Universitat de Girona
Escola Politècnica Superior

Incoming Exchange Student - Final Degree Project

Erasmus ☐ Techno ☑ Other (specify):

Degree course: Grau en Enginyeria Agroalimentària Pla 2010

Title: IMPROVING THE STABILITY OF AN HEMOGLOBIN-BASED NATURAL FOOD COLORANT BY MEANS ENCAPSULATION WITH CALCIUM ALGINATE

Document: Final Degree Project

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Delivered on (month/year): June – 2015

This final project entitled "IMPROVING THE STABILITY OF HEMOGLOBIN-BASED NATURAL FOOD COLORANT BY MEANS ENCAPSULATION WITH CALCIUM ALGINATE", has been carried out in the Food Technology Group of the University of Girona, Spain, under the supervision of Dr. Carmen Carretero and Mrs. Dalin Ly (Lecturer of Royal University of Agriculture).

Girona, 29th June, 2015

Dr. Carmen Carretero

Mrs. Dalin Ly

Acknowledgements

This final project about “the improving stability of hemoglobin-based natural food colorant by means encapsulation with calcium alginate” had been done in the University de Girona, UdG. I was incorporated in one of the research lines of the Laboratory of Food Technology.

During my first time of staying in Girona as well as in European country, I had good chance to make the best life experience here with all the people whom I just had a little bit time of knowing them. However, they are very close to me, friendly, kind especially, they usually give advice and explain me more as they can. They never blame me about my mistakes. So that, I would like to say thank you to every person who deals with this situation.

First of all, I would like to deeply thank to my family who has supported me everything related to my study. The most thankful is for my parents who’ve worked hard for survive all children even we are big family.

Second most especially, I would like to give thanks to my experienced and helpful lecturer, Dr. Carmen Carretero who has provided me with an important deal of guidance, counseling, working with me, advice and provide good opportunity to write the final project which is the first time of mine.

Special thanks for Dr. Dolos Parés who gave me the chance to work in Food Technology also revised my final work writing. As well as thanks for everyone whom I have done my work with and who help me for everything which I can’t do to Anna Maria, Monica, Anna Bonaterra, Elena, David, Guillem.

Lastly, I really would like to thank Prof. Emili Garcia-Berthou for gave me the special opportunity to carrying out my final project in Girona and Mrs. Raquel Solà G. as well for arranged everything for me before coming and going back. I also would like to give the most thanks to the financial support from the European Commission (Erasmus Mundus Project Techno I) who have supported me everything during my staying period in this new world and Royal University of Agriculture, Cambodia, for giving me this good chance.

Koemseang Sok

Summary

This work is circumscribed in a research project aimed at producing a natural red food colorant, stable during long-term storage at room temperature and with a wide range of applications, using hemoglobin from porcine blood as raw material.

Previous studies had shown that both spray-drying (which is the most frequently used preservation method for liquid products) and freeze-drying, promoted the oxidation of iron during dehydration, and the subsequent loss in the quality of hemoglobin powder color. To prevent oxidation reactions during dehydration, several potentially protective substances (i.e. antioxidants, chelating agents and stabilizers) were evaluated. At present, a spray-dried product showing excellent color characteristic by adding 3 different compounds, which act synergistically, has been achieved. However, further research is necessary in order to increase the stability of the product during storage at room temperature and to extend the applications to a wide range of food matrices.

The objective of this study was improving the stability of this hem-derivative product at acidic conditions and moderate temperatures by means of encapsulation with calcium alginate.

Commercial hygienically collected blood from local industrial slaughterhouse was used. Plasma was separated from red blood cells fraction by centrifugation. Then, red cells fraction was immediately hemolyzed to obtain hemoglobin (Hb) solution.

Firstly, spray-dried powder of hem-derivative from blood was produced as control for comparing some parameters, as color and color stability, water activity and moisture content, with capsules obtained in this experiment.

In this work, sodium alginate encapsulation system was developed. Hemoglobin solution was firstly stabilized by the same method used for spray drying control capsules, after the samples were mixed with 2% of sodium alginate solution (AS) at different concentrations (20:80, 40:60, and 60:40). Hb-derivative:AS solutions were dropped into 5% of $CaCl_2$ solution to find the best condition to obtain the Hb capsules.

The best combination of Hb and AS, having in mind the desired characteristics was 40:60. Different drying systems were assayed; freeze drying and fluidized bed drying. Several conditions of fluidized bed drying were assessed; 50% airflow (for 20, 30 and 40 min) and 70% airflow (for 10, 20 and 30 min). During fluid bed drying, capsules were mixed with poppy seeds (2:3 w:w) in order to improve the fluidization of wet capsules.

Spray-dried powder of control Hb-derivative was good enough and could be acceptable because of the high level of dry matter and a_w that can avoid of microbial spoilage, as well as the color, which was acceptably conserved during 3 months of storage. Whereas, the effect of freeze drying on encapsulated samples was not a good drying system, because Hb capsules turned into dark brown color during the dehydration process, even the 40:60 capsules showed the best color parameters. Fluidized bed drying was neither a good system to dry the capsules, due to the brownish and dark color acquired in all drying conditions. Even though drying at 70 % airflow for 30 min or at 50 % airflow for 40 min, were conditions which permitted to reach moisture contents and a_w values low enough to prevent microbial spoilage.

Scanning electron microscopy (SEM) was used to understand the behavior of capsules from different concentrations of Hb-derivative:AS.

Moreover, coating with chitosan was assessed as a system to improve stability of alginate capsules. Different stirring times of capsules in a 0.4% solution of chitosan in acetic acid 0.1M at pH 6 were assayed (5, 10, 15 and 30 min). The best results were reached in coating process during 30 min. SEM pictures showed that coating of chitosan is practically the same for 5 and 10 min of stirring. For 15 min, the coating is thicker, and become still more compact as longer is the stirring time up to 30 min.

Several conclusions were obtained from this study, although alginate encapsulation was not a good system to reach our objectives. Coating alginate capsules with chitosan could be a good alternative to stabilize Hb color. However, further studies are necessary to develop a best system to apply this method.

Abbreviations

–	AS	:	Alginate solution
–	Hb	:	Hemoglobin
–	HHP	:	High hydrostatic pressure
–	MTGasa	:	Microbial transglutaminase
–	NAM	:	Nicotinamide
–	NPN	:	Non-protein nitrogen
–	RBC	:	Red blood cell
–	SA	:	Sodium alginate
–	TCA	:	Trichloracetic acid
–	TN	:	Total Nitrogen

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1. Introduction

1.1. General introduction

Color is an important indicator of food quality. As we know coloring agents are extensively used by the food and beverage industries to make their products more attractive to consumers. Although both artificial and natural colorants are approved for use in human food, consumers today are increasingly demanding the use of natural products because of reported adverse reactions to some synthetic food dyes and their impurities (Ben Mansour et al., 2007; Gennaro, Abrigo, & Cipolla, 1994). Moreover, this physical property affects the perception of the other sensory characteristics (Clydendale, 1993) by permitting, in some products, the use of smaller quantities of other additives such as flavorings.

There are many natural sources of colorants; however, their commercial potential would be limited by the availability of the raw material (Walter et al., 1993; Martinez et al., 1998). In this case, blood hemoglobin from slaughtered animals could be an important source of natural red colorant due to the high quantities generated daily (Walter et al., 1993; Martinez et al., 1998). Blood is a rich source of iron and proteins of high nutritional and functional quality, it contains about 18% proteins, is sometimes referred to as 'liquid protein' (F.W. Putnam, 1975; Ockerman and Hansen, 2000). The cellular or red blood cell (RBC) fraction of porcine blood represents 40% of the blood (Nakamura, Hayakawa, Yasuda, & Sato, 1984; Wismer-Pedersen, 1988). An approximation of the amount of potential blood that can be derived from primary meat processing operations in Europe can be estimated from the number of slaughtered animals; which in 2012 were in excess of 308 and 42 million pigs and cattle, respectively (FAOSTAT, 2014). If we consider that 3 liters of blood can be collected from each pig and 10 liters from each calf, the annual available blood supply can exceed 1,344,000 tones. In China, about 1,500,000 tons of porcine blood is produced every year (Yu et al., 2004).

Hemoglobin is the protein that makes blood red. However, its red color is unstable and heavily dependent on the oxygenation/oxidation state of the hem iron. Deoxyhemoglobin, -the deliganded ferrous hemoglobin is purple red in color while oxyhemoglobin – the dioxygen ferrous form has a bright red color. When the hem iron is oxidized to Fe^{3+}, the hemoprotein is known as methemoglobin (metHb) and is an unstable brown hue (Jaffé, 1964; Saguer et al., 2003). The dehydration process, widely used to preserve animal blood fractions, accelerates hemoglobin autoxidation, probably due to the effects of temperature on both the protein denaturation that make the access of oxidizing agents to the hem pocket much easier and the autoxidation rate itself. Oxidation could be minimized by adding protective agents like chelating agents that are able to

form complexes when reacting with a free hem group, myoglobin, hemoglobin and other hemoproteins like leghemoglobin (Agte, Paknikar, & Chiplonkar, 1997; Akoyunoglou, Olctt, & Brown, 1963; Appleby, Wittenberg, & Wittenberg, 1973; Bertani, et al., 1999; Kendrick &Watts, 1969; Koizumi & Nonaka, 1974). Nicotinic acid and nicotinamide have been shown to be effective in preventing hemoglobin autoxidation during spray-drying (Saguer et al., 2003). Otherwise, despite the positive effects of nicotinic acid and nicotinamide, hemoglobin powder has a limited shelf life due to the oxidative browning that takes place during storage (Saguer et al., 2003).

1.2. Blood

1.2.1. Blood utilizing

Blood is mainly used in the feed industry in the forms of blood powder and blood meal (Matsumoto et al., 1995; M'ncenem et al., 1999). And also there are a lot of more kinds of blood-derived protein ingredients used as food additives and dietary supplements,

Table 1: Products of blood-derived protein ingredients used as additive and dietary supplements (source; Jack and Yun-Hwa, 2012)

Product	Company	Source of blood	Description
Fibrimex®	Sonac BV, Netherlands	Porcine or bovine	Thrombin and fibrinogen protein isolate
Plasma Powder FG	Sonac BV, Netherlands	Porcine or bovine	Plasma with increased fibrinogen concentration
Harimix (C, P or P^{+})	Sonac BV, Netherlands	Porcine or bovine	Stabilized hemoglobin
PP	Sonac BV, Netherlands	Porcine or bovine	Frozen or powder hemoglobin
Prolican 70	Lican Functional Protein Source, Chile	Bovine	Spray-dried bovine plasma concentrate
Prientin	Lican Functional Protein	Porcine	Spray-dried porcine

	Source, Chile		whole blood
Myored	Lican Functional Protein Source, Chile	Porcine or bovine	Natural colorant obtained from the red pigments of blood
ImmunoLin®	Proliant, USA	Bovine	Bovine serum concentrate
B7301	Proliant, USA	Bovine	Spray-dried bovine red blood cells
AproRed	Proliant, USA	Porcine	Stabilized hemoglobin
Aprofer 1000®	APC Europe, Spain	Porcine or bovine	Hem iron polypeptide
Proferrin®	Colorado Biolabs Inc., USA	Bovine	Hem iron polypeptide
Vepro 95 HV	Veos NV, Belgium	Bovine	Globin (hemoglobin with the hem group removed)
Plasma	Veos NV, Belgium	Bovine or porcine	Liquid, powder, frozen or flaked plasma

1.3. Hemoglobin

Hemoglobin (Hb) is the protein molecule in red blood cells that carries oxygen from the lungs to the body's tissues and returns carbon dioxide from the tissues back to the lungs. Hb is made up of four protein molecules (globulin chains) that are connected together. Hb of porcine blood constitutes 95% of the RBC dry weight and has good nutritional and functional properties (Nakamura, Hayakawa, Yasuda, & Sato, 1984; Toldrà, Busquets, Saguer, & Carretero, 2002; Toldrà, Elias, Parés, Saguer, & Carretero, 2004; Wismer-Pedersen, 1998).

There are two major parts of hemoglobin:

- **Hem:** a *porphyrin* made of four pyrrole rings with an Fe in the center. *Porphyrin* rings are found throughout biological systems and play many different roles including photosynthesis in

green plants, delivering O_2 in muscles (myoglobin) and transporting O_2 in blood (hemoglobin). The four pyrrole rings are linked so that the N atoms point toward the center of the rings (Fig.1).

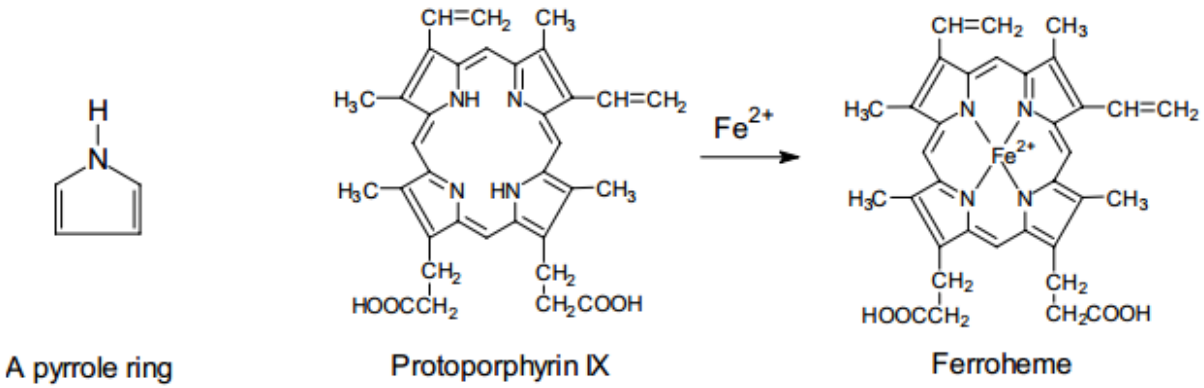

Figure 1: Ferroheme structure (Source; Russo and Sorstokke, 1973).

The hem group is what gives the red color to the hemoglobin. By changing the structure of the hem group, one can change the color of blood.

- **Globin:** It is the globular protein surrounding the hem group.

Nomenclature:

– Globin + hem (Fe^{2+}) = hemoglobin (red)

– Globin + hem (Fe^{3+}) = methemoglobin (brown)

1.3.1. Structure of hemoglobin

Hb is found in red blood cells. The Hb molecule is a tetramer consisting of 4 polypeptide chains, known as globins, which are usually:

- 2 alpha chains that are each 141 amino acids long
- 2 beta chains that are each 146 amino acids long

1.3.2. Solubility

Hemoglobin is soluble in water (1 part in 7 of water), and slowly soluble in glycerol. M. Toldrà et al. (2004) reported that the protein solubility of spray-dried RBC was significantly affected ($P < 0.05$) by both pressurization and pH (Fig. 2). Solubility at neutral pH (7) was lower than that at acid pH (4.5) in both untreated and pressurized samples. High hydrostatic pressure (HHP) treatment had a denaturing effect on the Hb, resulting in noticeable reduction in solubility at pH 7, close to the p*I* of the Hb (according to Stryer, 1995; 6.9 in oxyhemoglobin and 6.7 in desoxyhemglobin). Although

the decrease in solubility at neutral pH was higher than it was at pH 4.5 in pressurized RBC powder, as has previously been reported for liquid RBC.

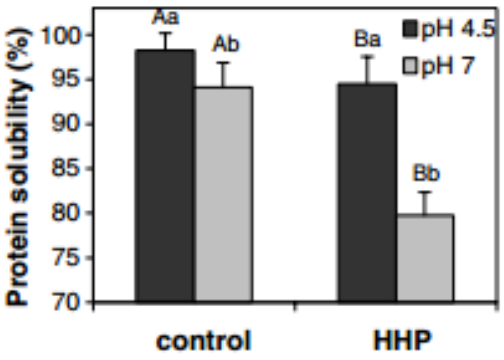

Figure 2: Protein solubility at pH 4.5 and pH 7 from 1% spray-dried red blood cell fraction (RBC) solutions obtained from untreated (control) and pressurized RBC. Error bars show mean confidence intervals (P¼95%). Different letters indicate significant differences (P<0:05) between treatment (capital letters) and pH (small letters). (Source: M. Toldrà et al., 2004)

1.4. Encapsulation

Encapsulation is a rapidly expanding technology with a lot of potential applications in areas including pharmaceutical and food industries. It is a process by which small particles of core materials are packed within a wall material to form capsules. Encapsulation method was employed to protect bioactive compounds (polyphenols, micronutrients, enzyme, antioxidants, and nutraceuticals) and in the finished application to protect them from adverse environment and also for the controlled release at targeted sites (Gouin, 2004).

1.4.1. Alginate and coating systems

Alginic acid, also called algin or alginate, is an anionic polysaccharide distributed widely in the cell walls of brown algae, where through binding with water it forms a viscous gum. In extracted form it absorbs water quickly; it is capable of absorbing 200–300 times its own weight in water (Raymond et al,. 2009). Its colour ranges from white to yellowish-brown. It is sold in filamentous, granular or powdered forms.

Alginic acid is a linear copolymer with homopolymeric blocks of (1-4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues, respectively, covalently linked together in different sequences or blocks. The monomers can appear in homopolymeric blocks of consecutive

G-residues (G-blocks), consecutive M-residues (M-blocks) or alternating M and G-residues (MG-blocks).

Alginate is classified as a hydrocolloid (a water-soluble biopolymer of colloidal nature when hydrated). The first scientific studies on the extraction of alginates from brown seaweed were made by the British chemist E.C. Stanford at the end of the 19th century, and the large-scale production of alginate was introduced 50 years later. Alginate is one of the most versatile biopolymers and is used in a wide range of food, pharmaceutical and specialty applications for; thickening, stabilizing, gelling and film forming.

It is most commonly used with calcium lactate or calcium chloride in the spherification process. Today, FMC Biopolymer is among the world's largest alginate manufacturers. Alginate is a natural polysaccharide that comprises from 30 to 60% of brown algae (on dry weight basis). Alginate has dietary fiber properties. It is applied with many products such as food, textile printing, welding rods, animal feed, pharmaceutical and cosmetic.

A coating is a covering that is applied to the surface of an object, usually referred to as the substrate. The purpose of applying the coating may be decorative, functional, or both. The coating itself may be an all-over coating, completely covering the substrate, or it may only cover parts of the substrate. Functional coatings may be applied to change the surface properties of the substrate, such as adhesion, wettability, corrosion resistance, or wear resistance. A major consideration for most coating processes is that the coating is to be applied at a controlled thickness, and a number of different processes are in use to achieve this control, ranging from a simple brush for painting a wall, to some very expensive machinery applying coatings in the electronics industry. Coatings may be applied as liquids, gases or solids.

1.5. Spray-drying

Spray drying is a method of producing a dry powder from a liquid or slurry by rapidly drying with a hot gas. This is the preferred method of drying of many thermally-sensitive materials such as foods and pharmaceuticals. A consistent particle size distribution is a reason for spray drying some industrial products such as catalysts. Air is the heated drying medium; however, if the liquid is a flammable solvent such as ethanol or the product is oxygen-sensitive then nitrogen is used (Mujumdar; 2007).

All spray dryers use some type of atomizer or spray nozzle to disperse the liquid or slurry into a controlled drop size spray. The most common of these are rotary disks and single-fluid high

pressure swirl nozzles. Atomizer wheels are known to provide broader particle size distribution, but both methods allow for consistent distribution of particle size (http://www.elantechnology.com/spray-drying/). Alternatively, for some applications two-fluid or ultrasonic nozzles are used. Depending on the process needs, drop sizes from 10 to 500 µm can be achieved with the appropriate choices. The most common applications are in the 100 to 200 µm diameter range. The dry powder is often free-flowing.

The most common spray dryers are called single effect as there is only one drying air on the top of the drying chamber (see n°4 on the schema in Fig.3). In most cases the air is blown in co-current of the sprayed liquid. The powders obtained with such type of dryers are fine with a lot of dusts and poor flow ability. In order to reduce the dusts and increase the flow ability of the powders, there is since over 20 years a new generation of spray dryers called multiple effect spray dryers. Instead of drying the liquid in one stage, the drying is done through two steps: one at the top (as per single effect) and one for an integrated static bed at the bottom of the chamber. The integration of this fluidized bed allows, by fluidizing the powder inside a humid atmosphere, to agglomerate the fine particles and to obtain granules having commonly a medium particle size within a range of 100 to 300 µm. Because of this large particle size, these powders are free-flowing.

The fine powders generated by the first stage drying can be recycled in continuous flow either at the top of the chamber (around the sprayed liquid) or at the bottom inside the integrated fluidized bed. The drying of the powder can be finalized on an external vibrating fluidized bed.

The hot drying gas can be passed as a co-current or counter-current flow to the atomizer direction. The co-current flow enables the particles to have a lower residence time within the system and the particle separator (typically a cyclone device) operates more efficiently. The counter-current flow method enables a greater residence time of the particles in the chamber and usually is paired with a fluidized bed system.

The physical-chemical properties of powders produced by spray drying depend on some process variables, such as the characteristics of the liquid feed (viscosity, flow rate) and of the drying air (temperature, pressure), as well as the type of atomizer (Barbosa-Cánovas, 2005).

In addition to a dehydration process, spray drying can also be used as an encapsulation method when it allows ‘active’ material being entrapped within a protective inert matrix. Compared to the other conventional microencapsulation techniques, it offers the attractive advantage of producing microcapsules in a relatively simple continuous processing operation.

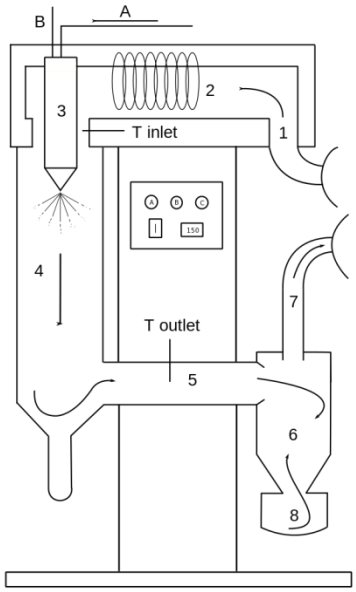

Figure 3: Laboratory-scale spray dryer. A=Solution or suspension to be dried in, B=Atomization gas in, 1= Drying gas in, 2=Heating of drying gas, 3=Spraying of solution or suspension, 4=Drying chamber, 5=Part between drying chamber and cyclone, 6=Cyclone, 7=Drying gas is taken away, 8=Collection vessel of product, arrows mean that this is co-current lab-spray-dryer

1.6. Freeze-drying

Freeze drying was developed to overcome the loss of the compounds responsible for flavor and aroma in foods, which are lost during conventional drying operations (Karel, 1975; Dalgleish, 1990). The freeze drying process consists mainly of two steps: (1) the product is frozen, and (2) the product is dried by direct sublimation of the ice under reduced pressure. Freeze drying, or lyophilization, was initially introduced in the 1940s on a large scale for the production of dry plasma and blood products (Rey, 1975). Later, antibiotics and biological materials were prepared on an industrial scale by freeze drying. Figure 4 shows a basic configuration of a freeze drying system.

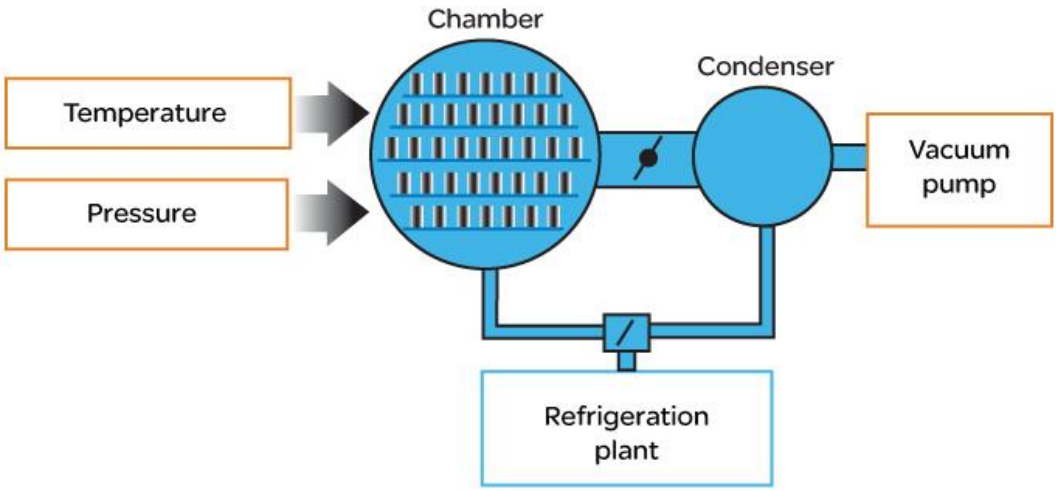

Figure 4: Basic freeze drying system. (Source: http://www.eurotherm.es/industries/life-sciences/applications/freeze-drying/).

Freeze drying has been shown to be an attractive method for extending the shelf life of foods (Ma and Arsem, 1982). The drying of food products in freeze drying has two main characteristics (Longmore, 1971):

- Virtual absence of air during processing: The absence of air and low temperature prevent deterioration due to oxidation or chemical modification of the product.
- Drying at temperatures lower than ambient temperature: Products that decompose or undergo changes in structure, texture, appearance, and/or flavor as a consequence of high temperature can be dried under vacuum with minimum damage.

Freeze-drying products that are properly packaged can be stored for an unlimited period of time, while retaining most of the physical, chemical, biological, and organoleptic properties of their fresh state. Freeze drying reduces loss of quality due to deleterious chemical reactions caused by enzymatic and nonezymatic browning. However, the oxidation of lipids, induced by the low moisture levels achieved during drying, is a major concern for freeze-dried products. Lipid oxidation reactions are controlled by packaging in oxygen-impermeable containers. Nonenzymatic browning is avoided because of the rapid transition from high to low moisture content during the process. The use of low temperature ranges also avoids protein denaturation in freeze-dried products (Okos et al., 1992).

Freeze-dried products can be reconstituted to their original shape and structure by the addition of liquid. The sponge like structure of the dried product allows a rapid rehydration process. The rehydrated product characteristics are similar to those in a fresh product. The porosity of freeze-

dried products allows for more complete and rapid rehydration than is possible with air-dried products.

The major disadvantages of freeze drying are the energy cost and the drying time (Liapis and Marchello, 1984). Some food products that are commercially freeze-dried include extracts (coffee and tea), vegetables, fruits, meats, and fish (Schwartzberg, 1982; Dalgleish, 1990). Freeze-dried products are lightweight (10% to 15% original weight) and do not require refrigeration. Moisture levels as low as 2% are reached with freeze drying. Steaks, fish, and chicken can be dried without crushing or shredding the product (Sacharow and Griffin, 1970).

1.6.1. Fundamentals of Freeze Dehydration

The process consists of two main stages: freezing and drying. Freezing must be very rapid to obtain a product with small ice crystals and in an amorphous state (Mellor, 1978). The drying process involves lowering the pressure to allow ice sublimation. Figure 5 presents the phase diagram of water, and Figure 6 presents the freeze drying steps.

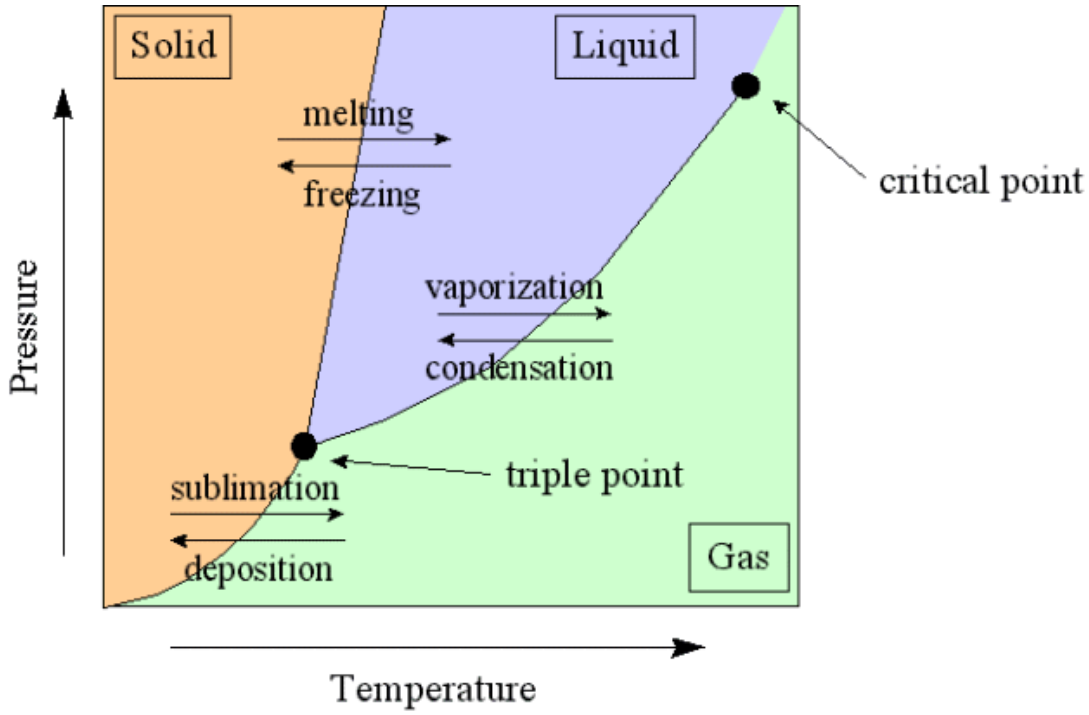

Figure 5: Phase diagram of water. (Source: http://epsc.wustl.edu/courses/epsc105a/).

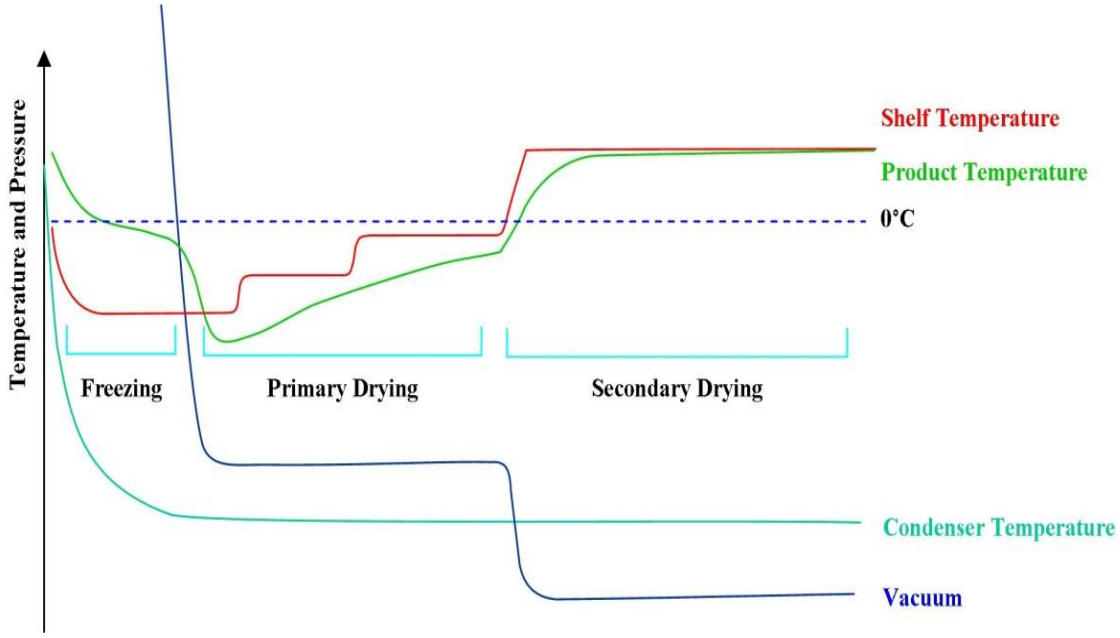

Figure 6: Freeze drying steps (Source: http://biopharma.co.uk/blog/2013/03/06/common-misconceptions-in-freeze-drying-part-1/).

Three important design variables to be considered in freeze drying are: (1) vacuum inside the chamber, (2) radiant energy flux applied to the food, and (3) the temperature of the condenser. The initial drying rate is high because there is little resistance to either heat or mass flux. However, a build-up of a resistive layer around the frozen material slows down the rate as the drying proceeds. The dry layer around the product serves as an insulation material that affects the heat transfer to the ice front. Also, the mass transfer from the ice front is reduced as the thickness of the dry layer is increased. This is because of a reduction in the diffusion process from the sublimation interface to the product surface.

1.6.1.1. Freezing Step

The freezing temperature and time on foodstuffs is a function of the solutes in solution (Schwartzberg, 1982). Pure water freezing temperature remains constant at the freezing point until all the water is frozen. In the case of food, the freezing temperature is lower than that of pure water. Because the solutes become more concentrated in the unfrozen portion of the mix, the freezing point temperature continually decreases until all the solution is frozen. At the end of the freezing process, the entire mass should become rigid, forming an eutectic consisting of ice crystals and food components (Mellor, 1978). The eutectic state is required to ensure the removal of the water by sublimation only and not by a combination of both sublimation and evaporation. Melting and

inadequate freezing should be avoided because the formation of frothy and gummy substances will appear in the final product.

The permeability of the frozen surface layer can be affected by the migration of soluble components during the freezing step. However, the removal of a thin surface layer of the frozen product, or freezing under conditions inhibiting the separation of the concentrate phase, result in better drying rates (Karel, 1975).

1.6.1.2. Drying Steps—Primary and Secondary Drying

Two drying steps can be identified during lyophilisation (King, 1970; Mellor, 1978). The primary drying step involves sublimation of ice under vacuum. The ice sublimes when the energy for the latent heat is supplied.

Because of the low pressure, the water vapor generated in the sublimation interface is removed through the outer porous layers of the product as shown in Figure 7 the condenser prevents the return of the water vapor to the product. The driving force for the sublimation is essentially the difference in pressure between the water vapor pressure at the ice interphase and the partial pressure of water vapor in the drying chamber. The energy for sublimation (latent heat) can be supplied by radiation and conduction through the frozen product, or by irradiating the water molecules with microwaves (Mellor, 1978; Arsem and Ma, 1990).

The secondary step (Figure 7) begins when no more ice (from unbound water) is in the product and the moisture comes from partially bound water in the drying material. At this time the heating rate must decrease to maintain the temperature of the product below 30 to 50 ^{o}C which will prevent collapse of the material (Mellor, 1978). If the solid matrix becomes too hot, the structure collapses, which in turn decreases the sublimation rate from the ice front within the product as discussed by Bellows and King (1973). The secondary drying step will take up to a third of the total drying cycle to desorb the moisture from the internal surface within the dried product.

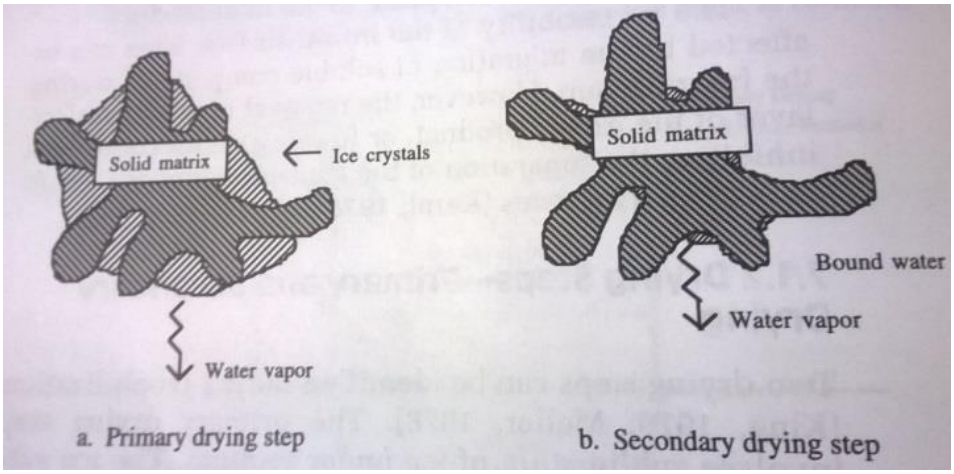

Figure 7: Removal of water during freeze drying (Adapted from, Mellor, 1978).

1.7. Fluidized Bed Dryers

Food particles are fluidized when the pressure drop across a particle bed balances the weight of the particles and the bed expands. The expansion results in the suspension of the particles in the air. The system behaves as a fluid when the Froude number is below unity (Karel, 1975):

$$\text{Froude} = \frac{U^2}{2\,gr}$$

where U is the air velocity, g is the gravitational constant, and r is the particle radius. Usually the air velocity is in the range of 0.05 to 0.75 m/s. The use of vibratory conveyors assists in maintaining particles suspended for products that are not easily fluidized (Heldman and Singh, 1981). The design of industrial fluidized beds is based on pilot plant testing and on-site operational experience (Masters, 1993). The advantages of fluidized bed technology include both, batch and continuous modes, large- and small-scale operations, equipment items with few mechanical moving parts, rapid heat and mass transfer rates between the product and the drying medium, and rapid mixing of solids which leads to nearly isothermal conditions throughout the fluidized layer. They are used in the dairy, food, and pharmaceutical industries for drying, cooling, coating, and agglomeration.

The fundamental aspect of fluidization and fluidized bed operations involves mixing, entrainment, segregation, and heat transfer. Materials suitable for fluidized bed operations can be somewhere between 20 µm and 10 mm to avoid excessive channeling and slugging with a narrow particle size and regular shape. The particles cannot be sticky at the processing temperature (Masters, 1993).

1.7.1. Batch Fluidized Bed

This type of dryer is used extensively where the capacity is small and the quality assurances are a concern. In a batch fluidized bed, the material to be dried is placed in a suitable container with a distribution plate (wire mesh supporting screen) and is subjected to a stream of heated air at a selected temperature until the desired moisture level is reached. The typical configuration of a batch type fluid bed dryer is shown in Figure 8. The temperature may be constant or reduced during the drying period (Williams-Gardner, 1971).

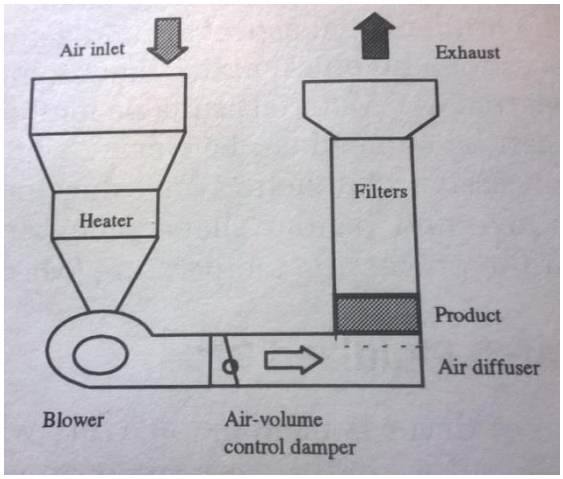

Figure 8: Batch fluidized bed dryer (Adapted from, Williams-Gardner, 1971).

1.8. Previous studies of research group

The research group of Food Tecnology began to address the issue of the use blood as raw material for the production of ingredients for the food industry in 1996. During this time the physical-chemical and microbiological characterisation of porcine blood and blood fractions have been carried out and studies on technological functionality of ingredients obtained from blood proteins subjected to different treatments have been conducted: spray-dried plasma and red-blood-cells, plasma sanitized by means of bactofugation and tangential microfiltration (Carretero and Parés, 2000), plasma and hemoglobin concentrate treated by high hydrostatic pressure (Parés et al., 1999, 2000 and 2001, Parés and Ledward, 2001; Toldrà et al., 2002 and 2004), both pressurised and non-pressurised, hemoglobin hydrolysates (Toldrà et al., 2002, 2011), fractionation of the main proteins of plasma (Dàvila et al., 2006, 2007a, 2007b and 2007c). Treatments with microbial transglutaminase (MTGasa) of plasma to improve its functional properties have also been carried out by testing the enzyme alone, combined with a reducing agent or assisted by high pressure (Saguer et al., 2004 and 2007, Fort et al., 2006, 2008 and 2009).

From the red-blood-cells, which is the most difficult to valorise, the group worked on the use of the cellular fraction to obtain decolorized haemoglobin hydrolysates. The enzymatic method developed in our laboratory allows the separation of a white protein hydrolysate and a residue that contains the entire haem group (Toldrà et al., 2005, 2011). The hydrolysate contains several peptide fragments covering a wide range of molecular weights that has poor techno-functional properties, but it shows some biological activities.

From 2004, the group has been working in obtaining better quality food ingredients from the functional point of view and harnessing the residue of the hemolgobin hydrolysis, by taking advantage of on the nutritional value of the hem group and the potential biological activity of the low molecular-weight peptides (Dàvila et al., 2006, 2007a, 2007b and 2007c; Saguer et al., 2004 and 2007, Fort et al., 2006, 2008 and 2009).

Currently the group is working in a project to develop applications for the use of blood derivatives in the formulation of meat products. This research as been supported by companies in the food industry (Hurtado et al, 2012; Parés et al, 2012).

As a good alternative to valorise, the cellular fraction has also been used to develop a system to obtain an hemoglobin-based stable red colorant by means of the addition of different chelating agents and antioxidants (Saguer et al., 2003; Salvador et al., 2009).

Results from recent trials considering new compounds as potential stabilizing agents seem to indicate that we are really close to getting a formulation for the patentable food colorant. A quite stable red haem-derivative has been developed, it conserves its bright red color during the spray drying process, and it is able to maintain color and properties for more than six months of storage in refrigerated conditions. Applications on meat nitrified products and foods with neutral or sligthly acidic pH has been succefully assessed. The last challenge is to reach the stability of Fe^{2+}, as well as its characteristic red color, in acidic conditions and after heating of not nitrified products.

2. Objective

This work is circumscribed in a research project aimed at producing a natural red food colorant, stable during long-term storage at room temperature and with a wide range of applications, using hemoglobin from porcine blood as raw material.

Previous studies had shown that both spray-drying (which is the most frequently used preservation method of liquid products) and freeze-drying, promoted the oxidation of iron during dehydration, and the subsequent loss in the quality of hemoglobin color. To prevent oxidation reactions during dehydration, several potentially protective substances (i.e. antioxidants, chelating agents and stabilizers) were evaluated. At present, a powdered product showing excellent color characteristic by adding 3 different compounds, which act synergistically, has been achieved. However, further research is necessary in order to increase the stability of the product during storage at room temperature and to extend the applications to a wide range of food matrices.

The objective of this study was improving the stability of this hem-derivative product at acidic conditions and moderate temperatures by means of encapsulation with calcium alginate. The work plan stages were:

- Production of spray-dried powder of hem-derivative from porcine blood.
- Preparation of alginate capsules by means of reaction of sodium alginate and Ca^{2+}
- Determination of color stability by CIEL*a*b* color parameters, water activity (a_w) and moisture measurements.

3. Material and Methods

3.1. Experimental design

The possible uses of red blood cells fraction (RBC) of blood mainly include two ways: (1) hydrolysate it to be used as colorless food ingredient, and (2) stabilize the red color, to be applied as coloring additive (Figure 9).

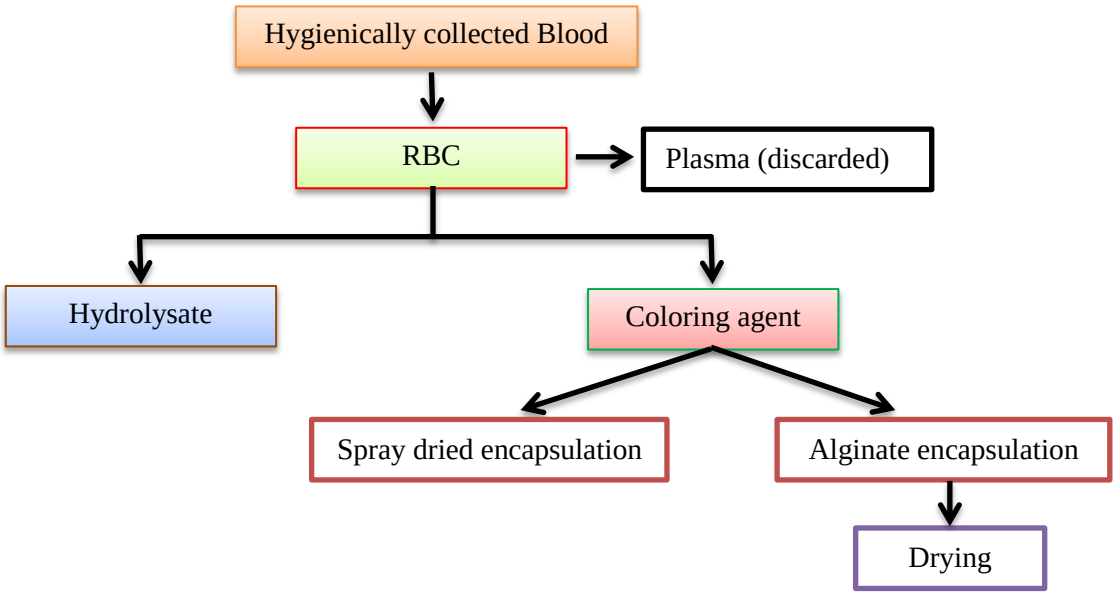

Figure 9: Some ways to upgrading RBC

Although it was not the objective of this work, firstly, I was given the opportunity to collaborate in the production of a RBC hydrolysate, which had to be used in another work to stabilize Hb color. So, I collaborated in the preparation and characterization of globin hydrolysates in order to learn an alternative system to upgrade the RBC fraction.

As reported before, the main objective of this work was trying to improve quality and stability of encapsulated red hem-derivative. Therefore, the first thing to do was to prepare and characterize the standard spray dried hem-derivative, which had been previously developed by the research group, to be used as comparative control of new experiments. So, two repetitions of hem-derivative were prepared and analyzed, and color evolution was followed for three months.

The next step was to develop the system to obtain alginate encapsulated RBC that had been never assayed before.

Firstly, different possibilities to dissolve sodium alginate (SA) in RBC as well as several concentrations were assessed (Figure 10). Afterwards, different ways to proceed to encapsulation

were evaluated. After several attempts the best system was found to be dropping SA/RBC solution by means of a peristaltic pump into a $CaCl_2$ solution, through a micropipette needle.

This way permitted us to obtain wet encapsulated spheres, which contained RBC-derivative and calcium alginate. To dry this product two systems were tested, freeze-drying (Figure 10) and fluidized bed drying (Figure 11).

Freeze-drying experiments were carried out in duplicate for three different AS/Hb concentrations (20/80, 40/60, and 60/40), whilst fluidized bed drying was applied to only one SA/RBC combination, specifically 40/60, and several time/airflow combinations of drying were assessed. Since the variability of the measurements was greater than in freeze-drying, all these experiments were made in triplicate.

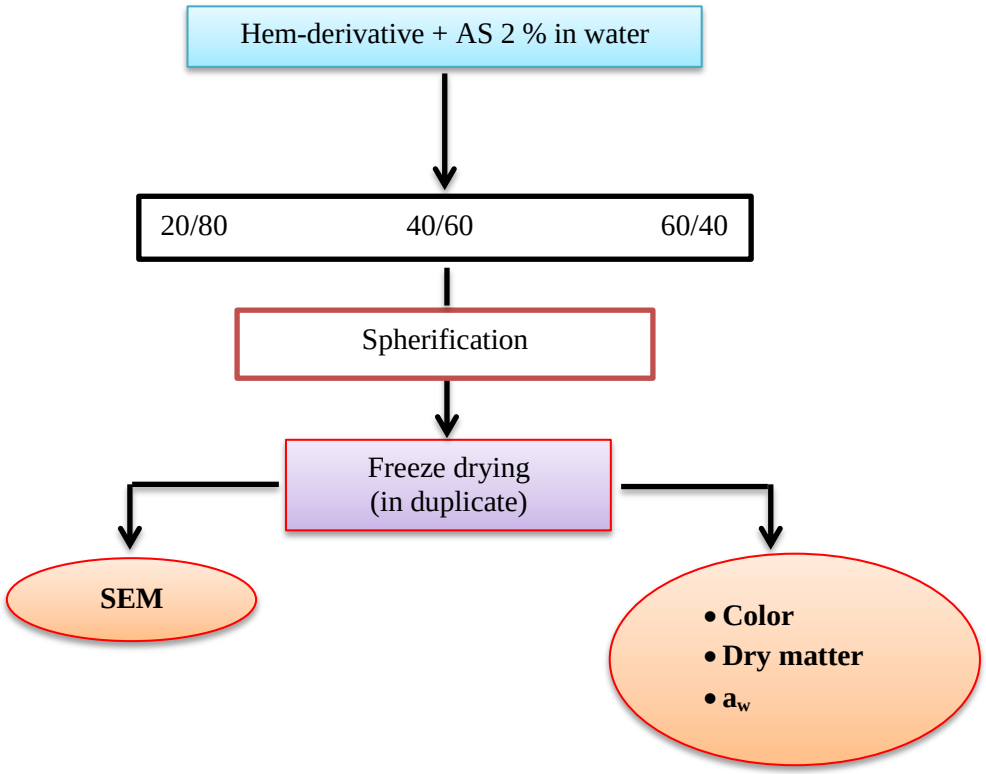

Figure 10: Flowchart representing experimental design of SA/RBC concentration.

Analyses of water contents, water activity, protein contents and color, were used to characterize each product, as well as Scanning Electron Microscopy (SEM) of the best ones, to understand the behavior as related to microstructure.

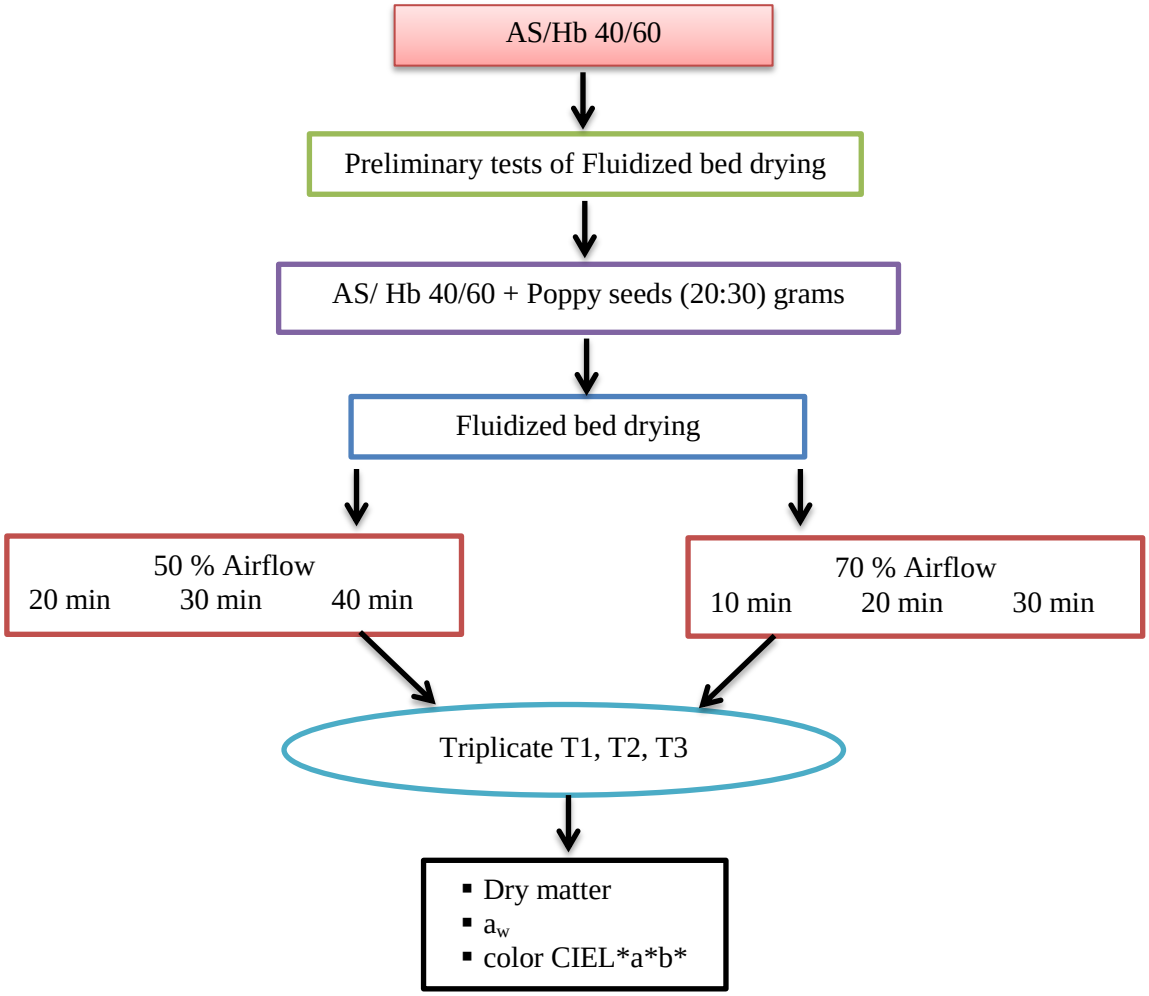

Figure 11: Diagram of experimental design to find the best conditions for fluidized bed drying.

3.2. Material

3.2.1. Red cell fraction of porcine blood

Commercial hygienic blood, containing polyphosphate 4% as anticoagulant, was collected from the refrigerated storage tank of a local industrial slaughterhouse (Norfrisa S.A., Riudellots de la Selva, Girona, Spain) in sterile containers. It was immediately transported to the laboratory and plasma was separated by centrifuging at 2530 x g and 5-10 ^{o}C for 15 min (Sorvall RC-SC plus, Dupont Co, Newton, Connecticut, USA). After centrifugation red cell fraction was immediately hemolyzed using a FPG 7400 high-pressure laboratory valve homogenizer (Stansted Fluid Power Ltd., Essex, UK) under the following conditions: inlet pressure, 5 bars; processing pressure, 10 MPa. Afterwards, the Hb solution was kept in the refrigerator or continuously to powder or encapsulate processing right away.

3.2.2. Reaction agents and equipment

Detailed description of all reaction agents and equipment that were used in this work are included in Annex I, as well as pictures of the main apparatus and machines.

3.3. Sample preparations

3.3.1. Hydrolysis processing of hemoglobin

The hydrolysate was made from RBC by following the flow chart showed below (Figure 12); in this case homogenization was not applied because hemolysis was made by osmotic shock when RBC was diluted with distillate water.

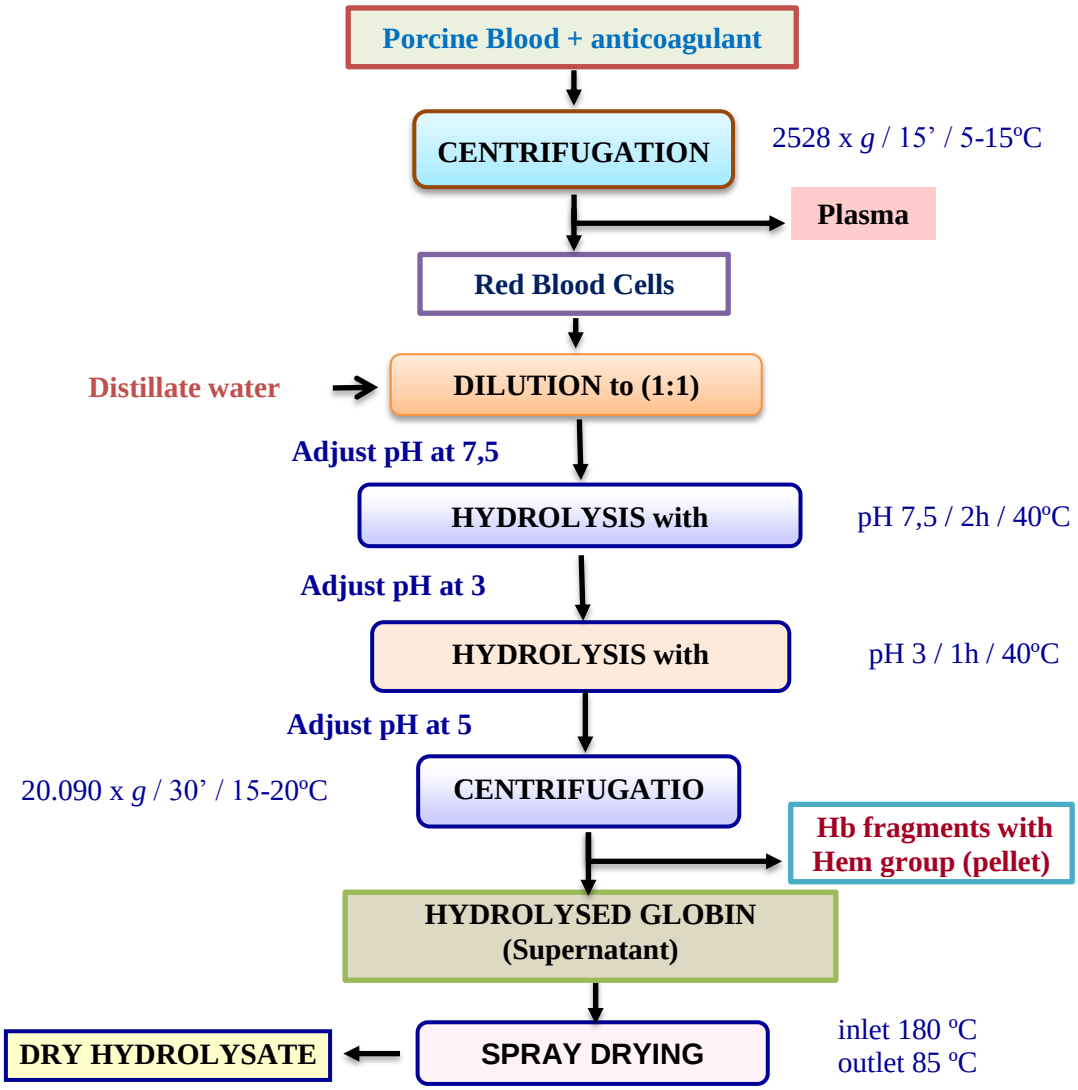

Figure 12: Flowchart of hemoglobin Hydrolysis process

Some parameters of the hydrolysate such as protein, non-protein nitrogen, water activity (a_w), ash and moisture were measured and compared to the results of previous experiments.

3.3.2. Spray-drying encapsulation

For this process, the pH of Hb was firstly increased until 8 by adding NaOH (10%) under continuous stirring by using a magnetic stirrer. 3.5% Nicotinamide (NAM) was then added whilst stirring for 5 min, followed by 10% of sucrose and 10% stabilizer A (polysaccharide) (this agent cannot be specified for confidentiality reasons).

After that a home hand blender (Braun, Barcelona, Spain) was used to stir RBC-derivative in order to oxygenate hem-groups until its color became bright red.

Then the sample was spray-dried in a Büchi Mini Spray Dryer model B-191 (Büchi Labortechnik AG, Flawil, Switzerland), at 120 °C of inlet temperature; feeding pump flow 65% (0.85 L/h); aspiration pump flow 100 % (60L/h); spray pressure 5 bar), outlet temperature have been maintained under 76 °C.

Processing yield, water activity, color, ash and moisture contents were analyzed after the drying process. The powder was kept at refrigeration temperature until use.

This preparation was made twice, with blood collected in different days under the same conditions.

3.3.3. Alginate Encapsulation

Solution to be encapsulated was Hb-derivative (pH 8), containing 3.5% NAM and 10% sucrose, prepared as explained in the previous paragraph. The obtained color of this hem-derivative was not considered satisfactory so 10 % of polysaccharide stabilizer was added to improve the color, having the product to be encapsulated finally the same composition than the spray-dried one.

- Alginate solution (AS): 2g of sodium alginate (SA) powder were dissolved in 100ml of deionized water and stirred over night at 25 ^{o}C to produce 2% (w/v) solution.
- Encapsulate Mixing: three different combinations were prepared; 20%, 40% and 60% of hemoglobin derivative with 80%, 60% and 40% of 2% sodium alginate solution, respectively. In the same way made for spray-drying we mixed all solutions by means of the home hand blender for about 2-3 min until they became bright red.

- Calcium chloride ($CaCl_2$) solution: it was prepared by dissolving $CaCl_2$ in distilled water at 5% (w/v) and stirring for 15 min at room temperature.

The AS/RBC-derivative mixing was then extruded through a 10 µl pipette needle connected to a peristaltic pump, and dropped into the $CaCl_2$ gelling solution, under continuous soft stirring. This way, spherical capsules of around 1.5 mm diameter were obtained. Excess of $CaCl_2$ was removed by rinsing whit distilled water.

3.3.4. Freeze-drying

All capsules were frozen and stored at -70 ^{o}C at least overnight. Frozen samples were placed in a laboratory scale freeze-dryer (Unitop HL, Virtis, Gardiner, NY, USA). Standard freeze-drying conditions were used, which consist of a primary drying (-10 ºC and 100 mT vacuum) for 24 h followed by 5 h of secondary drying (+15 ºC and 100 mT).

3.3.5. Fluidized-bed drying

Drying was performed in a Fluidized bed dryer STREA-1™ Aeromatic-Fielder (GEA Process Engineering Inc., Columbia, USA)

Several previous trials were carried out before make possible to use that system to dry alginate capsules. Samples were always prepared as 40% of RBC with 60% of alginate solution, as alginate spheres were quite sticky, the fluidization was very difficult, and poppy's seeds were used to facilitate fluidization during drying. The samples were dried at different airflow and time combinations. These experiments were repeated 3 times each one.

Water contents, water activity, protein and color were determined in all samples.

3.4. Analysis Methods

3.4.1. Water activity measurement

Products' water activity (a_w) was measured by automatic equipment *Lab* Master-aw (Novasina AG, Lachen, Sweden). Measuring was conducted on samples in plastic specific containers, placed into the hole of a_w measure. After sealing the chamber lid over the sample and waiting for vapor equilibrium a_w was directly displayed in the screen. The spent time for each measurement ranged from 5 to 10 min at 20 ^{o}C.

3.4.2. CIELab colorimetric parameters

Color measurements were taken with the MINOLTA CR-300 colorimeter (section 7.27) using diffuse illumination, a D_{65} illuminant and a 2^{o}-standard observer. CIEL*a*b* color parameters were determined as indicators of lightness (*L**), redness (*a**) and yellowness (*b**). Three random measurements per sample were taken and averaged. The colorimeter was calibrated before every measurement by using a standard white ceramic plate in the tri-stimuli (*Yxy*) system.

3.4.3. Moisture content

Moisture content is one of the most commonly measured properties of food materials. It is very important in food products for different reasons such as legal and labeling requirements, economic, microbial stability, food quality and food processing operation.

Moisture measurement was carried out by gravimetric official method. Sample and glass containers were weighed, placed into the oven at 100 ± 5 ^{o}C overnight and weighed again to know the remaining dry matter. We did all measurements in duplicate. The moisture content was determined by measuring the mass of the sample before and after the water being removed through evaporation:

$$\%\text{Moisture} = \frac{M_{\text{INITIAL}} - M_{\text{DRIED}}}{M_{\text{INITIAL}}} \times 100$$

Here, M_{INITIAL} and M_{DRIED} are the mass of the sample before and after drying, individually. The basic principle of this technique is that water has a lower boiling point than the other major components within foods, *e.g.,* lipids, proteins, carbohydrates and minerals.

3.4.4. Ash content

The ash content is a measure of the total amount of minerals present within a food, such as Fe, Ca, Na, K and Cl.

Ash was determined as the inorganic residue remaining after the water and organic matter have been removed by heating at 550 °C. Analytical techniques for providing information about the total mineral content are based on the fact that the minerals can be distinguished from all the other components within a food in some measurable way.

Determination of ash content was applied to hydrolysate Hb. The samples were previously dried, to measure the moisture content and after they were passed muffle at 550 ^{o}C, until white ash were obtained. Water and other volatile materials are vaporized and organic substances are burned in the presence of the oxygen in air to CO_2, H_2O and N_2. Most minerals are converted to oxides, sulfates, phosphates, chlorides or silicates.

The food sample is weighed before and after combustion to determine the concentration of ash present.

$$\% \text{ Ash (dry basis)} = \frac{M_{ASH}}{M_{DRY}} \times 100$$

where M_{ASH} refers to the mass of the ash sample, and M_{DRY} refer to the original masses of the dried samples.

3.4.5. Protein determination

The analysis of nitrogenous compounds was done by Kjeldahl method. There are three steps of protein measuring quantity of protein:

- **Digestion:** The sample to be analyzed is weighed into a digestion tube and then digested by heating it in the presence of sulphuric acid (an oxidizing agent which digests the food), and a catalyst (47.7% Na_2SO_4, 47.7% K_2SO_4, 2.8% TiO_2, 1.8% $CuSO_4$, MERCK) (to speed up the reaction) by using a semi-automatic system (Gerhart KB20, German). Digestion converts any nitrogen in the food (other than that which is in the form of nitrates or nitrites) into ammonia, and other organic matter to CO_2 and H_2O. Ammonia gas is not liberated in an acid solution because the ammonia is in the form of the ammonium ion (NH_4^+) which binds to the sulphate ion (SO_4^{-2}) and thus remains in solution:

  N (food) → $(NH_4)_2SO_4$ (1)

- **Neutralization:** After the digestion has been completed the digestion tube is connected to a receiving flask by a tube. The solution in the digestion flask is then made alkaline by addition of sodium hydroxide (40%), which converts the ammonium sulphate into ammonia gas:

  $(NH_4)_2SO_4 + 2\ NaOH \rightarrow 2\ NH_3 + 2\ H_2O + Na_2SO_4$ (2)

  The ammonia gas that is formed is liberated from the solution by distillation in water steam stream (Buchi K314, German) and moves out of the digestion tube and into the receiving flask – which contains an excess of boric acid. The low pH of the solution in the receiving

flask converts the ammonia gas into the ammonium ion, and simultaneously converts the boric acid to the borate ion:

$NH_3 + H_3BO_3$ (boric acid) → $NH_4^+ + H_2BO_3^-$ (borate ion) (3)

- **Titration:** The nitrogen content is then estimated by titration of the ammonium borate formed with standard hydrochloric acid, using Methyl Red solution 0.1 % (Panreac) as indicator to determine the end-point of the reaction.

  $H_2BO_3^- + H^+ \rightarrow H_3BO_3$ (4)

  The concentration of hydrogen ions (in moles) required to reach the end-point is equivalent to the concentration of nitrogen that was in the original food (Equation 3). The following equation can be used to determine the nitrogen concentration of a sample that weighs *m* grams using a 0.1 or 0.25M HCl acid solution for the titration:

$$\%N = \frac{x \text{ moles}}{1000 \text{ cm}^3} \times \frac{(v_s - v_b) \text{ cm}^3}{m \text{ g}} \times \frac{14 \text{ g}}{\text{moles}} \times 100 \quad (5)$$

  Where v_s and v_bI are the titration volumes of the sample and blank, and 14g is the molecular weight of nitrogen N. A blank sample is usually ran at the same time as the material being analyzed to take into account any residual nitrogen which may be in the reagents used to carry out the analysis. Once the nitrogen content has been determined it is converted to a protein content using the appropriate conversion factor: % Protein = F x % N; (F=6.25)

3.4.6. Non protein nitrogen

Non-protein nitrogen (or NPN) is a term used to refer collectively to components such as urea, biuret, and ammonia, which contain nitrogen but are not proteins. In our case, when proteins were broken down by proteases, some small peptides appeared, these peptides are considered as NPN, which was determined in the same way as protein (3.4.4) from a sample in which protein had been previously precipitated with trichloroacetic acid (TCA). The process was performed from 10 ml of liquid hydrolysate, adding 12.5 ml of TCA (25%) and filling up to 25 ml with distilled water, in order to precipitate the proteins, whilst peptides with molecular weight ≤ 10 kDa remained solubilized. The solution was filtered through Whatman filter number 1 and the soluble/non-protein nitrogen from 10 ml of the filtered solution was analyzed by Kjeldahl method.

3.5. Coating with Chitosan

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It is made by treating shrimp and other crustacean shells with the alkali sodium hydroxide. It has a number of commercial and possible biomedical uses. It can be used in agriculture as a seed treatment and bio-pesticide, helping plants to fight off fungal infections. In winemaking it can be used as a fining agent, also helping to prevent spoilage. In industry, it can be used in a self-healing polyurethane paint coating. In medicine, it may be useful in bandages to reduce bleeding and as an antibacterial agent; it can also be used to help deliver drugs through the skin.

As the color of alginate capsules was not good enough, we try to coat the spheres with chitosan. We used 0.4% solutions of low molecular weight chitosan (Deacetylated chitin, Poly "D-glucosamine") (ALDRICH, Iceland in 0.1 M acetic acid. adjusted to pH 6, as described in (Cook et al., 2011).

The alginate RBC spheres were then rinsed, and stirred in chitosan solution for 5, 10, 15 and 30 minutes. The color and protein content were determined in the wet spheres, before being freeze-dried.

3.6. Scanning Electron Microscopy (SEM)

Microstructure pictures were obtained using a Zeiss DSM 960 scanning electron microscope (SEM) (Carl Zeiss; Electron Optics Division, Oberkochem, Germany). Samples were processed as follows: samples were fixed in a double face adhesive tape, then they were sputter-coated with a layer of gold/palladium in an Emitech K550 (EMIntegrated Technology, Kent, UK) diode sputtering instrument. A minimum of two representative pictures of each sample were taken at each magnification, and two capsules from each treatment were observed by SEM.

3.7. Statistical data analysis

Samples from different days were considered as blocks in a randomized complete block design in which the treatments applied constituted the main factor affecting the hemoglobin oxidation and the color parameters analyzed. The statistical analyses were carried out using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL). Data were submitted to ANOVA using the general model procedure (Proc GLM); when a significant effect was obtained, the Tukey's test was used to compare sample means. The significance level for all tests was 0.05.

4. Results and Discussion

In dehydrated products, water is probably one of the main factors governing deterioration. It has been shown that the available moisture has a great influence on the stability of some pigments (Pasch and van Elbe, 1975; Kearsley and Katsaboxakis, 1980; Cohen and Saguy, 1983; Gloria et al., 1995). It is also known that water can play a protective effect against the oxidation of pigmented molecules by reducing the accessibility of oxygen (Martinez and Labuza, 1968; Ramakrishnan and Francis, 1979; Lajolo and Lanfer-Marquez, 1982). Moreover, at low values of water activity, trace metals become more reactive as they lose water (Labuza, 1980).

4.1. Control capsules

We prepared an encapsulated spray dried hem-derivative in order to use it as a control for further experiments.

4.1.1. Color

The color parameters were measured the same day of production, the second and third days and after, every seven days, for three months. The results are showed in table 2.

Table 2: CIEL*a*b* parameters of color through three months storage

Time of storage	Sample	L (lightness)		a* (red)		b* (yellow)	
		Mean	Sd	Mean	Sd	Mean	Sd
1 day	S 1	48.85	0.54	41.62	0.79	20.79	0.24
	S 2	48.85	0.16	43.76	0.10	22.93	0.03
2 days	S 1	48.95	0.18	43.03	0.48	22.35	0.36
	S 2	48.47	1.65	44.25	0.32	23.26	0.20
3 days	S 1	48.29	0.36	43.17	0.56	22.30	0.37
	S 2	47.32	1.10	44.37	0.67	23.31	0.41
7 days	S 1	46.62	1.03	42.01	1.01	21.60	0.65
	S 2	47.31	1.27	43.72	0.08	22.83	0.06
14 days	S 1	46.37	0.91	41.68	0.66	21.44	0.43
	S 2	45.31	0.90	41.25	0.90	21.47	0.47
21 days	S 1	45.33	0.88	42.06	0.89	21.83	0.60
	S 2	47.37	1.22	41.23	0.85	21.08	0.46
2 months	S 1	45.40	2.48	38.85	0.65	19.95	0.36
	S 2	45.03	0.76	39.00	0.26	20.08	0.19
3 months	S 1	46.71	0.69	36.56	0.47	18.33	0.05
	S 2	46.00	0.10	36.15	0.07	17.61	0.02

As can be seen in Table 2, samples maintained or slightly changed its lightness, red and yellow components, during the three first days of storage; while all color parameters decreased after longer storage time. After three months the redness decreased 2 to 7 points as compared to the initial samples, and similar changes were observed for L* and b* parameters. Nevertheless, the visually perceived red color can be considered acceptable after this time of storage.

4.1.2. Composition

Table 3 shows total dry matter and water activity (a_w) of S1 and S2 samples. As can be seen, both moisture and a_w of S1 were slightly higher than S2, which is coherent because higher moisture usually means less linked water. However, the differences cannot be considered as very significant. Previous studies carried out in this laboratory showed that small differences in remaining water contents are usually found among samples processed in different days under identical conditions.

In spite this, remaining water as well as a_w of both samples were considered low enough to avoid microbial spoilage.

Table 3: Total dry matter and a_w of spray dried encapsulated hem-derivatives

Components / Samples	Dry matter (%)		a_w
	Mean	Sd	
S1	94.87	0.05	0.190
S2	95.88	0.04	0.160

4.2. Hemoglobin Hydrolysate

As reported above an alternative way to valorize RBC is obtaining a decolorized product by means of enzymatic hydrolysis. In this case, hydrolyzed hemoglobin was prepared to be used as color stabilizer in the encapsulated red hem-derivative in another work. Hydrolysis was carried out following the method described by Toldrà et al. (2011), according the flowchart presented in section 3.3.1.

In order to calculate the percentage of non-protein nitrogen of the hydrolysate, i.e. peptides of molecular weight < 10 kDa, samples of the liquid product, before spray-drying, were analyzed for total nitrogen (TN) and soluble nitrogen after treatment with 12.5 % Trichloracetic acid (NPN). The result showed that 86.41 % of total nitrogen was NPN, this data agrees with previous studies of the research group (Toldrà, 2002).

Table 4 shows some composition parameters of hydrolyzed sample. The hydrolysate is a quite white powder with low a_w and high nitrogen contents, those a high percent corresponds to non-protein nitrogen, i.e. peptides with molecular weight below than10 kDa. For that it is very water-soluble and very hygroscopic.

Previous studies of the research group showed that the hydrolysate contributes to stabilize both a_w and color of hem-derivatives.

Table 4: Composition of Hb hydrolysates

a_w	Ash (%)		Dry matter (%)		Total Nitrogen (%)	
	Mean	Sd	Mean	Sd	Mean	Sd
0.203	8.98	0.11	97.23	0.06	14.45	0.36

4.3. Developing Alginate encapsulated samples

As this was the first time we asses to encapsulate RBC with calcium alginate, we had to assay every parameter or factor which could be involved in the encapsulation process and capsule's stability.

4.3.1. Dissolve Alginate into the blood

Firstly, we assayed dissolving alginate directly in hemoglobin solutions. The solution was too viscous due to the presence of Ca^{2+} in RBC that provoked the gelation of alginate, therefore we refuse this mixing. So another system had to be assayed to mix the alginate with the hemolyzed blood.

4.3.2. Mixing 2% Alginate water solution

In order to reduce viscosity alginate was previously dissolved in water at 2 % concentration. The solutions were always prepared at least one day before its use; to be sure that alginate was completely hydrated and homogenously dissolved in water. From this mother solution, different final concentrations of alginate in derivative Hb were evaluated (see Table 5).

Table 5: Concentration of blood and alginate

Hb (%)	AS 2% (%)
20	80
40	60
60	40

Different systems to drop these solutions into 5% calcium chloride to prepare coated spheres with calcium alginate were also assessed; i.e. syringe needle, pipette needle and Pasteur pipette. Having into account the homogeneity and size of spheres, we decided the best system was using the pipette needle, connected to the peristaltic pump (section 7.2.11) through a flexible silicone tube.

4.3.3. Protein and its solubility

With the aim to decide which would be the best conditions to continue the work, capsules containing different Hb:alginate ratios were analyzed for protein content and solubility in water. Results can be seen in table 6. 2 g of the capsules were stirred into 100 ml of distilled water for 30 minutes. Then, aliquots of 10 ml of the solution were used for TKN analysis. Every sample was done in duplicate.

Table 6: Protein contents and solubility of capsules with different Hb/Alginate ratios

Hb : AS (%)	Protein (%)	Solubility (%)
20	5.55	74.64
40	9.65	41.17
60	9.19	63.48

The aim of this experiment was to choice the ratio Hb:AS that allows obtaining spheres with the maximum protein content, and at the same time, as low solubility as possible, in order to minimize protein losses during the encapsulation process. Table 6 shows that the 20:80 capsules contained the lowest amount of protein showing the highest solubility. The 40:60 and 60:40 samples comparison shows that, although the amount of protein in capsules was practically the same, the 40:60 capsules showed higher ability to retain Hb during rinsing with water. Therefore, the best ratio for this

encapsulation process was considered to be 40:60 that is 40% of hemoglobin and 60 % of 2% alginate solution.

4.3.4. Freeze drying system of encapsulated spheres

Capsules made of different concentrations of Hb:AS (20:80, 40:60 and 60:40) were then freeze dried and color, dry matter, and aw, of dried capsules were measured (tables 7, 8 and 9) to study the effect of the dehydration system.

Table 7: Color parameters of freeze-dried capsules (rep 1)

Hb:Alginate ratio (%)	L		a*		b*	
T 1	Mean	Sd	Mean	Sd	Mean	Sd
20:80	39.67	0.97	30.81	1.03	14.24	0.64
40:60	37.80	0.42	33.44	0.37	14.85	0.32
60:40	37.52	0.92	34.42	0.72	14.97	0.41

Table 8 : Color parameters of freeze-dried capsules (rep 2)

Hb:Alginate ratio (%)	L		a*		b*	
T 2	Mean	Sd	Mean	Sd	Mean	Sd
20:80	38.41	0.13	31.96	0.27	14.29	0.16
40:60	40.75	0.71	36.33	1.38	16.10	0.70
60:40	40.30	0.10	34.53	0.54	14.98	0.22

As can be seen in Tables 7 and 8, the color of the alginate encapsulated product was not good as compared to the color parameters of the control capsules (Table 2). L* values were always lower than in the spray-dried capsules, meaning that the color of alginate capsules was darker. Parameters a* and b* were also lower than in control product, which indicates that the color was brown instead of bright red. The best color parameters were obtained in capsules 40:60, but the parameters being similar to the ones corresponding to control samples after three-month storage.

Table 9 : Dry matter and a_w of freeze-dried capsules, from two repetitions

Table 9 a: Treatment 1

Samples (T 1)	Dry matter %		a_w
	Mean	Sd	
S1 (20%)	92.62	1.16	0.156
S2 (40%)	94.90	0.60	0.133
S3 (60%)	93.72	0.29	0.152

Table 9 b: Treatment 2

Samples (T 2)	Dry matter %		a_w
	Mean	Sd	
S1 (20%)	93.25	2.11	0.156
S2 (40%)	96.10	0.08	0.105
S3 (60%)	98.13	0.12	0.102

Results of dry matter content and water activity of all samples are positive because they are low enough to guarantee the protection from microbial spoilage. Moreover, the sample 20:80, which contained largest amount of AS, showed the highest moisture content and a_w. These results confirmed the choice of the 40:60 as the best ratio to prepare the beads of Hb:A but also indicated that freeze-drying could not be suitable as dehydration system. The problem could be the poor stability of color due to so very low a_w, that can favor the oxidation of Fe^{2+} (Labuza, 1980). In consequence, this alginate encapsulation system was given up and other dehydration systems were studied.

4.3.5. Scanning Electron Microscopy of freeze dried samples

Two capsules of each sample were observed in SEM at different magnification levels in order to compare their aspect and understand the performance of the encapsulation process.

Figures 13, 14, and 15 show the pictures of samples containing 20, 40 and 60% of Hb, respectively.

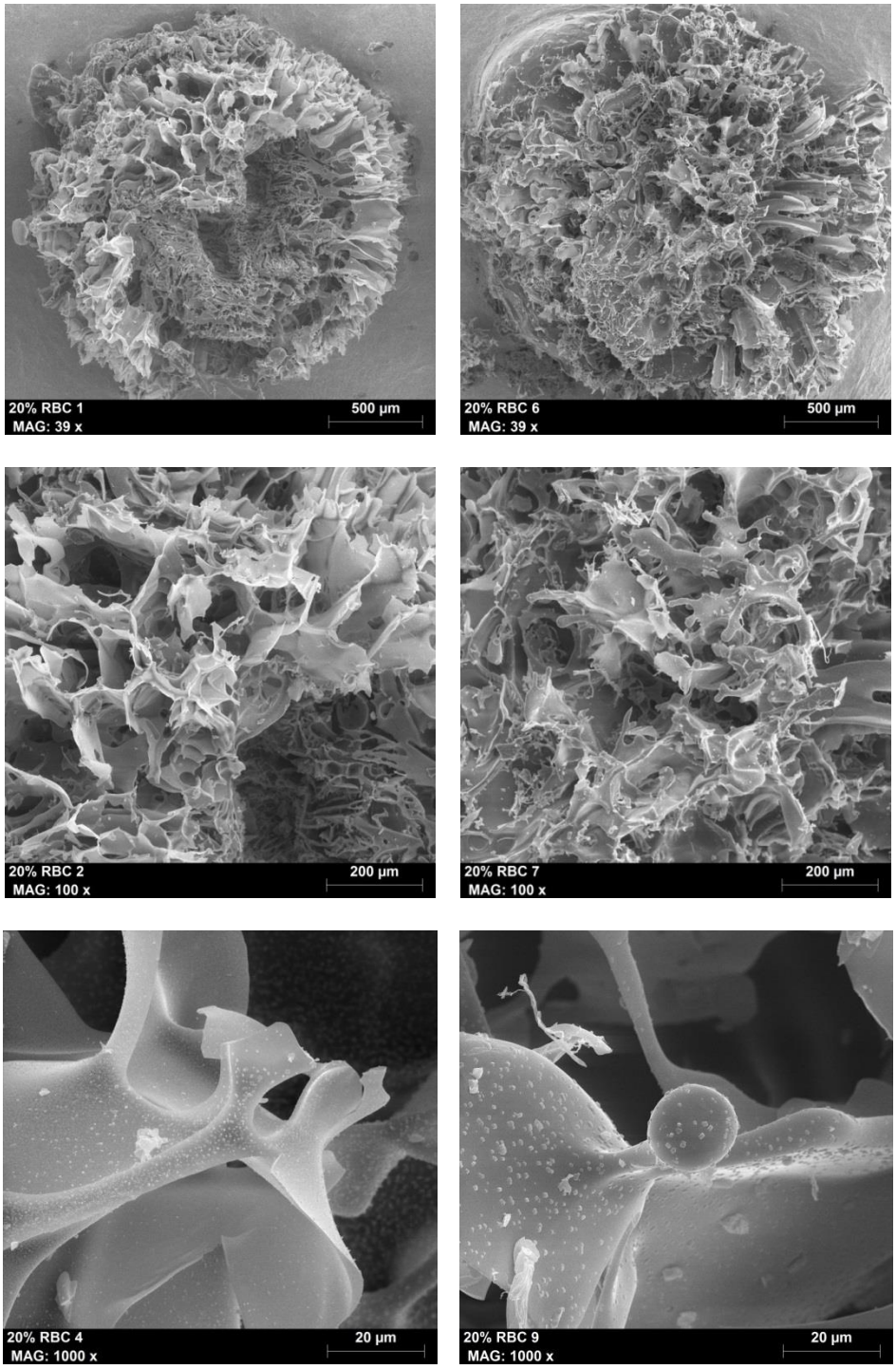

Figure 13: SEM picture of sample 20:80 % Hb:AS, at 39, 100, and 1000 magnification.

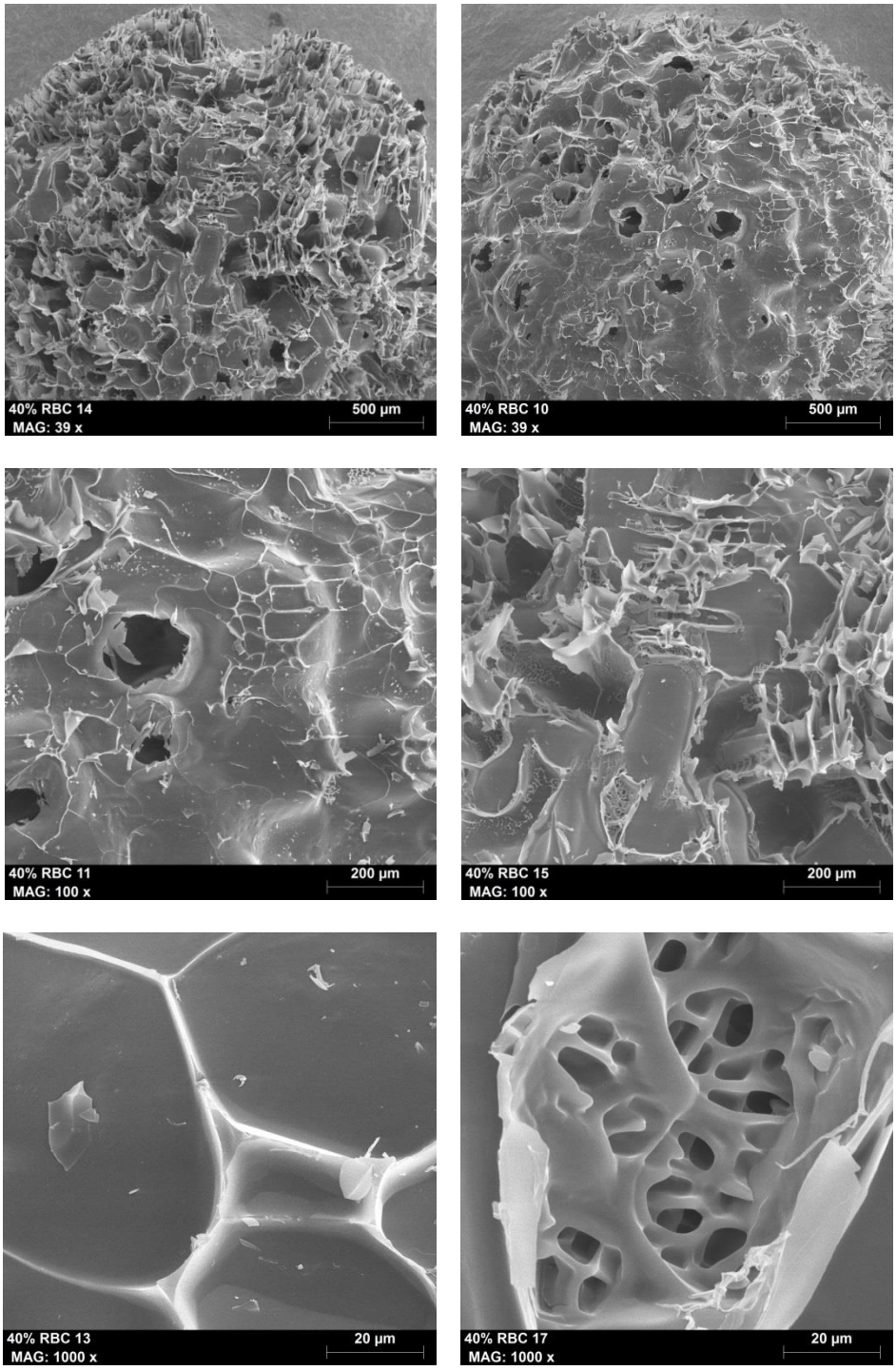

Figure 14: SEM picture of sample 40:60 % Hb:AS at 39, 100, and 1000 magnification.

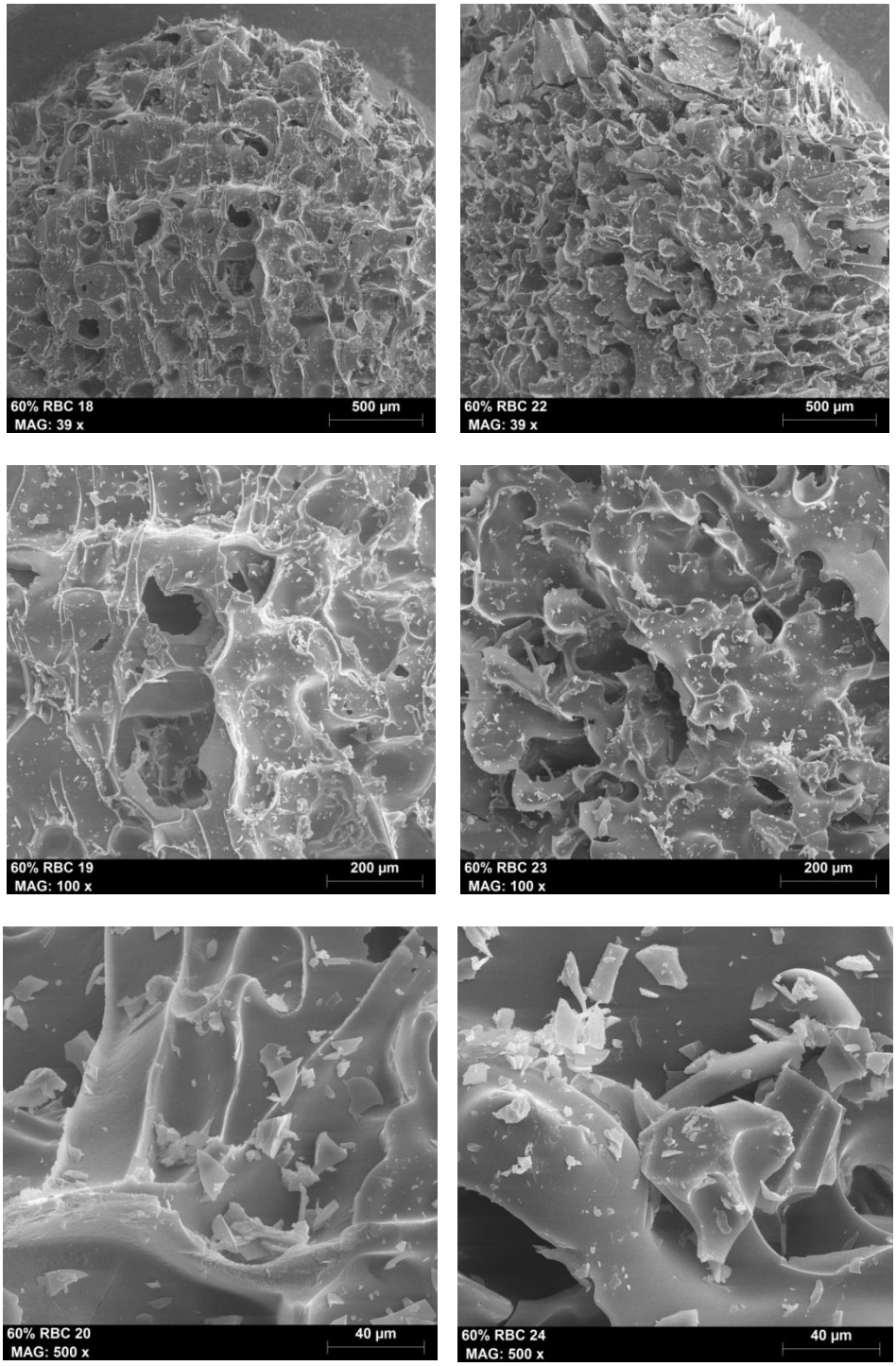

Figure 15: SEM picture of sample 60:40 % Hb:AS at 39, 100, and 1000 magnification.

When comparing pictures at x39 magnification from 20:80 and 40:60 samples (Fig 13 and 14) we observe that higher concentration of Hb resulted in larger capsules; while the size of the capsules did not increase further when the Hb concentration was raised from 40 to 60% (Fig 14 and 15).

Particles obtained from samples with high concentration of alginate show more and larger pores, which can explain the high solubility of the hemoglobin of these particles described in paragraph 4.3.3 and could lead to an increased susceptibility of iron to oxidation.

From the pictures in figures 14 and 15 we can see that the surface of the capsules at 40 and 60% of Hb content were very different from the 20% ones. The capsules looked more compact and showed a smoother surface. This may indicate that the protein, in this case Hb, interacts with alginate and modified the gelation behavior leading to a different structure. The interaction between negative charges of proteins and calcium-induced alginate gelation has been frequently reported. So, the ratios Hb:alginate had a great influence on the obtained structure.

x1000 magnification pictures showing details of surface confirmed this differences. Some crystals, probably originated from the excess of $CaCl_2$ used for gelation, can be seen in the pictures.

4.3.6. Drying by Fluidized bed dryer

Firstly, different combinations of time/air flow were applied on capsules put directly into the dryer chamber. However, the level of drying was not as good as expected for any of the combinations evaluated, probably due to the stickiness of the wet spheres, which made them difficult to fluidize. Therefore, the drying was assessed by adding poppy seeds which helped in the fluidization of the product and made possible to reach and acceptable level of drying. Capsules and seeds were mixed at a ratio 2:3 (w/w). Two airflow conditions, 50 and 70 % of pump power, were compared; and three different drying times: 20, 30 and 40 min for the first airflow (50) and 10, 20 and 30 min for the latter (70).

We found that the airflow affected inlet, product, and exhaust temperatures, as shown in Table 10.

Table 10 : Condition of Fluidized bed drying for all samples

	Sample	Inlet Temp		Product Temp		Exhaust Temp		Airflow %	Time (min)
		Start	End	Start	End	Start	End		
T 1	S 1	25.4	38.6	22.8	34.3	22.4	33.5	50	20
	S 2	35.8	40.6	27.3	36.8	28.9	36.7		30
	S 3	36.2	41.3	29.4	37.5	30.2	37.5		40
	S 4	36.9	45.8	30.1	40.5	30.6	39.9	70	10
	S 5	22.3	43.3	21.2	38.4	21.7	37.4		20
	S 6	37.6	46.4	28.5	42.7	28.9	41.4		30
T 2	S 1	20.2	35.9	20.4	31	20	30.7	50	20
	S 2	32.2	38.7	25.3	35.2	25.7	34.5		30
	S 3	35.7	39.3	28	36.5	29.6	35.7		40
	S 4	36.3	43.1	29.8	39.4	31	37.5	70	10
	S 5	38.4	44	29.9	32.2	30.9	39.7		20
	S 6	38.4	45	27.7	40.6	31	40.8		30

T 3	S 1	20.5	33.8	21.3	29.9	21.5	29.6	50	20
	S 2	30.2	36	25	32.7	25.3	32.7		30
	S 3	23.3	37.2	23.1	33.9	22.8	33.6		40
	S 4	33.8	42.3	28.1	37.1	29	36.5	70	10
	S 5	37.9	43.8	29.3	39.9	30.6	39.2		20
	S 6	40.3	45.8	31.2	42.3	33.6	41.5		30

T1, T2 and T3 correspond to three different batches of blood and S1-S6 to different aliquots from each encapsulated sample.

This table shows the influence of both time and airflow on the product and exhaust temperatures. A raise in temperatures of the product was favored by longer processing times and higher airflow. Among samples processed at the same airflow, the effects of processing time on the temperatures were significant. However, for the shorter processing time, the effects on temperatures were the same at both airflow conditions assayed.

On every dried sample, dry matter content, a_w, and color were analyzed, results are shown in tables 11 and 12.

Table 11: Dry matter content and a_w of the spheres after drying through a fluidized bed system

Sample	T 1			T 2			T 3			Airflow (%)	Time (mins)
	Dry matter (%)		a_w	Dry matter (%)		a_w	Dry matter (%)		a_w		
	Mean	Sd		Mean	Sd		Mean	Sd			
S 1	87.89	0.14	0.512	79.52	0.11	0.687	74.97	0.09	0.766	50	20
S 2	89.67	0.44	0.406	83.34	0.02	0.571	84.55	0.22	0.576		30
S 3	91.24	0.16	0.336	85.50	0.22	0.513	83.16	0.16	0.586		40
S 4	84.92	0.09	0.614	71.13	0.28	0.797	71.10	0.35	0.780	70	10
S 5	84.54	0.24	0.597	82.57	0.36	0.615	80.90	0.16	0.659		20
S 6	89.19	0.11	0.370	84.31	0.18	0.562	85.33	0.16	0.526		30

In general, dry matter was higher in samples treated for longer drying times. ANOVA analysis of the data (Annex II) showed significant differences ($P<0.05$) between treatments. The result of the Tuckey test gives three groups of means. 70% airflow for 10 min was the one that leads to samples with lower dry matter contents. 20 min treatments at 50 or 70% airflow allowed obtaining more dehydrated samples. And the most effective treatments were at least 30 min at both airflows.

Normally at higher process temperatures lower residual moisture and a_w were achieved. Some samples showed an exceptional behavior, having low moisture and high a_w, which could be due to hysteresis of adsorption/desorption processes (Labuza, 1980). Some samples had too high a_w (> 0.6) to warrant the stability and prevent microbial spoilage of the product during storage. Only the

longer treatments (S3 and S6) allowed to obtain samples with significantly low water activities ($P<0.05$) to ensure the stability.

Table 12: The effect of time and airflow of fluidized-bed drying on color parameters of the spheres (n=3)

Sample	Color parameter	T1	T2	T3	Airflow (%)	Time (min)
S1	L	31.77	25.32	25.11	50	20
	a*	22.92	11.53	15.72		
	b*	8.45	1.94	3.33		
S2	L	33.9	27.23	30.46		30
	a*	22.71	14.67	21.73		
	b*	8.82	4.03	7.79		
S3	L	33.78	30.41	25.92		40
	a*	19.81	17.32	12.2		
	b*	7.54	4.75	2.49		
S4	L	33.21	26.35	25.76	70	10
	a*	21.35	8.79	15.76		
	b*	7.36	0.08	3.11		
S5	L	32.38	26.54	25.93		20
	a*	24.78	7.51	9.54		
	b*	9.43	0.05	1.35		
S6	L	31.12	27.94	29.51		30
	a*	19.32	10.77	16.36		
	b*	7.22	1.82	6.12		

As can be seen in table 12, the color parameters of all samples were really worse than those of control capsules (Table 2). During the drying process the spheres turned into a too dark and brown color, whatever the airflow or the drying time. The color of these samples was not commercially acceptable, so this drying system was considered not good enough to improve the stability of Hb. The changes in color were due to the oxidation of iron, which happened at all conditions applied.

Further studies using nitrogen instead of air as drying fluid could be carried out before definitely discard this system.

4.3.7. Coating with chitosan

Coating with chitosan alginate capsules was assayed as a method to protect Hb against oxidation, as was described by Valenzuela, et al. (2014). Spheres were prepared as explained in section 4.3, and then they were coated with a chitosan solution for different times (section 3.5).

Color and protein content were measured in coated wet capsules, in order to decide what could be the best system to obtain them.

Table 13: Protein content in chitosan coated capsules

Time (min)	Protein (%)
5	1.51
10	1.46
15	1.30
30	1.19

In Table 13 can be seen that the percent of protein decreased as stirring time with chitosan increased, because the spheres incorporated more chitosan, and the relative contents in protein, logically, decreased.

Table 14: Color parameter of chitosan coated capsules

Sample	L*	a*	b*
control	15.92	25.96	5.96
5 min	18.88	18.49	3
10 min	17.41	31.97	8.62
15 min	21.53	31.97	8.62
30 min	26.54	33.46	11.17

Contrarily, as table 14 shows, the color of capsules was redder as increasing stirring time in chitosan solution. Although the a* parameter was never so high as was in the control capsules (section 4.1.1), the visual aspect of these spheres was really bright red. The discrepancy could be related to the fact that color measurements were conducted on wet samples. On the other hand more time were spheres in chitosan solutions, more stable was the color. The 30 min sample maintained red color after two weeks of refrigerated storage. These data are not shown in this work because it was not possible to carry out the complete experiment, but we think that this would be the way to improve the stability of color.

The SEM pictures of coated spheres permitted us to confirm this idea, as reported below.

4.3.8. Scanning Electron Microscopy of chitosan encapsulated samples

Before SEM observation, chitosan encapsulated spheres were freeze dried, and subsequently were prepared as described in section 3.6.

Two spheres from each sample were chosen to be observed, and pictures were taken at 39, 100, 500 and 1000 magnification.

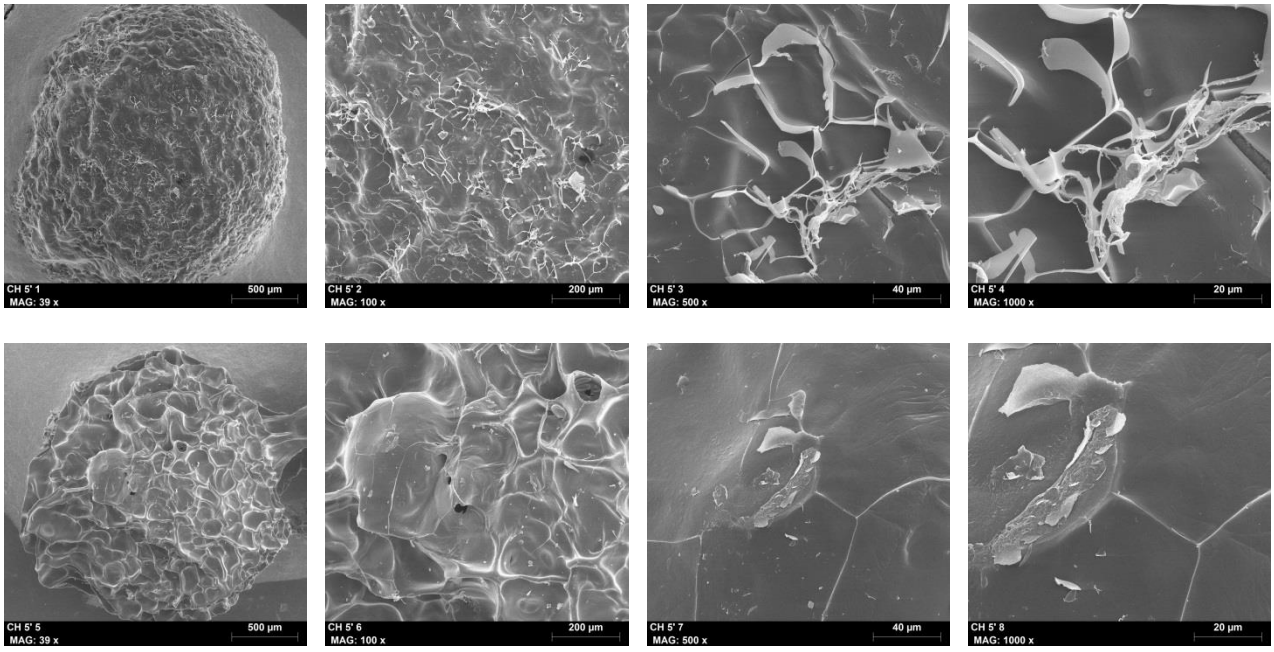

Figure 16 : SEM picture of 5 min coating with chitosan sample at 39, 100, 500 and 1000 magnification.

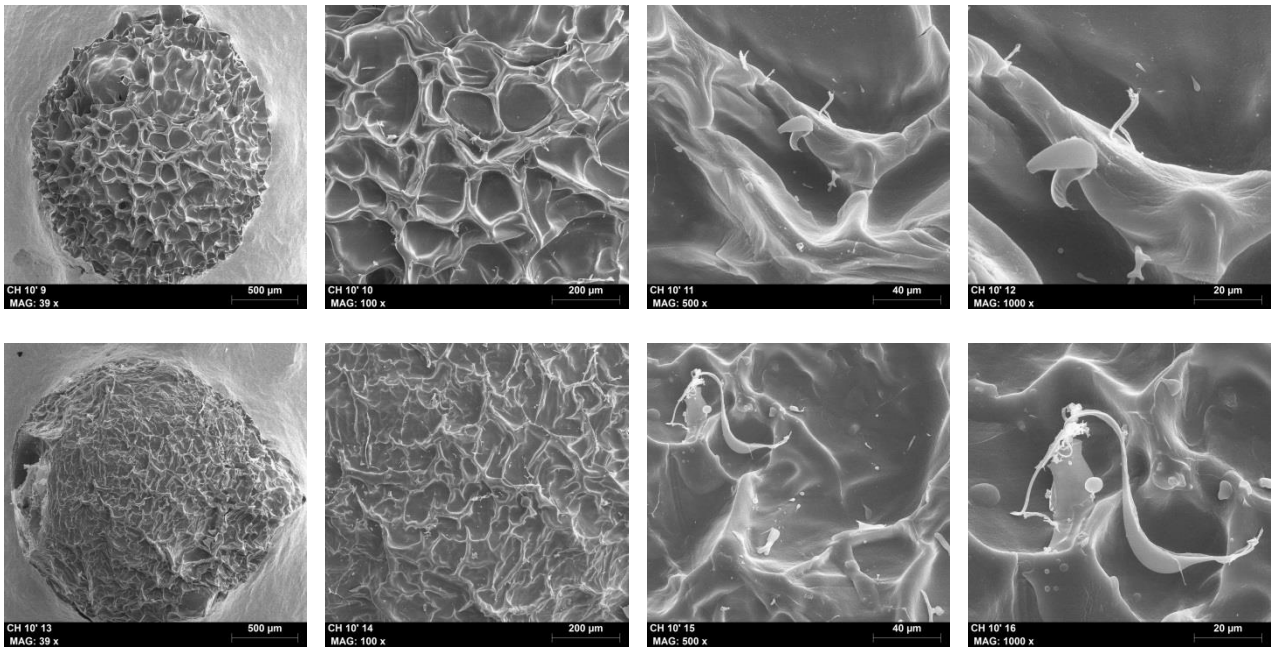

Figure 17 : SEM picture of 10 min coating with chitosan sample at 39, 100, 500 and 1000 magnification.

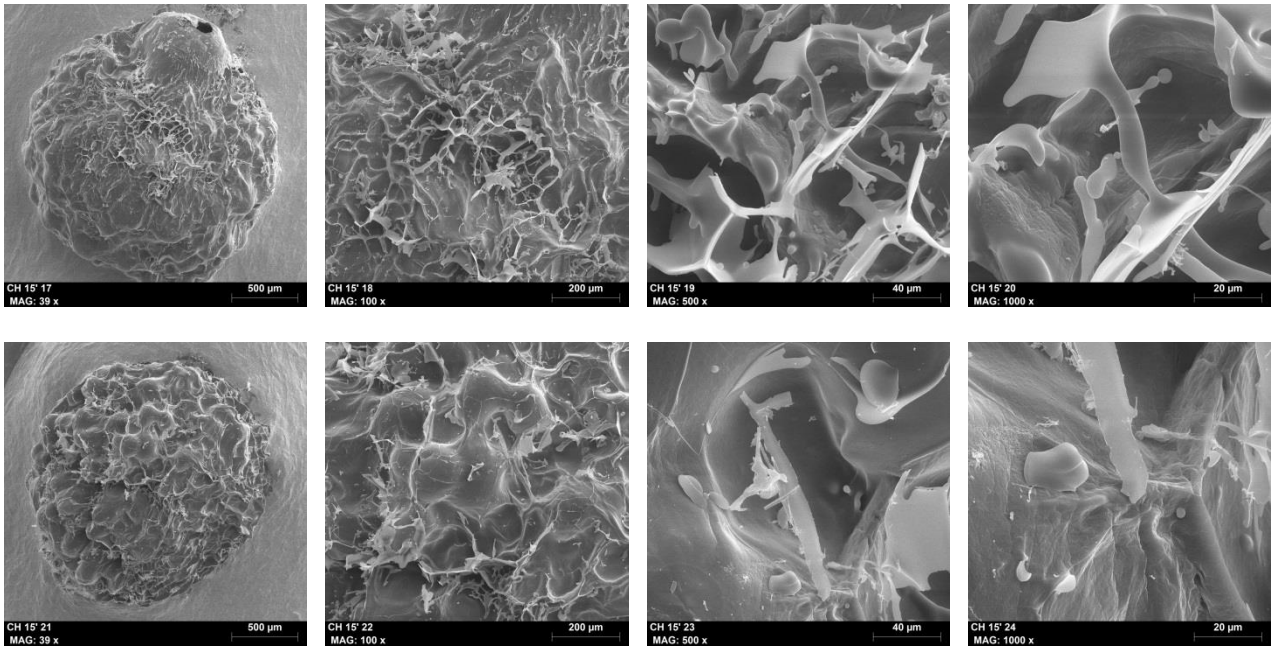

Figure 18: SEM picture of 15 min coating with chitosan sample at 39, 100, 500 and 1000 magnification.

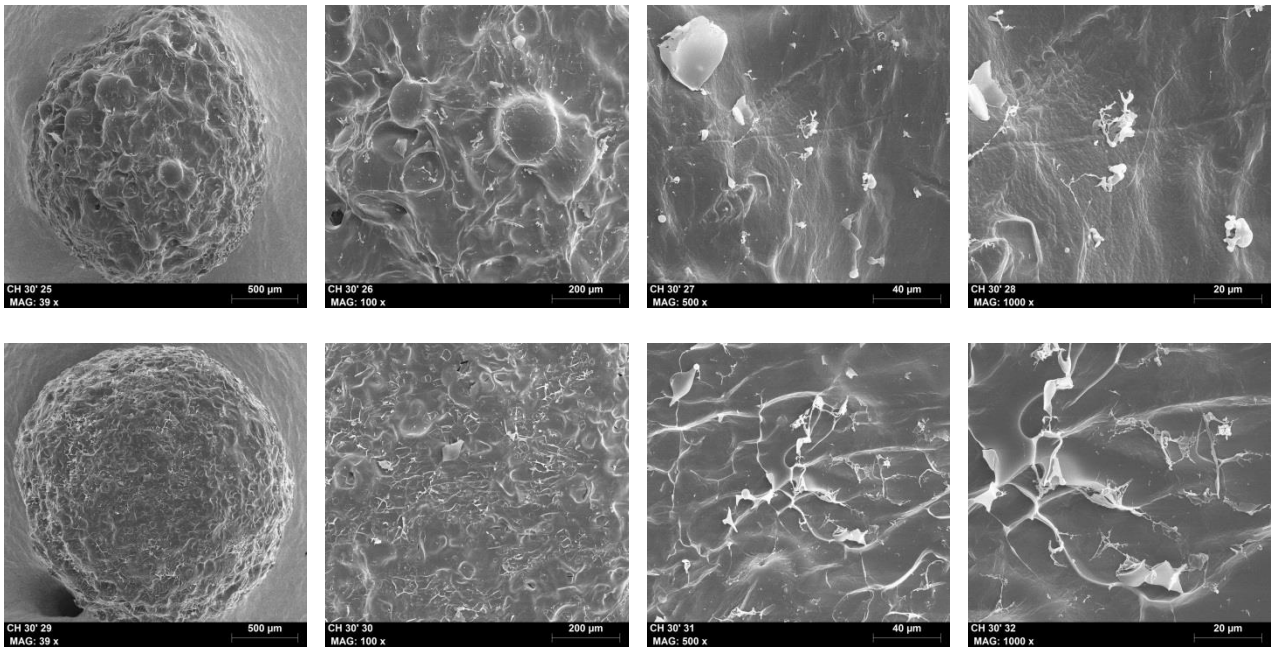

Figure 19 : SEM picture of 30 min coating with chitosan sample at 39, 100, 500 and 1000 magnification.

As can be observed in figures 16, 17, 18 and 19, the coating of chitosan on spheres is practically the same for 5 and 10 min of stirring. For 15 min, the coating is thicker, and become still more compact as longer is the stirring time. Consequently, the best color corresponded to samples coated for 30 min.

5. Conclusions

1. Color of control spray-dried capsules was bright red, with a* parameter in CIEL*a*b* measurements between 41 and 44 values.
2. Red color in control samples was quite preserved until 21 days storage, after two and three months, the red component (a*), as well as L* and b* decreased 4 and 6 units, respectively. In spite of that, the color was commercially acceptable at the end of storage.
3. The best system to prepare calcium alginate Hb capsules was, (1) mixing the hem-derivative with 2% alginate water solution, in the proportion 40:60; (2) dropping this mixture into a 5% $CaCl_2$ solution through a 10 µl pipette needle, by using a peristaltic pump; and (3) filtrating to remove the excess of liquid and recover the capsules.
4. Capsules containing 40:60 Hb:AS, showed the highest protein content, and the lowest protein solubility.
5. Freeze drying was not a good system to dry the capsules because they turned into brown and dark color during the dehydration process. Nevertheless, the 40:60 capsules showed the best color parameters as compared to 20:80 and 60:40 mixtures.
6. SEM pictures allowed seeing that higher alginate contents (80%) led to capsules that were smaller and more porous than those obtained with 40 or 60 % AS. The aspect of the surface of both kinds of capsules was very different, meaning that Hb interacted with alginate during calcium-induced gelation.
7. Fluidized bed drying system was difficult to apply because wet capsules were too sticky to be fluidized. The addition of poppy seeds was successfully used to help fluidization.
8. By using fluidizing bed dryer, only samples dried for 30 or 40 min at 70 or 50% airflow respectively, reached moisture contents, and consequently a_w values, significantly lower than the other process parameters evaluated. Moreover, these values were considered low enough to prevent microbial spoilage.
9. The brownish and dark color of fluidized bed dried capsules was not commercially acceptable, so the drying system was not considered appropriate to improve the stability of Hb.
10. Chitosan coating of alginate capsules can be a good system to improve color and stability, according to the preliminary studies carried out in this work. Nevertheless, further investigations are necessary to verify these results and to set the optimal process conditions.

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7. Annex I

7.1. Reaction agents

In these practical experiments, the chemical substances that were used in the process are:

- **Nicotinamide (99%):** from Sigma (St. Louis, MO, USA) it was used as a vitamin to increase the stability Fe^{2+} of blood. Nicotinamide is a water-soluble vitamin and is part of the vitamin B group.
- **Sucrose:** table sugar purchased in a local trade. It was used also to push the stability Fe^{2}.
- **Polysaccharide:** technical and structural characteristics of this reagent cannot be explained because of this project is confidential.
- **NaOH (10%):** was used to adjust pH of red cell fraction before adding other reagents. It was prepared by adding 10 g of NaOH to 90ml of distilled water, keeping on stirrer machine until it was dissolved, so that it can be used.
- **Alginate (2%):** 2% of alginate solution was prepared by adding 2 g of alginate powder to 100 ml of distilled water about one day before use. The solution was kept in the fridge until use.
- **$CaCl_2$ (5%):** it was prepared with 5 g of calcium chloride powder in 100 ml of distilled water. This solution was used to induce surface coagulation of alginate spheres.
- **Trypsin:** This reagent was used as an enzyme preparation and tenderizing for the production of protein hydrolysates. Trypsin (EC 3.4.21.4) 6.0 Anson units (AU)/g, provided from Novozymes (Novozymes A/S, Bagsvaerd, Denmark). Trypsin is a serine protease from the PA clan superfamily, found in the digestive system of many vertebrates, where it hydrolyses proteins (Rawlings and Barrett, 1994). Trypsin is produced in the pancreas as the inactive protease trypsinogen. Trypsin cleaves peptide chains mainly at the carboxyl side of the amino acids lysine or arginine, except when either is followed by proline. It is used for numerous biotechnological processes. The process is commonly referred to as trypsin proteolysis or trypsinisation, and proteins that have been digested/treated with trypsin are said to have been trypsinized. In the duodenum, trypsin catalyzes the hydrolysis of peptide bonds, breaking down proteins into smaller peptides. The peptide products are then further hydrolyzed into amino acids via other proteases, rendering them available for absorption into the blood stream. Tryptic digestion is a necessary step in protein absorption as proteins are generally too large to be absorbed through the lining of the small intestine. Trypsin is produced as the inactive zymogen trypsinogen in the pancreas. When the pancreas is stimulated by cholecystokinin, it is then secreted into the first part of the small intestine (the duodenum) via the pancreatic duct. Once in the small intestine, the enzyme enteropeptidase activates trypsinogen into trypsin by proteolytic cleavage. Auto catalysis can happen with trypsin using trypsinogen as the substrate. This activation mechanism is

common for most serine proteases, and serves to prevent autodegradation of the pancreas. Trypsin has an optimal operating pH of about 7.5-8.5 and optimal operating temperature of about 37.1°C.

➢ **Pepsin:** is a proteolytic enzyme preparation derived from animals or fish. Pepsin (EC 3.4.23.1) 596 units/mg from porcine stomach mucosa purchased from Sigma (Sigma-Aldrich Chemie Gmbh, Steinhem, Germany). It is an enzyme whose zymogen (pepsinogen) is released by the chief cells in the stomach and that degrades food proteins into peptides. It was discovered in 1836 by Theodor Schwann who also coined its name from the Greek word πέψις pepsis, meaning "digestion" (from πέπτειν peptein "to digest") (Florkin, 1957; Asimov, 1980). It was the first enzyme to be discovered, and, in 1928, it became one of the first enzymes to be crystallized, by John H. Northrop (1929). Pepsin is a digestive protease, a member of the aspartate protease family. Pepsin is one of three principal protein-degrading, or proteolytic, enzymes in the digestive system, the other two being chymotrypsin and trypsin. The three enzymes were among the first to be isolated in crystalline form. During the process of digestion, these enzymes, each of which is specialized in severing links between particular types of amino acids, collaborate to break down dietary proteins into their components, i.e., peptides and amino acids, which can be readily absorbed by the intestinal lining. Pepsin is most efficient in cleaving peptide bonds between hydrophobic and preferably aromatic amino acids such as phenylalanine, tryptophan, and tyrosine (Dunn, 2001). Pepsin is most active in acidic environments between 37 °C and 42 °C. Accordingly, its primary site of synthesis and activity is in the stomach (pH 1.5 to 2). Pepsin exhibits maximal activity at pH 3.0 and is inactive at pH 6.5 and above, however pepsin is not fully denatured or irreversibly inactivated until pH 8.0 (Johnston et al., 2007).

7.2. Equipment

7.2.1. The Centrifuge

Separation of plasma from the RBC fraction was conducted by using a Sorvall RC 50 Plus (Dupont, Newtown, USA) centrifuge (Figure 20) which has specifications as below:

- Run speed
  - Speed Selection Range (rpm) 50 to 21000
  - Speed Control Accuracy ±1% or 100rpm, whichever is greater
- Maximum Relative Centrifuge Force 51070 *g*
- Run Temperature
  - Temperature Selection Range -20 to +40 ^{o}C

- Temperature Control Range +2 to +40°C[2,3]
- Temperature Control Accuracy ±1°C3,4

- Run Time Selection Range 0 to 99 hr, 59 min or Hold
- Ambient Temperature Range +15 to +38°C[3]
- Mass (weight) 308 kg (680 Ib)

Figure 20: Centrifuge Sorvall RC 50 Plus

7.2.2. Homogenizer

A continuous high pressure valve homogenizer FPG 4755 (Stansted Fluid Power Ltd., Essex, UK) was used to hemolyze RBC after centrifugation of blood (Fig. 21).

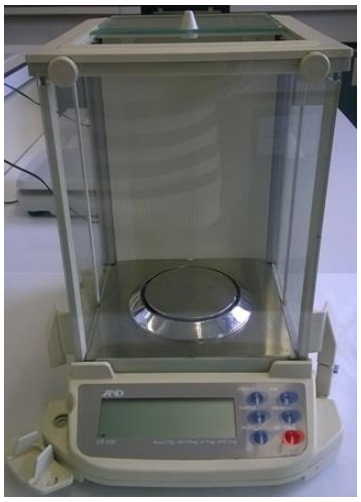
Figure 21 : Homogenizer Stansted FPG 4755

7.2.3. Electric Balances

Two electric balances were used to weight the samples and reagents (Figure 22):

- ***SCALTEC*** *(Instruments Gmb, Goettingen, Germany)*: it can weight maximum until 4200g, minimum 0.1g and its d = 0.1g (Figure 22b)
- ***A & D*** (A & D instruments LTD, Japan), (Figure 22a). It can work as below information:
  - Max: 210g
  - Min: 10mg
  - e = 1mg
  - d = 0.1mg

It was used to weight reaction agent and preparing sample for moisture, ash and protein measurement.


(a)


(b)

Figure 22: (a) A & D, (b) SCALTEC

7.2.4. Magnetic Stirrer

The magnetic stirrer (AGIMATIC-E, Spain (Figure 23) was used to help for mixing and dissolving reaction agents during preparation and adjusting pH of the samples.

Figure 23: Magnetic Stirrer

7.2.5. pH Meter

This electric pH meter (CRISON *GLP 22*, Alella, Barcelona) (Figure 24) was used to measure pH of the red blood cell during adjusting pH, and preparing other solutions.

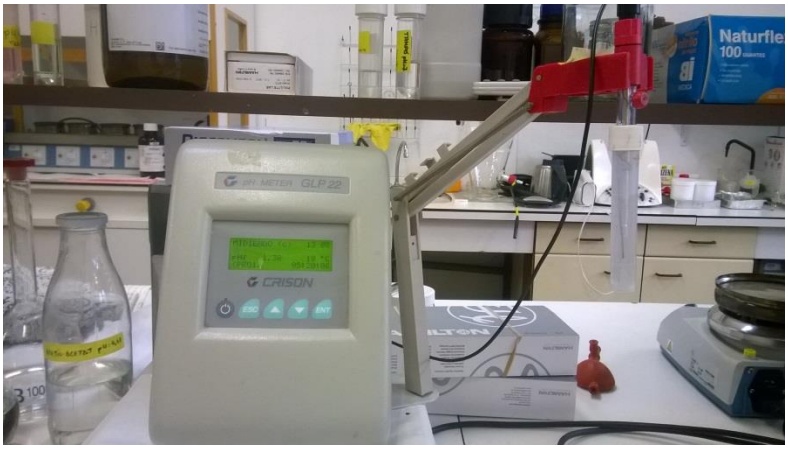


Figure24: pH Meter

7.2.6. Drying Oven

This oven (J.P. SELECTA, S.A., Abrera, Barcelona) (Figure 25) was used to dry the product until constant weight to calculate the moisture content. It was set at 100±5 ^{o}C.

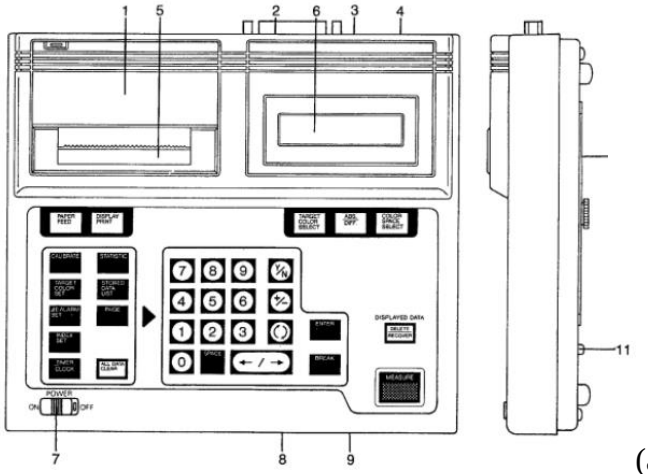
.

Figure25: Drying oven

7.2.7. Colorimeter

The equipment was used to measure the color of the sample is Minolta Chroma Meter CR-300 (Japan), is compact tristimulus color analyzer for measuring reflective colors of surfaces. This Chroma Meter consists of the measuring head and the Data Processor DP-301. The measuring head of the Chroma Meter CR-300 has an 8mm-diameter measuring area and uses diffuse illumination and a 2^{o} viewing angle (specular component included) for accurate measurements of a wide variety of subjects. CIE is *Commission Internationale de l'Eclarirage*.

Absolute measurements can be displayed as Yxy (CIE 1931), L*a*b* (CIE 1976), L*C*H^{o}, Hunter Lab, or XYZ tristimulus values; color difference can be displayed as △(Yxy), △(L*a*b*)/△E*ab, △(L*C*H*)/△E*ab, or Hunter △(Lab)/△E values. Data can be converted between color systems or between absolute and difference measurements by the data processor and each measurement can also be printed out in all color spaces if desired. The data can be printed out at the time of measurement. Figure 26 (a and b) will be shown about the illustration and real picture in the manual:



1. Paper chamber
2. Connector
3. Remote-control socket
4. AC-adapter socket
5. Printer
6. Display
7. POWER switch
8. ΔE ALARM output socket
9. RS-232C terminal
10. Battery-chamber cover
11. Viewing-angle adjustment lever

(a)


(b)

Figure26: "a" schema and "b" Colorimeter

7.2.8. Spray-Dryer

A Büchi Mini Spray Dryer model B-191 (Büchi Labortechnik AG, Flawil, Switzerland) was used for drying the HB solutions.

Figure 27: Spray-dryer Büchi

7.2.9. Freezer

After finished dropping, all types of encapsulated samples for freeze drying were transferred into a ultra-low temperature freezer model MDF-U5386S (Sanyo, Japan) asset at -70 ^{o}C. The samples were kept in it over night before going forward to the freeze drying process.

➢ **Specifications**

- Temperature range -50 ^{o}C to -86 ^{o}C (in 1 ^{o}C increments)
- Maximum cooling performance -86 ^{o}C (Ambient temperature 30 ^{o}C)
- External dimensions (W × D × H) 890 × 867 × 1990 (mm) / 35.0 × 34.1 × 78.3 (inch)
- Internal dimensions (W × D × H) 630 × 600 × 1280 (mm) / 24.8 × 23.6 × 50.4 (inch)

7.2.10. Freeze-dryer

This freeze-dryer (Unitop HL, Virtis, Gardiner, NY) (Fig.28) was used to dry all frozen spheres. During freeze drying it took also long time as frozen time it was about one day of finishing the whole operation. .

Figure 28: Virtis freeze-dryer

7.2.11. Peristaltic pump

This pump MiniPuls 3 model M312 (Gilson S.A.S., Villiers le Bel, France) was used to drop RBC to make encapsulated spheres by alginate and calcium chloride solution.

Figure 29: Peristaltic pump

7.2.12. Fluidized bed dryer

This Fluidized bed dryer STREA-1™ Aeromatic-Fielder (GEA Process Engineering Inc., Columbia, USA); was used to dry the bead products with alginate by different air flows of drying.

Figure 30: Fluidized bed dryer STREA-1™

8. Annex II. Results of statistical analyses

8.1. Water activity (a_w)

Tests of Between-Subjects Effects

Dependent Variable: a_w

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	6,094	1	6,094	111,390	,009
	Error	,109	2	,055[a]		
Treatment	Hypothesis	,160	5	,032	16,389	,000
	Error	,019	10	,002[b]		
Sample	Hypothesis	,109	2	,055	28,104	,000
	Error	,019	10	,002[b]		

a. MS (Sample)

b. MS (Error)

a_w

Tukey HSD[a,b]

Treatment	N	Subset 1	Subset 2	Subset 3
3	3	,4783		
6	3	,4860		
2	3	,5177	,5177	
5	3		,6237	,6237
1	3			,6550
4	3			,7303
Sig.		,874	,111	,108

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square (Error) = ,002.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = 0,05.

8.2. Dry matter

Tests of Between-Subjects Effects

Dependent Variable: Dry matter

Source		Type III Sum of Squares	Df	Mean Square	F	Sig.
Intercept	Hypothesis	123973,782	1	123973,782	1121,774	,001
	Error	221,032	2	110,516[a]		
Treatment	Hypothesis	270,312	5	54,062	7,840	,003
	Error	68,960	10	6,896[b]		
Sample	Hypothesis	221,032	2	110,516	16,026	,001
	Error	68,960	10	6,896[b]		

a. MS (Sample)

b. MS (Error)

Dry matter

Tukey HSD[a,b]

Treatment	N	Subset 1	Subset 2
4	3	75,7167	
1	3	80,7933	80,7933
5	3	82,6700	82,6700
2	3		85,8533
6	3		86,2767
3	3		86,6333
Sig.		,071	,154

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 6,896.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = 0,05.

8.3. Color L* parameter

Tests of Between-Subjects Effects

Dependent Variable: L

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	15175,143	1	15175,143	251,838	,004
	Error	120,515	2	60,257[a]		
Treatment	Hypothesis	21,207	5	4,241	1,617	,242
	Error	26,223	10	2,622[b]		
Sample	Hypothesis	120,515	2	60,257	22,979	,000
	Error	26,223	10	2,622[b]		

a. MS (Sample)

b. MS (Error)

8.4. Color a* parameter

Tests of Between-Subjects Effects

Dependent Variable: a

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	4762,555	1	4762,555	30,443	,031
	Error	312,888	2	156,444[a]		
Treatment	Hypothesis	56,989	5	11,398	,973	,479
	Error	117,187	10	11,719[b]		
Sample	Hypothesis	312,888	2	156,444	13,350	,002
	Error	117,187	10	11,719[b]		

a. MS (Sample)

b. MS (Error)

8.5. Color b* parameter

Tests of Between-Subjects Effects

Dependent Variable:b

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	407,837	1	407,837	7,175	,116
	Error	113,676	2	56,838[a]		
Treatment	Hypothesis	22,534	5	4,507	1,501	,273
	Error	30,033	10	3,003[b]		
Sample	Hypothesis	113,676	2	56,838	18,925	,000
	Error	30,033	10	3,003[b]		

a. MS (Sample)

b. MS (Error)