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 hem-based food colorant by means of spray-drying encapsulation

Document: Final Degree Project

Student (Name & Surname): **Senghak CHHUN**

EPS Advisors: Carmen Carretero and Dolors Parés

Department: Enginyeria Química, Agrària i Tecnologia Agroalimentària

Delivered on (month/year): **May 2016**

The final project entitled "IMPROVING THE STABILITY OF AN HEM-BASED FOOD COLORANT BY MEANS OF SPRAY-DRYING ENCAPSULATION" has been carried out in the Laboratory of Food Technology, High Polytechnic School (EPS), University of Girona, Spain, under the supervision of Dr. Carmen Carretero and Dr. Dolors Parés, Professors of University of Girona, Spain and Mr. Rithy Chrun, Lecturer of Royal University of Agriculture, Cambodia.

Girona, 13th May, 2016

Dr. Carmen Carretero and Dr. Dolors Parés

Mr. Rithy Chrun

Acknowledgment

This final project about "IMPROVING THE STABILITY OF AN HEM-BASED FOOD COLORANT BY MEANS OF SPRAY-DRYING ENCAPSULATION" had been successfully completed at the University of Girona, and it was part of a research line of the food technology group.

Firstly, through the strong cooperation between Royal University of Agriculture, Cambodia and the European Commission under the Techno II Project-Erasmus Mundus Action, I was given the opportunity to conduct the above project under the supervision by Prof. Dr. Carmen Carretero and Prof. Dr. Dolors Parés. Thus, I would like to give my profound thanks to both professors for their valuable counselling as well as other kind assistance within the laboratory and written work; and also my home university, the European commission, and the University of Girona.

Moreover, I would like to give my special thanks to Dr. Monica Toldrà, Dr. Elena Saguer, Dr. Anna Maria, and other colleagues in the research group of food technology who helped me with document collection, statistical data analysis, sample collection as well as other more things during conducting research, and also thank to a local industrial slaughterhouse for kind sample donation.

However, I would like to deeply thank to Prof. Dr. Laura Verheyleweghen, Prof. Dr. Emili Gracia-Berthou, Mrs. Raquel Solà, Mrs. Particia Eyskens and their colleagues for their document arrangement for my stage here.

Finally, I would like to give my great gratitude to my family and relatives for their strong supports and motivation for me in order to continue my study until now.

Summary

This work is part of a research project that aims to produce a natural red food colorant, stable during long-term cold storage, with a wide range of applications, using hemoglobin from porcine blood as raw material. The objective of this study was specifically to assess the suitability of the spray-drying technology to microencapsulate the hem-derivative into a protective matrix, aiming at obtaining a red powder able to maintain its red color in a wide range of pH. To achieve the objective, three separate formulations of haemoglobin derivatization, were prepared by addition of 3.5% nicotinamide as well as, 10% sucrose and the convenient quantity of encapsulation polysaccharide (E1 or E2) in A and C experiments, and 6% of sucrose, 0,1% of E1 and 4 % of a chelating agent in experiment B. Every hem-derivative was then encapsulated and dried in a spray-dryer. After dehydration process, several quality parameters of each encapsulation product, such are the proximate composition, the color, and the water activity, were used to assess the suitability of the encapsulation method and the matrix that leaded to the better quality characteristics. Moreover, gelatine gels were used as a model system to test the coloring power of both encapsulated products at different pH conditions. The results showed that all the spray-drying encapsulation of hem-derivatives permitted obtaining powders showing water content and water activity (a_w) low enough to warrant the microbiological stability without favoring iron oxidation reactions. Immediately after dehydration, all the products exhibited an acceptable bright red color, whatever the encapsulation agent used in the derivatization of hemoglobin. The colorant containing polysaccharide E1 showed better color stability than the two products containing polysaccharide E2. The red color of the E1 powder remained constant over 2-month chill storage. The partial substitution of sucrose by a chelating agent in the products containing the polysaccharide E2 negatively affected the color stability of the dehydrated products. At pH 6-7 all the gelatins containing the hem-colorants showed a bright red color. Nevertheless, only the formulation with combination of chelating agent and polysaccharide E2 was able to preserve the red color at pH below 5. At pH 4.5 the formulation with the same combination did not succeed to complete stabilize redness during storage, since a 30% decrease in a* values occurred after 7-day chill storage. However, final a* values of these gelatins were even 3-fold higher than the initial ones in gelatins containing E1 or E2 without the chelating agent. At pH above 5, although there were significant differences in b* and L* parameters, the a* coordinates showed to be similar for all the treatments at the end of 7-day chill storage, the mean reduction from the initial values being in the range from 6 to 11%.

Abbreviations

a_w	: Water activity
AOAC	: Association of Official Analytical Chemists
CIE	: Commission Internationale de l'Eclairage (International Commission On Illumination
FDA	: Food and Drug Administration
Hb	: Hemoglobin
RBC	: Red blood cells

Table of contents **Page**

1. Introduction

1.1. Food colorants

Color is an important quality attribute of foods. The objective of adding color to foods is to make them appealing, to counteract the loss of color during processing, to improve the quality and also to influence the consumer to buy a product (Madhava & Sowbhagya, 2012). It plays a key role in food choice by influencing taste thresholds, sweetness perception, food preference, pleasantness, and acceptability. Color, in a quantitative sense, has been shown to be able to replace sugar and still maintain sweetness perception in flavored foods (Clydesdale, 1993). There are four classifications of color available on the global food color market; they are synthetic (42%), natural (27%), natural identical (20%) and caramel (10%) (Downham & Collins, 2000).

Chemically synthesized colors are complex organic chemicals that were originally derived from coal tar, but now from petroleum. Companies like using them because they are cheaper, more stable, and brighter than most natural colorings. Many artificial dyes have been banned because of their adverse effects on laboratory animals. Actually, many studies have been reported that the use of certain synthetic dyes can result in carcinogenicity, genotoxicity, and neurotoxicity (Kobylewski & Jacobson, 2010). However, according to the U.S Food and Drug Administration (2015), there are 9 certified food dyes to be utilized as food additives, including Blue 1, Blue 2, Citrus Red 2, Green 3, Orange B, Red 3, Red 40, Yellow 5, and Yellow 6. For Europe, there are 40 colors permitted to be used in certain foods with specific quantitative limits (Food Standards Agency, 2014).

Natural colorants for food are taken from inexhaustible sources; those are plant material, insects, algae, cyanobacteria, and fungi (Mortensen, 2006). At present, the demand for natural dyes is increasing worldwide due to the increased awareness on therapeutic and medicinal properties and their benefits among public and also because of the recognized toxicity of synthetic colors. Among all the natural dyes, plant-based pigments have medicinal values so are mostly preferred (Chaitanya, 2014).

1.2. *CIELAB* system

CIE stands for *Comission Internationale de l'Eclairage* (International Commission on Illumination). *CIELAB* system is one of the methods being widely used for color measurement in food industries. Its color space is defined by three axes: *L**, *a**, *b** where L*, the central vertical axis, running from 100 to 0 values, indicates the level of lightness or darkness, the a* value indicates redness (positive values) or greenness (negative values), and the b* value yellowness (positive values) or blueness (negative values) (MacDougall, 2002). The color axes (a and b) are based on the fact that a color cannot be both red and green, or both blue and yellow, because these colors oppose each other. For both axes, zero is neutral gray (Figure 1.1).

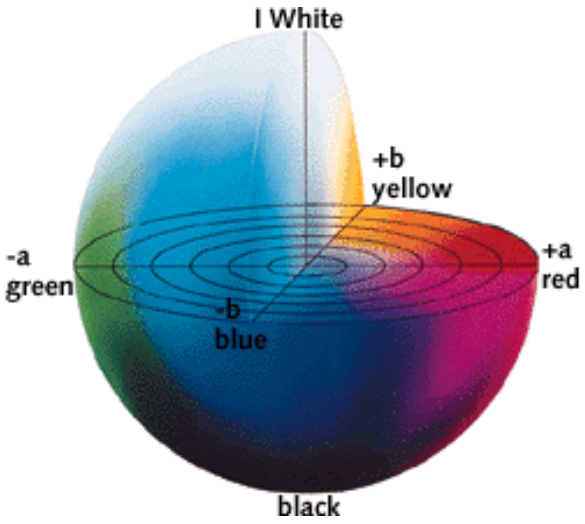


Figure 1.1 CIELAB system. Source: Walter (2016)

1.3. Animal blood

Blood is composed of blood cells (40-45%) suspended in a liquid called blood plasma (up to 60%). The cellular elements present in blood are mainly red blood cells (also called RBC or erythrocytes), together with white corpuscles (leucocytes) and platelets. RBC and plasma can be easily separated by centrifuging. Within the RBC fraction, total protein content ranges from 28 to 38%, and hemoglobin (Hb) is the major protein constituent. Plasma is a straw yellow liquid with 6-8% of proteins: albumin (up to 60%), globulins (40%) and fibrinogen (around 3%).

The proteins from blood are used commercially due to its high nutritional and functional quality (Satterlee, 1975). Blood proteins contain a wide range of essential amino acids; for example, Márquez et al. (2005) reported that porcine blood proteins contain isoleucine (0.69%), lysine (5.84%) and methionine (0.96%); and have good functional properties, whereas hem-iron of Hb can be used as food supplement in addressing iron deficiency (Ofori & Hsieh, 2012). Proteins from porcine blood are abundant; nonetheless, they are commonly discarded as waste which may cause environmental pollution and it is very costly in reducing this impact. So, making porcine blood into high value-added products is highly desired and also an ideal solution (Yu et al., 2006). Yearly China produces approximately 1,500,000 tons of porcine blood which is comprised of protein equivalent to that of 2,000,000 tons of meat or 2,500,000 tons of whole eggs (Wang et al., 2007). In 2013, there were 246 541 000 pigs slaughtered in Europe (Eurostat, 2014). With high volume of animal blood produced annually, the bulk of blood proteins have mainly been used as food ingredients by the meat industry; however, there are many other applications (Table 1.1) which take the advantage from the renewable source including the productions of baked products, dietary supplements and functional foods (Ofori & Hsieh, 2012).

Table 1.1 Examples of animal blood use in food applications

Blood fraction	Function	Food product	Reference
Whole animal blood	Protein source	Spanish blood sausage	Santos et al. (2003)
Bovine hemoglobin	Iron fortification	Cookies	Walter et al. (1993)
Bovine globin and plasma	Fat replacer	Ham pate	Viana et al. (2005)
Animal plasma	Fat replacer	Bologna (fermented) sausage	Cofrades et al. (2000)
Porcine transglutaminase, fibrinogen, thrombin	Binder	Restructured meat products	Tseng et al. (2006)
Porcine plasma	Protease inhibitor	Surimi	Visessanguan et al. (2000)
Chicken plasma	Protease inhibitor	Surimi	Rawdkuen et al. (2004)
Bovine plasma	Egg white replacer	Cakes	Lee et al. (1991) Myhara & Kruger (1998)
Processed bovine plasma	Emulsifier, Stabilizer	Minced meats	Furlán et al. (2010)

Source: Adapted by Bah et al. (2013)

1.4. Hemoglobin

The hemoglobin molecule is a tetrameric protein, formed from four subunits (α_1 α_2 β_1 β_2). Each subunit consists of a polypeptide chain (Figure 1.2), which is the protein or globin part of hemoglobin, and a heme prosthetic group. The number and sequence of amino-acid residues make both α and β chains distinct from each other. In different species, several amino acid sequences are also found in hemoglobin. Normally, the total molecular weight of the hemoglobin is approximately 67000 with some 10000 atoms inside, and 4 of which are iron; the rest are C, H, 0, N, S. (Weissbluth, 1974).

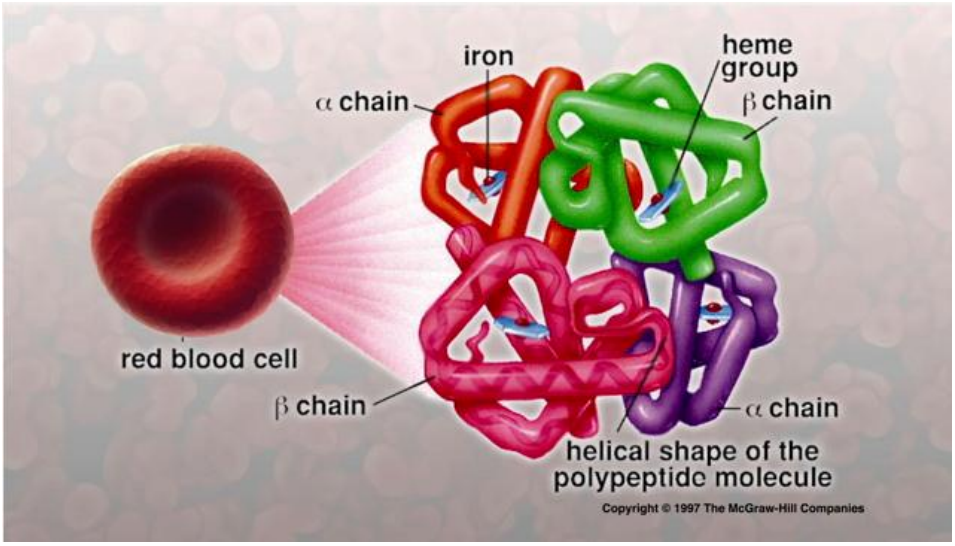


Figure 1.2 The hemoglobin molecule. Source: Mader (2002)

The heme group is responsible for the Hb red color, and consists of an Fe^{2+} (ferrous) ion in the center of a large heterocyclic organic ring (porphyrin), made up of four pyrroles (Figure 1.3). By taking advantage from the red color of the Hb, the RBC can be applied to different food commodities as a natural coloring agent (Toldrà et al., 2004). However, the color of the heme group is unstable and heavily dependent on the oxygenation/oxidation state of the iron. Deoxyhemoglobin, the deliganded ferrous Hb, is purple red while oxyhemoglobin, the dioxygen ferrous form, has a bright red color. Moreover, the Fe^{2+} in the Hb can be easily oxidized to the Fe^{3+} state, resulting in methemoglobin, with brown coloration (Parés et al., 2011).

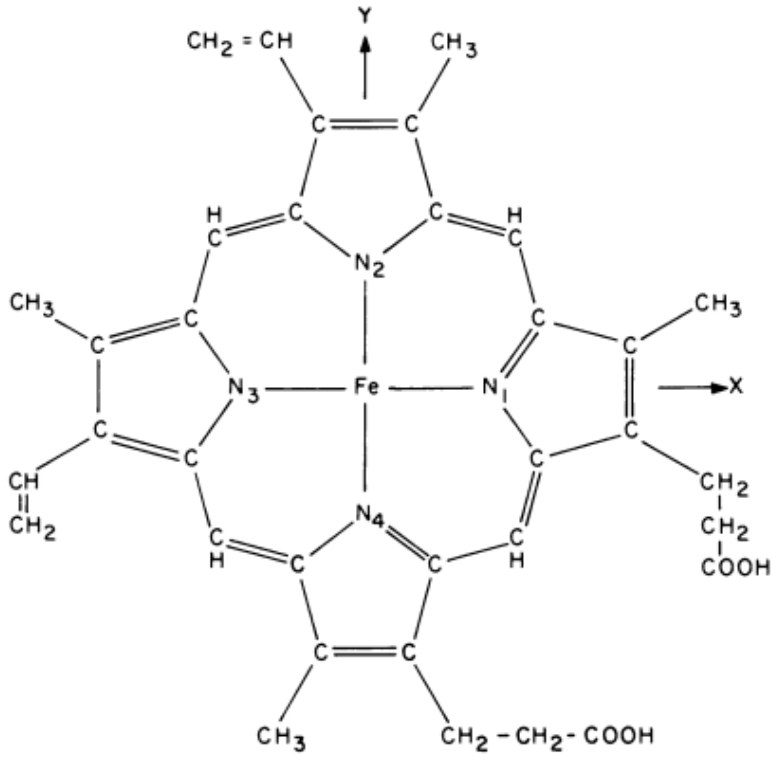


Figure 1.3 The heme complex. Source: Weissbluth (1974)

Freeze-drying and spray-drying have been employed as RBC preservation and dehydration systems in order to obtain a bright red powder that can be used as a natural food colorant; however, the application of freeze-drying for this objective is very costly because commercial scale freeze dryers are expensive and production outputs are low, so the spray-drying technology is considered a good alternative due to its higher production rates, and lower operative costs. Compared to freeze-drying, the cost of spray-drying method is 30–50 times cheaper (Gharsallaoui et al., 2007).

1.4.1. Hemoglobin color stabilization

As explained before, the bright red color of hemoglobin depends on the molecule remaining in the reduced ferrous form while the undesirable brown color results from oxidation of oxyhemoglobin (red) and deoxyhemoglobin (purple). High air temperatures, as used in the spray-drying process, enhance ferrous to ferric oxidation. Moreover, oxidation of hemoglobin can also

take place during the storage of the spray-dried powder. It has been reported a significant increase of the browning index of the animal blood color during the spray-drying process and also that this value keeps rising during the powder storage (Saguer et al., 2003). High temperatures speed up the rate of hemoglobin oxidation probably due to the effects on both the protein denaturation, that makes the access of oxidizing agents to the heme pocket much easier, and the autoxidation rate itself (Salvador et al., 2009).

Addition of protective agents to form hemoglobin complex, by means of spray-drying or freeze-drying microencapsulation, could be effective in minimizing the oxidation of hemoglobin as well as improving the stability of its red color (Salvador et al., 2009). The introduction of protective agents like carbon monoxide (Fontes et al., 2003; Fontes et al., 2010), nicotinic acid and nicotinamide (Saguer et al., 2003), nicotinic acid, nicotinamide and glucose (Salvador et al., 2009), L-cysteine and lactose (Zhou et al., 2012) and sodium alginate (Valenzuela et al., 2014) have been used for this purpose. Sgarbieri et al. (1999) also demonstrated that carboxymethylcellulose heme complex contains desirable red color with high iron bioavailability when using it as iron supplementation in foods.

1.5. Microencapsulation

Microencapsulation is a process of packing an active substance within another substance, ultimately resulting in the form of particles with the diameters between a few nanometers and a few millimeters. The substance being packed is called the core material, the active agent, fill, internal phase, or payload phase whereas the substance used for encapsulation system is called the coating, membrane, shell, carrier material, wall material, external phase or matrix (Zuidam & Shimoni, 2010).

There are two main types of structures formed by the encapsulation processes: the single-and the multiple-core microcapsules. In the single-core capsules, the core material is enveloped in the wall matrix. Sometimes, the central area may be void (Figure 1.4). A double-coating process in which the capsules were overlaid with edible wax using the hot melt process resulted in double encapsulated particles (Figure 1.5) (Onwulata, 2012). A wide range of encapsulated products

have been successfully provided in the fields of the pharmaceutical and cosmetic industries, and food industry.

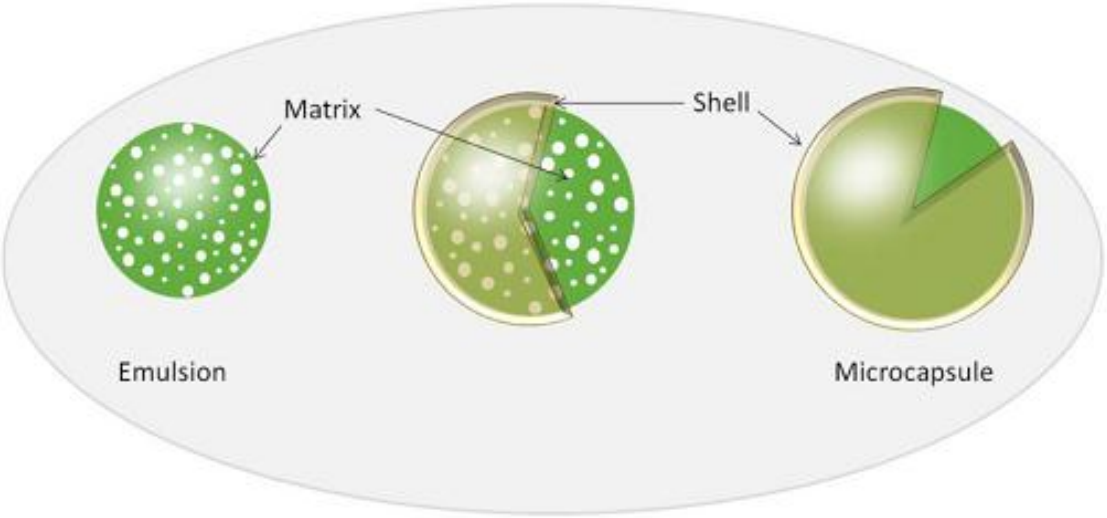


Figure 1.4 Encapsulation scheme showing emulsion and single-coated microcapsules capsules with voids. Source: Onwulata (2012)

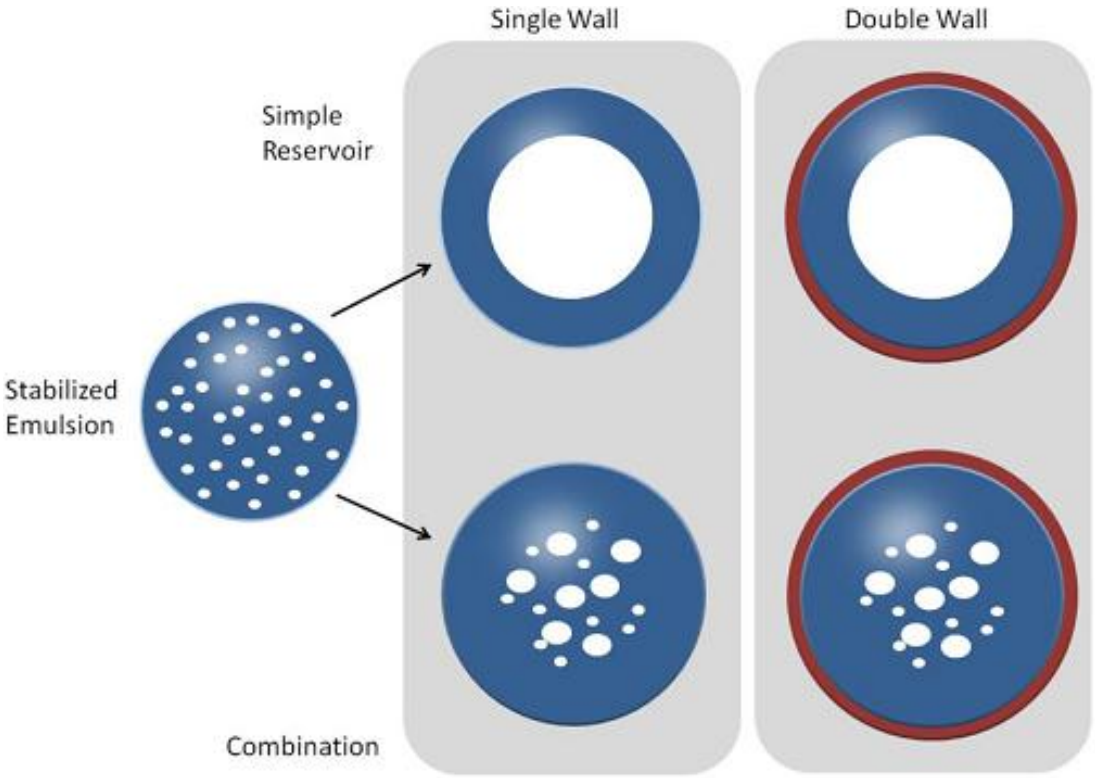


Figure 1.5 Encapsulation schemes showing the emulsions and single-and double-coated capsules. Source: Onwulata (2012)

There are many different methods being exploited in the production of microcapsules, but generally they are divided into three major groups (1) physical processes: spray-drying, extrusion and spray-coating, (2) physical and chemical processes: simple or complex coacervation and entrapment in liposomes, and (3) chemical processes: interfacial polymerization and molecular inclusion (Yáñez Fernández et al., 2002). The three categories of microencapsulation systems have been used by researchers for aroma products (Zuidam & Heinrich, 2010), fish oil (Beindorff & Zuidam, 2010), iron and other micronutrients for food fortification (Zimmermann & Windhab, 2010), carotenoids (Ribeiro et al., 2010), enzymes and peptides (Meesters, 2010) and probiotics for use in food products (Manojlović et al., 2010). Microencapsulation is essential for the stability of these bioactive compound-containing products since it protects bioactive compounds from adverse conditions and is also used for the controlled release at targeted sites (Gouin, 2004).

There are many wall materials suitable for microencapsulation in the food industry in regard to their origins: from plants, marine, microbes and animals. These materials can be (1) carbohydrate polymers, which consist of starch and its derivatives, cellulose and its derivatives, plant exudates (i.e gum arabic, gum karaya, mesquite gum, soluble soybean), plant extracts (galactomannans, soluble soybean), polysaccharide, carrageenan, alginate, xanthan, gellan, dextran and chitosan, (2) proteins, gluten (corn), isolate (pea, soy), caseins, whey proteins, and gelatin, or (3) lipids, fatty acids/alcohols, glycerides, waxes, and phospholipids (shellac) (Wandrey et al., 2010).

The application of these coating materials is based on their protective properties, and the characteristics of the molecules to be packed. Chávarri et al. (2010) showed that the microencapsulation of *Lactobacillus gasseri* and *Bifidobacterium bifidum* with alginate and chitosan is an effective means of delivery the probiotic bacteria into the colon and enhancing their survival during the adverse conditions of the gastro-intestinal tract. Similarly, chitosan-coated alginate beads also provided the increased survivability for microencapsulated *Lactobacillus acidophilus* and *Lactobacillus casei* (Bhandari et al., 2004). The result from both above studies are also proposed by Vandamme and Gbassi (2012) who argued that probiotic encapsulation technology has the potential to protect microorganisms and to deliver them into the gut. Bunjes and Strasdat (2013) claimed that calcium alginate beads show positive effects when entrapping lipid nanoparticles.

1.6. Spray-drying technology

Spray-drying is an essential unit operation that enables the transformation of a fluid substance (solutions, suspensions, dispersions or emulsions) into powder by spraying the feed into a hot drying medium, with the aims of preservation, ease of storage, transport, handling and other cost-effective goals (Bhandari et al., 2008). This technology is greatly successful in powder production and has an appealing feature associated with slight destruction of thermal-sensitive products during manufacturing (Bahnasawy et al., 2010).

Many different industries such as pharmaceutical, agrochemical, light and heavy chemicals, detergent, pigment, biotechnology and ceramic have used this technique and is the method most commonly used to dry liquid foods and slurries. Dairy production is, among all the food industries, the major area employing such a drying process to make powder from a wide range of milk and other milk-based produce. The production capacity of the spray-dryer, which ranges from a few hundred grams per hour to several tons per hour, is considered one of its unique characteristics (Bhandari et al., 2008). The final product can be in the form of free-flowing powder of individual particles, agglomerates or granules depending upon the physical and chemical properties of the feed, the dryer design, and the final powder properties (Michael, 1993).

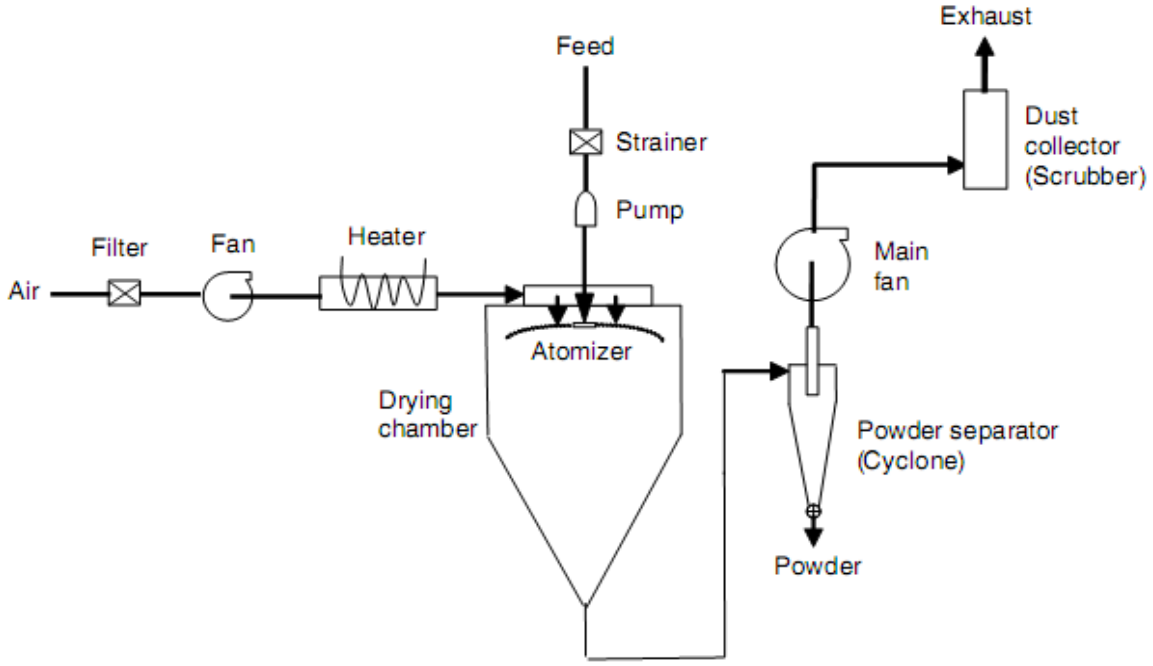


Figure 1.6 Scheme of a single-stage spray drying system. Source: Bhandari et al. (2008)

A common spray-dryer should comprise not less than four fundamental parts, which are drying gas supply and heating system, atomization system, drying chamber, and powder separators (Bhandari et al., 2008) (Figure 1.6). In order to achieve the transformation of feed from a liquid form into dried particles, there are basically four steps (Ronald, 1997) involved, apart from the concentration stage which is normally applied to the feedstock prior the introduction into the spray-dryer (More Swati & Wagh, 2014). The four stages of the process are as follows.

– **Atomization of the feed:** The feed is converted into a fine mist or spray. This stage generates the optimum condition for evaporation to a dried product consisting of the desirable attributes. Generally it is carried out using one of three principle tools: a rotary (centrifugal) atomizer, a single-fluid (or pressure) nozzle, or a two-fluid (pneumatic) nozzle (Figure 1.7). A rotary atomizer contains a disk, bowl, or wheel with the diameters of over 50-300 mm. The liquid is fed onto the disk near its center of rotation, and due to the rotational speeds in the range 50 000-10 000 rpm, it is broken up into droplets. A single-fluid nozzle features a small orifice, with a diameter from 0.4- 4.0 mm, through which the feed is pumped at high pressure of 5.0- 50.0 MPa. A grooved core insert, sited

Figure 1.7 Different atomization systems. Source: Kodi (2011)

before the orifice, imparts a spinning motion to the liquid, producing a hollow cone of spray. A two-fluid nozzle consists of an annular opening through which a gas, usually air, exits at high velocity. The feed exits through an orifice concentric with the air outlet. A Venturi effect is created and the liquid is converted into a spray. The feed pumping pressure is lower than that required in a single-fluid nozzle (Brennan, 2012).

- **Spray-gas contact**: The gas generally used is air, and this step is then called droplet-air contact, or more rarely an inert gas as nitrogen. In the chamber, atomized liquid is brought into contact with hot gas, resulting in the high evaporation of the water contained in the droplets in a matter of a few seconds.
- **Evaporation of moisture:** It is also known as droplet drying. Moisture evaporation takes place in two stages. During the first stage, there is sufficient moisture in the droplet to replace the liquid evaporated at the surface and evaporation takes place at a relatively constant rate. The second stage begins when there is no longer enough moisture to maintain saturated conditions at the droplet surface, causing a dried shell to form at the surface. Evaporation then depends on the diffusion of moisture through the shell, which increases in thickness.
- **Particle separation:** Cyclones, bag filters, electrostatic precipitators or scrubbers are often used to remove the dried product from the wet air in an economical and pollutant-free manner. Relying on the design, the final product can be separated at the base (as in a flat-bottomed dryer), and fines are collected in some type of collection equipment.

Spray-drying is used by the food and other industries not only as a preservation technology but also as an encapsulation technique. In fact, spray-drying is the most common and cheapest technique to produce microencapsulated food materials. Equipment is readily available and production costs are lower than most other methods. The encapsulation of food ingredients by spray drying are normally performed with the involvement of some common external matrixes such as gum arabic, maltodextrin, whey protein (concentrate or isolate), gelatin, sodium caseinate, modified starches and chitosan (Table 1.2). Moreover, this widely used dehydration process is also effectively applicable with nanoencapsulation of food nanoparticles by operation of nano spray dryer (Anandharamakrishnan & Padma Ishwarya, 2015).

Table 1.2 Experimental conditions for the encapsulation of some different food ingredients by spray-drying

Encapsulated ingredient	Wall material	Feed T (^oC)	Air inlet T (^oC)	Airoutlet T (^oC)	Reference
Anhydrous milk fat	Whey proteins/lactose	50	160	80	Young et al. (1993)
Ethyl butyrate ethyl caprylate	Whey proteins/lactose	5	160	80	Rosenberg & Sheu (1996)
Oregano, citronella and marjoram flavors	Whey proteins/milk proteins	NR	185–195	85–95	Baranauskiené et al. (2006)
Soya oil	Sodium caseinate/ carbohydrates	NR	180	95	Hogan et al. (2001)
Calcium citrate calcium lactate	Cellulose derivatives /polymethacrylic acid	NR	120–170	91–95	Oneda & Ré (2003)
Lycopene	Gelatin/sucrose	55	190	52	Shu et al. (2006)
Fish oil	Starch derivatives/ glucose syrup	NR	170	70	Drusch et al. (2006)
Cardamom essential oil	Mesquite gum	Room T	195–205	105–115	Beristain et al. (2001)
Arachidonyl $_L$-ascorbate	Maltodextrin/gum arabic /soybean polysaccharides	NR	200	100–110	Watanabe et al. (2004)
Cardamom oleoresin	Gum arabic/modified starch/maltodextrin	NR	176–180	115–125	Krishnan et al. (2005)
Bixin	Gum arabic/maltodextrin /sucrose	Room T	180	130	Barbosa et al. (2005)
$_D$-Limonene	Gum Arabic/maltodextrin /modified starch	NR	200	100–120	Soottitantawat et al.(2005a)
$_L$-Menthol	Gum arabic/modified starch	NR	180	95–105	Soottitantawat et al. (2005b)
Black pepper oleoresin	Gum arabic/modified starch	NR	176–180	105–115	Shaikh et al. (2006)
Cumin oleoresin	Gum arabic /maltodextrin /modified starch	NR	158–162	115–125	Kanakdande et al. (2007)
Fish oil	Sugar beet pectin/glucose syrup	NR	170	70	Drusch (2006)
Caraway essential oil	Milk proteins/whey proteins/maltodextrin	NR	175–185	85–95	Bylaité et al. (2001)
Short chain fatty acid	Maltodextrin/gum arabic	NR	180	90	Teixeira et al. (2004)

T: temperature, NR: not reported

Source: Adapted by Gharsallaoui et al. (2007)

1.7. Previous studies of the research group

The research group of Food Technology began to address the issue of the use of blood as raw material for the production of ingredients for the food industry in 1996. During this time the physical, chemical and microbiological characterization 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 & Parés, 2000), plasma and hemoglobin concentrate treated by high hydrostatic pressure (Parés et al., 1999, 2000 & 2001, Parés & Ledward, 2001; Toldrà et al., 2002 & 2004), both pressurized and non-pressurized, hemoglobin hydrolysates (Toldrà et al., 2011), fractionation of the main proteins of plasma (Dàvila et al., 2006, 2007a & 2007b; Dàvila & Parés, 2007; Toldrà et al., 2008). 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 & 2007, Fort et al., 2007, 2008 & 2009).

From the red-blood-cells, which is the most difficult to valorize, the group worked on the use of the cellular fraction to obtain decolorized hemoglobin hydrolysates. The enzymatic method developed in the laboratory allows the separation of a white protein hydrolysate and a residue that contains the entire heme group (Toldrà et al., 2005). 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 heme group and the potential biological activity of the low molecular-weight peptides (Toldrà et al., 2008).

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

As a good alternative to valorize, the cellular fraction has also been used to develop a system to obtain a 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 they are really close to getting a formulation for the patentable food colorant. A quite stable red hem-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 slightly acidic pH has been successfully 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 part of a research project that aims to produce a natural red food colorant, stable during long-term storage, with a wide range of applications, using haemoglobin from porcine blood as raw material.

The objective of this study was specifically to assess the suitability of the spray-drying technology to microencapsulate the hem-derivative into a protective matrix, aiming at the obtaining of a red powder with increased pH stability.

To achieve this objective the study considered two stages:

1. Two different polysaccharides (E1 and E2) were tested as protective matrices in the spray-drying process. Several quality parameters, such are the proximate composition, the color, and the water activity of the encapsulated products, were used to assess the suitability of the encapsulation method and the matrix that leaded to the better quality characteristics.
2. Gelatin gels were used as a model system to test the coloring power and the stability of both encapsulated products at different pH conditions.

3. Methodology

3.1. Experimental design

The productions of a natural red food colorant by means of two different spray-drying encapsulation systems (A and B) were carried out, using hemoglobin from porcine blood as raw material. Each experiment was conducted three times, with blood collected from a local industrial abattoir under the same conditions but on different days. The proofs of application of coloring at three different pH conditions were performed by using gelatin gels as model medium. Analyses of moisture content, water activity, ash content, protein content, and color, were used to characterize each encapsulated product. In gelatins only color parameters were analyzed.

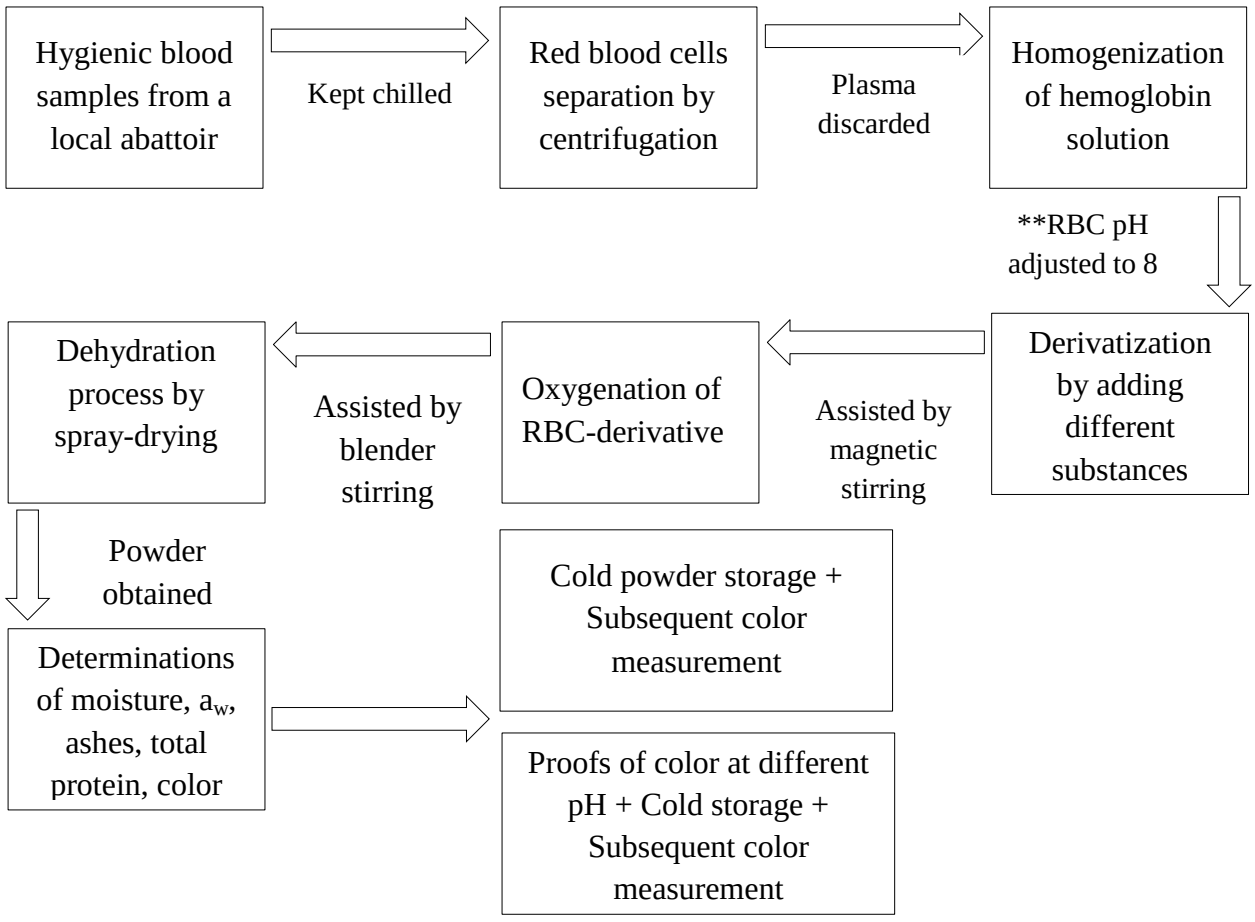


Figure 3.1 Experimental design

******Before RBC pH adjusted to 8, RBC samples were taken for moisture, ashes, and total protein determinations.

3.2. Materials

3.2.1. Reagents and equipment

Description in detail of reagents and equipment employed in the work is shown in Annex I, together with pictures of the main apparatus and machines.

3.2.2. Preparation of hemoglobin solutions

The hygienic porcine blood samples, in which polyphosphate 4% had been added as anticoagulant, were collected from a refrigerated storage container of a local industrial abattoir (Norfrisa S.A., Riudellots de la Selva, Girona, Spain), using sterile containers. Blood was kept chilled during transport to the laboratory and until processing. The red blood cells fraction was the sediment obtained by centrifuging at 2530 x g at 4 -10 °C for 15 min (Sorvall RC-SC plus, Dupont Co, Newton, Connecticut, USA), and decanting the supernatant (plasma). To obtain Hb solutions, red blood cell samples were immediately hemolyzed using a high-pressure laboratory valve homogenizer FPG 7400 (Stansted Fluid Power Ltd., Essex, UK) at 5-bar inlet pressure, 10 MPa processing pressure, and 15 ^{o}C and 25 ^{o}C inlet and outlet temperature, respectively.

3.3. Spray-drying encapsulation systems of hemoglobin solutions

3.3.1. Preliminary studies

Before conducting the encapsulation experiments, some hemoglobin samples containing different concentrations of the polysaccharide E1 were spray-dried. Measurements of the moisture content, water activity and color parameters of the dried products were used to assess the suitability of E1 as encapsulation matrix, as well as to determine the spray drying conditions.

3.3.2. Preparation of hem-derivatives

Before the dehydration process, Hb solutions were adjusted to pH 8 by the addition of NaOH (10%) under continuous stirring using a magnetic stirrer. Afterwards, the derivatization was carr-

ied out by adding different substances, as indicated in Table 3.1.

Table 3.1 Description of derivatization in each experiment

	Experiment A	Experiment B	Experiment C**
Nicotinamide (NAM)	3.5 %	3.5%	3.5%
Sucrose	10%	6%	10%
Chelating agent	-	4%	-
E1	0.5%	-	-
E2	-	0.1%	0.1%

** The results from experiment (C) actually derived from another study and were used here to be compared with that of the two other experiments above (A and B).

Nicotinamide (NAM) (Agrōs organics, New Jersey, USA) was added whilst stirring for 5 min, followed by sucrose and the different encapsulation agents (E1 and E2).

E1 and E2, as well as the chelating agent used in experiment B, cannot be specified for confidentiality reasons.

A home hand blender (Braun, Barcelona, Spain) was used to stir RBC-derivatives in order to completely dissolve the encapsulation agent and to oxygenate heme groups until its color became bright red.

3.3.3. Spray-drying process

The samples were immediately spray-dried using a Büchi Mini Spray Dryer model B-191 (Büchi Labortechnik AG, Flawil, Switzerland), at a 120 ^{o}C inlet temperature; feeding pump flow 50-65% (0.85L/h); aspiration pump flow 100% (60L/h); spray pressure 5 bars; and the outlet temperature ranged from 69-72 ^{o}C.

3.4. Effect of pH on color

In order to determine the effect of pH on color, gelatin gels at pH 4.5, 5.5, and 6.5 were prepared. Firstly, three different acidic solutions were heated using the microwave oven, and then 5% (w/v) of gelatin 99% was dissolved in these solutions under continuous heating and stirring. Afterwards, these gelatin solutions were cooled below 40 ºC and the different powders were added (0.05% w/v). Finally, the solutions were transferred into Petri dishes and kept cooling until solidification. Duplicates with the same thickness were used to assess the CIEL*a*b* color parameters of the gelatins.

3.5. Analytical determinations

3.5.1. Water activity (a_w)

Water activity is defined as the ratio of the vapor pressure of water in a material to the vapor pressure of pure water at the same temperature.

Water activity (a_w) at 20 ºC of the powders was measured by automatic equipment *Lab* Master a_w (Novasina AG, Lachen, Sweden). Samples were put in plastic specific containers and placed into the hole of the measuring chamber. The chamber lid was then sealed and the value of vapor equilibrium (a_w) was directly displayed on the screen of the device within approximately 20 min. This measurement was done in duplicate.

3.5.2. Moisture content

Moisture content is the quantity of water contained in a material. In food materials is very significant in many aspects including the design of technological processes, stability and shelf-life of foodstuffs, economic reasons, product specifications and other legal regulations (Isengard, 2001).

Moisture measurements were performed by means of a gravimetric method (AOAC, 1995). Samples and crucibles were initially weighed, and then placed into the oven at 100 ± 5 ^{o}C overnight. Afterwards, they were reweighed in order to obtain the remaining dry matter as well as

the amount of water evaporated. The moisture content was calculated as follows:

$$\%\text{Moisture} = \frac{M_{Initial} - M_{Dried}}{M_{Initial}} \times 100$$

where $M_{Initial}$ and M_{Dried} refer to the mass of the sample before and after drying, respectively. Each measurement was performed in duplicate

3.5.3. Ash content

The ash content in a food material refers to the total amount of inorganic constituents including Fe, Ca, Na, K and Cl. Mainly, ashes are defined as the inorganic substances remaining after heating samples at 550 ^{o}C, in order to make sure that water and other volatile elements are vaporized and organic matters are burnt in the presence of oxygen in air into CO_2, H_2O and N_2. Most minerals are converted into oxides, sulfates, phosphates, chlorides or silicates (source: http://people.umass.edu/~mcclemen/581Ash&Minerals.html).

The determination of ash content was carried out in duplicate using the official method (AOAC, 1995) by incinerating the samples in a muffle. First, the samples, which were previously dried for the moisture content measurement, were placed into the muffle. Samples were initially heated at 150 ^{o}C for 1 hour, and then at 250 ^{o}C for 1 hour, and finally at 550 ^{o}C overnight. The mass of the samples after incineration was recorded and the mass of sample before drying (section 3.5.2) was again used in order to obtain the ash content via the following equation.

$$\%\ \text{Ash (wet basis)} = \frac{M_{Ash}}{M_{Initial}} \times 100$$

where M_{Ash} is the residue after heating at 550 ^{o}C, and $M_{Initial}$ is the mass of samples before drying (section 3.5.2).

3.5.4. Protein content

The analysis of nitrogen content was determined by Kjeldahl method (AOAC, 1995). Total amount of nitrogen was then multiplied by a factor (6.25) to obtain the approximate protein content. There are three principal stages for measuring the total protein as follows:

- **Digestion:** The food sample is weighed into a digestion tube and digested by heating it in the presence of sulphuric acid and a catalyst (47.7% Na_2SO_4, 47.7% K_2SO_4, 2.8% TiO_2, 1.8% $CuSO_4$, MERCK) by employing a semi-automatic system (Gerhart KB20, German). This process converts any nitrogen in food (except that which is in the form of nitrates or nitrites) into ammonia, and other organic substances into CO_2 and H_2O. Ammonia gas is not liberated in an acid solution as the ammonia is in the form of the ammonium ion (NH_4^+) which binds the sulphate ion (SO_4^{-2}) and finally remains in the solution:

  N (food) $\rightarrow$ $(NH_4)_2SO_4$ (1)

- **Neutralization:** The digestion tube is attached to a receiving flask by a tube after completion of the digestion process. The solution in the digestion flask is subsequently made alkaline by addition of sodium hydroxide (40%), which converts the ammonium sulphate into ammonia gas:

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

  The ammonia gas formed is released by distillation in the water steam stream (Büchi K314, German) and moves out of the digestion tube 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 into the borate ion:

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

- **Titration:** The estimate of nitrogen content is done by titration of the ammonium borate formed with standard hydrochloric acid, using mixed indicator to determine the end-point of the reaction.

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

  The concentration of hydrogen ions (in equivalents) required to reach the end-point is equivalent to that of nitrogen that was in the initial food sample (Equation 3). The following equation can be used to determine the nitrogen concentration of a sample that

weighs *m* grams using HCl solution for the titration:

$$\%N = \frac{V \times N}{m} \times \frac{14}{1000} \times 100$$

where *V* is the titration volume of the sample, and *N* is the normality of HCl solution. 14.01 is the molecular weight of nitrogen N. Once the nitrogen content has been determined, it is converted into the total protein content using the appropriate conversion factor: % Protein=F × %N; (F=6.25).

3.5.5. CIELab colorimetric parameters

Color measurements were performed by the MINOLTA CR-300 colorimeter (Minolta Co., Ltd., Japan) 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 each measurement by utilizing a standard white ceramic plate in the tri-stimuli (*Yxy*) system.

3.6. Statistical data analysis

The statistical package SPSS 23.0 for Windows (SPSS Inc., Chicago, IL) was used to carry out the statistical analyses. In order to determine the effect of both kind of encapsulating agent and pH on the color of gelatin gels prepared from dried hemoglobin, color data were submitted to ANOVA using the general linear model procedure (Proc GLM) and the Tukey test to compare means. The significance level for all tests was α= 0.05.

4. Results and discussion

4.1. Composition of raw materials and final products

Table 4.1 indicates the components of red blood cells fraction as raw materials and the dried hemoglobin as final products, from each experiment; that included moisture content, water activity, ashes and total protein.

Table 4.1 Composition of hemoglobin solutions (raw materials) and dried hemoglobin powders (final products) in each experiment (mean ± s.d, n=3)

Experiment	Types of sample	Moisture (%)	a_w	Ash (%)	Protein (%)
A	Final product	5.23 ± 0.51	0.25 ± 0.03	3.01 ± 0.50	71.79 ± 3.13
	Raw material	67.81 ± 0.16	n.d	1.22 ± 0.38	31.05 ± 0.55
B	Final product	7.52 ± 0.73	0.23 ± 0.03	7.13 ± 0.33	72.05 ± 3.62
	Raw material	67.18 ± 2.32	n.d	1.28 ± 0.06	30.09 ± 2.15
C	Final product	6.47 ± 0.67	0.23 ± 0.01	2.11 ± 1.07	74.67 ± 3.04
	Raw material	n.d	n.d	n.d	n.d

n.d=no data

As can be seen in Table 4.1, the raw materials collected on different days but under identical conditions, in both experiment A and B, had very similar composition, which consisted of approximately 67% of water content, 1.20% of total ashes and 31% of total protein. Nevertheless, the total protein content in final products from experiments A and B (aprox. 72%), was slightly lower than the value obtained in experiment C, which was 74.67 %.

Although the a_w value in the three powders was practically the same; the moisture content in the product from the experiment A seems to be lower. This fact can be related to the characteristics of the encapsulation agent used in this case (E1), which probably has lower water binding capacity when compared to E2, used in the experiments B and C. The great difference among the ash percentage of the sample corresponding to experiment B with respect to the products from experiments A and C can be attributed to the mineral content of the chelating agent used to partially substitute the sucrose in the formulation of this particular hem-derivative.

The protein content of our dried products was about 11-13% lower than the value reported by Fontes et al. (2010) for other dried blood powders, which was 85.70 ± 1.44%, probably due to the substances that were added to stabilize the color. Besides this, the water activity obtained was in the optimal range (0.2-0.4), which was adequate not only to prevent iron from becoming more reactive to oxidation (Labuza, 1980) but also to avoid microbial spoilage (Labuza & Altunakar, 2007). Moreover, the residual humidity of the products ranged from 5 to 7%, these results agree with previous data from Saguer et al. (2003) in which 6% residual moisture was found in similar hem-derivatives.

4.2. Chromatic parameters of spray-dried products

Table 4.2 illustrates the chromatic parameters of dried hem-based food colorants obtained from three separate encapsulation processes on the day of productions (day 0). Looking in detail at the values in Table 4.2 we can see that the product of experiment C showed the highest value of redness (a*) 42.52 ± 1.31, which accounted for approximately 3 and 6 units over those of the two other experiments A and B, respectively. Similarly, it also showed the higher value of yellowness (b*) but a lightness (L*) which was some 3 units below that of the experiment A, and roughly 1 unit above that of the experiment B. Overall, and despite the mentioned differences, we can say that all spray-dried powders were visually acceptable, showing a bright red color, on day 0.

Table 4.2 Chromatic parameters of hemoglobin powders at the day of production (day 0)

Experiment	L*	a*	b*
A	48.00 ± 2.87	39.20 ± 0.64	19.76 ± 0.88
B	44.28 ± 1.37	36.52 ± 1.16	16.77 ± 1.11
C	45.63 ± 2.07	42.52 ± 1.31	21.30 ± 0.67

mean ± s.d, n=3

The redness of the dried hemoglobin obtained from the three encapsulation systems was quite similar to a freeze- hemoglobin powder containing l-cysteine and α-lactose, reported by Zhou et al. (2012). This author obtained color parameters which were approximately 37 (redness), 59

(lightness) and 42 (yellowness) on the day of production, that is levels of brightness and yellowness higher than those from our products. Anyway, the studies using nicotinic acid and nicotinamide (Saguer et al., 2003); and nicotinamide and glucose (Salvador et al., 2009), produced the lower qualities of spray-dried red blood cells with color parameters of about 27 (a*), 35 (L*) and 15 (b*); and 33 (a*), 42 (L*) and 18 (b*), respectively, thus it can be concluded that the current encapsulation systems resulted effective in preventing hemoglobin from oxidation during dehydration process.

4.2.1. Evolution of chromatic parameters of spray-dried hemoglobin during storage

Figure 4.1 shows the variation of each color indicator in the dried powder obtained from experiment A during 58-day storage in the refrigerator. The color was considerably constant over the whole storage period and remained practically unchanged during the first 9 days. Only a slight decrease in each color parameter from 48.00 to 46.72 (L*), from 39.20 to 36.13 (a*) and from 19.76 to 17.87 (b*) was observed within the entire time of storage.

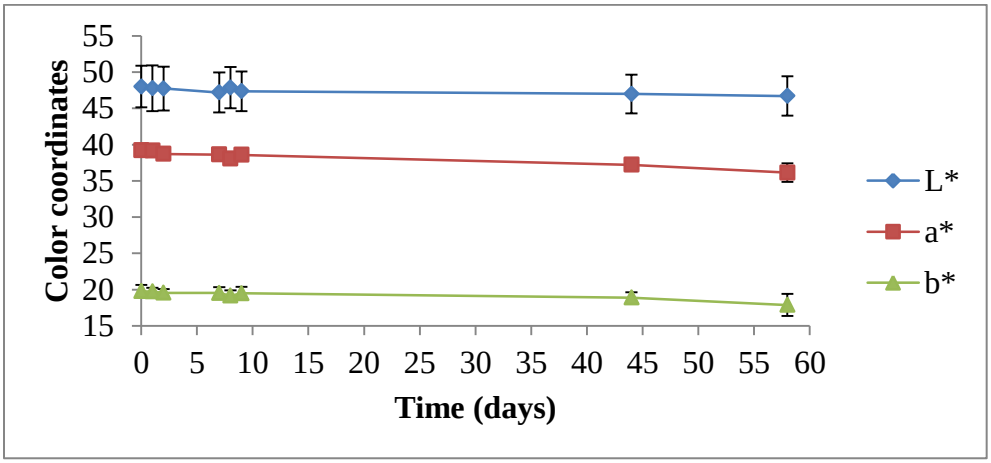


Figure 4.1 Changes in the color coordinates (CIE L*a* b*) of the spray-dried hem-derivative A through 2-month storage (mean ± s.d, n=3)

Figure 4.2 illustrates the changes of the three color parameters, L*, a*, b*, of dried hemoglobin obtained from experiment B during the 21-day refrigerated storage. Within the first 2 days after the production, the color parameters remained stable; however, afterwards both a* and b*

decreased until reaching 6 and 3 units below the original values at the end of the storage, respectively. In contrast, the lightness increased by 3 units above the initial value.

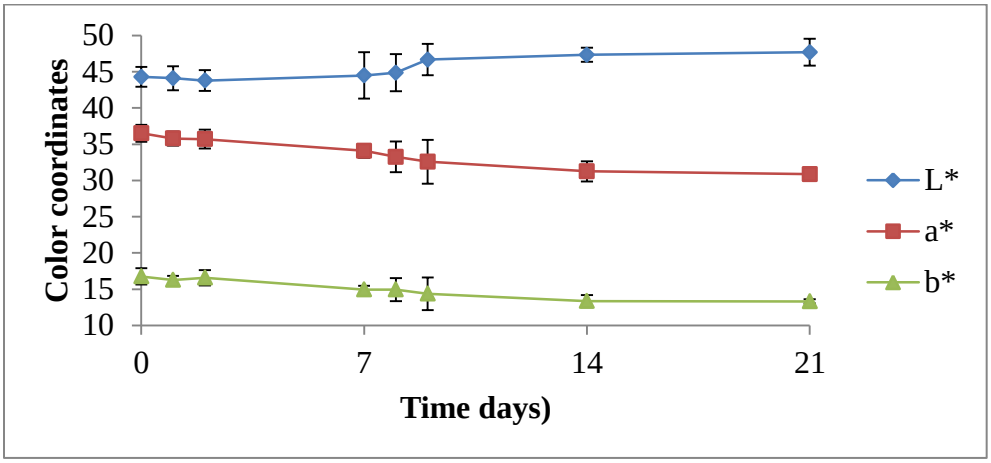


Figure 4.2 Changes in the color coordinates (CIE L*a* b*) of the spray-dried hem-derivative B through 21-day storage (mean ± s.d, n=3)

Figure 4.3 shows the evolution of chromatic parameters of the hem-derivative obtained from experiment C during the 14-day cold storage. As can be seen in the figure, the values of L*, a* and b* remained stable up to 7 days after its production. However, at the end of storage the lightness was slightly increased (approximately 1 unit from the initial value), whereas decreases of about 7 units in the redness and 5 units in the yellowness coordinates occurred.

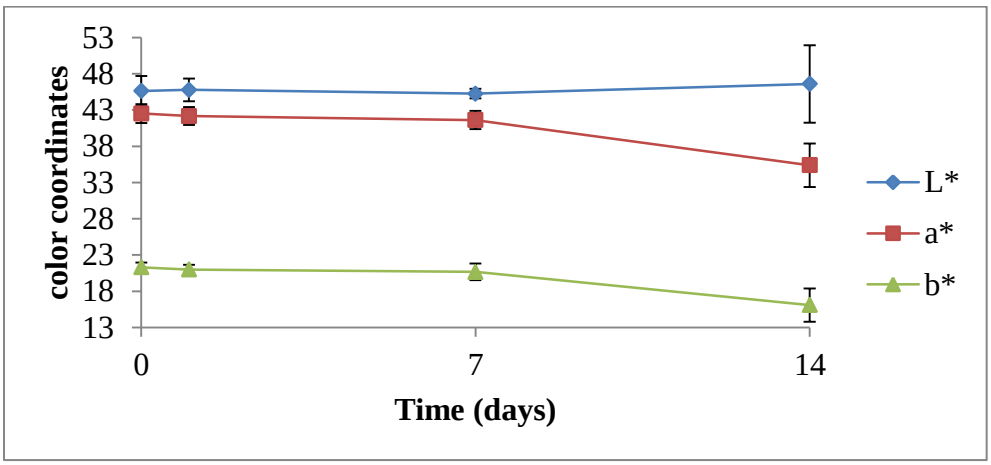


Figure 4.3 Changes in the color coordinates (CIE L*a* b*) of the spray-dried hem-derivative C through 14-day storage (mean ± s.d, n=3)

From the results illustrated in the Figures 4.1, 4.2 and 4.3, it could be seen that within the 14-day refrigerated storage, the redness of the dried pigmented powder in experiment A was constant, while this parameter considerably declined some 7 units in the encapsulation system C, although both (A and C) kept their brightness unchanged. The color coordinate (b*) of the pigment from system A also remained constant whereas there was a drop of 5 units in that from system C. Anyway, as described above, within the entire storage period of 14 days, both experiments resulted in satisfactory red colorants. On the other hand, system B did not show such a good stability as compared to the A and C systems.

4.3. Effect of pH on color powder

Table 4.4 shows the effect of pH on colorimetric parameters of gelatin gels containing dried hem-derivatives from each experiment. Although three pH ranges (4.5, 5.5 and 6.5) were used to determine the effect of pH on color, as described in the methodology section, only results from gels at pH 4.5 and 6.5 are actually presented. The pH 5.5 results were excluded because they could not be considered consistent enough due to a too broad range of variability between repetitions.

Table 4.4 Colorimetric parameters of gelatin gels containing 0.05% (w/v) hem-derivatives

(a) pH 4.5

Experiment	L*	a*	b*
A*	28.17 ± 2.37^{a}	7.79 ± 0.59^{a}	2.97 ± 0.16^{a}
B	31.66 ± 3.01^{a}	35.46 ± 2.74^{b}	18.99 ± 3.75^{b}
C	27.62 ± 0.81^{a}	8.84 ± 2.61^{a}	2.91 ± 1.83^{a}

(b) pH 6.5

Experiment	L*	a*	b*
A*	37.37 ± 1.95^{a}	48.51 ± 2.55^{a}	28.53 ± 2.66^{a}
B	33.99 ± 3.10^{a}	45.41 ± 4.08^{a}	26.69 ± 4.57^{a}
C	44.89 ± 0.17^{b}	50.98 ± 0.34^{a}	32.15 ± 0.40^{a}

mean ± s.d, n=3, *n=2

Different letters mean significant differences ($p<0.05$)

As shown in Table 4.4(a), at pH 4.5 the color of gels containing the product from the experiments A and C was quite similar. Both showed significant lower a* and b* values as compared to those in experiment B ($p<0.05$). All three gels had the same lightness value. Visually, the gels B were the only ones which showed a satisfactory bright red color while the color of gels A and C reflected a brown hue related to iron oxidation.

On the one hand, it can also be observed in Table 4.4(b) that at pH 6.5 all gels showed a high bright red color without significant differences in the red and yellow components; although the lightness of the gels from experiment C showed to be significantly higher ($p<0.05$).

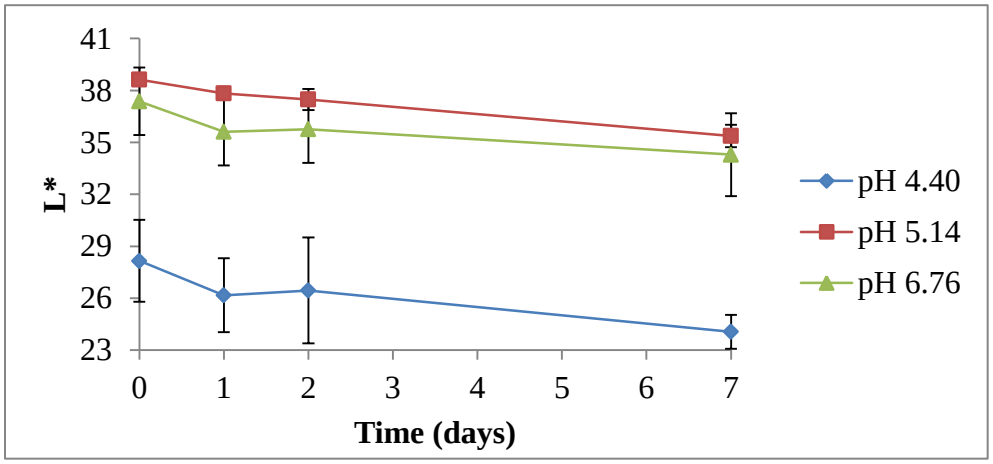
From these results it can be concluded that all color parameters were strongly influenced by pH. Based on the Turkey's test, redness values (a*) were significantly higher at pH 6.5 than at pH 4.5 ($p<0.05$), in agreement with the results described in the studies carried out by Fontes et al. (2010) and Saguer et al. (2003). It is known that a pH drop accelerates the rates of hemoglobin oxidation (Richards & Hultin, 2000; Undeland et al., 2004), which definitely explained the undesirable change in color.

The best protection from color changes at pH 4.5 was achieved in experiment B, in which polysaccharide E2 combined with a chelating agent were used; thus it seems that this combination works better in preventing hemoglobin from being oxidized than the polysaccharide matrix E1 (experiment A) and the polysaccharide E2 without the chelating agent (experiment C).

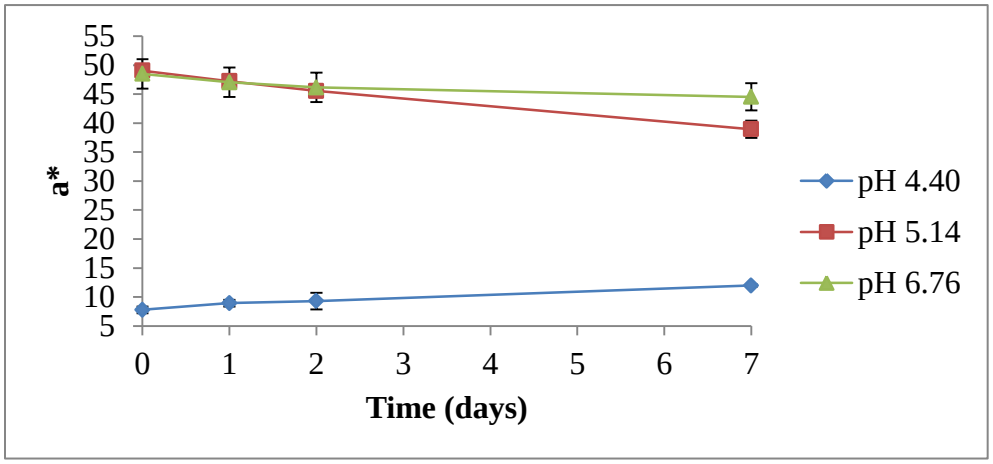
4.3.1. Effect of pH on color evolution during refrigerated storage

Figure 4.5 shows the colorimetric changes of gelatin gels containing the colorant A during one-week cold storage as influenced by pH (4.40, 5.14 and 6.76). It can be observed in Figure 4.5(a) that there was a decrease in lightness of about 4 units at pH 4.4, and 3 units at both pH 5.14 and 6.76, within the whole storage time. Similarly, from Figure 4.5(b), redness (a*) dropped about 11 units at pH 5.14 and 4 units at pH 6.76; but surprisingly at pH 4.4 the a* value increased in almost 4 units. The yellowness (b*) was seen to decrease at pH 5.14 and 6.76, about 6 and 4 units, respectively, while it increased 3 units at pH 4.4. Overall, at the pH range 5-6, the gels containing the colorant A retained a slightly bright red color at the end of storage time, nevertheless, they became dark brown at pH below 5.

(a)



(b)



(c)

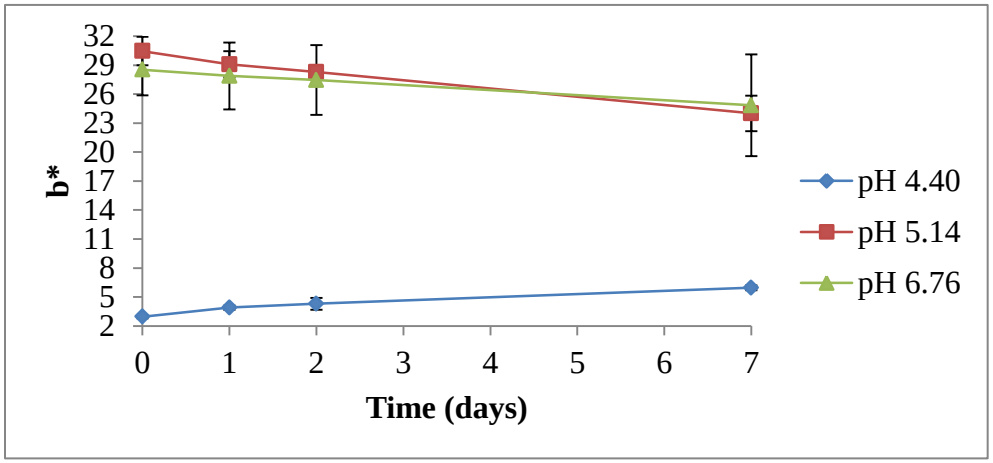
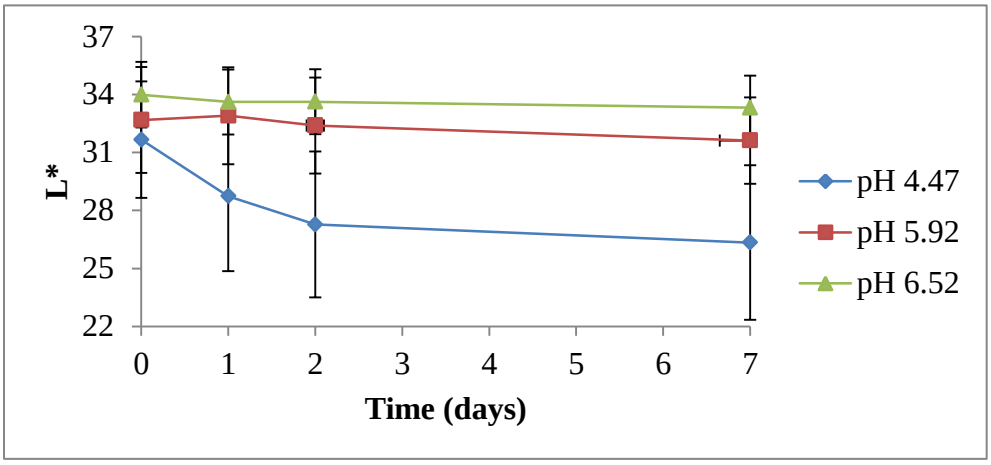


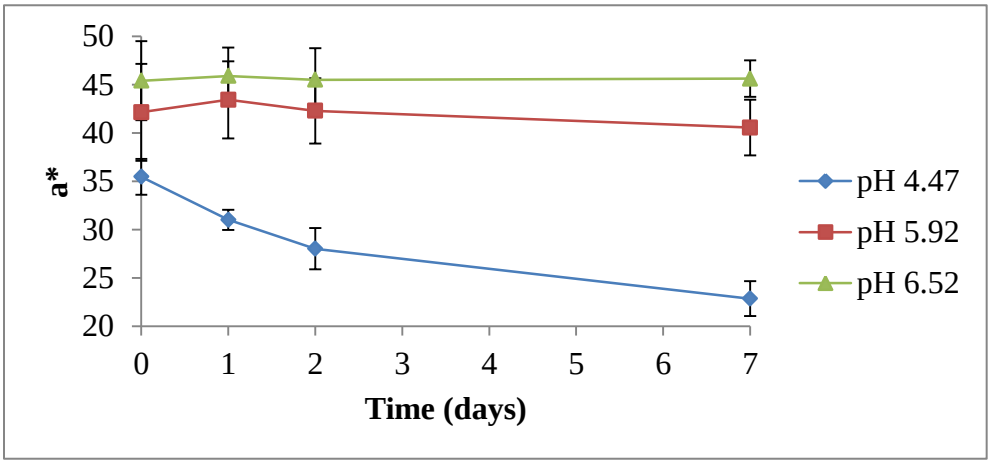
Figure 4.5 Effect of pH on color characteristics of gels containing the colorant A during one-week refrigerated storage (mean ± s.d, n=2)

Figure 4.6 shows the variation on colorimetric parameters of the gelatin gels containing the hem-derivative from experiment B at different pH conditions during 7-day cold storage.

(a)



(b)



(c)

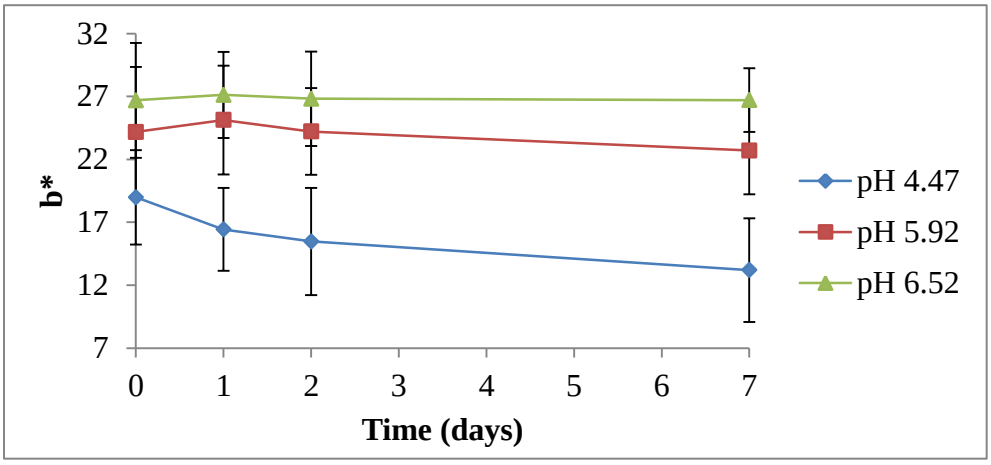


Figure 4.6 Effect of pH on color characteristics of gels containing the colorant B during one-week refrigerated storage (mean ± s.d, n=3)

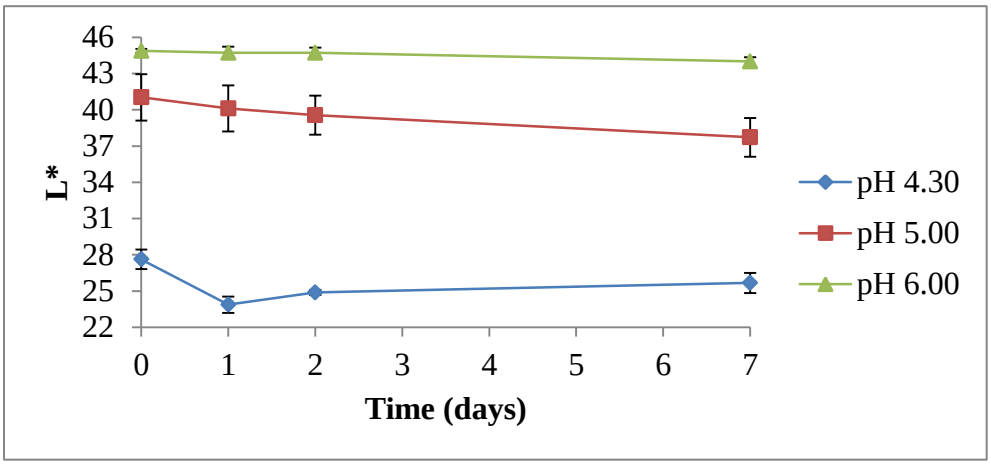
As can be seen in Figure 4.6 (a), a slight decrease in lightness, of about 5 units, occurred during the storage, in samples at pH 4.47, while this parameter remained practically constant in gelatins at pHs 5.92 and 6.52. It can also be observed in Figure 4.6 (b) that the redness declined 2 units at pH 5.92 and remained relatively stable at pH 6.52. However, there was a significant decrease of 12 units in redness at pH 4.47. From Figure 4.6 (c) we can observe that the yellowness dropped about 5 units at pH 4.47, and 2 units at pH 5.92, but practically leveled off at pH 6.52. Thus, during the entire refrigerated storage the gelatins were slightly bright red at 5-6 pH ranges, but at lower pH they became browner as the storage time increased, despite the slightly red color they had on day 0.

Figure 4.7 shows the evolution of colorimetric parameters of the gelatin gels containing the hem-derivative from experiment C, at different pH conditions during 7-day refrigerated storage.

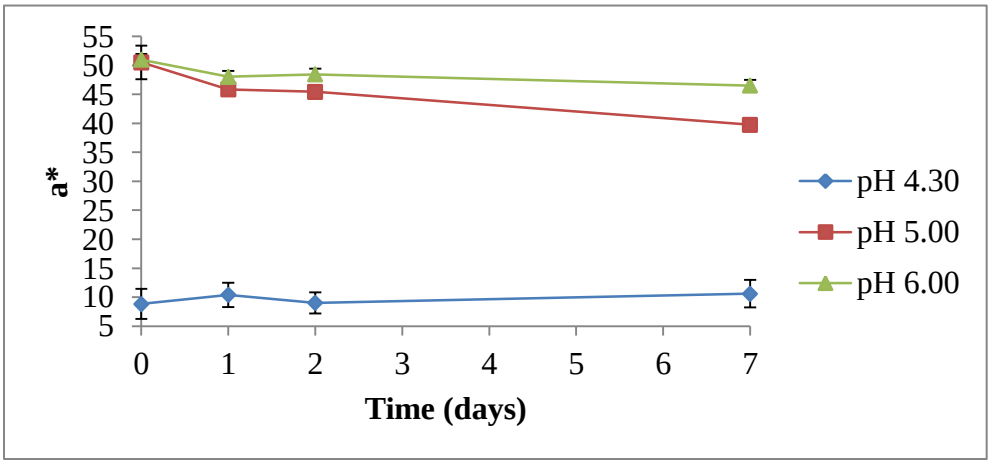
As can be seen in Figure 4.7(a), during storage the lightness of the gels at pH 4.3 and 5.0 declined 2 and 4 units, respectively; while this parameter was stable in gels at pH 6. At pH 4.3 the redness of the gels, Figure 4.7(b), showed very low values as compared to gels at pH 5 and 6. Although a decrease of about 11 units (pH 5) and 4 units (pH 6) were observed, both gels maintained a bright red color at the end of storage. Figure 4.7(c) shows that within the same storage period the yellowness of the gels slightly decreased at pH 5, remained relatively

unchanged at pH 6, and increased about 2 units at pH 4.3. Thus, at the end of storage, the gelatin gels in this experiment were dark brown at pH 4.3, and remained bright red at pHs 5-6.

(a)



(b)



(c)

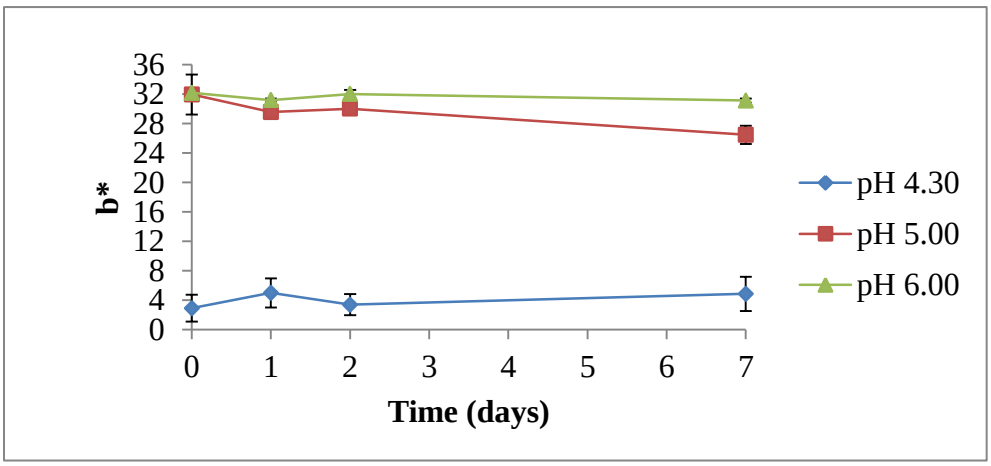


Figure 4.7 Effect of pH on color characteristics of gels containing the colorant C during one-week refrigerated storage (mean ± s.d, n=3)

It can also be noted from Figures 4.5, 4.6 and 4.7 that none of the formulations tested (E1, E2+ chelating agent, and E2) were effective enough to satisfactorily maintain a red color of cold-stored gelatins at pH below 5. At higher pH ranges (5-6) the color of all gels kept their redness and showed quite stable brightness.

Table 4.5 shows the values of the colorimetric parameters of gelatins containing the three hem-colorants after 7 days of cold storage.

At pH 4.5 [Figure 4.5(a)], the gels containing powders from experiment B (E2+chelating agent) showed significantly higher redness values ($p<0.05$) as compared to those of experiments A (E1) and C (E2). There were no significant differences in L* and b* values among the three experiments.

Table 4.5 Color parameters of gelatin gels at the end of 7- day cold storage

(a) pH 4.5

Experiment	L*	a*	b*
A*	24.06 ± 0.98^{a}	11.98 ± 0.27^{a}	5.95 ± 0.25^{a}
B	26.34 ± 4.00^{a}	22.86 ± 1.80^{b}	13.32 ± 4.12^{a}
C	25.67 ± 0.83^{a}	10.60 ± 2.38^{a}	4.85 ± 2.33^{a}

(b) pH 6.5

Experiment	L*	a*	b*
A*	34.29 ± 2.39^{a}	44.55 ± 2.37^{a}	24.01 ± 5.26^{a}
B	33.32 ± 2.81^{a}	45.62 ± 1.89^{a}	$26.71 \pm 2.52^{a,b}$
C	44.01 ± 0.36^{b}	46.52 ± 0.38^{a}	$31.10 \pm 0.29^{b,c}$

mean ± s.d (n=3), *n=2

Different letters mean significant differences ($p<0.05$)

At pH 6.5 [Figure 4.5(b)], there were no significant differences in redness but the encapsulation agent E2 (experiment C) led to significantly higher L* and b* values as compared to the gels containing the encapsulation agent E1 ($p<0.05$).

Thus, it can be concluded that the three hem-derivatives could be successfully used as red colorants at mild acidic conditions (pH range 6-7). At pH<5 only the polysaccharide E2 together with the chelating agent (experiment B) is able to preserve the red color but it does not show a satisfactory stability because it becomes slightly brown after 7-day storage.

Consequently, further studies with the encapsulation system B, using a combination of the polysaccharide E2 and a chelating agent, will be carried out in order to find the formulation that leads to a more stable colorant.

5. Conclusions

1. For the three formulations tested, spray-drying encapsulation of hem-derivatives permitted obtaining powders showing water content and water activity (a_w) low enough to warrant the microbiological stability without favoring iron oxidation reactions.

2. Immediately after dehydration, all the products exhibited an acceptable bright red color, whatever the encapsulation agent used in the derivatization of hemoglobin.

3. The colorant containing polysaccharide E1 (experiment A) showed better color stability than the two products containing polysaccharide E2 (experiments B and C). The red color of the E1 powder remained constant over 2-month chill storage.

4. The partial substitution of sucrose by a chelating agent in the products containing the polysaccharide E2 (experiments B and C) negatively affected the color stability of the dehydrated products.

5. At pH 6-7 all the gelatins containing the hem-colorants showed a bright red color. Nevertheless, only the formulation corresponding to experiment B (chelating agent + polysaccharide E2) was able to preserve the red color at pH below 5.

6. At pH 4.5 the formulation corresponding to experiment B (chelating agent + polysaccharide E2) did not succeed to complete stabilize redness during storage, since a 30% decrease in a* values occurred after 7-day chill storage. However, final a* values of these gelatins were even 3-fold higher than the initial ones in gelatins containing E1 or E2 without the chelating agent.

7. At pH above 5, although there were significant differences in b* and L* parameters, the a* coordinates showed to be similar for all the treatments at the end of 7-day chill storage, the mean reduction from the initial values being in the range from 6 to 11%.

6. Bibliography

- Anandharamakrishnan, C., & Padma Ishwarya, S. Spray drying techniques for food ingredient encapsulation (1st ed.), John Wiley & Sons, Ltd (2015).

- AOAC (Association of Official Analytical Chemists). Official methods of analysis of AOAC international (16th ed.), Arlington, VA, Washington DC. (1995).

- Bah, C.S.F., Bekhit, A.E.A., Carne, A., & McConnell, M.A. (2013). Slaughterhouse blood: an emerging source of bioactive compounds. *Comprehensive Reviews in Food Science and Food Safety*, *12*(3), 314-331.

- Bahnasawy, A.H., Okasha, A.E., & Gonbeej, E.E. (2010). Performance evaluation of a laboratory scale spray dryer. *Process Engineering*, *27*(1), 326-346

- Barbosa, M.I.M.J., Borsarelli, C.D., & Mercadante, A.Z. (2005). Light stability of spray-dried bixin encapsulated with different edible polysaccharide preparations. *Food Research International*, *38*(8-9), 989–994. http://doi.org/10.1016/j.foodres.2005.02.018

- Baranauskienė, R., Venskutonis, P.R., Dewettinck, K., & Verhé, R. (2006). Properties of oregano (Origanum vulgare L.), citronella (Cymbopogon nardus G.) and marjoram (Majorana hortensis L.) flavors encapsulated into milk protein-based matrices. *Food Research International*, *39*(4), 413-425. http://doi.org/10.1016/j.foodres.2005.09.005

- Beindorff, C.M., & Zuidam, N.J. (2010). Microencapsulation of fish oil. In: Zuidam, N.J., & Nedović, V.A. (eds.), *Encapsulation technologies for active food ingredients and food processing*, Springer Science + Business Media, LLC, pp. 161-186.

- Beristain, C.I., García, H.S., & Vernon-Carter, E.J. (2001). Spray-dried encapsulation of cardamom (Elettaria cardamomum) essential oil with mesquite (Prosopis juliflora) gum. *LWT - Food Science and Technology*, *34*(6), 398-401. http://doi.org/10.1006/fstl.2001.0779

- Bhandari, B., Krasaekoopt, W., & Deeth, H. (2004). The influence of coating materials on some properties of alginate beads and survivability of microencapsulated probiotic bacteria. *International Dairy Journal*, *14*, 737-743.

- Bhandari, B.R., Patel, K.C., & Chen, X.D. (2008). Spray drying of food materials–process and product characteristics. In: Chen, X.D., & Mujumdar, A.S. (eds), *Drying technologies in food processing* (1st ed.), Blackwell Publishing Ltd, pp. 113-189.

- Brennan, J.G. (2002). Evaporation and dehydration. In: Brennan, J.G., & Grandison, A.S. (eds), *Food Processing Handbook* (2nd ed), Wiley-VCH Verlag GmbH and Co. KGaA, pp. 77-130.

- Bunjes, H., & Strasdat, B. (2013). Incorporation of lipid nanoparticles into calcium alginate beads and characterization of the encapsulated particles by differential scanning calorimetry. *Food Hydrocolloids*, *30*, 567-575.

- Bylaite, E., Nylander, T., Venskutonis, R., & Jönsson, B. (2001). Emulsification of caraway essential oil in water by lecithin and β-lactoglobulin: emulsion stability and properties of the formed oil–aqueous interface. *Colloids and Surfaces B: Biointerfaces, 20*(4), 327-340. http://doi.org/10.1016/S0927-7765(00)00212-5

- Carretero, C., & Parés, D. (2000). Improvement of the microbiological quality of blood plasma for human consumption purposes. In: Pandalai, S.G. (ed.), *Recent research developments in agricultural & food chemistry* (vol 4), Research Singpost, Trivandrum, India, pp.203-216. ISBN: 81-7736-013-2.

- Chaitanya, L.G. (2014). Food coloring: the natural way. *Research Journal of Chemical Sciences, 4*(2), 87-96.

- Chávarri, M., Marañón, I., Ares, R., Ibáñez, F.C., Marzo, F., & Carmen Villarán, M. D (2010). Microencapsulation of a probiotic and prebiotic in alginate-chitosan capsules improves survival in simulated gastro-intestinal conditions. *International Journal of Food Microbiology*, *142*, 185–189.

- Cofrades, S., Guerra, M.A., Carballo, J., Fernandez-Martin, F., & Colmenero, F.J. (2000). Plasma protein and soy fiber content effect on bologna sausage properties as influenced by fat level. *Journal of Food Science, 65*(2), 281–287. doi:10.1111/j.1365-2621.2000.tb15994.x

- Clydesdale, F.M. (1993). Color as a factor in food choice. *Critical Reviews in Food Science and Nutrition, 33*(1), 83-101.

- Dàvila, E., & Parés, D. (2007). Structure of heat-induced plasma protein gels studied by fractal and lacunarity analysis. *Food Hydrocolloids, 21*(2), 147–153. doi:10.1016/j.foodhyd.2006.02.004

- Dàvila, E., Parés, D., & Howell, N.K. (2007a). Studies on plasma protein interactions in heat-induced gels by differential scanning calorimetry and FT-Ramam spectroscopy. *Food Hydrocolloids, 21,* 1144-1152.

- Dàvila, E., Saguer, E., Toldrà, M., Carretero, C., & Parés, D. (2007b). Surface functional properties of blood plasma protein fractions. *European Food Research and Technology*, *226,* 207-214.

- Dàvila, E., Zamora, L.M., Pla, M., Carretero, C., & Parés, D. (2006). Identification and antagonistic activity of lactic acid bacteria occurring in porcine blood from industrial slaughterhouses – A preliminary study. *International Journal of Food Microbiology, 107,* 207–211. doi:10.1016/j.ijfoodmicro.2005.08.022

- Downham, A., & Collins, P. (2000). Colouring our foods in the last and next millennium: A review of colours used in our food and drink. *International Journal of Food Science and Technology*, *35*, 5-22.

- Drusch, S. (2006). Sugar beet pectin: A novel emulsifying wall component for microencapsulation of lipophilic food ingredients by spray-drying. *Food Hydrocolloids*. doi:10.1016/j.foodhyd.2006.08.007.

- Drusch, S., Serfert, Y., Van Den Heuvel, A., & Schwarz, K. (2006). Physicochemical characterization and oxidative stability of fish oil encapsulated in an amorphous matrix containing trehalose. *Food Research International*, *39*(7), 807–815. http://doi.org/10.1016/j.foodres.2006.03.003

- Eurostat (2014). Pig farming in the European Union: considerable variations from one Member State to another. ISSN:2314-9647 Available from: http://ec.europa.eu/eurostat/statistics-explained/index.php/Pig_farming_sector_-_statistical_portrait_2014 (Accessed on 01 April 2016)

- FDA (2015). Listing of color additives subject to certification, 21 CFR 74.101-74.706. Available from: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=74 (Accessed on 28 March 2016)

- Fontes, P.R., Gomide, L.A.M., Ramos, E.M., Stringheta, P.C., & Parreiras, J.F.M. (2003). Color evaluation of carbon monoxide treated porcine blood. *Meat Science*, *68*, 507-513.

- Fontes, P.R., Gomide, L.A.M., Fontes, E.A.F, Ramos, E.M., & Ramos, A.L.S. (2010). Composition and color stability of carbon monoxide treated dried porcine blood. *Meat Science*, *85*, 472-480.

- Food Standards Agency (2014). Current EU approved additives and their E numbers. Available from: https://www.food.gov.uk/science/additives/enumberlist#toc-1 (Accessed on 04 April 2016)

- Fort, N., Carretero, C., Parés, D., Toldrà, M., & Saguer, E. (2007). Combined treatment of porcine plasma with microbial transglutaminase and cysteine: Effects on the heat-induced gel properties. *Food Hydrocolloids*, *21*, 463–471. doi:10.1016/j.foodhyd.2006.05.007

- Fort, N., Kerry, J.P., Carretero, C., Kelly, A.L., & Saguer, E. (2009). Cold storage of porcine plasma treated with microbial transglutaminase under high pressure: Effects on its heat-induced gel properties. *Food Chemistry*, *115*, 602–608.

- Fort, N., Lanier T.C., Amato, P.M., Carretero, C., & Saguer, E. (2008). Simultaneous application of microbial transglutaminase and high hydrostatic pressure to improve heat induced gelation of pork plasma. *Meat Science*, *80*, 939-943.

- Furlán L.T.R, Padilla, A.P., & Campderrós, M.E. (2010). Functional and physical properties of bovine plasma proteins as a function of processing and pH, application in a food formulation. *Advance Journal Food Science and Technology*, *2*(5), 256–267.

- Gharsallaoui, A., Roudaut, G., Chambin, O., Voilley, A., & Saurel, R. (2007). Applications of spray-drying in microencapsulation of food ingredients: An overview. *Food Research International*, *40*, 1107–1121.

- Gouin, S. (2004). Microencapsulation: Industrial appraisal of existing technologies and trends. *Trends in Food Science and Technology*, *15*(7-8), 330-347.

- Kodi (2016). Available from http://kodichina.com/newsshow.asp?nclassname =60&nid=22 (Accessed on 15 April 2016)

- http://people.umass.edu/~mcclemen/581Ash&Minerals.html (Accessed on 20 April 2016)

- Hurtado, S., Saguer, E., Toldrà, M., Parés, D., & Carretero, C. (2012). Porcine plasma as polyphosphate and caseinate replacer in frankfurters. *Meat Science*, *90*(3), 624–628. doi:10.1016/j.meatsci.2011.10.004

- Hogan, S.A., McNamee, B.F., O'Riordan, E.D., & O'Sullivan, M. (2001). Emulsification and microencapsulation properties of sodium caseinate/carbohydrate blends. *International Dairy Journal*, *11*(3), 137–144. http://doi.org/10.1016/S0958-6946(01)00091-7

- Isengard, H.D. (2001). Water content, one of the most important properties of food. *Food control*, *12*(7), 395-400.

- Kanakdande, D., Bhosale, R., & Singhal, R.S. (2007). Stability of cumin oleoresin microencapsulated in different combination of gum arabic, maltodextrin and modified starch. *Carbohydrate Polymers*, *67*(4), 536–541. http://doi.org/10.1016/j.carbpol.2006.06.023

- Kobylewski, S., & Jacobson, M.F. Food dyes: A rainbow of risks. *Center for Science in the Public Interest*. (2010).

- Krishnan, S., Bhosale, R., & Singhal, R. (2005). Microencapsulation of cardamom oleoresin: Evaluation of blends of gum arabic, maltodextrin and a modified starch as wall materials. *Carbohydrate Polymers*, *61*(1), 95–102. http://doi.org/10.1016/j.carbpol.2005.02.020

- Labuza, T.P. (1980). The effect of water activity on reaction kinetics of food deterioration. *Food Technology*, 36-41.

- Labuza, T.P. & Altunakar, B. (2007). Diffusion and sorption kinetics of water in foods. In: Barbosa-Cánovas, G.V., Fontana, A.J., Schmidt, S.J., & Labuza, T.P. (eds), *Water activity in foods: fundamentals and applications* (1st ed), Blackwell publishing, pp. 215-238.

- Lee, C.C., Johnson, L.A., Love, J.A., & Johnson, S. (1991). Effects of processing and usage level on performance of bovine plasma as an egg-white substitute in cakes. *Cereal Chemistry*, *68*(1), 100-104.

- MacDougall, D.B. (2002). Colour measurement of food: Principles and practice. In: MacDougall, D.B. (ed), *Colour in food: Improving quality* (1st ed), Woodhead Publishing Limited and CRC Press LLC, 33-63.

- Mader, S.S. Inquiry into Life (10th ed), McGraw-Hill (2002).

- Madhava, N.M., & Sowbhagya, H.B., (2012). Technological advances in food colors. *Chemical Industry Digest.*, 79-88.

- Manojlović, V., Nedović, V.A., Kailasapathy, K., & Zuidam, N.J. (2010). Encapsulation of probiotics for use in food products. In: Zuidam, N.J., & Nedović, V.A. (eds.), *Encapsulation technologies for active food ingredients and food processing*, Springer Science + Business Media, LLC, pp. 269-302.

- Márquez, E., Bracho, M., Archile, A., Rangel, L., & Benítez, B. (2005). Proteins, isoleucine, lysine and methionine content of bovine, porcine and poultry blood and their fractions. *Food Chemistry*, *93*, 503–505.

- Meesters, G.M.H. (2010). Encapsulation of enzymes and peptides. In: Zuidam, N.J., & Nedović, V.A. (eds.), *Encapsulation technologies for active food ingredients and food processing*, Springer Science + Business Media, LLC, pp. 253-268.

- Michael, J.K. (1993). Spray drying and spray congealing of pharmaceuticals. In: *Encyclopedia of pharmaceutical technology*, Marcel Dekker INC, New York, pp. 207-221.

- More Swati, K., & Wagh, M.P. (2014). Review on spray drying technology. *International Journal of Pharmaceutical, Chemical and Biological Sciences, 4*(2), 219-225.

- Mortensen, A. (2006). Carotenoids and other pigments as natural colorants. *Pure and Applied Chemistry, 78*(8), 1477–1491.

- Myhara, R.M., & Kruger, G. (1998). The performance of decolorized bovine plasma protein as a replacement for egg white in high ratio white cakes. *Food Quality and Preference*, *9*(3), 135–138. doi:10.1016/S0950-3293(97)00046-3

- Ofori, J.A., & Hsieh, Y-H.P. (2012). The use of blood and derived products as food additives. In: El-Samragy, Y. (ed.), *Food additive*, In Tech. ISBN: 978-953-51-0067-6

- Oneda, F., & Ré, M. (2003). The effect of formulation variables on the dissolution and physical properties of spray-dried microspheres containing organic salts. *Powder Technology*, *130*(1-3), 377–384. http://doi.org/10.1016/S0032-5910(02)00239-5

- Onwulata, C.I. (2012). Microencapsulation and functional bioactive foods. *Journal of Food Processing and Preservation*, *37*, 510–532.

- Parés, D., & Ledward, D.A. (2001). Emulsifying and gelling properties of porcine blood plasma as influenced by high pressure processing. *Food Chemistry*, *74*, 139-145.

- Parés, D., Saguer, E., & Carretero, C. (1999). Aplicación de la alta presión hidrostática sobre el plasma de sangre porcina de mataderos. *Ibérica, Actualidad Tecnológica, 417*, 156-159.

- Parés, D., Saguer, E., & Carretero, C. (2011). Blood by-products as ingredients in processed meat. In: Kerry, J.P., & Kerry, J.F. (eds), *Processed meats: Improving safety, nutrition and quality*, Woodhead Publishing Limited, pp. 218-242.

- Parés, D., Saguer, E., Pap, N., Toldrà, M., & Carretero, C. (2012). Low-salt porcine serum concentrate as functional ingredient in frankfurters. *Meat Science*, *92*, 151–156. doi:10.1016/j.meatsci.2012.04.029

- Parés, D., Saguer, E., Toldrà, M., & Carretero, C. (2000). Effect of high pressure processing at different temperatures on protein functionality of porcine blood plasma. *Journal of Food Science*, *65*, 486-490.

- Parés, D., Saguer, E., Toldrà, M., & Carretero, C. (2001). High hydrostatic pressure as a method to reduce microbial contamination of porcine blood plasma. *Food Science and Technology International*, *7*, 117-121.

- Rawdkuen, S., Benjakul, S., Visessanguan, W., & Lanier, T.C. (2004). Chicken plasma protein: Proteinase inhibitory activity and its effect on surimi gel properties. *Food Res Intl*, *37*, 156–65.

- Ribeiro, H.S., Schuchmann, H.P., Engel, R., Walz, E., & Briviba, K. (2010). Encapsulation of carotenoids. In: Zuidam, N.J., & Nedović, V.A. (eds.), *Encapsulation technologies for active food ingredients and food processing*, Springer Science + Business Media, LLC, pp. 121-252.

- Richards M.P., & Hultin H.O. (2000). Effect of pH on lipid oxidation using trout hemolysate as a catalyst: A possible role for deoxyhemoglobin. *Journal of Agricultural and Food Chemistry*, *48*, 3141-3147.

- Ronald C.D. (1997). Spray-drying innovative use of an old process: Design elements. Available from: http://www.naturalproductsinsider.com/articles/1997/05/spray-drying-innovative-use-of-an-old-process.aspx (Accessed on 11 April 16)

- Rosenberg, M., & Sheu, T-Y. (1996). Microencapsulation of volatiles by spray-drying in whey protein-based wall systems. *International Dairy Journal*, *6*, 273–284.

- Saguer, E., Altarriba, S., Lorca, C., Parés, D., Toldrà, M., & Carretero, C. (2003). Color stabilization of spray-dried porcine red blood cells using nicotinic acid and nicotinamide. *Food Science and Technology International*, *9*(4), 301-307.

- Saguer, E., Fort, N., Mompió, M., & Carretero, C. (2004). Efecto de la adición de cisteína sobre las propiedades gelificantes del plasma porcino a pH ácido. *Alimentación: equipos y tecnología*, *23*, 86- 91.

- Saguer, E., Fort, N., Parés, D., Toldrà, M., & Carretero, C. (2007). Improvement of gelling properties of porcine blood plasma using microbial transglutaminase. *Food Chemistry*, *101*, 49-56. doi:10.1016/j.foodchem.2005.11.050

- Salvador, P., Toldrà, M., Parés, D., Carretero, C., & Saguer, E (2009). Color stabilization of porcine hemoglobin during spray-drying and powder storage by combining chelating and reducing agents. *Meat Science, 83*, 328-333.

- Santos, E.M., González-Fernández, C., Jaime, I., & Rovira, J. (2003). Physicochemical and sensory characterisation of Morcilla de Burgos, a traditional Spanish blood sausage. *Meat Science, 65*(2), 893–8. doi:10.1016/S0309-1740(02)00296-6

- Satterlee, L.D. (1975). Improving utilization of animal by-products for human foods-A review. *Journal of Animal Science, 41(*2), 687-697.

- Sgarbieri, V.C., Duarte, R.T., & Carvalho Simões, M.C. (1999). Bovine blood components: fractionation, composition, and nutritive value. *Journal of Agriculture and Food Chemistry, 47*, 231−236

- Shaikh, J., Bhosale, R., & Singhal, R. (2006). Microencapsulation of black pepper oleoresin. *Food Chemistry, 94*(1), 105–110. http://doi.org/10.1016/j.foodchem.2004.10.056

- Shu, B., Yu, W., Zhao, Y., & Liu, X. (2006). Study on microencapsulation of lycopene by spray-drying. *Journal of Food Engineering*, *76*(4), 664–669. http://doi.org/10.1016/j.jfoodeng.2005.05.062

- Soottitantawat, A., Bigeard, F., Yoshii, H., Furuta, T., Ohkawara, M., & Linko, P. (2005a). Influence of emulsion and powder size on the stability of encapsulated d-limonene by spray drying. *Innovative Food Science & Emerging Technologies*, *6*(1), 107–114. http://doi.org/10.1016/j.ifset.2004.09.003

- Soottitantawat, A., Takayama, K., Okamura, K., Muranaka, D., Yoshii, H., Furuta, T., Linko, P. (2005). Microencapsulation of l-menthol by spray drying and its release characteristics. *Innovative Food Science & Emerging Technologies*, *6*(2), 163–170. http://doi.org/10.1016/j.ifset.2004.11.007

- Teixeira, M.I., Andrade, L.R., Farina, M., & Rocha-Leão, M.H.M. (2004). Characterization of short chain fatty acid microcapsules produced by spray drying. *Materials Science and Engineering: C, 24*(5), 653–658. http://doi.org/10.1016/j.msec.2004.08.008

- Toldrà, M., Busquets, A., Saguer, E., Parés, D., & Carretero, C. (2002). Effects of high hydrostatic pressurised treatment on porcine blood red cells fraction. *Food Science and Technology International, 8,* 41–48. doi:10.1177/1082013202008001916

- Toldrà, M., Carbó, N., & Parés, D. (2005). Valorisation of a hemic residue obtained from the enzymatic hydrolysis of the porcine red blood cells fraction. Books of Proceedings of the EFFoST Conference 2005. Innovations in traditional foods (INTRADFOOD 2005).Volume II: 1007-1010. Ed. Universidad Politécnica de Valencia.

- Toldrà, M., Dàvila, E., Saguer, E., Fort, N., Salvador, P., Parés, D., & Carretero, C. (2008). Functional and quality characteristics of the red blood cell fraction from biopreserved

porcine blood as influenced by high pressure processing. *Meat Science*, *80*(2), 380-388. doi:10.1016/j.meatsci.2007.12.025

- Toldrà, M., Elias, A., Parés, D., Saguer, E., & Carretero, C. (2004). Functional properties of a spray-dried porcine red blood cell fraction treated by high hydrostatic pressure. *Food Chemistry*, *88*, 461-468.

- Toldrà, M., Parés, D., Saguer, E., & Carretero, C. (2011). Hemoglobin hydrolysates from porcine blood obtained through enzymatic hydrolysis assisted by high hydrostatic pressure processing, *Innovative Food Science and Emerging Technologies*, *12*(4), 435–442. doi:10.1016/j.ifset.2011.05.002

- Tseng, T., Tsai, C., Yang, J., & Chen, M. (2006). Porcine blood plasma transgluataminase combined with thrombin and fibrinogen as a binder in restructured meat Asian-Australasian. *Journal of Animal Sciences*, *19*(7), 1054-1058.

- Undeland, I., Kristinsson, H.G. & Hultin, H.O. (2004). Hemoglobin-mediated oxidation of washed minced cod muscle phospholipids: Effect of pH and hemoglobin source. *Journal of Agricultural and Food Chemistry*, *52*, 4444−4451.

- Valenzuela, C., Hernández, V., Morales, M.S., Neira-Carrillo, A., & Pizarro, F. (2014). Preparation and characterization of heme iron-alginate beads. *Food Science and Technology*, *59*, 1283-1289.

- Vandamme, T., & Gbassi, G.K. (2012). Probiotic encapsulation technology: from microencapsulation to release into the gut. *Pharmaceutics*, *4*, 149-163.

- Viana, F.R., Silva, V.D.M., Delvivo, F.M., Bizzotto, C.S., & Silvestre, M.P.C. (2005). Quality of ham pâté containing bovine globin and plasma as fat replacers. *Meat Science*, *70*(1), 153–60. doi:10.1016/j.meatsci.2004.12.013

- Visessanguan, W., Benjakul, S., & An, H. (2000). Porcine plasma proteins as a surimi protease inhibitor: effects on actomyosin gelation. *J Food Sci*, *65*, 607-611.

- Walter, J. (2016). Digression colorimetry. Available from: https://www.techfak.uni-bielefeld.de/~walter/lehre/dm2/vis/Farbmetrik.html (Accessed on 20 April 2016)

- Walter, T., Hertrampf, E., Pizarro, F., Olivares, M., Llaguno, S., Letelier, A., Vega, V., & Stekel, A. (1993). Effect of bovine-hemoglobin-fortified cookies on iron status of schoolchildren: a nationwide program in Chile. *Am J Clin Nutri*, *57*, 190–204.

- Wandrey, C., Bartkowiak, A., & Harding, S.E. (2010). Materials for encapsulation. In: Zuidam, N.J., & Nedović, V.A. (eds.), *Encapsulation technologies for active food ingredients and food processing*, Springer Science + Business Media, LLC, pp 31-100.

- Wang, J.Z., Zhang, M., Ren, F. Z., Han, B.Z., Wang, L., Chen, S.W., & Humera, A. (2007). Changes of chemical and nutrient composition of porcine blood during fermentation by Aspergillus oryzae. *World J Microbiol Biotechnol*., *23*, 1393–1399

- Watanabe, Y., Fang, X., Adachi, S., Fukami, H., & Matsuno, R. (2004). Oxidation of 6-*O*-arachidonoyl -ascorbate microencapsulated with a polysaccharide by spray-drying. *Lebensmittel-Wissenschaft und-Technologie*, *37*, 395–400.

- Weissbluth, M. Hemoglobin: cooperativity and electronic properties (1st ed), Springer-Verlag Berlin· Heidelberg· New York (1974).

- Yáñez Fernández, J., Salazar Montoya, J.A., Chaires Martínez, L., Jiménez Hernández, J., Márquez Robles, M., & Ramos Ramírez, E.G. (2002). Aplicaciones biotecnológicas de la microencapsulación. *Avance y Perspectiva*, *21*, 313-319.

- Young, S. L., Sarda, X., & Rosenberg, M. (1993). Microencapsulating properties of whey proteins. 1. microencapsulation of anhydrous milk fat. *Journal of Dairy Science*, *76*(10), 2868–2877. http://doi.org/10.3168/jds.S0022-0302(93)77625-0

- Yu, Y., Hu, J., Miyaguchi, Y.; Bai, X., Du, Y., & Lin, B. (2006). Isolation and characterization of angiotensin I-converting enzyme inhibitory peptides derived from porcine hemoglobin. *Peptides*, *27*, 2950-2956.

- Zhou, C., Wang, H., Chen, Y., & Chen, C. (2012). Effect of L-cysteine and lactose on color stability of porcine red blood cell during freeze-drying and powder storage. *Food Science and Biotechnology*, *21*(3), 669-674.

- Zimmermann, M.B., & Windhab, E.J. (2010). Encapsulation of iron and other micronutrients for food fortification. In: Zuidam, N.J., & Nedović, V.A. (eds.), *Encapsulation technologies for active food ingredients and food processing*, Springer Science + Business Media, LLC, pp. 187-210.

- Zuidam, N.J., & Heinrich, E. (2010). Encapsulation of Aroma. In: Zuidam, N.J., & Nedović, V.A. (eds.), *Encapsulation technologies for active food ingredients and food processing*, Springer Science + Business Media, LLC, pp. 127-160.

- Zuidam, N.J., & Shimoni, E. (2010). Overview of microencapsulates for use in food products or processes and methods to make them. In: Zuidam, N.J., & Nedović, V.A. (eds.), *Encapsulation technologies for active food ingredients and food processing*, Springer Science + Business Media, LLC, pp. 3-30.

7. Annex I

7.1. Reagents

The chemical substances used for the productions of spray-dried hemoglobin are:

- **NaOH (10%):** This solution was prepared in order to adjust pH of red blood cell fraction prior to addition of other reagents, to the 8 pH, and this led the hem-protein to be negatively charged.
- **Nicotinamide (99%):** This substance was from Agrōs organics, New Jersey, USA, and it is the amide of nicotinic acid (vitamin B_3). In the experiment, it was used to link Fe^{2+} ions in hemoglobin solutions with N in the nicotinic acid part in order to prevent this ferrous ion from being oxidized.
- **Sucrose:** This table sugar was brought from a local company, and used to achieve the dried hemoglobin without high water activity that could lead to microbial spoilage during powder storage since it has a good water retention capacity, and it can protect hem group against oxidation.
- **Chelating agent:** This chemical compound was from Scharlau, Sentmenat, Spain. It was used to bind free water and Fe^{2+}, and also as acidity regulator which is quite important with the powder application to acidic foods since it resists pH changes.
- **Polysaccharide E1:** It was purchased from Sigma Aldrich, St. Louis, Missouri, USA and it was dissolved with 0.1M acetic acid to get a 2% concentration before adding to hemoglobin solutions. With its positive charge, it was used to form a strong bond with the negatively charged red blood cells, which resulted in the microcapsules with this polysaccharide as matrix material, and hemoglobin as core material. In this capsule form, the dried hemoglobin could be able to stay more stable than the dried ones without any protecting substance since the wall material protected oxygen penetration.
- **Polysaccharide E2:** It was kindly donated by a local food industry and this coating material is hydrophilic, and once it was added and mixed well the hemoglobin solutions which consists of hydrophilic protein; subsequently formed the colloids. During spray-drying process, the evaporation of free water occurred; the particles of capsule were formed in which the hem-based protein was the core materials, being entrapped by this polysaccharide.

7.2. Equipment

7.2.1. The centrifuge

The separation of plasma from the red blood cells fraction was performed by utilizing a centrifuge (Sorvall RC-SC plus, Dupont Co, Newton, Connecticut, USA) (Figure 7.1) with its specifications as follows:

- Run speed
  - Speed selection rage (rpm) 50 to 21000
  - Speed control accuracy ±1% or 100 rpm, whichever is greater
- Maximum relative centrifuge force 51070 g
- Run temperature
  - Temperature selection range -20 to +40 ^{o}C
  - Temperature control range +20 to +40 ^{o}C[2,3]
  - Temperature control accuracy ±1 ^{o}C[3,4]
- Run time selection range 0 to 99 hr, 59 min or hold
- Ambient temperature range +15 to 38 ^{o}C[3]
- Mass (weight) 308kg (680Ib)

Figure 7.1 The centrifuge

7.2.2. Homogenizer

The homogenization of red blood cell fractions was done by using a continuous high pressure valve homogenizer FPG 7400 (Stansted Fluid Power Ltd., Essex, UK) (Figure 7.2) after the completion of blood centrifugation.

Figure 7.2 Homogenizer

7.2.3. Electronic scales

Samples and reagents were weighed by using two electronic scales (Figure 7.3). They both were also employed for the preparation of samples for moisture, ash and protein determination.

- **SCALTEC** (Instruments Gmb, Goettingen, Germany): Its maximum and minimum capacity is 4200g, and 0.1g, respectively, and d=0.1g [Figure 7.3(a)].
- **A&D (**A&D instruments Ltd., Japan) [Figure 7.3(b)]: It can operate as the below information:
    - Maximum capacity: 210g
    - Minimum capacity: 10mg
    - e=1mg
    - d=0.1mg

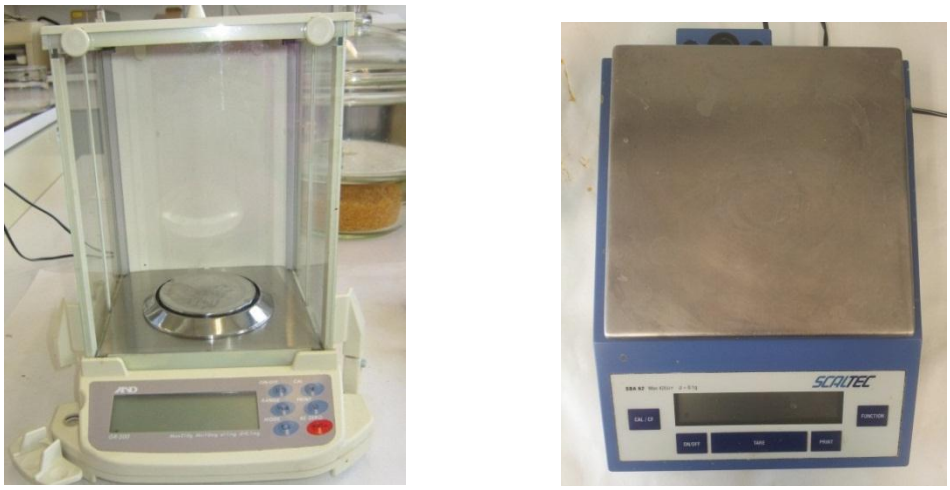


Figure 7.3 (a) A&D electronic scale; (b) SCALTEC electric scale

7.2.4. Spray-dryer

A Büchi Mini Spray Dryer model B-191 (Büchi Labortechnik AG, Flawil, Switzerland) (Figure 7.4) was chosen for the dehydration of hemoglobin solutions.

Figure 7.4 Spray-dryer

7.2.5. Magnetic stirrer

The magnetic stirrer (AGIMATIC-E, Spain) (Figure 7.5) was used for the operation of mixing and dissolving reagents during RBC pH adjustment and derivatization of hemoglobin.

Figure 7.5 Magnetic stirrer

7.2.6. pH meter

The electric pH meter (CRISON *GLP 22,* Alella, Barcelona) (Figure 7.6) was used for measuring the pH of hemoglobin solutions during pH adjustment and the preparation of other solutions.

Figure 7.6 pH meter

7.2.7. Drying oven

The oven (J.P. SELECTA, S.A., Abrera, Barcelona) (Figure 7.7) was used for the determination of moisture content, and set at 100 ± 5 ^{o}C.

Figure 7.7 Drying oven

7.2.8. Muffle furnace

The muffle furnace (JP Selecta Naber, Japan) (Figure 7.8) was used for ash determination, and it was set at 150 ^{o}C for 1 hour, 250 ^{o}C for 2 hours and finally 550 ^{o}C.

Figure 7.8 Muffle furnace

7.2.9. Distillation unit

The distillation unit (Büchi K314, German) (Figure 7.9) was used for the ammonia gas by distillation in the water steam stream.

Figure 7.9 Distillation unit

7.2.10. Colorimeter

The colorimeter Minolta Chroma Meter CR-300 (Japan) (Figure 7.10) was selected for the measurement of colorimetric parameters, and it is a compact tristimulus color analyzer for measuring reflective colors of surfaces. This equipment is comprised of the measuring head and the Data Processor DP-301. The measuring head of the Chroma Meter CR-300 contains 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.

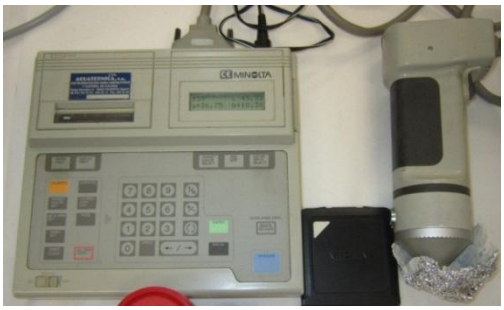


Figure 7.10 Colorimeter

8. Annex II

8.1. Statistical analysis of color parameters of gels containing hem-derivatives on day 0

Overall results for parameter L* -Day 0

Tests of Between-Subjects Effects

Dependent Variable: L*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	584,888[a]	5	116,978	24,259	,000
Intercept	17780,973	1	17780,973	3687,393	,000
Treatment	44,634	2	22,317	4,628	,038
pH	355,598	1	355,598	73,743	,000
Treatment * pH	167,668	2	83,834	17,385	,001
Error	48,221	10	4,822		
Total	19233,296	16			
Corrected Total	633,109	15			

a. R Squared = ,924 (Adjusted R Squared = ,886)

Results for parameter L* at pH 4.5

Tests of Between-Subjects Effects

Dependent Variable: L*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	27,779[a]	2	13,889	2,767	,155
Intercept	6553,753	1	6553,753	1305,741	,000
Tractament	27,779	2	13,889	2,767	,155
Error	25,096	5	5,019		
Total	6906,738	8			
Corrected Total	52,875	7			

a. R Squared = ,525 (Adjusted R Squared = ,336)

Results for parameters L* at pH 6.5

Tests of Between-Subjects Effects

Dependent Variable: L*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	184,523[a]	2	92,262	19,948	,004
Intercept	11582,818	1	11582,818	2504,386	,000
Treatment	184,523	2	92,262	19,948	,004
Error	23,125	5	4,625		
Total	12326,558	8			
Corrected Total	207,648	7			

a. R Squared = ,889 (Adjusted R Squared = ,844)

L*

Tukey HSD[a,b,c]

Treatment	N	Subset	
		1	2
B	3	33,9900	
A	2	37,3700	
C	3		44,8867
Sig.		,267	1,000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 4,625.
a. Uses Harmonic Mean Sample Size = 2,571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0,05.

Overall results for parameter a*-Day 0

Tests of Between-Subjects Effects

Dependent Variable: a*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4950,354[a]	5	990,071	162,668	,000
Intercept	16632,429	1	16632,429	2732,693	,000
Treatment	480,484	2	240,242	39,472	,000
pH	3691,319	1	3691,319	606,480	,000
Treatment * pH	938,540	2	469,270	77,101	,000
Error	60,865	10	6,086		
Total	22880,224	16			
Corrected Total	5011,219	15			

a. R Squared = ,988 (Adjusted R Squared = ,982)

Results for parameter a* at pH 4.5

Tests of Between-Subjects Effects

Dependent Variable: a*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1372,395[a]	2	686,197	164,170	,000
Intercept	2326,339	1	2326,339	556,569	,000
Treatment	1372,395	2	686,197	164,170	,000
Error	20,899	5	4,180		
Total	4149,825	8			
Corrected Total	1393,294	7			

a. R Squared = ,985 (Adjusted R Squared = ,979)

a*

Tukey HSD[a,b,c]

Treatment	N	Subset	
		1	2
A	2	7,7900	
C	3	8,8433	
B	3		35,4633
Sig.		,834	1,000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 4,180.
a. Uses Harmonic Mean Sample Size = 2,571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0,05.

Results for parameter a* at pH 6.5

Tests of Between-Subjects Effects

Dependent Variable: a*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	46,629[a]	2	23,314	2,917	,145
Intercept	17997,408	1	17997,408	2251,609	,000
Treatment	46,629	2	23,314	2,917	,145
Error	39,966	5	7,993		
Total	18730,400	8			
Corrected Total	86,595	7			

a. R Squared = ,538 (Adjusted R Squared = ,354)

Overall results for b*-Day 0

Tests of Between-Subjects Effects

Dependent Variable: b*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2168,697[a]	5	433,739	51,704	,000
Intercept	5397,622	1	5397,622	643,422	,000
Treatment	143,649	2	71,825	8,562	,007
pH	1674,554	1	1674,554	199,615	,000
Treatment * pH	385,608	2	192,804	22,983	,000
Error	83,889	10	8,389		
Total	8073,513	16			
Corrected Total	2252,586	15			

a. R Squared = ,963 (Adjusted R Squared = ,944)

Results for parameter b* at pH 4.5

Tests of Between-Subjects Effects

Dependent Variable: b*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	483,410[a]	2	241,705	34,736	,001
Intercept	529,660	1	529,660	76,118	,000
Treatment	483,410	2	241,705	34,736	,001
Error	34,792	5	6,958		
Total	1159,201	8			
Corrected Total	518,202	7			

a. R Squared = ,933 (Adjusted R Squared = ,906)

b*

Tukey HSD[a,b,c]

Treatment	N	Subset	
		1	2
C	3	2,9067	
A	2	2,9650	
B	3		18,9867
Sig.		1,000	1,000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 6,958.
a. Uses Harmonic Mean Sample Size = 2,571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0,05.

Results for parameter b* at pH 6.5

Tests of Between-Subjects Effects

Dependent Variable: b*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	45,847[a]	2	22,923	2,334	,192
Intercept	6542,515	1	6542,515	666,284	,000
Treatment	45,847	2	22,923	2,334	,192
Error	49,097	5	9,819		
Total	6914,312	8			
Corrected Total	94,944	7			

a. R Squared = ,483 (Adjusted R Squared = ,276)

8.2. Statistical analysis of color parameters of gels containing hem-derivatives on day 7

Overall results for parameters L* – 7th day

Tests of Between-Subjects Effects

Dependent Variable: L*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	787,243[a]	5	157,449	28,004	,000
Intercept	15097,247	1	15097,247	2685,178	,000
Treatment	105,078	2	52,539	9,345	,005
pH	541,477	1	541,477	96,306	,000
Treatment * pH	101,234	2	50,617	9,003	,006
Error	56,224	10	5,622		
Total	16764,229	16			
Corrected Total	843,467	15			

a. R Squared = ,933 (Adjusted R Squared = ,900)

Results for parameter L* at pH 4.5

Tests of Between-Subjects Effects

Dependent Variable: L*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6,316[a]	2	3,158	,459	,656
Intercept	4960,199	1	4960,199	720,877	,000
Treatment	6,316	2	3,158	,459	,656
Error	34,404	5	6,881		
Total	5250,372	8			
Corrected Total	40,720	7			

a. R Squared = ,155 (Adjusted R Squared = -,183)

Results for parameter L* at pH 6.5

Tests of Between-Subjects Effects

Dependent Variable: L*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	199,996[a]	2	99,998	22,914	,003
Intercept	10678,526	1	10678,526	2446,906	,000
Treatment	199,996	2	99,998	22,914	,003
Error	21,820	5	4,364		
Total	11513,856	8			
Corrected Total	221,817	7			

a. R Squared = ,902 (Adjusted R Squared = ,862)

L*

Tukey HSD[a,b,c]

Treatment	N	Subset	
		1	2
B	3	33,3200	
A	2	34,2900	
C	3		44,0067
Sig.		,862	1,000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 4,364.
a. Uses Harmonic Mean Sample Size = 2,571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0,05.

Overall results for parameter a* global – 7th day

Tests of Between-Subjects Effects

Dependent Variable: a*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3899,450[a]	5	779,890	252,407	,000
Intercept	14213,946	1	14213,946	4600,273	,000
Treatment	126,215	2	63,108	20,424	,000
pH	3568,396	1	3568,396	1154,894	,000
Treatment * pH	137,747	2	68,874	22,291	,000
Error	30,898	10	3,090		
Total	18925,575	16			
Corrected Total	3930,348	15			

a. R Squared = ,992 (Adjusted R Squared = ,988)

Results for parameter a* at pH 4.5

Tests of Between-Subjects Effects

Dependent Variable: a*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	259,258[a]	2	129,629	36,228	,001
Intercept	1769,304	1	1769,304	494,469	,000
Treatment	259,258	2	129,629	36,228	,001
Error	17,891	5	3,578		
Total	2209,081	8			
Corrected Total	277,149	7			

a. R Squared = ,935 (Adjusted R Squared = ,910)

a*

Tukey HSD[a,b,c]

Treatment	N	Subset	
		1	2
C	3	10,5967	
A	2	11,9800	
B	3		22,8567
Sig.		,703	1,000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 3,578.
a. Uses Harmonic Mean Sample Size = 2,571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0,05.

Results for parameter a* at pH 6.5

Tests of Between-Subjects Effects

Dependent Variable: a*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4,705[a]	2	2,352	,904	,462
Intercept	16013,038	1	16013,038	6155,491	,000
Treatment	4,705	2	2,352	,904	,462
Error	13,007	5	2,601		
Total	16716,493	8			
Corrected Total	17,712	7			

a. R Squared = ,266 (Adjusted R Squared = -,028)

Overall results for parameter b* - 7th day

Tests of Between-Subjects Effects

Dependent Variable: b*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1693,114[a]	5	338,623	55,462	,000
Intercept	4799,391	1	4799,391	786,076	,000
Treatment	59,442	2	29,721	4,868	,033
pH	1432,945	1	1432,945	234,697	,000
Treatment * pH	124,156	2	62,078	10,168	,004
Error	61,055	10	6,106		
Total	6920,544	16			
Corrected Total	1754,169	15			

a. R Squared = ,965 (Adjusted R Squared = ,948)

Result for parameter b* at pH 4.5

Tests of Between-Subjects Effects

Dependent Variable: b*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	118,767[a]	2	59,384	6,632	,039
Intercept	493,714	1	493,714	55,139	,001
Treatment	118,767	2	59,384	6,632	,039
Error	44,770	5	8,954		
Total	708,863	8			
Corrected Total	163,537	7			

a. R Squared = ,726 (Adjusted R Squared = ,617)

No significant differences according to the Tukey test

b*

Tukey HSD[a,b,c]

Treatment	N	Subset
		1
C	3	4,8500
A	2	5,9500
B	3	13,2000
Sig.		,055

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 8,954.
a. Uses Harmonic Mean Sample Size = 2,571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0,05.

Results for parameter b* at pH 6.5

Tests of Between-Subjects Effects

Dependent Variable: b*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	64,830[a]	2	32,415	9,952	,018
Intercept	5738,621	1	5738,621	1761,927	,000
Treatment	64,830	2	32,415	9,952	,018
Error	16,285	5	3,257		
Total	6211,682	8			
Corrected Total	81,115	7			

a. R Squared = ,799 (Adjusted R Squared = ,719)

b*

Tukey HSD[a,b,c]

Treatment	N	Subset	
		1	2
A	2	24,0100	
B	3	26,7133	26,7133
C	3		31,1000
Sig.		,293	,086

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 3,257.
a. Uses Harmonic Mean Sample Size = 2,571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0,05.