatment of proteins did not have a considerable effect on the size distribution of ASX PPI NPs. Z-potential values were highly negative for all the samples (Fig.8 B), and no significant difference was observable between the samples.

EE% ranged from 94-96%, with the lowest value exhibited by the sample N, and the highest by the sample H. Sample H displayed also the lowest amount of superficial ASX, 0.026%, and sample N the highest, 0.0325%. The sASX was in general higher in comparison with SPI, but still very low.

ASX PPI NPs	EE%	sASX %
Non-treated	94±2.6	0.033±0.0005
Heat	96±0.5	0.027±0.0001
pH	93±1.4	0.028±0.0012
pH + heat	95±0.3	0.027±0.0003

Tab.2. Effects of the pretreatments on encapsulation efficiency (EE%) and superficial ASX % of ASX PPI NPs

3.2.3. RPI

With the goal of identifying novel non-allergenic and gluten free protein candidates we tried to employ also rice protein isolate (RPI). As shown in Fig.10, it was impossible to obtain ASX NPs with the use of this protein as emulsifier: large amounts of non-encapsulated ASX and opalescence were observed for all the samples, probably because of the presence of aggregated or non-solubilized proteins. To our knowledge no data are reported in literature involving this type of matrix for nanoparticle production by emulsification-solvent evaporation technique. The works of *Shi et al., 2015* and *Shi et al., 2017* reported the ability of RPI to bind tea catechins, but this occurred only following pre-treatment and isolation of the soluble protein fraction of the rice bran, due to the high amount of fiber and fat. Therefore, in our study the absence of a purification step aiming at removing the non-soluble protein fraction, i.e. glutelins, could have negatively affected the process. This protein fraction is characterized by high presence of disulfide bond and high molecular weight. These characteristics make RPI a scarce emulsifier and thus not suitable for NPs production. Similar findings were discussed also in the work of O'Sullivan et al. 2016, who demonstrated that the use of ultrasonication was not able to reduce the size of RPI aggregates. In that case the presence of this insoluble aggregate was attributed to the occurrence of carbohydrates and to the denaturation of protein during the preparation of the isolate (*Mujoo, Chandrashekar, & Ali, 1998;O'Sullivan et al., 2016*).

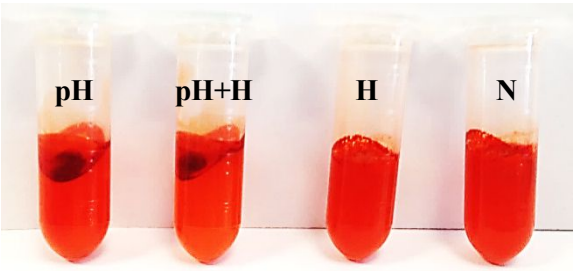


Fig.10. Appearance of the ASX suspensions using RPI as emulsifier.

The obtained data suggest that SPI and PPI could be used for the production on NPs with satisfactory results in terms of small particle size, narrow PDI and stability to coalescence. In both cases, the pre-treatment of proteins by heating and/or adjusting the pH did not lead to a significance variation of the encapsulation performance. For this reason, in the following experiment we decided to avoid these treatments.

3.3. Evaluation of ASX release by simulated digestion

In vitro release of ASX from ASX SPI NPs and ASX PPI NPs, was assessed by simulated gastrointestinal digestion and the results were compared to ASX WPC NPs. These experimental data are important to study possible differences in the release profiles and thus in the bioaccessibility of encapsulated active molecule. Ideally, the release of ASX should occur in the intestine where absorption is supposed to take place. The time-dependent release of ASX is shown in Fig.11. At time 0 ASX release from ASX WPC NPs was the highest, accounting for 36% of the total amount. ASX SPI and PPI NPs showed similar patterns, with 22-23% release. This initial release for all the formulations could be due to the acidic conditions of the gastric medium (pH 3) that could destabilize the NPs structure. Liu and co-workers (2016) already observed aggregation phenomena of sodium caseinate nano-emulsions at pH 2, suggesting that some irreversible droplet aggregation occurred when the pH of the initial samples is adjusted from neutral to acidic condition (*Liu, Chen, Cheng, & Selomulya, 2016*).

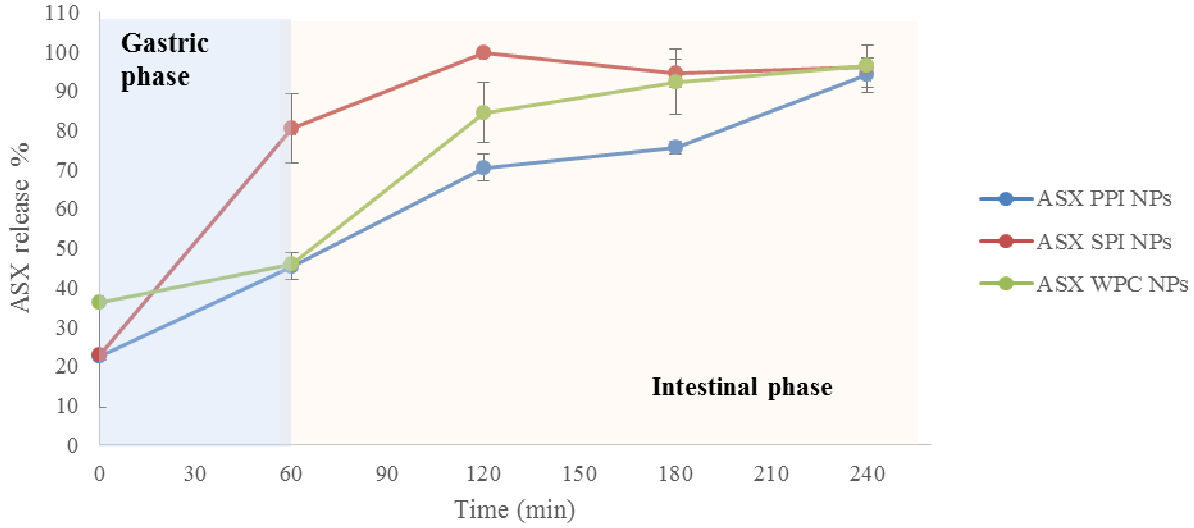


Fig.11. ASX release from ASX WPC NPs, ASX SPI NPs and ASX PPI NPs during *in-vitro* simulated digestion.

To further confirm this hypothesis, the average size of ASX PPI NPs was analyzed by microscopy and DLS (Fig.12), showing the formation of aggregates in the GS, a phenomenon already observed also by Overduin and coworkers, 2015. It was impossible to obtain information about the particles size due to the high PDI (>1) and presence of aggregates in the sample. At the end of the GS the release of ASX from ASX WPC and PPI NPs was comparable, i.e. 45% and 46 % respectively. Much higher was the release from ASX SPI NPs, that accounted for 80% of total ASX. For both

ASX WPC and PPI NPs during the intestinal stage (IS) the release of ASX increased constantly within the first 3 hours except for the SPI formulation that, due to the large amount of ASX released during GS, showed a plateau after the first hour of IS reaching 99.7% release. At the end of the IS the amount of ASX released from the three formulation was equivalent ranging from 96 and 94%.

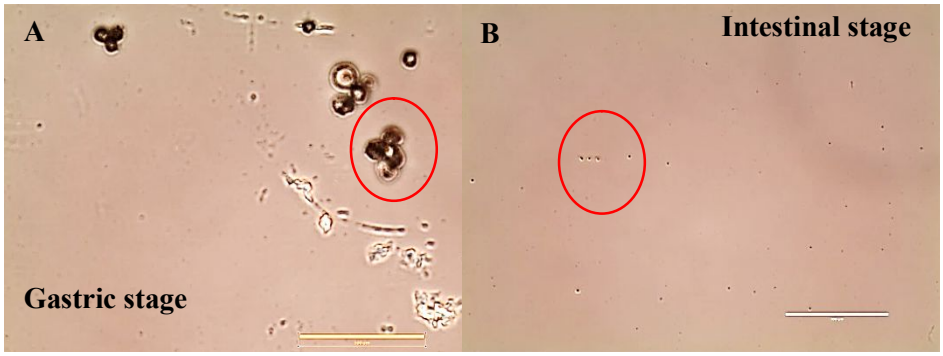


Fig.12. Optical microscope image showing the agglomeration of ASX NPs during gastric stage (A) and (B) their disappearance in the intestinal stage.

4. Conclusions

In this work three plant protein isolates from soy, pea and rice were tested as matrices for the nano-encapsulation of ASX oleoresin from *H.p*, by emulsification-solvent evaporation method. SPI and PPI gave the best results allowing for the production of NPs with dimensions in the range of 90-130 nm, while RPI where not suitable probably because of the low solubility and emulsifying properties of the proteins. Attempts aiming at optimizing the process for SPI and PPI revealed that neither the thermal pre-treatment nor the solubilization at pH far from the isoelectric point of the proteins before encapsulation, caused significant modifications of the encapsulation capacity of the matrices. The release profiles of ASX from SPI and PPI NPs were compared with the one of WPC NPs. WPC and PPI NPs displayed similar release patterns, while SPI NPs showed a burst release of most of all the ASX at the end of the gastric stage. Therefore, PPI seems to be the most suitable candidate as plant alternative emulsifier for the production of ASX NPs.

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Chapter 4.

Antioxidant capacity of astaxanthin nanoparticles

Abstract

Astaxanthin (ASX) was enclosed through emulsion-solvent evaporation technology in a protein shell formed by whey protein (WPC). The antioxidant capacity (AOC) of the obtained nanoparticles (NPs) was characterized by colorimetric assay (ABTS) and by the development of a flow cytometry assay based on J774A.1 mice monocyte macrophages cell line aiming at evaluating the capacity of ASX to reduce chemically induced oxidative stress. Both the assays showed that WPC ASX NPs possess higher AOC compared to *H.p.* oleoresin. Confocal laser scanning microscopy (CLSM) and flow cytometry ware employed to gain more information about the cell uptake of NPs. The results showed that the transport mechanism was characterized by an energy-dependent model in Caco2 and HepG2 cell lines.

Key words: *astaxanthin, nanoencapsulation, antioxidant activity, ABTS, cellular antioxidant activity, cytotoxicity, cell uptake.*

1. Introduction

Reactive oxygen/nitrogen species (ROS/RNS) are the products of normal metabolic respiration process. An excess of this species in the body could produce a damage of the DNA, proteins and lipids generating oxidative stress that can lead to cells death (*Tandon, Sharma, Mahajan, & Bardi, 2005*). Oxidative stress is described as an imbalance between the production of radical species and the functionality of endogenous antioxidant protection systems (*Sies, 1991*; *López-Alarcón & Denicola, 2013*). ROS and RNS are not only dangerous, these molecules are important player in fundamental functions like cells signaling and biosynthetic reactions. However, a strong imbalance between the production of oxidative species is considered to have a role in the process of aging and several degenerative diseases, such as cancer, atherosclerosis and cardiovascular disease (*Meagher & Rader, 2001*; *Storz, 2005*).

Carotenoids have been investigated due to their capability to act as ROS quencher in different biological systems. Among them, astaxanthin (ASX) (3,3'-dihydroxy-4,4'-dione-β,β'-carotene) showed strong antioxidant and anti-inflammatory properties (*Liu, Mao, Zhou, & Guarnieri, 2016*). Antioxidant activity of ASX was reported to be 10-100 times higher than vitamin E and β-carotene. ASX possesses also higher ability in the protection of lipids from photo-oxidation compared to lutein and β-carotene (*Naguib, 2000*; *Kurashige, Okimasu, Inoue, & Utsumi 1990*). With the growing perception of the consumer of a link between diet and human health, and the increasing tendency to consume supplements in order to enrich the diet, ASX due to its recognized multiple health effects and superior antioxidant activity has become one of the important health-promoting ingredients in functional foods and beverages. However ASX use is limited by its tendency to degrade at different conditions such as: light, presence of oxygen, extreme temperature and pH (*Anarjan & Tan, 2013a; Tamjidi, Shahedi, Varshosaz, & Nasirpour, 2017*; *Bustos-Garza, Yáñez-Fernández, & Barragán-Huerta, 2013*). This characteristic led to search for new technologies aiming at the stabilization of ASX that in the last years produced different solutions, mainly in the field of nano- and micro-encapsulation. In particular, nano-particles containing ASX were developed in the past in the form of inclusion complex with β-cyclodextrin (*Chen, Chen, Guo, Li, & Li, 2007*; *Yuan, Du, Jin, & Xu, 2013*), liposome, solid lipid nanostructures (*Tamjidi, Shahedi, Varshosaz, & Nasirpour, 2014*), nano-emulsions with different mixture of proteins and polysaccharides (*Anarjan & Tan, 2013b*). Often these studies were focused only at the "design" of

the encapsulation process but very little is known about the behavior of the particles, their biological activity and toxicity in biological system.

Chapters 2 and 3 described the production of ASX nanoparticles (ASX NPs) enveloped in a proteins shell. The present study focuses on the analysis of the antioxidant activity of ASX NPs through ABTS colorimetric assay. This method is recognized as simple, reproducible and sensitive but it does not allow to gain information about the direct effect of the antioxidant substance in a complex system, such as a cell. The use of cellular systems could allow to gain new data about the cellular uptake and the potential cytotoxicity of the developed nano-carrier. Indeed, although the NPs produced in the previous chapter are formed by a biodegradable and GRAS matrix, there are some concerns about the safety and potential toxicity of nano-carriers (*Conner & Schmid, 2003*). To evaluate these aspects, cytotoxicity, uptake efficiency and its mechanism were evaluated in this study.

2. Material and methods

2.1. Chemical and reagents

Whey protein concentrate (WPC) was gently provided by I.T.A.L.I (Reggio-Emilia, Italy). Protein composition (w:w) was 80%. *Haematococcus pluvialis* powder was obtained from a local producer (Italy). Ethyl acetate, Fluorescein isothiocyanate (FITC), methanol, 2′,7′-Dichlorofluorescin diacetate (DCFH-DA), Dimethyl sulfoxide (DMSO), trypsin, 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS), Potassium persulfate (PPS), Trolox were purchased from Sigma-Aldrich (St. Louis, MO, US).

2.2. Astaxanthin extraction from *H.p.*

H.p. oleoresin was obtained following the protocol proposed by Bustos-Garza and co-workers with minor modifications (*Bustos-Garza et al., 2013*). Briefly, the algae powder were pretreated by mixing 5 g of algae and 1 ml of 3 M HCl and treating the sample in a microwave oven for 1 min at 100 W (*Sarada, Vidhyavathi, Usha, & Ravishankar, 2006*). The pretreated algae were extracted with 25 ml of ethyl acetate in a tube with a screw cap, for 60 min under agitation at 50 °C in a thermal bath. The solid portion was separated by centrifugation at 3000 g for 10 min to eliminate the biomass. The oleoresin was kept in the dark at 4 °C until use.

2.3. Astaxanthin nanoparticles preparation

Astaxanthin nanoparticles (ASX NPs) were produced following the method described previously by (*Yi, Lam, Yokoyama, Cheng, & Zhong, 2015*) with some modifications. WPC was dissolved in distilled water at concentrations of 1%. The solution was stirred for 30 min at room temperature without pH modification. ASX extract from *H.p.* in ethyl acetate were combined with the protein solution at a ratio of 9:1. A fine emulsion was produced using an ultra-sonicator for 10 min at a potency of 10 W (Microson ultrasonic cell distruptor XL). At the end of the process ethyl acetate was removed using nitrogen flux in the dark. WPC ASX NPs was kept in the dark at 4 °C until use.

2.4. Spectrophotometric analysis

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

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

Where [A] is the concentration of ASX expressed as mg/ml; A480 the sample absorbance at 480 nm; DF: dilution factor; E(1%;1cm): ASX percent solution extinction coefficient [$(g/100\ ml)^{-1}\ cm^{-1}$] in ethyl acetate (2150); d: the optical path (cm).

2.5. Surface charge and average diameter

Zeta-potential, mean diameter and polydispersity index (PDI) of WPC-ASX nanoparticles were analyzed by dynamic light scattering (DLS) principles using a Malvern Zetasizer (Nano-ZS; Malvern Instruments, Worcestershire, U.K). Prior the analysis the sample were diluted 80 times to avoid multiple scattering effect. PDI value ranging from 0 to 1, indicated the distributions of the particle sizes, value close to 0 indicated a uniform population of particles and a value close to 1 indicated a wide variety among particles dimensions. Zeta potential give an important information about particles stability, for values closer to 0 the system is considered not stable, due to the absence of a net charge that can contrast the aggregation process of NPs. The obtained results are the average of at least three measurements.

2.6. ABTS assay

For ABTS assay we followed the procedure proposed by Thaipong and Co-workers, (2006) with some modifications. The working solution was prepared from a stock solution formed by 7.4 mM ABTS solution and 2.6 mM potassium persulfate (PPS) solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted opportunely with methanol (for the *H.p.* oleoresin), or PBS (for ASX NPs) to obtain an absorbance of 0.75 units at 734 nm. Fresh

ABTS solution was prepared for each assay. Samples (20 µl) were loaded in a 96-well plate and left to react with 180 µl of the ABTS solution for 2 h in a dark condition. Then the absorbance was taken at 734 nm using a plate reader. The standard curve was linear between 25 and 600 µM Trolox. Results were expressed in µM Trolox equivalents (TE)/g mass. Additional dilutions of the samples were eventually performed in order to fit the standard curve.

2.7. Cell culture

The experiments dealing with cells have been carried out in collaboration with the laboratory of animal cell cultures of the University of Verona directed by Prof. Roberto Chignola. The utilized cell lines were from ATCC (*American Type Culture Collection* Rockville, Maryland, USA).

Monocyite macrophages cell line (J774A) from adult mice, **HepG2** (human hepatocellular carcinoma, ATCC HB-8065, cell type: epithelial) and **Caco2** (human colorectal adenocarcinoma, ATCC HT-B37, cell type: epithelial), were cultured in RPMI 1640 medium (Biochrom AG, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine (Sigma, St. Louis, MO., USA) and 35 mg/l of gentamycin. Cells were grown at 37°C in a humidified 5% CO_2 atmosphere in T75 culture flasks (Greiner Bio-One, Frickenhausen, Germany) and periodically were diluted with fresh medium to avoid starvation. The concentration of viable cell was measured by an haemocytometer in presence of trypan blue (0.1% in PBS, Biochrom AG).

2.7.1. Cytotoxicity assay

HepG2 cells were initially seeded in 96-wells culture plates at a density of 25000 cells in 200 µl of complete medium. After 6 h 25 µl of medium was removed and substituted with a same volume of antioxidant, and incubated for 30 minutes at 37°C. The antioxidant was represented by *H.p.* oleoresin dissolved in 1% DMSO and WPC ASX NPs both tested at the following concentrations: 0.2, 0.1 and 0.05 mg/ml. Methanol 20% was used as negative control, RPMI medium as positive control and DMSO 1% to test if even in small quantity it could affect cell vitality. Cells were counted using light phase-contrast microscopy (Olympus IX51; Olympus, Tokyo, Japan), using a Burker chamber, and the vital dye trypan blue (dilution 1:2 v/v) to exclude dead cells. ATP was determined by the luciferine/luciferase method using CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega, Madison, WI, USA), following the manufacturer's instructions. Emitted light

was measured using a microplate luminometer (FL_x 800, Bio-tek Instruments) and data were expressed in luminescence arbitrary units.

2.7.2. Cellular antioxidant activity by flow cytometry

Adult mice macrophages cell line J774A were seeded at a density of $1x10^5$ cells in 2 ml of RPMI medium. After an incubation of 24 h at 37°C 1 ml of the medium was removed and 500 µl of DCFH-DA 50 µM and 500 µl of antioxidant were added. The tested antioxidants were: *H.p.* oleoresin dissolved in 1% DMSO and WPC ASX NPs dissolved in RPMI both at the concentration of 14, 7, 3.5, 1.75, 0.875 and 0.438 µg/ml of *H.p.* oleoresin. As a control, also the antioxidant activity of native WPC protein was analyzed at concentrations of 25, 12.5 and 6.25 µg/ml. After incubation with the antioxidant, the medium was removed and a same volume (500 µl) of the oxidant species was added to the plate (600 µM ABAP and 0.002% H_2O_2 were tested as different stressing conditions). The treated cells were incubated for 30 minutes at 37°C. After incubation the cells were scraped from the plate to obtain a cellular suspension for the cytofluorimetric analysis. The suspension was kept at 4°C until use. The cells associated fluorescence was measured using a Guava easyCyte TM 5 flow cytometer v.2.7 software (Merck Millipore, Billerica, MA, U.S). The cytometer was equipped with 488 nm, 20 mW, blue laser light, and forward scatter (FSC) photodiode and side scatter (SSC) photomultiplier. Green fluorescence 525/30 filter, yellow 583/26 and red 680/30 filters allow analysis of fluorescence emissions from samples. Calibration of the cytometer was routinely checked using the Guava EasyCheck kit (Merck Millipore, Billerica, MA, U.S.) according to the manufacturer's instructions. The raw data were exported, and then processed and analyzed by Mathematica software.

2.7.3. Confocal microscopy

The uptake of WPC ASX NPs was analyzed by confocal microscopy. To this purpose, WPC ASX NPs were labelled with fluorescein isothiocyanate (FITC). In particular, 10 mg of lyophilized WPC ASX NPs were resuspended in 1.695 µl of carbonate buffer pH 7.3. Ten milligrams of FITC was resuspended in 1 ml of DMSO. Eighty µl of FITC concentrated solution was added to the WPC ASX NPs solution and left to react for 2 h in the dark. The non-reacted FITC was removed using a PD Mini-Trap desalting column with Sephadex G-25 resin (Supelco, Bellefonte, PA, U.S.).

The following cell lines were used: Caco2 (human colorectal adenocarcinoma, ATCC HT-B37, cell type: epithelial), HepG2 (human hepatocellular carcinoma, ATCC HB-8065, cell type: epithelial). Cells were routinely cultured at 37 °C in a humidified 5% CO2 atmosphere, in RPMI 1640 (Biochrom AG, Berlin, Germany) supplemented with 2 mM glutamine (Sigma-Aldrich, St.Louis, MO, USA), 35 mg/l gentamycin (Biochrom AG) and 10% heat-inactivated fetal bovine serum (Biochrom AG). Cells were seeded into the wells of glass bottom µ-Slide IbiTreat chambers (Ibidi GmBH, Martinsried, Germany) at the initial cell density of $5 \cdot 10^4$ cells/well in 200 µl complete growth medium and incubated for 24 hours at 37 ºC. A volume of 50 µl of FITC-labelled WPC ASX NPs solution (green fluorescence) was then added, and the cells were further incubated at 37 ºC for 1 hour. Cells were carefully washed with PBS, fixed with 4% (w/v) paraformaldehyde for 30 min and, after washings with PBS and quenching with 50 mM NH_4Cl, permeabilized with PBS-0.1% Triton X-100. Cells were then stained with rhodamine-phalloidin (to label F-actin, red fluorescence; Cytoskeleton, Denver, CO, USA) for 30 min and then with DAPI (to label nuclei, blue fluorescence; Sigma-Aldrich, St.Louis, MO, USA) for 10 min. Images at Δz=0.5 µm were collected using the SP5 confocal microscope from Leica Microsystems (Mannheim, Germany) with 63x objective (HCX PL APO λ blue 1.4NA OIL). Image analyses were performed with ImageJ v. 2.0.0 software.

2.7.4. Cell nanoparticles uptake

To gain information on the mechanism involved in the cellular uptake, the experiment was conducted in the presence of a blocking condition, i.e. at low temperature. Indeed, Caco2 and HepG2 cells were incubated with FITC-labelled WPC ASX NPs (0.02 mg/ml) at 4°C for 30 minutes and at 37°C as a control.

3. Results and discussion

ASX NPs were obtained through emulsion solvent-evaporation technique with whey protein concentrate as encapsulant matrix. The obtained particles, were stable in solution, characterized by a highly negative Z-potential value (-28.5 mV), an average diameter of 90-100 nm) and a low polydispersity index (PDI) (0.245), underlining the presence of a slightly polydisperse population of NPs.

3.1. Antioxidant capacity

The antioxidant capacity of a given molecule can be attributed to different mechanisms which are for example the binding of transition metals, radical scavenging activity and, in the particular case of carotenoids, the possibility to quench radical species due to their highly unsaturated nature. In the case of ASX some works have suggested that its radical scavenging activity is mediated by the transfer of hydrogens or electrons, and in the case of the quenching of singlet oxygen, by energy transfer between the electrophilic singlet oxygen and the polyene chain (*Zhang, Sun, Sun, Chen, & Chen, 2014*).

A concentration of 0.2 mg/ml of ASX from *H.p.* oleoresin was shown to have a Trolox Equivalent Antioxidant Capacity (TEAC) value of 30 (expressed as mmol Trolox/kg extract), between 6 and 9 times lower than the values found in literature, probably because of the low extraction efficiency of ASX or for the differences in the algae batch used (*Jaime et al., 2010*; *Cerón et al., 2006*). WPC ASX NPs displayed an 11-folds higher antioxidant capacity than *H.p.* oleoresin tested at the same concentration. This result could be explained considering the antioxidant properties of whey proteins, already described in literature (*Yi et al., 2015*) and the small diameter of the NPs, if compared to the crystalline form of non-encapsulated *H.p.* oleoresin, that might increase the surface to volume ratio. The AOC of WPC native proteins were tested and found to contribute for the 74.2% of the total WPC ASX NPs antioxidant capacity. This was probably due to the free radical scavenging activity properties of cysteine, tyrosine, tryptophan, phenylalanine and histidine residues present in the proteins structure (*Singh & Sarkar, 2011*).

3.2. Cellular antioxidant activity of WPC ASX NPs

The AOC of WPC ASX NPs was tested also through the use of model cell line. Initially, the cytotoxicity of *H.p.* oleoresin and WPC NPs were tested at three different concentrations (0.2, 0.1 and 0.05 mg/ml) on HepG2 cells. The obtained results (Fig.1) showed negligible toxicity effect of WPC ASX NPs and a slight viability decrease of the cells treated with *H.p.* oleoresin at 0.1 mg/ml.

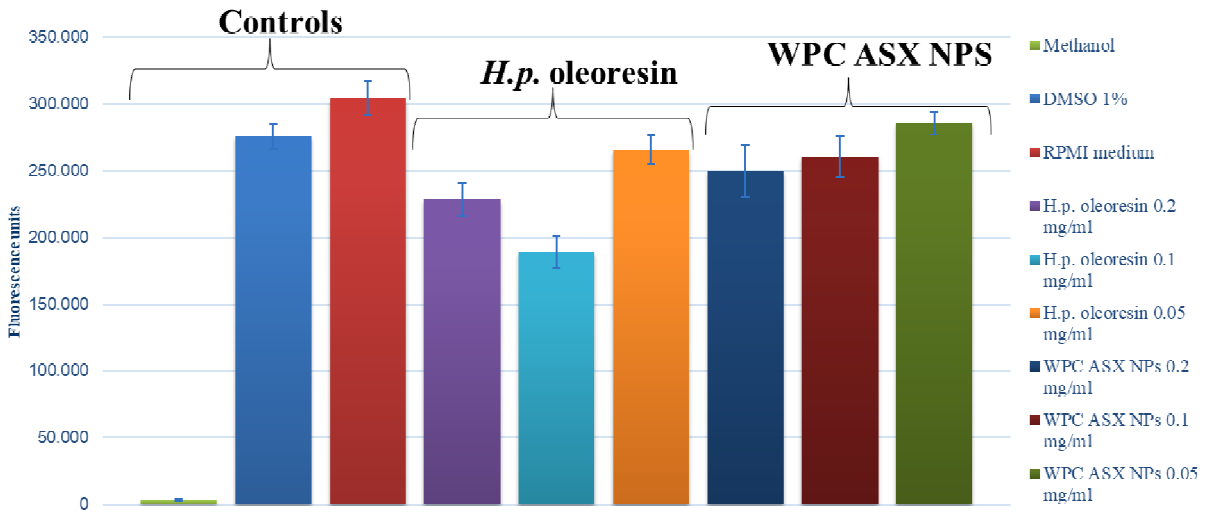

Fig.1 Cell viability of HepG2 cells incubated at different concentrations of *H.p.* oleoresin,WPC ASX NPs and WPC NPs.

Cellular antioxidant activity (CAA) was developed by Wolfe and co-workers (*Wolfe & Liu, 2007*). It is one of the most employed antioxidant techniques used to study the effect of antioxidant substances in cells systems in presence of highly reactive species. Although this technique is well established into microplate experimental set up using HepG2 cells, our attempts to apply this system to our samples did not lead to consistent and reproducible results (data not shown). For this reason, and because the method previously mentioned does not provide any information about cells viability during the experiments, a different approach based on flow cytometry was chosen. This approach gives the opportunity to discriminate between viable and non-viable cells due to their light forward and side scattering properties. To perform the assay, a macrophages cell line from adult mice (J774A.1) was selected. Compared to HepG2, frequently employed for the CAA assay, J774A.1 cells present higher phagocytic activity and are easier to manipulate. Moreover, they are naturally capable to produce high amounts of ROS. The first step to develop the method was to identify an appropriate stimulus for the generation of ROS by the cells. Macrophages cells were

subjected to different treatments: thermal shock, and incubation with different chemicals, e.g. ABAP, H_2O_2. The generation of ROS would then oxidize DCFH-DA with the concomitant emission of green fluorescence. Among the treatments, as shown in Fig.2**,** the fluorescence distribution relative to the treatment with ABAP (Fig.2-B) and thermal shock (Fig.2-C) were close to the signal produced by the control sample (Fig.2-A). In the case of cells treated with H_2O_2 (Fig.2.D) the fluorescence signal was largely increased in respect to the control, underlining a higher production of ROS species by these stressed cells. For this reason the treatment with H_2O_2 was chosen to induce a strong and homogeneous production of ROS species, in terms of time.

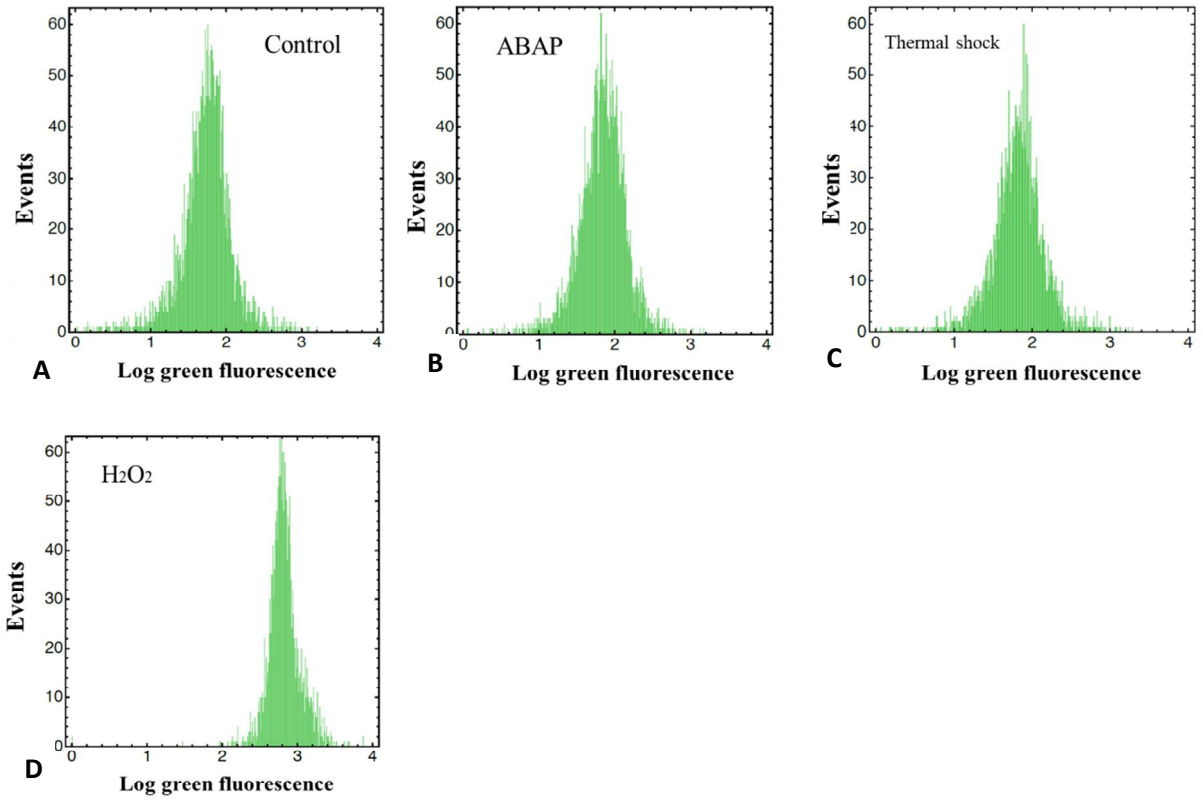

Fig.2. Cells fluorescence unit variance in response to different radical generator.

As shown from the fluorescence data, in Fig.3-A WPC ASX NPs were able to inhibit the fluorescence emission in a dose-dependent manner more effectively than WPC alone in the form of native proteins and *H.p.* oleoresin. In particular, WPC ASX NPs showed an AOC respectively 4 times higher than *H.p.* oleoresin at the maximum concentration tested (14 µg/ml) and 5 times higher than 1% WPC solution. It was interesting to observe that, differently from ABTS, by this system WPC did not show antioxidant properties, and all the activity seems to be related to ASX. The higher CAA showed by the nano-encapsulated system might derive from the higher uptake of ASX

by the cells in respect of the *H.p.* oleoresin form. In this contest, another important point to underline is the fact that the dispersion of *H.p.* oleoresin solubilized in DMSO could have formed aggregate when in contact to aqueous media that had led to the formation of aggregate with larger size in respect to the NPs system reducing in this way the absorption of the active ingredient inside the cells.

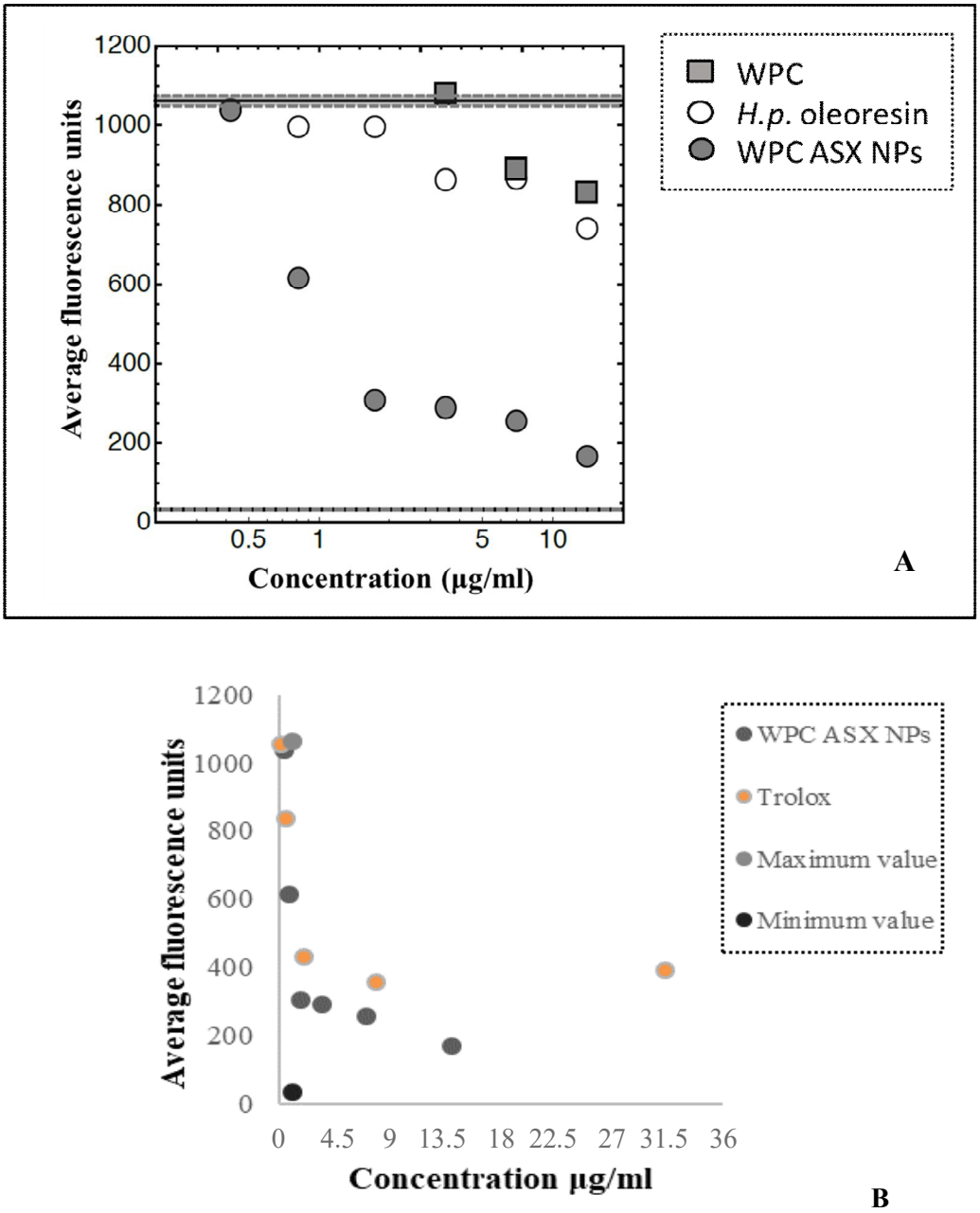

Fig.3 Cellular antioxidant activity tested in adult mice macrophages cells (J774A.1) via flow cytometry with A) WPC ASX NPs, *H.p.* oleoresin and WPC and B) the comparison between the antioxidant properties of Trolox and WPC ASX NPs.

In different study it was demonstrated that the bioavailability of encapsulated system is inversely proportional to the size, in part confirming the previous hypothesis (*Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013; Wang et al., 2017*). The antioxidant capacity of NPs was compared also with Trolox, as shown in Fig.3-B. ASX in the form of NPs showed higher antioxidant activity at all the tested concentrations.

3.3. Cellular uptake of WPC ASX NPs

Testing the bioavailability of NPs by *in vivo* systems is difficult to perform from a practical and ethical point of view. In this way, in the last decade, *in vitro* models, such as cellular uptake analysis through confocal microscopy systems had gained much interest due to the fact that it can provide useful information about the potential fate of NPs in a complex system. It is also relatively easy, quick to perform, and it allows for relatively inexpensive screenings of multiple samples.

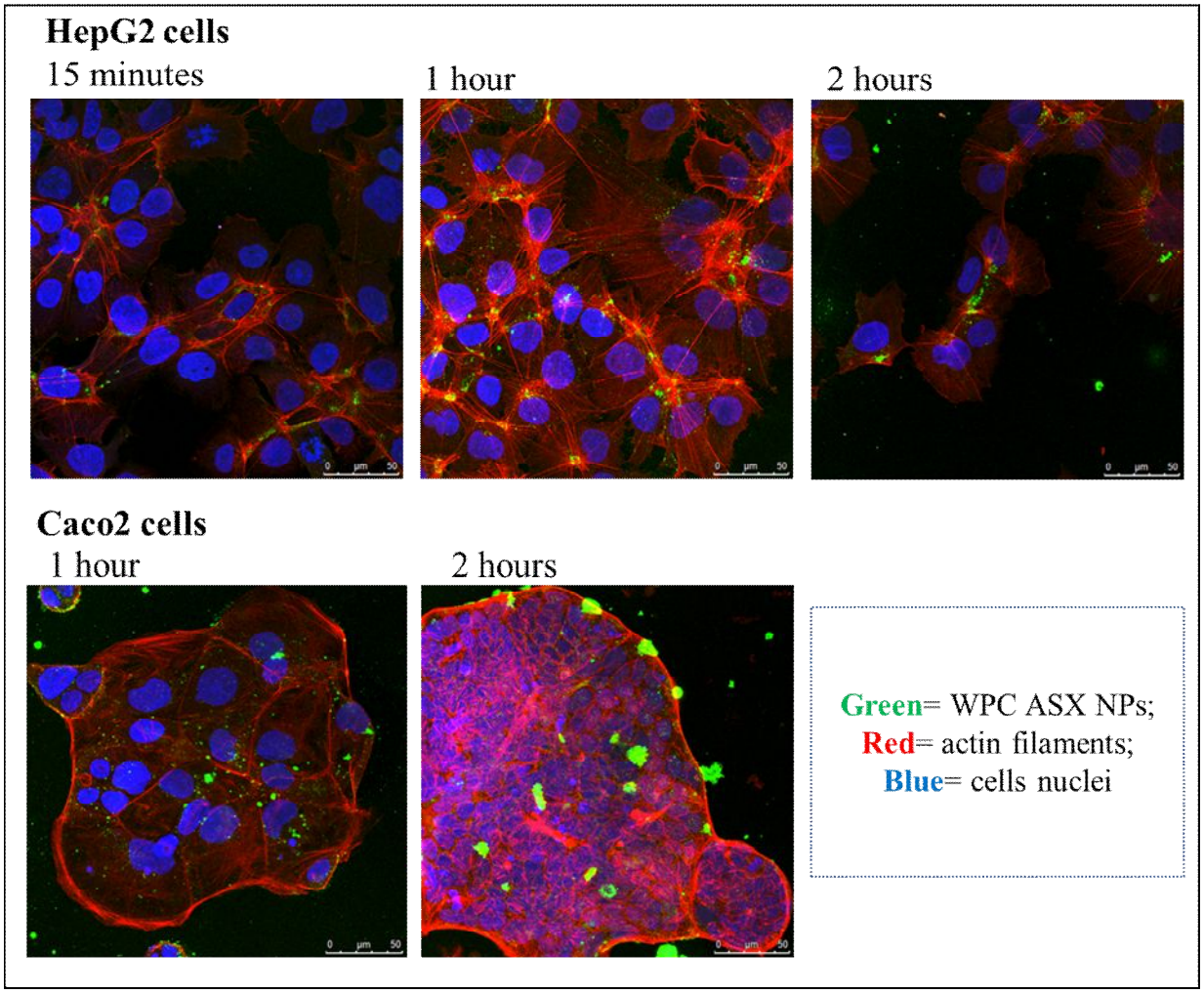

Fig.4 CLSM images of HepG2 and Caco2 cells incubated for different times with labelled FITC WPC ASX NPs.

In Fig.4 the micrograph pictures obtained by confocal microscope are observable. In particular, concerning HepG2 cells, WPC ASX NPs are visible inside the cells after 15 minutes of incubation underling that the uptake process of NPs is very fast, the accumulation seems to proceed later on during the first and the second hour of incubation as can be observed by the slight increase of fluorescence (Fig. 4).

The cell uptake of NPs seems to follow a precise pattern showing a strong accumulation of NPs along actins filaments. Actin polymerization and rearrangement plays an important role during endocytic internalization, in particular in clathrin- and caveolae-mediated process (*He et al., 2013*). WPC ASX NPs uptake is visible also in Caco2 cells. NPs appear to accumulate close to the cell membrane leading to bigger aggregates after 2 hours. It was already reported that the higher presence of carboxylic groups and surface charge of protein NPs correlate with an increased cell uptake (*He et al., 2013*; *Gåserød, Jolliffe, Hampson, Dettmar, & Skjåk-Bræk, 1998*).

3.4. Inhibition study

The cellular uptake of NPs with diameters around 100-150 nm has been already described in literature as endocytic internalization (*Conner & Schmid, 2003*). To confirm this kind of mechanism also in the case of WPC ASX NPs, the uptake was tested in presence of an endocytic blocking condition for both HepG2 and Caco2 cells. At 4°C, the condition tested, all the energy-dependent processes, and thus also endocytosis, are inhibited. In Fig.5 HepG2 cells showed a NPs uptake inhibition percentage of 86%, Caco2 cells showed an inhibition of 68%. The lower uptake could be due to the reduced cell activity and to the scarce fluidity of the cell membrane. The uptake still measured could be explained by the presence of residual ATP, that can be used for the transportation of the NPs in the cells. In particular, during endocytic internalization it was reported the polymerization and rearrangement of actin filaments when the process was caveolae- and clathrin- mediated, this seems to validate our previous observations about the peculiar positioning of NPs along actin filament during cellular uptake in the case of HepG2 cells (Fig.4).

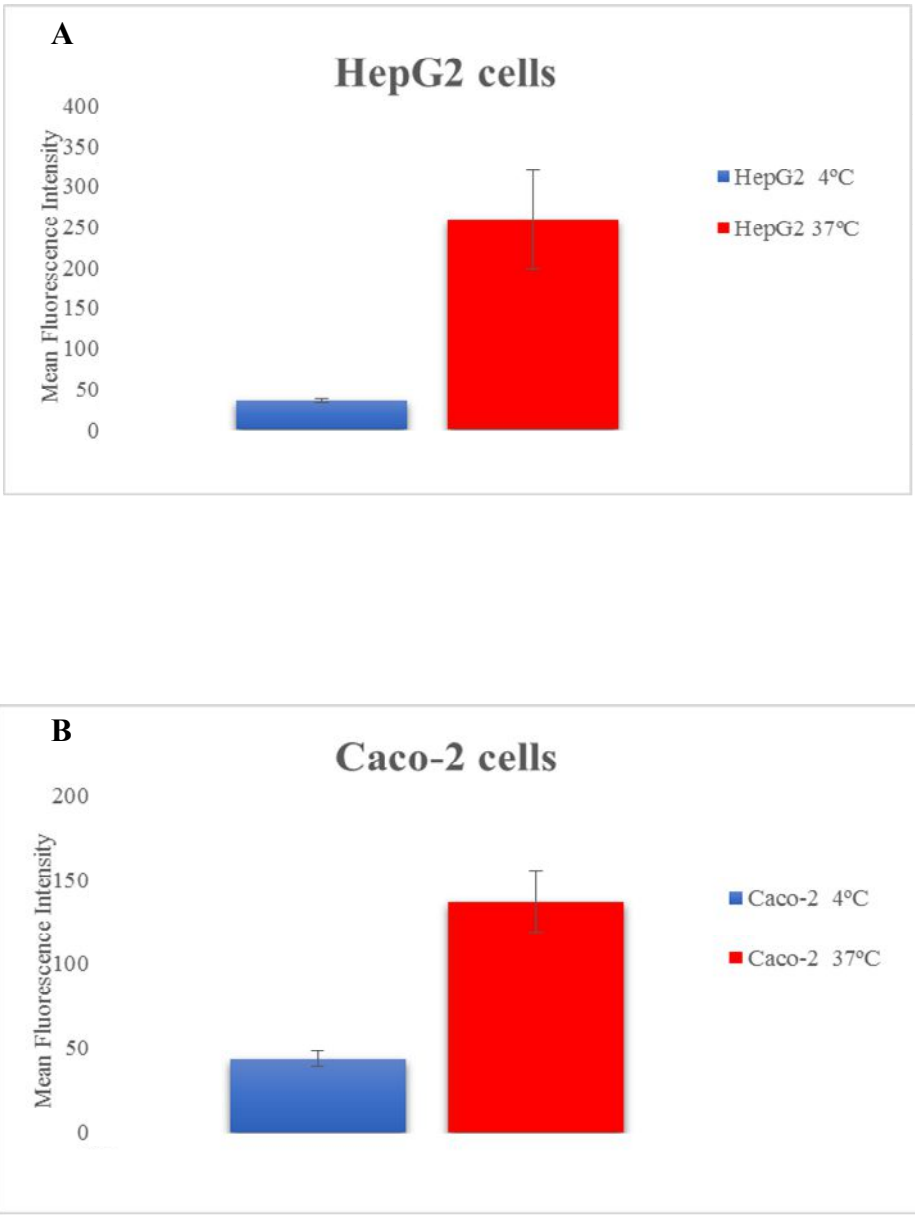

Fig.5 Cellular uptake inhibition of WPC ASX NPs in presence of a blocking condition (4°C) in A) HepG2 and B) Caco2 cells.

To obtain a deep understanding of the mechanism behind the uptake of WPC ASX NPs more investigation will be required, e.g. by using other blocking agents such as, genistein, that is reported to inhibit F-actin during internalization and clathrin-mediated uptake (*Iversen, Skotland, & Sandvig, 2011*). Another possibility is represented by polarized cells, like those constituting the Caco2 monolayer, which better resemble the complexity found in epithelial cell layers (*Sandvig & van Deurs, 2005)*.

4. Conclusions

ASX NPs were characterized by a protein shell, a diameter around 100 nm and a negative surface charge. NPs showed a TEAC 11 times higher than ASX tested at the same concentration. WPC, the constituent of the membrane surrounding the oily core of oleoresin seemed to contribute for the 70% to the total antioxidant capacity. The results of the cellular antioxidant activity were in agreement with ABTS confirming that WPC ASX NPs possess superior activity in comparison to non-encapsulated oleoresin. It is noteworthy that in this case the contribute of WPC to the total antioxidant capacity of WPC ASX NPs was definitely minor since in the form of native proteins they produced a strong fluorescent signal, proving their incapacity to counteract DCFH oxidation. These results confirm that the analysis *in vitro* of the antioxidant capacity of specific molecules/extracts should be confirmed by different approaches. The uptake by HepG2 and Caco2 cells analyzed through confocal laser microscopy revealed peculiar mechanisms. Indeed, in the case of HepG2, the deposition of NPs along actin filament lead us to hypothesize the involvement of endocytic energy-dependent transport mechanism. The data obtained by the cellular uptake inhibition test confirmed a decreased uptake for both HepG2 and Caco2 cells in presence of an endocytic blocking condition.

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Chapter 5.

Conclusions

In the present work the oleoresin extracted from *Hametacoccus pluvialis* was successfully nano-encapsulated through emulsion solvent-evaporation technique using proteins as encapsulant material. This approach allowed to disperse in water a strongly hydrophobic molecule like astaxanthin maintaining the light absorption characteristics of the carotenoid in the visible spectrum. The process was optimized varying different parameters to identify the conditions leading to the best compromise between the lowest achievable diameter, the highest stability and the greater payload. In particular the size around 100 nm is the most crucial objective to achieve since this would improve the bioavailability of the encapsulated molecule. The use of ethyl acetate is merely of processing aid, since it is totally removed from the product after encapsulation and can be easily recovered and used for the subsequent production. Even though it is an organic solvent, ethyl acetate is characterized by low toxicity in comparison to dichloromethane that other authors employed for this type of encapsulating approach. Finally being largely used for other food processes it is not difficult to find food-grade forms of this solvent on the market.

Form a technical point of view, the proposed protocol is easier in comparison to other works proposed in literature. Indeed satisfactory results were obtained without the need to 1) employ solvent-saturated water, 2) solubilize the proteins in buffered solutions 3) thermally pre-treat the proteins.

The NPs produced with whey protein concentrate (WPC) showed a scarce solubility in the pH range of 3,5-5,5, that correspond to the isoelectric point of whey proteins. This problem could be solved including a coating step using polysaccharides gum arabic. However, NPs showed higher stability toward UV light, metal-induced oxidation and heat degradation with respect to the *H.p.* oleoresin. Simulated digestion of WPC NPs showed a very high ASX bioaccessibility (90%) with 75% conversion to the free form that represents the most bioavailable ASX form. NPs showed a higher antioxidant activity compared to *H.p.* oleoresin.

To study the antioxidant capacity of the NPs a flow-cytometry-based cellular assay was developed. Indeed, routinely used assays like ABTS do not take into account the complexity of a cell system that could help to understand for instance whether the NPs are toxic or are truly absorbed. The encapsulates showed higher antioxidant capacity in comparison to the oleoresin when analyzed both by ABTS and flow cytometry. The study of the uptake of the NPs in cell model systems through confocal laser microscopy and flow cytometry indicated that the process probably follows an energy-dependent mechanism.

During the project we focused our attention in the research of further encapsulant matrices alternative to whey proteins in order to better comply with specific consumers cultural and religious needs, and/or in order to achieve an allergen-free ingredient. For this purpose, we selected three plant protein isolates from soy (SPI), pea (PPI) and rice (RPI). Out of these three matrices, SPI and PPI gave the best results allowing for the production of NPs with dimensions in the range of 90-130 nm, while RPI did not lead to the production of NPs, probably because of the low solubility and emulsifying properties of proteins constituting the isolate.

The release profiles of ASX from SPI and PPI NPs were compared with the ones of WPC NPs. WPC and PPI NPs displayed very similar release patterns, while SPI showed a burst release of most of the ASX at the end of the gastric stage. Therefore, PPI seems to be the most suitable candidate as plant alternative emulsifier for the production of ASX NPs. These results suggest that the use of different protein sources could allow to achieve a controlled release of the active ingredient, since specific molecules may need to be absorbed in different parts of the gastrointestinal tract.

Taken together, all these results show that the developed nanoparticles can be considered a good candidate carrier for the delivery of high amounts of ready-to-absorb astaxanthin for human diet. ASX NPs could be applied in different markets, e.g. the production of functional beverages, supplements in the forms of mono-dose sticks or sachets. To this purpose the formulations should be converted into more stable dried forms.