UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA
DEPARTAMENTO DE NUTRICIÓN, BROMATOLOGÍA Y TECNOLOGÍA DE LOS ALIMENTOS

TESIS DOCTORAL

Hyperbaric storage of foods at room temperatura
Characterization in strawberry juice

Almacenamiento hiperbárico a temperatura ambiente
Caracterización del zumo de fresa

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

Ana María Bermejo Prada

Directoras

Bérengère Guignon
Laura Otero García

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Hyperbaric storage of foods at room temperature: characterization in strawberry juice

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Hyperbaric storage of foods at room temperature: characterization in strawberry juice

Almacenamiento hiperbárico de alimentos a temperatura ambiente: caracterización del zumo de fresa

Thesis report submitted by Ana María Bermejo Prada to qualify for the Ph.D. degree at the Complutense University of Madrid

Under the supervision of Bérengère Guignon, Ph.D., and Laura Otero García, Ph.D.
Institute of Food Science, Technology and Nutrition (ICTAN-CSIC)

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Memoria que presenta Ana María Bermejo Prada para optar al grado de Doctor por la Universidad Complutense de Madrid

Bajo la dirección de la Dra. Bérengère Guignon y la Dra. Laura Otero García
Instituto de ciencia y Tecnología de Alimentos y Nutrición (ICTAN-CSIC)

Madrid, 2014

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CERTIFY/CERTIFICAN:

That the present Thesis Report entitled "**Hyperbaric storage of foods at room temperature: characterization in strawberry juice**", submitted by ANA MARÍA BERMEJO PRADA to qualify for the Ph.D. degree, has been carried out at the Institute of Food Science, Technology and Nutrition (ICTAN-CSIC) under their supervision, and that, once accomplished, they grant their permission to defend the dissertation in a public examination by the corresponding Thesis committee.

Que la presente Memoria titulada "**Almacenamiento hiperbárico de alimentos a temperatura ambiente: caracterización del zumo de fresa**", presentada por ANA MARÍA BERMEJO PRADA para optar al grado de Doctor, ha sido realizada en el Instituto de Ciencia y Tecnología de Alimentos y Nutrición, (ICTAN-CSIC) bajo su dirección, y que, hallándose concluida, autorizan su presentación para que pueda ser juzgada por el tribunal correspondiente.

In witness thereof, the parties hereby sign the present document in Madrid on the 21 of October of 2014.

Y para que así conste a los efectos oportunos, firman la presente certificación en Madrid, a 21 de octubre de 2014.

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RESUMEN

Desde tiempos inmemoriales, la conservación de alimentos ha sido una de las principales preocupaciones del ser humano. A lo largo de los siglos, se han desarrollado numerosas técnicas de conservación, pero actualmente la más común en los países desarrollados es la refrigeración. Sin embargo, la cadena de frío presenta importantes problemas de sostenibilidad que hacen que la innovación sea necesaria tanto para mejorar como para diversificar los métodos de conservación disponibles, y así proporcionar soluciones nuevas al mercado mundial de alimentos. Tecnologías novedosas, como la alta presión, ofrecen oportunidades interesantes a la industria alimentaria. Así, el almacenamiento de alimentos a presiones relativamente bajas está comenzando a recibir atención. Este denominado almacenamiento hiperbárico es distinto del procesado de alimentos por alta presión, ya implantado en la industria, debido a que se utilizan presiones más moderadas (normalmente menores de 220 MPa) durante tiempos más prolongados (semanas o meses). Sus principales ventajas frente a métodos tradicionales como la refrigeración radican en que, durante el almacenamiento, no se necesita control de la temperatura y, además, la tecnología es respetuosa con el medio ambiente. Sin embargo, la información disponible acerca de este método de almacenamiento, ya patentado, es más bien escasa. Por lo tanto, el objetivo de esta Tesis fue caracterizar el almacenamiento hiperbárico de alimentos a temperatura ambiente. Para ello, se eligió como objeto de estudio el zumo de fresa por su relativa simplicidad y por su importancia tanto en la producción frutícola española como en la industria de ingredientes alimenticios. La caracterización del almacenamiento hiperbárico se abordó desde el punto de vista del concepto de Calidad Total, tratando de cubrir el mayor número de aspectos posibles, desde la seguridad y la calidad del producto hasta el diseño de los equipos, sin olvidar las cuestiones económicas y medioambientales.

El experimento típico en esta Tesis consistió en almacenar zumo de fresa a diferentes niveles de presión, hasta un máximo de 220 MPa, durante distintos tiempos, hasta 15 días, a una temperatura fija de 20 °C. Tras el almacenamiento, se estudiaron en detalle los principales parámetros de calidad del zumo (carga microbiana, viscosidad, color,

aroma y sabor), así como algunos de los mecanismos implicados en su degradación. Además, para explorar las posibilidades de aplicación industrial, se propuso un diseño de equipo y se calcularon las dimensiones y el peso de la vasija. Para completar el estudio de viabilidad, se estimaron los costes del almacenamiento hiperbárico y su impacto ambiental y se compararon con los de la refrigeración.

Los resultados mostraron que el almacenamiento hiperbárico a temperatura ambiente (25-220 MPa/20 °C) es un método eficaz para inhibir el crecimiento microbiano en el zumo de fresa. Además, la presión consiguió atenuar las pérdidas de color, viscosidad, aroma y sabor del zumo, al menos, durante 15 días y, una vez finalizado el almacenamiento hiperbárico, el zumo se mantuvo estable durante, al menos, 15 días adicionales en refrigeración.

Por otra parte, el estudio detallado de los cambios inducidos por la presión en la carga microbiana, color y viscosidad puso de manifiesto que tanto el nivel de presión como el tiempo de almacenamiento afectaron a los mecanismos implicados en el crecimiento microbiano, la degradación, enzimática y no enzimática, del color y las pérdidas de viscosidad.

Respecto a la viabilidad de este método de almacenamiento a escala industrial, se pusieron en evidencia tanto sus ventajas como sus limitaciones. Se estimó que, para presiones de trabajo inferiores a 155 MPa, el tamaño de la instalación y la gestión logística son viables. El análisis de costes reveló que el almacenamiento hiperbárico es más caro que el almacenamiento refrigerado. Esto es debido a la importante inversión inicial necesaria, ya que el consumo de energía es insignificante. En cambio, la huella de carbono del almacenamiento hiperbárico a temperatura ambiente es 26 veces menor que la de la refrigeración convencional, lo que confirma, en términos cuantitativos, que este método es más respetuoso con el medio ambiente.

Por lo tanto, el almacenamiento hiperbárico a temperatura ambiente se revela como un nuevo método apto para la conservación de zumo de fresa, al menos durante 15 días, tanto desde el punto de vista de la calidad del producto como de su viabilidad a escala industrial.

ABSTRACT

Since time immemorial, food preservation has been at the heart of human society concerns. Many storage techniques have been developed over centuries being refrigeration the most common one today in developed countries. However, the cold chain poses serious problems of sustainability, and innovation is necessary to improve and diversify the available storage methods as well as to provide new solutions to the global food market. Novel technologies, such as high pressure, offer interesting opportunities to the food industry. Food storage at room temperature under relatively low pressures is just starting to receive some attention. This so-called hyperbaric storage is different from the current high-pressure processing of food already implemented in the industry in that much lower pressures (usually lower than 220 MPa) are intended to be used and for much longer times (weeks or months). Claimed advantages are that no temperature control is needed, that no energy is required during the storage period, and that this is an environment-friendly technology. However, the available information about this patented storage method is rather scarce. Thus, the objective of this Thesis is to characterize hyperbaric storage of food at room temperature. For this purpose, strawberry juice was chosen as the object of study because of its relative simplicity and its importance in Spanish fruit production and in the food ingredient industry. Then, the characterization of hyperbaric storage was addressed from the point of view of the Total Quality concept, trying to cover as many aspects as possible from safety and quality to equipment design, economic, and environmental issues.

A typical experiment consisted in storing strawberry juice at different pressure levels up to a maximum of 220 MPa during different times up to 15 days and at a fixed temperature of 20 °C. After storage, the main safety and quality parameters (microbial load, viscosity, color, aroma, and flavor), as well as some of their mechanisms of degradation, were examined in depth. Besides, to explore the industrial implementation possibilities, an equipment design was proposed and the vessel mass and dimensions were calculated. The associated cost of storage and its environmental impact were also computed in comparison to the refrigeration case.

Results showed that hyperbaric storage at room temperature (25-220 MPa/20 °C) is an efficient method to inhibit microbial growth in strawberry juice. Moreover, pressure was effective to attenuate viscosity, color, and sensory (aroma and flavor) deterioration in raw strawberry juices stored for 15 days. Besides, the juice remained stable when refrigerated for 15 additional days after hyperbaric storage. Thorough studies of the changes in microbial load, color, and viscosity induced by storage evidenced that both the pressure level and the storage time affected mechanisms involved in microorganism growth, color enzymatic and non-enzymatic degradations and viscosity losses.

Regarding the feasibility and viability of the storage method at industrial scale, both positive evidences and limitations were established. Installation size and logistics management were deemed more feasible for vessels working at pressures below 155 MPa. The cost analysis revealed that hyperbaric storage is more expensive than cold storage due to the huge initial investment whereas energy consumption is negligible. By contrast, the carbon footprint of hyperbaric storage at room temperature was more than 26 times lower than that of conventional refrigeration confirming in quantitative terms the environment-friendly character of this method.

Therefore, hyperbaric storage at room temperature is a novel storage method valid for the preservation of strawberry juice, at least for 15 days, from the point of view of the product quality and of the applicability of the method at industrial scale.

Chapter 1: Introduction

INTRODUCTION

1.1. Food preservation

1.1.1. Current issues and challenges

It is well known that all foods have a shelf life[1] determined by their rate of deterioration. Food deterioration can be defined as a series of continuous detrimental changes taking place in a food item which may affect product integrity, result in a reduction of its quality, and/or alter its edibility. Depending on the rate of deterioration, shelf life can vary from a few days to several months or even years and foods can be classified as perishable, semi-perishable and shelf-stable.

Among these categories, perishable foods occupy an important position in terms of quantity, public health, and industrial management, as explained below. They are important in quantity because, perishable, are almost all foods in their raw state and, virtually, all foods before being processed. Moreover, perishable foods use to be characterized by a higher nutritional quality than semi-perishable or shelf-stable foods (for example, orange compared with pasteurized orange juice and with crystallized orange slices). Thus, they have an essential place in consumers' diet who are more and more aware of their role for health. In return, the microbial quality of this food category is much less stable and risks of foodborne illness can exist. In case of quality incident, human and economic repercussions can be dramatic. For all these reasons, food quality control and food production chain efficiency are at the heart of food international organizations and manufacturers' concerns. The industrial means required to ensure perishable food preservation are consequently especially huge.

Food deterioration is initiated just after harvest, fishing or butchery and, then, it progressively increases during manufacturing, transportation, retail distribution and home storage. Thus, food quality is conceived as a dynamic state that declines continuously until food becomes unsuitable for sale or consumption. This makes a difference with other industrial production chains where quality is (almost) unaffected

[1] length of time during which a food is suitable for sale and consumption.

by the time variable. In the food industry, at all stages of food production, manufacture, storage, and distribution, several complementary preservation strategies are applied to slow down quality deterioration processes. These strategies comprise all the technological or (bio)-chemical actions that aim at eliminating or reducing the activity of deterioration agents. They involve technologies which are often high energy consuming, mobilize natural resources and, in consequence, pose serious problems of sustainability, particularly in the context of market globalization. Therefore, the primary challenge for food manufacturers is to develop and employ methods that comply with food sensory, nutritional, and safety quality criteria while remaining economic and sustainable.

1.1.2. Processing methods

The oldest strategy for food preservation relies on leaving microorganisms without the basic elements for their survival (nutrients, water, oxygen, heat…). In this way, the reduction of water activity is attained, for example, by drying, salting, concentration or crystallization. Recent advances also include lyophilization and osmotic dehydratation. Other methods alter the characteristics or the composition of the food, hindering the growth of microorganisms. For example, the acidification of the food, which limits microbial growth by lowering pH of the medium, could be reached by means of both fermentation (pickling, acid fermentation) and addition of acid substances, such as citric acid, tartaric acid, or fumaric acid, among others. Some processing methods are based on substances with antimicrobial effect like smoke in the smoking process or ethanol in the alcoholic fermentation. Besides of all these methods, addition of different chemical preservatives is widely used to conserve food quality. The products obtained by using all these preserving techniques are usually shelf-stable but their nutritional and sensory quality characteristics are very far from those of the natural food.

Improvements in food quality characteristics were gained with the rise of thermal technologies in the 19th and 20th centuries. Currently, thermal processing (e.g. pasteurization, sterilization) is probably the most common method to avoid, or at least, to retard microbial, physical, chemical and biochemical reactions associated with food spoilage. This is the prevailing method due to its availability, cost, and effectiveness.

However, although heating food effectively reduces levels of microorganisms and inactivates some deleterious enzymes, such processing can cause thermal degradation reactions leading to off-flavors, destruction of nutrients, and other product quality losses.

Nowadays, consumers have a growing preference for convenient, fresh-like, healthy, free-additives, minimally-processed food products with natural flavor and taste, and with an extended shelf life (Yordanov & Angelova, 2010). This is a new challenge for food technologists since they have to develop a new generation of food products having quality attributes superior to those existing in the traditional market. To reach these demands, in recent years, alternative non-thermal technologies have been proposed and thoroughly investigated. Non-thermal food preservation technologies can be defined as those in which temperature is not the main factor in the inactivation of microorganisms and enzymes. In this way, these technologies provide safe, fresher-tasting, nutritive foods without the use of heat or chemical preservatives. Some of these non-thermal technologies recently investigated are high-pressure processing (HPP), pulsed electric field processing, high-intensity pulsed light technology, radio frequency electric fields, oscillating magnetic field pulses, ultrasounds, gamma irradiation, ultraviolet irradiation, non-conventional chemical reagents, and natural bio-preservatives together with active packaging. Among them, HPP is one of the most studied and it has become an industrial reality, although there are still many aspects under research.

1.1.3. Storage methods

With or without a previous preservation treatment, all foods need specific conditions of storage until their consumption in order to extend their shelf life as much as possible. These conditions will define the storage method and they are selected so as to avoid or, at least, to retard microbiological (growth of microorganisms), physiological (e.g. ripening, senescence, and respiration), biochemical (e.g. browning reactions, lipid oxidation, and pigment degradation), and/or physical (e.g. moisture loss) changes in foods.

Shelf-stable foods can be simply preserved at room conditions provided that these remain in a relatively fresh, dry, dark, and stable location over the storage period. By contrast, perishable foods require a rigorous control of storage conditions. Strategies for food preservation during storage are based on the modification of the environmental parameters (e.g. temperature, atmospheric gases) where the food product is stored and, thus, some examples of storage methods are refrigeration, freezing, vacuum sealing, and controlled atmospheres. These strategies can be applied alone or combined and storage at low temperature (-20 °C to 5 °C) is, with any doubt, the most employed in developed countries through the food cold chain. Cold chain covers from the initial chilling or freezing of the raw ingredients to the domestic storage of the final food product with the aim of preserving the safety and quality of foods. However, the adequate management of these cold chains is difficult, expensive, and energy consuming. As an example, about 50 % of total energy in the food industry is consumed by refrigeration related facilities. So, taking into account that approximately 40 % of all food requires refrigeration, it is estimated that 15 % of the electricity consumed worldwide is used for refrigeration (James & James, 2010).

Moreover, due to CO_2 emissions and the effects of some refrigerants, refrigeration is partially responsible of global warming and climatic change, nowadays considered important threats to our planet (Tassou, Lewis, Ge, Hadawey, & Chaer, 2010).

For all these reasons, many efforts have been made in the last decades in the agro-food industry to improve the performance of conventional refrigeration systems, to find new environmental friendly refrigeration technologies, and also to look for new energy saving opportunities in food preservation (Masanet, 2008; Tassou et al., 2010; Ullah, Saidur, Ping, Akikur, & Shuvo, 2013). Many progresses are still needed to meet with sustainability concerns and new approaches are desirable. As for food processing, innovation is necessary to improve and diversify the available storage methods. In this way, new technologies such as high pressure may open new way forward, even for storage purposes. In the following Section, an overview of this technology is provided.

1.2. High-pressure technology in the food industry

1.2.1. High pressure: different levels and applications

Pressure is present in a large number of applications in food technology. Some of these applications, those that employ the lowest levels of pressure, were developed many years ago. Thanks to the technological advances of the last decades, the pressure level that the industrial equipment can now reach has increased notably and, nowadays, it is close to one gigaPascal. By increasing order of pressure, applications go from pressure cooking, supercritical fluid extraction, and extrusion-cooking - at pressures below 50 MPa - to waterjet cutting, (ultra-) high-pressure homogenization, and high hydrostatic pressure processing - at pressures up to about 600 MPa (Guignon, 2011). A succinct description of the most outstanding characteristics of these technologies is given below.

Pressure cooking. It consists in cooking the food under pressures higher than the atmospheric one. Since the food is cooked in a sealed vessel (pressure cooker), the steam generated produces an increment of pressure inside the vessel (regulated around 0.2 MPa) and the increment of water boiling point (around 120 °C). In this way, pressure cooking allows food to be cooked faster than with conventional boiling.

Supercritical fluids (SCFs). In the food industry, carbon dioxide, nitrogen, or argon in supercritical state are employed mainly in extraction processes. The supercritical state is reached when temperature and pressure are raised above the critical point. For example, in the case of CO_2, the critical point is located at 31.1 °C and 7.38 MPa. The SCFs present many advantages over conventional organic solvents; they possess a higher diffusion coefficient and a lower surface tension resulting in an easier penetration into the structure of the solid matrix to release the solute. Decaffeinated coffee is an emblematic example of this application.

Extrusion-cooking. In this process, a dough is pushed through an orifice of given shape (die). The machine is called extruder and consists of tightly fitting screw rotating within a stationary barrel. The product exits the extruder through the die where it usually puffs and changes its texture due to the release of steam and normal forces.

The mechanical and thermal actions generate a pressure level from 1 to 5 MPa. This technique is highly employed in the manufacture of pasta, ready-to-eat cereals, snacks, pet foods, and textured vegetable protein (Harper, 1981).

Waterjet cutting. This application employs a very high-pressure (200-400 MPa) water jet to cut materials. The water flows from a pump, through capillary tubing and finally exits at high velocity from a nozzle forming a cutting head. It makes it possible to cut any kind of food in optimal hygienic conditions.

High-pressure homogenization (HPH). High pressure is also employed to homogenize liquid foods. The typical homogenization pressure used in the industry is around 20 MPa, but current developments in the design of homogenizers allow for homogenization at much higher pressures of up to 500 MPa, depending on the design of the ultra-high pressure homogenizer (Dumay et al., 2013). Texture, taste, flavor, and shelf life characteristics of food emulsions are improved, especially in the case of dairy products like milk, cream, and ice cream. Consumer acceptance of some products has also been reported to be enhanced (Paquin, 1999). When using the highest pressure levels, products can even be sterilized as it has been reported for liquid foods such as milk (Amador-Espejo, Suàrez-Berencia, Bárcenas, & Trujillo, 2014; Poliseli-Scopel, Hernández-Herrero, Guamis, & Ferragut, 2014).

Processing at high hydrostatic pressure or, shortly, high-pressure processing (HPP). HPP consists in subjecting foods to elevated pressures - ranging from 200 MPa to 600 MPa - during a short period of time (in the order of minutes). HPP is usually carried out at room temperature but moderate, high, or low temperatures are also possible (at least at laboratory scale) to get additional effects. Pressure is lethal to many microorganisms leading to a microbial load reduction equivalent to that obtained after a pasteurization process. But, in contrast with pasteurization, pressure has almost no impact on most of the molecules responsible for nutritional and organoleptic properties. Hence, products from HPP require refrigeration but they present excellent fresh-like quality.

From all the above information, it is clear that high pressure is a consolidated technology in the food industry. Engineers are able to build different kinds of high-pressure equipment for a quite large variety of objectives. Besides, HPH and HPP are among the youngest technologies used for food preservation and they offer the most promising commercially viable alternative to traditional thermal processing. Compared to HPH, HPP is more versatile because it is not limited to liquid foods and signs exist that it could serve as a starting point to develop a new storage method for foods. More details about HPP are given below to establish a basis on which to develop the idea that pressure could be used for storage purposes too.

1.2.2. High hydrostatic pressure processing

HPP is one of the non-thermal food preservation technologies most extensively investigated to date. It extends product shelf life with a minimal impact on product quality. It has already been implemented in the food industry since, in April 1990, the first commercial high-pressure processed products (strawberry, kiwi, and apple jams) appeared in Japan (Hayashi, 1992). In the early 1990s, only a few high-pressure units were available for use, most of these were at research centers or universities. But, at the beginning of the 21st century, an important increase in the number of high-pressure units was observed, and since then, the number of high-pressure units has been growing exponentially around the world (see histogram in Figure 1.1). Thus, in mid-2013, there were around 220 high-pressure units installed in food industries and more than the half were installed in America (see circular graph in Figure 1.1). Nowadays, the main markets are North America, the European Union, Japan, Korea, Australia, and New Zealand, but HPP equipment may also be found in Peru, Chile, and China.

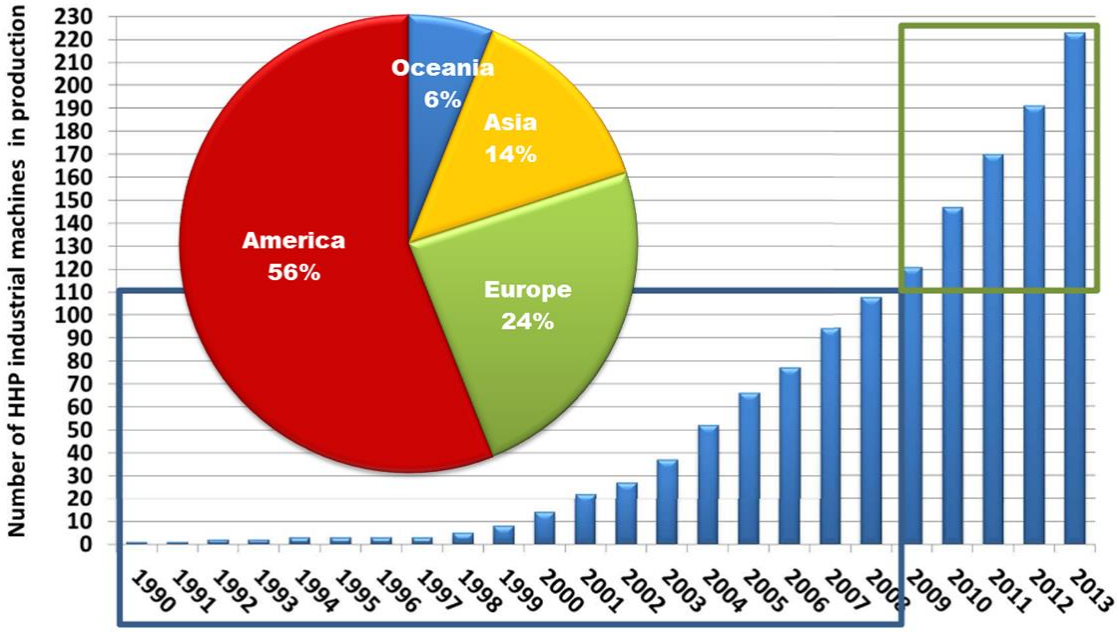

Figure 1.1. *Worldwide growth of the HPP installations and distribution of HPP equipment sorted by continents (Courtesy of Hiperbaric, Burgos, Spain).*

Of all the machines installed around the world, 43 % are dedicated to process fruit and vegetable products (Figure 1.2). This high percentage is due to the fact that avocado industry has been one of the main drives for this innovative technology (Balda, Aparicio, & Samson, 2012). For similar historical reasons, fruit preparations, smoothies, and fruit juices are also an important segment. Meat products industry ranks second with 26 % of the machines installed around the world in connection with the need to reduce the risk of contamination with *Listeria monocytogenes*. A significant number of high-pressure units is also employed in the fish and seafood industry with various applications such as opening bivalves, shucking meat from crustaceans, and sanitation of ready-to-eat products.

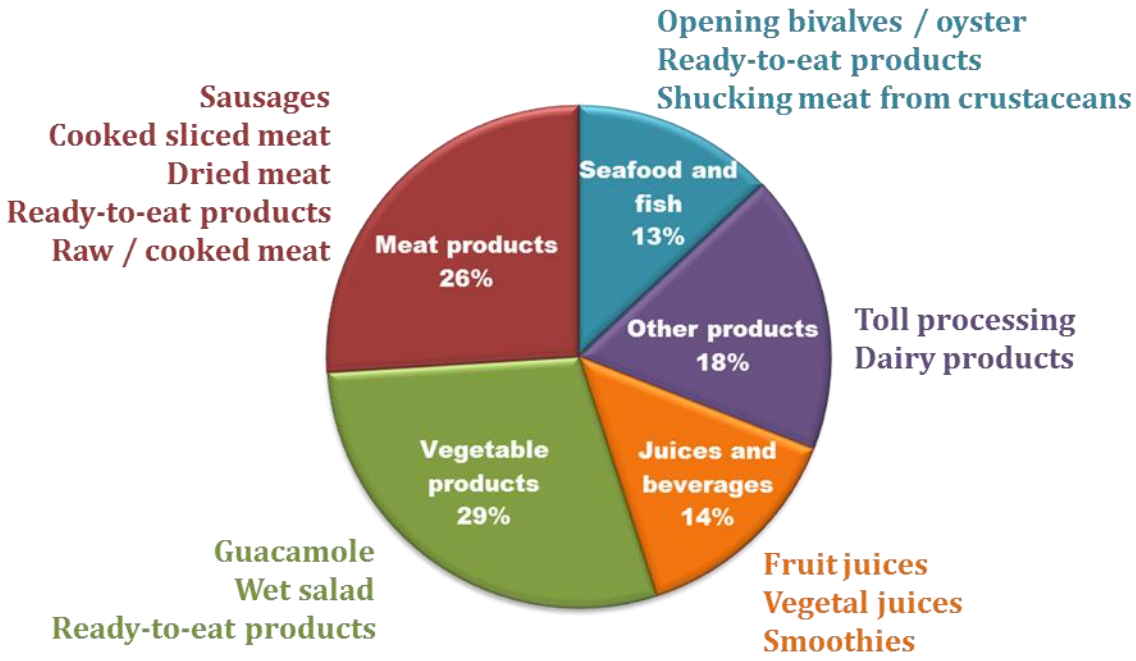

Figure 1.2. *Distribution of HPP equipment in the food industry (Courtesy of Hiperbaric, Burgos, Spain).*

The rest of commercial applications include rice cakes, sauces, dairy foods, and a series of new functional products, such as a drink made with colostrum and milk (coldplus.com), for which HPP is especially suitable (Oey, Lille, Van Loey, & Hendrickx, 2008). The range of high-pressure processed products available on the market is really extensive (Figure 1.3) and, in 2012, the global production was more than 350,000 tons (Hiperbaric, personal communication, February 2014).

Figure 1.3. *High-pressure processed products available on the market.*

For processing, food products are packaged in a flexible packaging and loaded into a high-pressure chamber (Figure 1.4). The vessel is sealed and filled with pressurizing fluid. Pressure is generated by pumping the pressurizing fluid, usually water, inside the closed chamber until the target pressure is reached. Then, the pump is stopped, the valves are closed, and the pressure can be maintained without further energy input. After holding the product for the desired time at the target pressure, the vessel is decompressed by releasing the pressure-transmitting fluid. The processed products are removed and cold stored.

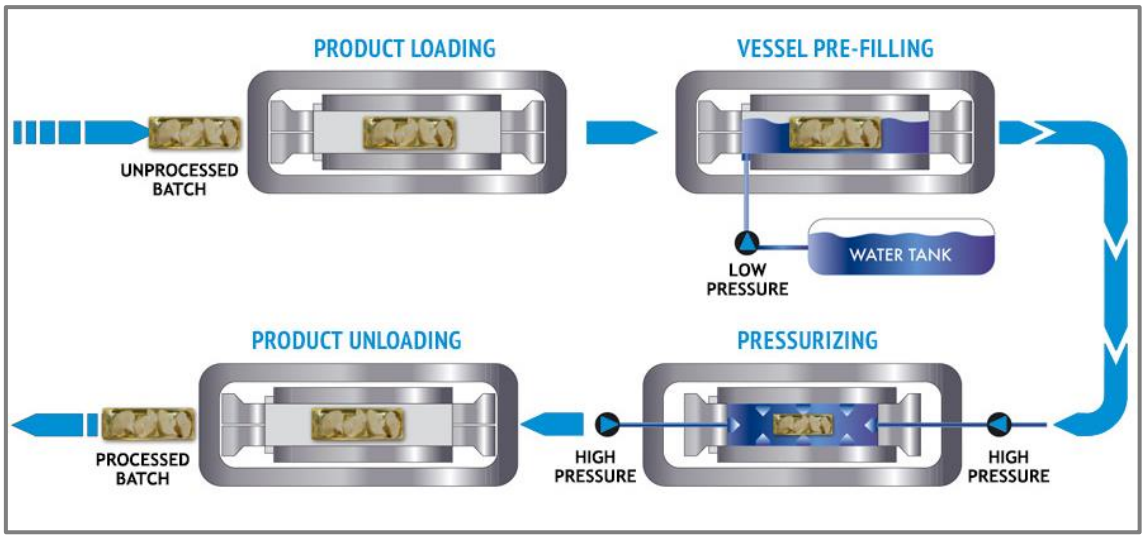

Figure 1.4. *Diagram of operation of a HPP unit. Source: Hiperbaric, Burgos, Spain (www.hiperbaric.com).*

There are mainly two general scientific principles of direct relevance to the use of high hydrostatic pressures in food processing (Hogan, Kelly, & Sun, 2005):

The isostatic rule (Pascal's principle): pressure is instantaneously and uniformly transmitted throughout a fluid (Figure 1.5). Thus, any food placed in water receives the same pressure over its entire surface when the water is compressed. Consequently, liquid foods but also solid foods of any geometry can be processed by HPP without losing their initial shape.

Le Chatelier's principle: when a system at equilibrium is disturbed, the system responds in a way that tends to minimize the disturbance. This means that pressure favors reactions that result in a decrease in volume, but opposes reactions that involve an increase in volume.

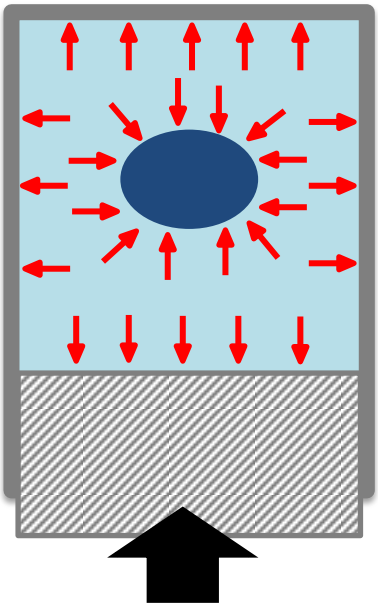

Figure 1.5. *The principle of isostatic processing.*

As a result of Le Chatelier's principle, HPP (at moderate temperature) has a limited effect on covalent bonds since their rupture would tend to cause a volume increase. Therefore, small molecules responsible for sensory and nutritional properties suffer no significant losses. This represents a unique characteristic of this technology compared to thermal processing. Pressure only affects weaker bonds such as van der Waals forces, electrostatic interactions, and hydrogen bridges, while covalent bonds remain unaffected. Such weak bonds exist in the molecular structure of proteins and in several supramolecular structures present in foods such as starch granules or cellular membranes, among others. By changes or rupture of these non-covalent interactions due to pressure, protein structure (*e.g.* enzymes) and molecular interactions are modified leading finally to a global dysfunction of microbial cells. Thus, HPP efficiently inactivates most pathogenic and spoilage vegetative microorganisms. The extent of inactivation depends on the type of microorganism, food composition, pressure level, and duration of the treatment as well as pH, temperature, and water activity (Cheftel, 1995). However, spore inactivation requires a combination of elevated pressure and high temperature. In addition to microorganisms, high-pressure treatments are also able to inactivate many food enzymes, reducing in many cases the degradation of food quality and nutritional value during the subsequent storage. As

biological activity of enzymes arises from an active site, even small changes in the active site can lead to loss of activity or to changes in its functionality (Rastogi, Raghavarao, Balasubramaniam, Niranjan, & Knorr, 2007). Again here, the effects of pressure vary extensively depending on the type of enzyme, pH, medium composition, temperature, etc. (Hendrickx, Ludikhuyze, Van den Broeck, & Weemaes, 1998). While some enzymes can be inactivated at room temperature by a few hundreds of MPa, others can withstand up to 1,000 MPa. There are even evidences that high pressure can induce stabilization and activation of some enzymes at relatively low pressure (100-200 MPa) (Eisenmenger & Reyes de Corcuera, 2009). So far, pressure effects have been determined only for relatively few enzymes and food systems, and knowledge on this subject is still incomplete (Mújica-Paz, Valdez-Fragoso, Samson, Welti-Chanes, & Torres, 2011).

It is clear from the above that HPP has reached a great level of maturity as a food preservation treatment. Sales of equipment are continuously growing, numerous products are on the international market, and the science behind this process is still in progress. Opportunities exist to develop further this technology as it will be highlighted in the following Sections.

1.2.3. Opportunities of high hydrostatic pressure in the food industry

The technical development of equipment has been determinant in the breakthrough of high pressure in the food industry. Additional sophistications are expected in parallel with research advances and industrial interests. For example, the combination of high pressure with high temperature is at its initial stage of implementation in the industry. In fact, in 2009, the US Food and Drug Administration (FDA) approved the commercial use of pressure-assisted thermal sterilization for low-acid foods (Processing, 2009; Somerville & Balasubramaniam, 2009). Pressure-assisted thermal processing leads to sterilization of food products, prolonging considerably their shelf life without needs for refrigeration. Moreover, the inactivation of bacterial spores is achieved with less quality losses than in conventional sterilization methods. The combination of high pressure and low temperature has also permitted new applications in the form of pressure supported freezing and thawing processes. Increasing pressure reduces the

melting point of water. Hence, by a suitable management of pressure and temperature, foods can be brought to supercooled conditions that induce quasi-instantaneous and uniform ice nucleation throughout the product, whatever its form and size. Thus, in high-pressure shift freezing, small ice crystals are formed that reduce structural damage compared with traditional freezing (Otero & Sanz, 2012). In high-pressure assisted thawing, phase change is faster than in conventional thawing and the quality of the final product is improved (Le Bail, Chevalier, Mussa, & Ghoul). In both cases, microbial load is reduced which is another advantage over traditional freezing and thawing. Also of great interest are the physical modifications of structure and function that high pressure can produce in foods. Hence, high-pressure processing can be used to develop new products with novel characteristics. For instance, the pressure-induced modification of casein micelles size and denaturation of serum proteins may result in dairy products with novel textures (Trujillo, Capellas, Saldo, Gervilla, & Guamis, 2002). These are only some examples of the immense potential of high hydrostatic pressure for the food industry. Many other applications have been described (Rastogi et al., 2007) and probably some still remain to be discovered.

There are several features which make high pressure attractive for the industry and can be determinant when deciding to transform these opportunities into a commercial reality or not. The first one is the instantaneous and uniform character of pressure. As compared with other conventional technologies such as thermal processing, the great advantage of hydrostatic pressure is that, unlike heat, it is transmitted immediately and uniformly in all directions through the medium surrounding the food. Therefore, pressure is the same at any given position and time, independently of the product size and geometry (Oey et al., 2008). This allows for short treatment times, particularly when comparing thermal processing and HPP of solid foods. However, HPP is not exempt of thermal effects. Attention must be paid to temperature changes during pressure built up caused by adiabatic heating. Temperature gradients can develop during the pressure holding step, giving rise to treatment heterogeneities with potential microbiological risks or over-processing effects, for example (Otero, Ramos, de Elvira, & Sanz, 2007). Fortunately, several strategies have been proposed to overcome this inconvenient or even to take advantage of it (Grauwet et al., 2012). A

second attractive feature of hydrostatic pressure is that scaling up of laboratory and pilot plant findings to commercial production is both simple and safe because pressure effects are theoretically independent of the equipment and product geometry and size (Mújica-Paz et al., 2011). A third argument in favor of high pressure is the low energy input needed. High-pressure processing only requires energy during the compression phase (Hogan, Kelly, & Sun, 2005). In contrast, the initial investment cost of the equipment is high. Nevertheless, it has been reduced up to three folds in the last decade thanks to the technical improvements (Balda et al., 2012) and thus, it is expected that the downward trend continues. A last point to be convinced of opportunities for high pressure in the food industry is the impact on the environment. It is a clean and environmentally friendly technology. This is practically a waste-free processing and the pressurization fluid most employed is water (Hogan, Kelly, & Sun, 2005; Hugas, Garriga, & Monfort, 2002; Toepfl, Mathys, Heinz, & Knorr, 2006).

All these advantages explain why a wide variety of novel HPP products have reached consumers in a very short time and why the corresponding market keeps on growing. Among these opportunities, a novel application of high pressure in the food industry could be hyperbaric storage as an innovative technique for food preservation. This technique consists in subjecting a product to a pressure higher that the atmospheric one **during a long period of time**, i.e. the storage period (for example, weeks or months). As for HPP, pressure is transmitted by a liquid medium (usually water) but the pressure levels suitable for storage are considerably lower, commonly **between 25 and 220 MPa** compared to 400-600 MPa for HPP. Storage can be carried out at low temperature but preferably **at room temperature**. Indeed, storage of perishable foods at room temperature would avoid all issues related with the cold chain while pressure effects would allow satisfying consumers' expectations about food quality.

To sum up, on one side, we have observed that storage methods for food preservation are limited and that innovation is desirable in this field (Section 1.1.3). On the other side, we have shown that high-pressure technology is a source of innovation for which real opportunities exist in the current context of the total quality challenge faced by the

food industry. Thus, storage of food under pressure at room temperature appears as a way worthy of being explored.

1.3. Hyperbaric storage as an innovative food preservation technique

1.3.1. History

The effectiveness of hyperbaric storage for food preservation has been known for more than forty years when, in 1968, the research submarine Alvin of the Woods Hole Oceanographic Institution sank below 1540 m of water, 135 miles southeast of Woods Hole, Massachusetts. Ten months later, Alvin was brought to the surface and, surprisingly, edible foods for the crew (sandwiches, bouillon, and apples) were practically untouched by decay. However, when kept under refrigeration at 3 °C and atmospheric pressure, the foods spoiled in a few weeks. Subsequent investigations demonstrated that the pressure and temperature conditions at 1540 m below the surface of sea, 15 MPa and 3-4 °C, were responsible for the good degree of conservation of the food items (Jannasch, Eimhjellen, Wirsen, & Farmanfarmaian, 1971).

Previously, in 1967, successful experiments of underwater storage of cereal grains, at 30 m below the surface of Lake Biwa, had been made in Japan (Mitsuda, Kawai, & Yamamoto, 1972). Because the main purpose of this study was to keep temperature constant over time, the researchers did not pay attention to the pressure effect. Nevertheless, retrospectively, pressure (0.3 MPa) may have been involved in such good results of preservation.

Ten years later, in 1977, Charm, Longmaid, and Carver (1977) proposed cold storage under pressure as a new method for food preservation. The authors tested storage pressures up to 40 MPa and temperatures ranging from 23 °C to -3 °C. As pressure lowers the freezing point of water, non-frozen storage could be achieved at subzero temperatures under pressure. Charm et al. (1977) proved that pressure inhibited microbial growth and, below a certain critical temperature, it also reduced peroxidase and trypsin activity. They found that fish, chicken, and beef stored, in a non-frozen state, for 30 days at -3 °C and 24 MPa were not significantly different, microbiologically

and organoleptically, from frozen controls held at atmospheric pressure and -20 °C for the same period. Non-frozen storage at subzero temperature under pressure substantially extended the shelf life of these highly perishable foods and avoided damage produced by ice crystals.

In the 90s', other researches also showed that storage under pressure (50-200 MPa) at subzero temperature could be an effective preservation technique with interesting advantages over freezing (Deuchi & Hayashi, 1992; Kalichevsky, Knorr, & Lillford, 1995). Temperatures employed for storage under pressure can be higher than those used conventionally without impairing quality aspects. However, although substantial energy savings can be achieved as compared with standard storage in frozen state, this method is still expensive. Energy costs remain notable throughout the period of food storage. Therefore, greater energy savings are necessary to be a viable method. This could only be attained by elimination of cold and demonstration of pressure efficiency in preserving food also at room temperature.

In 1997 and later, in 2000, two patents about hyperbaric storage were published (US patent No. 5,593,714 and No. 6,033,701, respectively). These patents describe how a huge variety of foods, food ingredients, and cooked foods are stored under pressure (up to 250 MPa), at room temperature (18-23 °C), from hours to days (up to 8 days). Successful and unsuccessful results are enumerated by the author. Unfortunately, the methodology and scientific justification are missing (Hirsch, 1997; 2000).

Currently, hyperbaric storage at room temperature can be considered to be at its beginning. In fact, to our best knowledge, up to 2012, there was only one scientific paper about this technology in the literature (Ko & Hsu, 2001). In this study, the effectiveness of high pressure storage at room temperature (50-300 MPa/25 °C/12 h) for preserving tilapia fillets was tested. It is concluded that storage pressures greater than or equal to 200 MPa maintained the freshness of tilapia meat by reducing the putrefactive rate and the initial microbial load for, at least, 12 hours. In the course of this Thesis some more publications about hyperbaric storage of melon and watermelon juices have been published, but knowledge about this new technology is still very scarce in any case (Fidalgo et al., 2013; Queirós et al., 2014; Santos et al., 2014).

1.3.2. State-of-the art

In the literature, one can find numerous studies about the effect of high pressure on the quality parameters of a large variety of food items. However, these studies cover only HPP, meaning that the reported pressure effects on foods are due to short stays (minutes) under relatively high pressures (above 200 MPa). The case of relatively low pressures such as those suitable for hyperbaric storage has usually been overlooked because the highest pressures were always faster to inactivate microorganisms and enzymes. Also, studies including pressure holding times longer than hours are scarce since they were judged unproductive and thus industrially uninteresting for processing purposes. So, as it can be deduced from above, the available information about hyperbaric storage of foods is still limited and the effect of low pressures and long times on food characteristics is unknown or, at least, largely incomplete. In particular, studies carried out at room temperature are almost inexistent. We can only report a few more researches for which foods were placed under high pressure and room temperature conditions for long periods, but the focuses are often somewhat different. These are investigations on fruits and vegetables postharvest life extension, on cheese ripening, on Japanese seafood meal preparation, on dried food hygienization, and on non-thermal inactivation of spores.

Fruits and vegetables postharvest life extension. The main purpose of pressure application is to control postharvest decay of fresh horticultural crops, and consequently, to extend the shelf life and maintain the product quality. It is important to highlight here some big differences from the studies previously reported. The first difference is that the food product is still alive. The second one is that, in this case, the pressure applied is much lower, ranging from 0.1 to 3.5 MPa, in order to avoid irreversible damage to the fresh product. And, the third one is that pressure is built up by means of compressed air. The published studies made cover different storage times (from 5 days until 4 weeks), at both refrigerated (Baba, Ito, Ikeda, & Manago, 2008; Robitaille & Badenhop, 1981; Yang, Balandran-Quintana, Ruiz, Toledo, & Kays, 2009) and room temperatures (Baba & Ikeda, 2003; Liplap, Boutin, LeBlanc, Vigneault, & Vijaya Raghavan, 2014; Liplap, Vigneault, Toivonen, Charles, & Raghavan, 2013; Robitaille & Badenhop, 1981). The apparent effects of pressure are a decrease in the

respiration rate, ethylene production, and weight loss, a slowing down of the ripening process, and the reduction of chilling injuries and of bacterial growth (Baba & Ikeda, 2003; Liplap et al., 2014; Liplap, Toussaint, et al., 2013; Liplap, Vigneault, et al., 2013; Robitaille & Badenhop, 1981). In spite of the differences with the hyperbaric storage method proposed in this Thesis, it is interesting to stand out that, even at very low pressure levels and room temperature, quality benefits are noticed for the preservation of vegetal products.

Dried food sanitization. Dried microorganisms are particularly resistant to high hydrostatic pressure effects. Nevertheless, Espinasse, Perrier-Cornet, Marecat, and Gervais (2008) proved that dried cells were sensitive to pressurized gases. These authors performed high-pressure treatments on *Saccharomyces cerevisiae* at 150 MPa and 25 °C with holding times up to 12 months, and using nitrogen, argon, or helium. The higher growth inhibition in presence of pressurized inert gases than in absence of a gas atmosphere had already been reported by other authors (Arao, Hara, Suzuki, & Tamura, 2005).

Cheese ripening. On the fringe of food preservation, Yokoyama, Sawamura, and Motobayashi (1992) proposed to use high pressure to accelerate the ripening of cheese. Cheddar cheese was maintained under pressure, from 5 to 300 MPa, for 3 days and 25 °C and compared to untreated cheese and to 6-months old commercial Cheddar. Free amino acid (FAA) levels and taste of cheese stored at 50 MPa were comparable to those of 6-months old cheese. Besides, addition of lipase and protease to the cheese curd at salting resulted in a Parmesan-type cheese equivalent to a commercial control in terms of levels of FAA and flavor scores after 3 days of storage at 50 MPa and 25 °C. These results suggest that the application of high pressure could reduce ripening times significantly. However, in later investigations, O'Reilly, O'Connor, Murphy, Kelly, and Beresford (2000) and Saldo, McSweeney, Sendra, Kelly, and Guamis (2002) concluded that, although levels of proteolysis in general increased in treated Cheddar cheese (50 MPa/3 days/25 °C) and caprine milk cheese (50 MPa/3 days/14 °C) when compared with control cheeses, they were not as high as those suggested by Yokoyama et al. (1992). Therefore, the application of relatively low pressure during long times in order

to accelerate cheese ripening should be further studied to throw light on the effectiveness of this method. Once more, it can be highlighted that even low pressures are able to induce changes in foods.

Japanese seafood preparation. A particular application of high pressure is the autolytic hydrolysis under hydrostatic pressure (AHHP), that is, the use of high pressure to induce autolysis in order to improve the sensory characteristics of the product while inhibiting bacterial growth. This application is not widely known and the information is scarce since it is being developed by Japanese groups and the research works are in Japanese language. For example, Shigeta, Aoyama, Okazaki, Matsui, and Namba (2008) found that the best conditions for AHHP to produce seasoning of unsalted squid liver were 60 MPa and 50 °C for 24-48 h. In another work performed by Okazaki, Shigeta, and Aoyama (2007), "Shiokara" of sea cucumber's guts produced by AHHP (60 MPa/30 °C/24 h) were found to present better sensory characteristics than by conventional autolysis.

Non-thermal inactivation of spores. It is known that pressure can induce germination of spores. Once germinated, these spores can be killed. This was found to be possible at pressures lower than 100 MPa if a relatively long holding time and mild temperatures are employed (Aoyama, Shigeta, Okazaki, Hagura, & Suzuki, 2004). In a subsequent study, Aoyama, Shigeta, Okazaki, Hagura, and Suzuki (2005) reported that *Bacillus subtilis* spores pressurized at 60 MPa and 40 °C for 24 hours are germinated due to pressure and the germinated spores are then inactivated before changing into vegetative cells. These works put forward the importance of the pressure holding time in the inactivation of microorganisms.

1.3.3. Investigation needs

Initial studies available in the literature show that hyperbaric storage could be an effective technique for food preservation and even this method has already been patented. When applied at room temperature, the potential advantages of hyperbaric storage are evident but, up to date, there are no industrial applications of this kind of storage. Before the implementation of a new technology in the industry, it is

mandatory to study if it is viable in terms of product safety and quality, as well as, in economic and environmental terms.

Firstly, it is necessary to examine the effectiveness of the method for preserving food quality and safety and to compare it with other existing techniques. Scientific studies about the effect of relatively low pressures (< 200 MPa) applied during long time on food quality characteristics mainly concern safety aspects (see Section 1.3.1). For this reason, pressure effects on other food quality parameters such as sensory or nutritional properties should also be examined after storage.

But interest must not be restricted to the effects observed after hyperbaric storage. The real interest should be focused in knowing how pressure affects the biological, chemical, and biochemical agents responsible for food degradation. Understanding how pressure is able to alter the activity of degradation agents in food throughout the storage should allow for a better management of this method. Virtues and weaknesses should be put in evidence in order to be able to adapt storage conditions in the best possible way.

Once the effects of hyperbaric storage at room temperature on quality and safety parameters are globally studied, it is fundamental to assess the economic viability of the method at industrial scale. This is a prerequisite to evaluate risks and to decide whether the investment could be profitable. One of the main limitations of hyperbaric storage is the high capital cost of the equipment. But, pressure requirements for hyperbaric storage are considerably less severe (pressure level up to 220 MPa) than those currently employed in the food industry for high-pressure processing (pressure level up to 600 MPa). Consequently, technical specifications and constraints are less drastic and the construction should be less expensive. Moreover, equipment cost should also reduce because of the exponential expansion of HPP in the industry and the expected future innovations in this field. In fact, innovations performed during the last years in equipment design have made possible a decreasing trend in equipment costs from 1996 up to now. The identification of new high-pressure applications with proven advantages over current technologies will also contribute to this aim (Balasubramaniam, Farkas, & Turek, 2008; Hogan et al., 2005). On the other hand,

hyperbaric storage at room temperature should be notably advantageous from the point of view of energy savings. Energy is only spent during compression, no additional energy is required to maintain the product under pressure for long times. Therefore, hyperbaric storage of food commodities in industrial size vessels, at room temperature, should involve important economic savings in storage and distribution. Unfortunately, to our knowledge, this point has never been addressed. Furthermore, to date, no design has been proposed for such peculiar industrial high-pressure equipment. Thus, it would be necessary to study in depth the economic viability of hyperbaric storage, taking into account the initial investment, its amortization, the maintenance cost of the installation, and the energy consumption during storage.

Finally, an environmental impact evaluation is also of great interest since, nowadays, there is a growing environmental concern and both global warming and climatic change are considered important threats to our planet. Therefore, diminution of wastewater, gas emissions, and energy consumption are factors that increasingly attract food processors' attention. As an example, it is believed that the cold chain of the food industry is responsible for approximately 2.5 % of global greenhouse gas emissions through direct and indirect (energy consumption) effects (Evans et al., 2014). Hyperbaric storage at room temperature does not need refrigeration facilities and, therefore, it could contribute to diminish greenhouse gas emissions.

1.3.4. Object of study

As revealed from the previous research review and conclusions reported above, existing research on hyperbaric storage is scarce and of limited access due to intellectual property protection. This Thesis proposes to develop a methodological study on hyperbaric storage. To do so, we decided to focus our study on only one food and then to investigate in depth the main characteristics of hyperbaric storage at room temperature for this food. After preliminary storage tests on different foods and for obvious reasons of simplification needs, a liquid matrix was found appropriate for this initial characterization. The selection of **strawberry juice** as the object of study for hyperbaric storage is largely explained in the following Sections.

1.3.4.1. Selection and relevancy

Although fruits and vegetables are generally consumed fresh, nowadays, there is a growing increase of fruits and vegetables minimally processed. Changes in consumption habits of population are the main responsible of this increase in the demand of minimally processed food, with organoleptic and nutritional characteristics similar to unprocessed products, without chemical preservatives, microbiologically safe, and easy to prepare and consume. These new habits have led to an increase in the consumption of prepared fruit and vegetable juices.

Driven by the rising demand of healthy products, the global juices market got an annual growth rate of 3.4 % between 2008 and 2012 (MarketLine, 2013). In 2012, the European Union represented approximately 15.2 % of global juice and nectars consumption. Focusing on Spain, the consumption in 2012 reached the 10 % of UE (AIJN, 2012).

In general, juices and nectars from citrus fruits and apples dominate the market. Nevertheless, in the last decades, new types of fruit juice products have come onto the market, including those elaborated from strawberry. Though strawberry juice is rarely consumed alone, it is employed in mixes and as an ingredient in other products (coulis, sauces, jams, dairy desserts, etc.). As an example, Figure 1.6 shows some strawberry juice products that are available on the international market and the assortment is increasing every year.

In Spain, strawberry is a crop of special relevancy. According to the Food and Agriculture Organization (FAO) of the United Nations, world production of strawberries exceeds 4.5 million tons since 2012. The largest producers among countries where statistical data are available are the United States, Turkey, Spain, Egypt, and Republic of Korea. In 2011, a little more than 30 % of the total production came from the United States and approximately 25 % came from the European Union. Within the European Union, Spain ranks the first position with 24.3 % of the European Union total production, followed by Poland, Germany, and Italy with 15.4 %, 14.3 %, and 13.9 %, respectively. Therefore, it is obvious that strawberry fruit and strawberry products, such as strawberry juice, are of great economic interest for Spain.

Figure 1.6. *Some examples of strawberry juice products currently available on the market.*

Besides, strawberry juice is a highly perishable food. The acidic nature of the product prevents the growth of the main foodborne pathogens, but some of them and many spoilage microorganisms can grow. During storage, both microbial growth and biochemical reactions quickly produce juice deterioration. Therefore, thermal processing and/or cold storage are usually employed to preserve strawberry juice products.

Thus, considering that strawberry juice is a simple liquid matrix, widely employed as a food ingredient, and highly perishable, it was selected as a food model that can be representative of juices and other food ingredients.

1.3.4.2. Quality parameters

The quality of strawberry juice can be defined by several factors such as microbial load, color, viscosity, soluble solids, acidity, aroma, taste, and nutritive factors (minerals, vitamins, etc.). Among them, microbial load, color, viscosity, aroma, and flavor are especially important because they determine consumer acceptance.

Microbial load is of primary interest, mainly due to safety implications, but also because it can seriously affect all the other quality factors. Besides, the limits of microbial load in fruit juices are regulated by the legislations. For example, the Spanish legislation establishes a maximum acceptable value for total aerobic mesophilic counts of 10^5 CFU·g^{-1}. As previously mentioned, the low pH of the strawberry juice prevents the growth of the main foodborne pathogens, but a number of spoilage yeasts and molds and a few acid-tolerant bacteria can grow in the juice.

C**olor** is another important quality characteristic of strawberry juices because this visual property is the first evaluated by consumers. However, the attractive red bright color of strawberry juice is not stable and can be degraded. Previous studies indicated a wide range of parameters and conditions which can affect the color stability of strawberry juice during processing and storage. Temperature, L-ascorbic acid content, and pH-value as well as the presence of certain metal ions, light, oxygen, and the progression of non-enzymatic browning reactions have been related to color changes (Gössinger et al., 2009).

Viscosity is another important quality parameter of strawberry juice which mainly affects mouth feel. It also plays an essential role in holding the solid portion of the juice, that is the cloud, in suspension and avoiding phase separation. Viscosity losses in cloudy juices are attributed to the precipitation of pulps and to the degradation of pectin, through both enzymatic and non-enzymatic mechanisms (Sila et al., 2009). It is widely known that viscosity of strawberry juice decreases during storage (Cao et al., 2012) and the storage temperature plays a relevant role. Besides, factors such as fruit genotype, maturity degree, or processing method also have an influence (Tiziani & Vodovotz, 2005).

Finally, the pleasant **aroma** and **flavor of strawberry juice** are also important quality attributes and they are determinant for consumer acceptance. Studies demonstrate that strawberry aroma contains over 360 identified volatile compounds (Schieberle & Hofmann, 1997). The characteristic aroma is mainly determined by a complex mixture of esters, aldehydes, alcohols, and sulphur compounds. Despite the numerous compounds present in strawberry flavor, only a few of them significantly contribute to

the conception of the overall flavor (Zabetakis & Holden, 1997). Besides, organic acids and sugars are other key components in the perception of flavor (Pelayo, Ebeler, & Kader, 2003; Pérez, Olías, Espada, Olías, & Sanz, 1997). Various authors have proved that the aroma of strawberry juices, both fresh and processed, changes drastically during storage mainly due to enzymatic activity (Aguiló-Aguayo, Oms-Oliu, Soliva-Fortuny, & Martín-Belloso, 2009; Golaszewski, Sims, O'Keefe, Braddock, & Littell, 1998; Siegmund, Derler, & Pfannhauser, 2001). In addition to aroma, the flavor of the strawberry juice changes during storage. Sensory analysis performed by Golaszewski et al. (1998) showed that fresh attributes decreased while off-flavor attributes increased during strawberry juice storage.

1.4. Summary

The primary challenge for food manufactures is to cover a growing consumer demand towards minimally-processed food, ensuring the safety and quality criteria, while remaining economic and sustainable. To reach these demands, numerous novel technologies have been proposed and investigated in the area of food processing in contrast to the scarce innovation that has been proposed in the area of food storage. Among them, high hydrostatic pressure processing is one of the newest technologies studied up to date and it has already been implemented in the food industry. A novel application of high pressure in the food industry could be hyperbaric storage as an innovative technique for food preservation, preferably at room temperature, to avoid all the disadvantages of the cold chain.

Hyperbaric storage consists in subjecting a product under pressures, higher than the atmospheric one (25-220 MPa), during all the storage period. Energy is only required to build up the pressure while, during storage, no additional energy is necessary to maintain it. In hyperbaric storage at room temperature, no temperature control should be needed and, therefore, energy costs must be low. Despite the substantial energy savings, there are no industrial applications, the available information about hyperbaric storage of food is still limited, and the effect of relatively low pressures applied during long times on food characteristics is insufficient. In order to implement

hyperbaric storage at room temperature in the food industry, firstly, it is mandatory to study if it is viable in terms of product safety and quality. Then, it is fundamental to assess the economic viability to decide if the investment could be profitable. Finally, it is also convenient to study the environmental impact due to growing environmental concern.

This Thesis pretends to address as many aspects of this new technology as possible. With this aim, strawberry juice has been selected as the object of study because it is a simple food matrix, widely employed as a food ingredient, and highly perishable.

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Chapter 2: Hypothesis, objectives, and work plan

HYPOTHESIS

Human society has always had to deal with food deterioration. Many storage strategies have been applied to slow down quality degradation processes. Among them, low temperature is, undoubtedly, the most employed in developed countries through the food cold chain. However, despite its effectiveness for preserving the food quality, the cold chain presents several disadvantages. Its adequate management is difficult, expensive, and energy consuming. Moreover, cold storage represents a threat to the environment since it is partially responsible for global warming and climate change. For these reasons, innovation is necessary to improve and diversify the available storage methods. New storage methods have to be found and tested.

In this way, food storage under pressure at room temperature appears as a method worthy of being explored. High pressure is nowadays a reality in the food industry and it is present in a huge number of applications. It is well known that pressure is lethal for many microorganisms, but it has almost no impact on most of the molecules responsible for nutritional and organoleptic properties. Moreover, high pressure is a clean and environmentally friendly technology. Therefore, in the last years, high-pressure processing has been gaining in popularity with an increasing number of food processors distributed all over the world. The emergence of this technology is mainly due to its capability to extend the shelf life of foods with a minimal impact on product quality. Nevertheless, preservation by high-pressure processing is not the only high-pressure application in the food field, and opportunities still exist to develop further this technology. Hyperbaric storage at room temperature could be one of these high-pressure opportunities as an alternative to cold storage.

This storage strategy consists in maintaining foods under a relatively low pressure, usually lower than 220 MPa, during weeks or months with no temperature control. In hyperbaric storage, pressure is intended to be used as a limiting factor for food deterioration, just like temperature in refrigeration. Since no refrigeration is additionally necessary, all drawbacks associated to it would be eliminated.

Although the effectiveness of hyperbaric storage for food preservation was first evidenced in 1968 at low temperature, the available information about hyperbaric storage, especially at room temperature, is almost nonexistent. In fact, to our best knowledge, there was only one scientific paper, published in 2001, about the effectiveness of high pressure storage at room temperature for preserving tilapia fillets during 12 h. Furthermore, although two patents about hyperbaric storage at room temperature have been published, the scientific evidences of its effectiveness, working mechanisms, and industrial viability are lacking. Even so, it is possible to find in the literature some indications that this storage strategy could be potentially interesting for preserving foods.

Considering the benefits that could be achieved by discovering an alternative and complementary storage method to refrigeration, the present Thesis poses the possibility that hyperbaric storage at room temperature could be a novel storage method valid for the preservation of food products during weeks or even months. It is postulated that this method may offer certain important advantages (e.g. sustainability) over conventional storage methods. The founding principles behind this idea are that: high pressure acts over the agents of food deterioration while the quality attributes are well preserved, the use of room temperature eliminates all the concerns related with the cold chain, and high pressure is an environmentally friendly technology.

In more specific words, the main hypotheses at the origin of this Thesis are that: (1) hyperbaric storage at room temperature could be effective to preserve the quality characteristics of high added-value fruit juices, such as strawberry juice, (2) the performance of the method over time could be explained by the inhibitory effect of pressure on the strawberry juice microflora growth, endogenous enzymes activity, and other biochemical degradation mechanisms, and (3) the method could be feasible and viable for storing strawberry juice in industrial quantities in terms of design, costs and environmental impact.

OBJECTIVES

The main objective of this Thesis, stemming from the hypothesis defined in the previous section, is **to characterize hyperbaric storage at room temperature in the frame of the preservation of strawberry juice**. To achieve this goal, the characterization of hyperbaric storage will be addressed from the point of view of the Total Quality concept. Thus, not only the usual microbiological and organoleptic qualities but also the equipment design and economic and environmental issues will be taken into account. The different aspects of this storage method that are addressed can be organized around three poles: the effectiveness of the method for strawberry juice preservation, the effect of the pressure level and storage time on the evolution of quality characteristics of the juice, and the feasibility at industrial scale. From this distinction, three partial objectives emerge:

1. To evaluate the effectiveness of hyperbaric storage at room temperature, as an innovative technique for preserving strawberry juice, compared with cold storage, as the most common method used up to date. The hyperbaric storage characteristics explored under this first objective are: quality, stability after hyperbaric storage, and acceptability of the stored juice.
2. To analyze the effect of pressure level and storage time on the most relevant quality parameters of strawberry juice. The quality parameters examined under this second objective are microbial growth, color, and viscosity.
3. To assess the viability of hyperbaric storage at room temperature for preserving strawberry juice at industrial scale. The hyperbaric storage characteristics evaluated under this third objective are equipment design, storage costs, and environmental impact.

WORK PLAN

In order to reach the objectives proposed, the following general strategic approaches are adopted:

- The maximum pressure is set to 220 MPa. This pressure level represents the maximum pressure of interest in hyperbaric storage at subzero temperatures. Although hyperbaric storage at room temperature has, in principle, no pressure limitations, target pressures higher than 220 MPa would make the implementation of the method at industrial scale difficult.
- Temperature is set constant to 20 °C as an approximation to room temperature. Although the best advantage of hyperbaric storage at room temperature is that temperature control is not needed, in this Thesis, temperature is fixed to 20 °C to avoid potential temperature effects in replicated experiments.
- The maximum period of storage is set to 15 days for all the studies performed. Although longer storage times are possible and interesting, the limitation of storage time is mandatory in order to be able to cover as much characteristics as possible over the time allocated to this Thesis.

More specifically, it is intended to address each partial objective as follow:

1. To achieve the first objective, storage experiments will be conducted at different pressures and at room temperature. Main safety and quality parameters (microbial load, viscosity, color, aroma, and flavor) will be measured and compared with those of juices stored at atmospheric pressure and 5 °C. This will allow for standing out the effectiveness and advantages of hyperbaric storage over refrigeration. Besides, the stability of the juices after decompression will be also studied. This **first objective** will be addressed in Chapter 4 **"Effectiveness of hyperbaric storage at room temperature for strawberry juice preservation: Comparison with refrigeration"** through the following parts:

- Part 4.1: Effectiveness of hyperbaric storage at room temperature for preserving the most relevant quality parameters of strawberry juice.
- Part 4.2: Effectiveness of hyperbaric storage at room temperature for preserving the volatile profile of strawberry juice.
- Part 4.3: Effectiveness of hyperbaric storage at room temperature for preserving the sensory attributes of strawberry juice.

2. To achieve the second objective, the focus will be placed on three quality parameters: microbial load, color, and viscosity. The effect of pressure level and storage time on these quality parameters will be analyzed in detail by studying the evolution of these properties during storage as well as the mechanisms responsible for their degradation. The **second objective** will be developed in Chapter 5 **"Effect of pressure level and storage time on some of the most relevant quality parameters of strawberry juice"** through the following parts:
   - Part 5.1: Effect of hyperbaric storage at room temperature on microbial growth.
   - Part 5.2: Effect of hyperbaric storage at room temperature on color.
   - Part 5.3: Effect of hyperbaric storage at room temperature on pectin-methylesterase activity and serum viscosity.

3. To reach the third objective, several industrial applicability criteria will be addressed, namely the logistic, economic, and ecological ones. The equipment design, the cost analysis of the storage method, and its environmental impact will be studied to define and discuss the domain of hyperbaric storage feasibility and viability. This **third objective** will be dealt with in Chapter 6 **"Application of hyperbaric storage at room temperature at industrial scale: Feasibility and viability study"** through the following parts:

- Part 6.1: Equipment design for hyperbaric storage at industrial scale.
- Part 6.2: Cost analysis of hyperbaric storage in comparison with refrigeration.
- Part 6.3: Evaluation of the environmental impact of hyperbaric storage in comparison with refrigeration.

The work plan of this Thesis is reflected using keywords in the experimental design illustrated in Figure 2.1 on the next page.

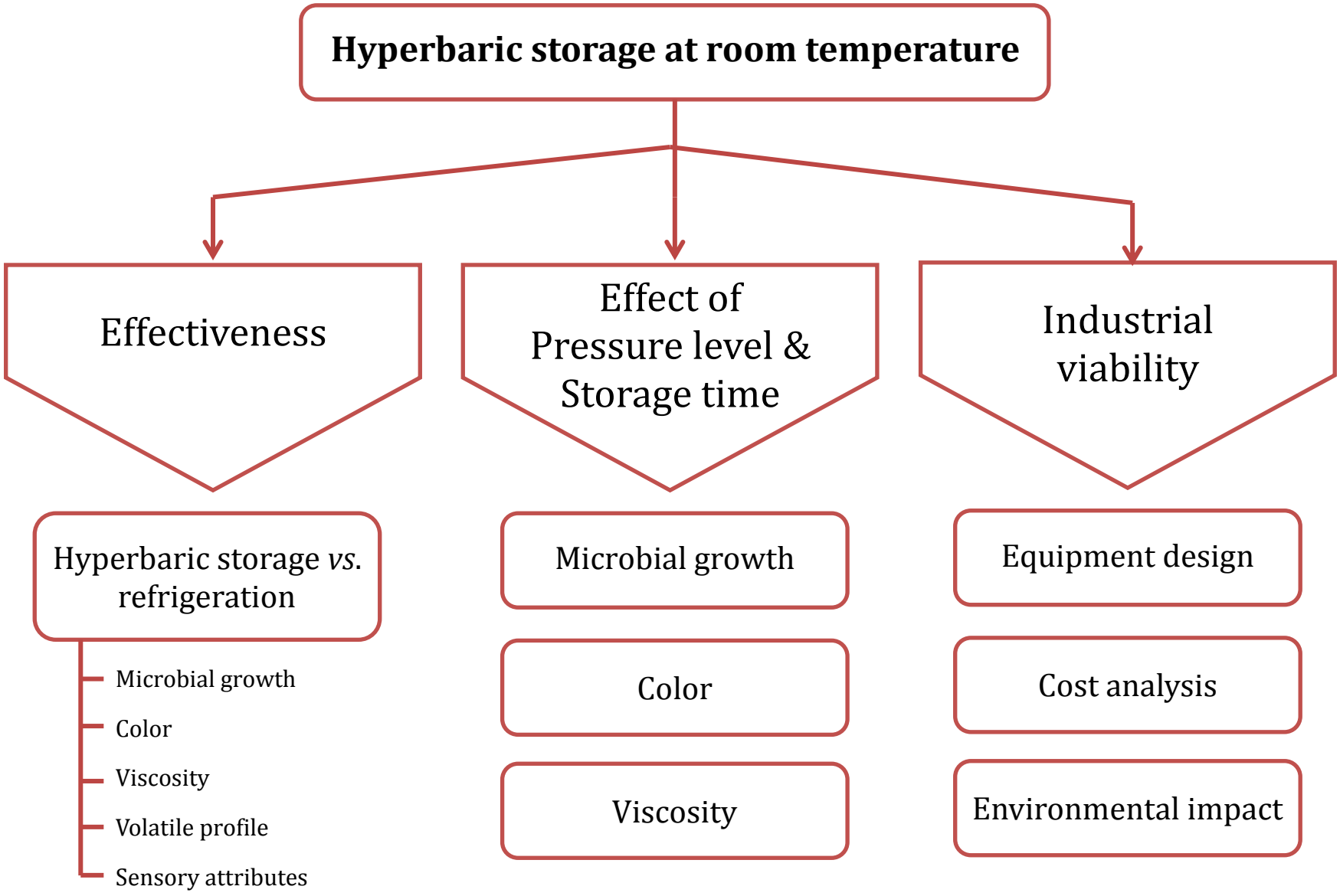

Figure 2.1. *Experimental design of the Thesis.*

Chapter 3: Materials and methods

MATERIALS AND METHODS

3.1. Materials

3.1.1. Strawberry juices

All strawberries (*Fragaria x ananassa* Duch.) employed in this Thesis were purchased at commercial maturity from local suppliers. Different cultivars were used depending on the market availability.

Strawberry juices were always prepared exactly the same way for each experiment. Firstly, the strawberries were washed with tap water, green parts were discarded, and fruits were processed with a juicer (Moulinex Frutti Pro, Moulinex, France). The liquid obtained was then centrifuged at 3,500 *g* and 4 °C for 10 min (Sorvall Evolution RC Superspeed centrifuge, Thermo Scientific, Spain). Finally, the supernatant was subsequently collected and filtered through a 0.1 mm pore diameter sieve.

3.1.2. Chemicals

All reagents and solvents were of analytical grade. The chemicals employed in this Thesis are listed below.

- Acetic acid glacial (Panreac, Barcelona, Spain).
- Acetone (QP, Panreac, Barcelona, Spain).
- Ammonium acetate (≥ 98.0 %, Sigma-Aldrich, St. Louis, USA).
- Apple pectin (70-75 % esterification, Sigma-Aldrich, St. Louis, USA).
- Folin-Ciocalteu's reagent (Panreac, Barcelona, Spain).
- Gallic acid (3, 4, 5-Trihydroxybenzoic acid, Sigma-Aldrich, St. Louis, USA).
- Hydrochloric acid (37 %, QP, Panreac, Barcelona, Spain).
- Hydrogen peroxide (6 % w/v, (20 vol.) stabilized (BP), Panreac, Barcelona, Spain).
- Methanol (≥ 99.5 % (GC), Sigma, Sigma-Aldrich, St. Louis, USA).
- 4-methyl-chatecol (Sigma-Aldrich, St. Louis, USA).
- 2,4-pentanedione (Acetylaceton ≥ 99.0 %, Sigma-Aldrich, St. Louis, USA).

- *Pichia pastoris* (EC. 1.1.3.13, Sigma-Aldrich, St. Louis, USA).
- Phosphate buffer solution (pH 7.4, 0.1 M, Panreac, Barcelona, Spain).
- Potassium chloride (≥ 99.5 %, Panreac, Barcelona, Spain).
- p-phenylenediamine (Sigma-Aldrich, St. Louis, USA).
- Polyvinylpolypirrolidone (Sigma-Aldrich, St. Louis, USA).
- Potassium metabisulfite (≥ 97.0 %, Sigma-Aldrich, St. Louis, USA).
- Sodium acetate trihydrate (≥ 99.0 %, Sigma-Aldrich, St. Louis, USA).
- Sodium di-hydrogen phosphate 1-hydrate (Panreac, Barcelona, Spain).
- Sodium carbonate anhydrous (Panreac, Barcelona, Spain).
- Sodium chloride (≥ 99.5 %, Sigma-Aldrich, St. Louis, USA).
- Sodium hydroxide (Panreac, Barcelona, Spain).
- Sodium phosphate dibasic (≥ 99.0 %, Sigma-Aldrich, St. Louis, USA).
- Tris(hydroxymethyl)aminomethane (ACS reagent, ≥ 99.8 %, Sigma-Aldrich, St. Louis, USA).
- Triton X-100 (Sigma-Aldrich, St. Louis, USA).

3.2. High pressure equipment

3.2.1. Pilot-plant high-pressure storage system

Hyperbaric storage experiments were carried out in a pilot-plant high-pressure storage system (model V1, Institute of High Pressure Physics, Unipress Equipment Division, Poland) (Figure 3.1). It was composed of two independent high-pressure stainless steel vessels (Figure 3.2), two pressure control units, and a high-pressure pump (model BP3, Institute of High Pressure Physics, Unipress Equipment Division, Poland).

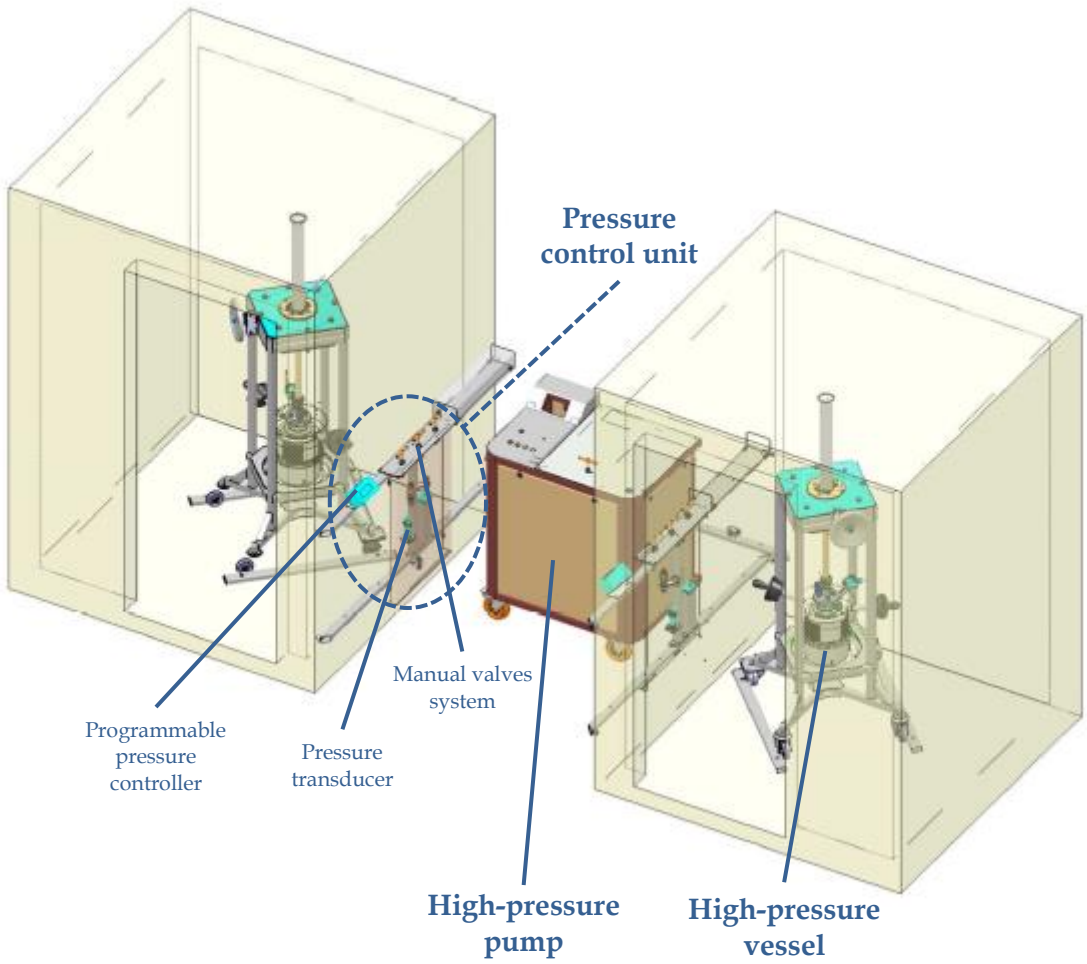

Figure 3.1. *Scheme of the pilot-plant high-pressure storage system V1. Image by courtesy of Institute of High Pressure Physics, Unipress Equipment Division, Poland.*

Each high-pressure vessel was located in an individual thermostatic chamber and was connected, via a feed-through in the chamber wall, to its pressure control unit by means of a high-pressure capillary tube (Figure 3.1). Dimensions of each vessel were internal diameter 100 mm, height 130 mm, and working volume 1 L. The pressure control unit is composed of a pressure sensor to measure the pressure level inside the vessel, a pressure controller to set up and display the pressure, and a set of manual valves that allows keeping the pressure inside the vessels during storage and depressurizing the system when the storage is finished. The high-pressure pump was able to reach a maximal pressure of 250 MPa and was commanded by a programmable controller with a control panel. A mixture of propylene glycol (44 %, v/v) in water was used as the compression fluid.

Figure 3.2. *High-pressure stainless steel vessels used for hyperbaric storage in this work.*

Temperature was measured in each pressure vessel by a metal sheathed thermocouple, type T, located at its geometric center. Pressure produced in the high-pressure vessel by the intensifier was measured by a strain gauge transducer (0-400 MPa, SH-1, WIKA, Germany). All sensor measurements were recorded every 30 s by a data acquisition system (MW100 Data Acquisition Unit, Yokogawa Electric Corporation, Tokyo, Japan). An example of temperature and pressure evolution during hyperbaric storage for 15 days is showed in Appendix 1.

3.2.2. Lab-scale high-pressure equipment

The isothermal/isobaric experiments for simulating enzyme behavior under pressure were carried out in a lab-scale high-pressure equipment U111 (working ranges: 0…700 MPa, -40 °C … +100 °C; Institute of High Pressure Physics, Unipress Equipment Division, Poland) (Figure 3.3). This equipment was composed of a hydraulic power unit (01/5200145, Rexroth Bosch Group Ltd, Poland), an intensifier, and a CuBe alloy vessel (Figure 3.4). Dimensions of the vessel were: internal diameter 30 mm, height 64 mm and working volume 45 mL. An electric power and control unit (X US/2003-Unipress, Poland) allowed programming the pressure and managing the system. Silicone oil (SilOil Type M40.165.10, Huber Kältemaschimenbau GmbH, Germany) was

used as a compression fluid. Homogeneous temperature in the inner volume of the vessel and temperature equilibration after pressure build-up was achieved by immersing the vessel in a tank filled with water. This water was continuously circulating between the tank and a thermostatic bath (Haake F3-K, Fisons Instruments, Karlsruhe, Germany) maintained 0.5 °C above the target temperature (to compensate heat losses).

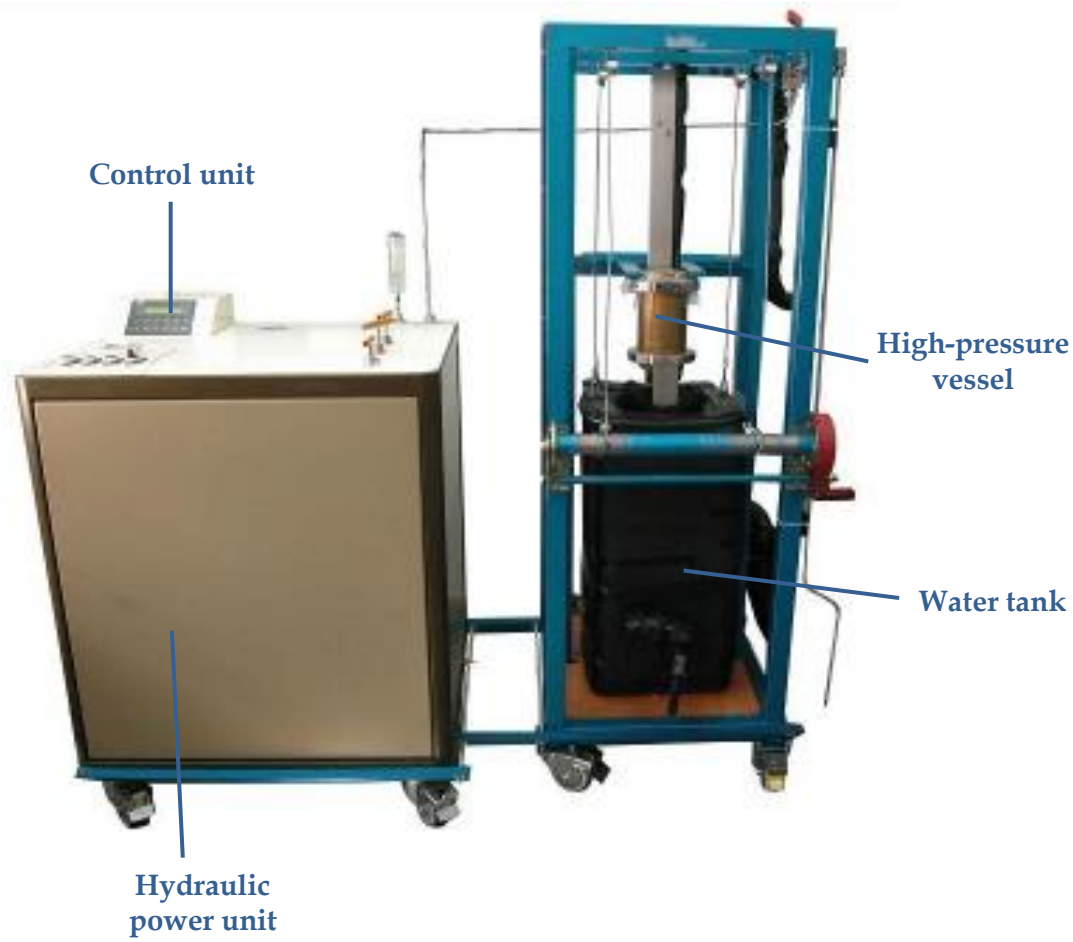

Figure 3.3. *Lab-scale high-pressure equipment used for nearly-isothermal/isobaric experiments.*

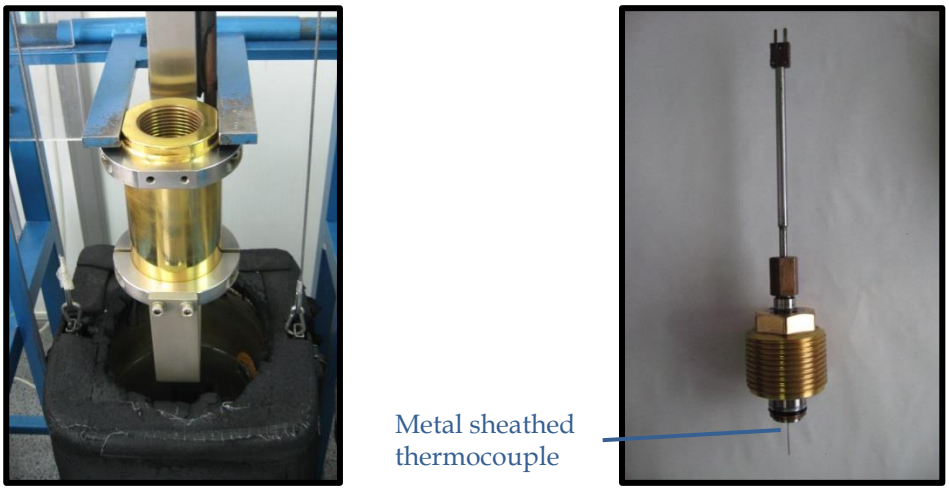

Figure 3.4. *CuBe alloy vessel (left) and screw cap of the vessel (right) used for nearly-isothermal/isobaric experiments.*

Temperatures in the sample and in the vessel were controlled by two metal sheathed thermocouples (TC Ltd., England), type T: one located at the geometric center and the other at the bottom of the vessel. The thermocouples, with a sheath diameter of 1 mm, had a response time of 0.15 s. Pressure produced in the high pressure vessel was measured by a strain gauge transducer (EBM6045 V-0-10 GmbH, KGT Kramer, Dortmund, Germany). All sensor measurements were recorded every 0.5 s by a data acquisition system (MW100 Data Collector, Yokogawa, Tokyo, Japan).

3.3. Methods

3.3.1. Physicochemical determinations

In this section, the methods used to characterize strawberry juice quality parameters and analyze their variation throughout storage are compiled.

3.3.1.1. Determination of total titratable acidity

Total titratable acidity (TA) was determined using an automatic titrator (Titrando 907, Metrohm, Herisau, Switzerland) according to the method described by Friedrich (2001). In brief, strawberry juice was mixed with distilled water (1:3, v/v) and the diluted juice was titrated with a standardized 0.1 N NaOH solution to pH = 8.2. TA was calculated according to equation (3.1) and expressed in g citric acid·mL^{-1} of juice:

$$\mathrm{TA} = \frac{V \times N \times meq.\ wt \times 100}{1000 \times v} \tag{3.1}$$

where V is the volume of sodium hydroxide solution used for titration (mL); N is the normality of sodium hydroxide solution (here, 0.1 N); *meq. wt*. is the milliequivalent weight of the standard acid (*meq. wt.* (citric acid) = 60); and v is the sample volume (mL).

3.3.1.2. Color measurements

A CM-3500d spectrophotometer managed by the color data software CM-S100w SpectraMagic™ (Konica Minolta, Japan) was utilized for color measurement. Strawberry juice color was characterized objectively according to the L^* (lightness), a^* (redness-greenness), and b^* (yellowness-blueness) color parameters in the CIELab uniform color space defined by the Commission Internationale de l'Eclairage. The spectrophotometer operated in the reflectance specular-included mode with an aperture size of 8 mm in diameter. Measurements were made with the D65 standard illuminant and the ultraviolet component of the illumination was included. Illuminating and viewing configurations complied with the CIE diffuse/8° geometry. The instrument was calibrated with black and white (No. 14671004) standards before each series of analyses.

For each sample color analysis, a glass Petri-dish (42 mm internal diameter) was filled with 10 mL of juice and closed with its cap. In each Petri-dish, five measurements were performed: one at its center and four at radial positions distributed 90 degrees apart and the obtained L^*, a^*, and b^* values were averaged. From these mean values, the total color change ΔE^*, hue angle $h°$, and chroma C^* parameters were also calculated. The equations for their calculation are given below:

$$\Delta E^* = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \tag{3.2}$$

Where L_0^*, a_0^*, and b_0^* are values for the juice before the storage.

$$h^\circ = \arctan\left(\frac{b^*}{a^*}\right) \tag{3.3}$$

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad (3.4)$$

3.3.1.3. Determination of total phenolics

Total phenolics (TP) content was determined using the Folin-Ciocalteau method described by Waterhouse (2002) with some modifications. This method is based on the chemical reduction of the Folin reagent, a mixture of tungsten and molybdenum oxides. The products of the metal oxide reduction have a blue color that exhibits a maximum light absorption at 765 nm. The intensity of the light absorption at that wavelength is proportional to the concentration of phenols.

Phenolic compounds were extracted with a solution composed of methanol, distilled water (Type I, Milli-Q system, Millipore, Billerica, MA, USA), and acetone (respective volume fractions: 0.6, 0.3, and 0.1). For extraction, one volume of juice sample was mixed with two volumes of this solution and stirred. Then, the mixture was centrifuged at 14,400 *g* and 4 °C for 10 min (ref. 1468R, Finsen-R, Bunsen S. A., Madrid, Spain) and the supernatant was collected. For the assay, 100 μL of the extract was mixed with 750 μL of Folin reagent and incubated for 6 min at room temperature in the dark. After the incubation, 100 μL of sodium carbonate saturated solution was added and the sample was incubated again for 30 min at 36.7 °C. The absorbance of the sample was read at 765 nm (Genesys 10S UV-Visible, Thermo Scientific, Madrid, Spain). TP content was quantified from a standard curve prepared using gallic acid and it was expressed as mg of gallic acid equivalent (GAE) per liter of juice (mg $GAE \cdot L^{-1}$).

3.3.1.4. Determination of total monomeric anthocyanins

Total monomeric anthocyanins (TMA) content was determined by using the pH differential method of Giusti and Wolstad (2001) with slight modifications. This method is based on reversible structural transformation of the anthocyanin chromophore as a function of pH. The colored oxonium form predominates at pH 1.0 and the colorless hemiketal form at pH 4.5. By using optical spectroscopy, the differences of absorbance caused by the change of pH can be quantified. This method

permits an accurate and rapid measurement of the TMA content, even in the presence of polymerized degraded pigments and other interfering compounds.

For the extraction of the anthocyanin, 1 mL of juice was mixed with 2.5 mL of methanol. After mixing, the solution was centrifuged at 14,500 *g* and 4 °C for 10 minutes (ref. 1468R, Finsen-R, Bunsen S. A., Madrid, Spain) and the supernatant collected. Then, two dilutions of the extract (1:10, v/v) were prepared: one with 0.025 M potassium chloride buffer, pH 1, and the other with 0.4 M sodium acetate buffer, pH 4.5. Finally, the absorbance of each solution was measured in a spectrophotometer (Genesys 10S UV-Visible, Thermo Scientific, Madrid, Spain) at 510 nm and at 700 nm (to correct for haze).

The TMA content was calculated according to the following equation:

$$\left[Pg-3-glu\right]=\frac{\left[\left(A_{510}-A_{700}\right)_{pH1}-\left(A_{510}-A_{700}\right)_{pH4.5}\right]\times MW\times DF\times 1000}{\left(\varepsilon\times L\right)} \qquad (3.5)$$

where A_{510} and A_{700} are the absorbance values measured at 510 nm and 700 nm, respectively; *MW* is the molecular weight of pelargonidin-3-glucoside (*i.e.* 433 g·mol^{-1}); *DF* is the dilution factor of the sample; ε is the molar absorptivity of pelargonidin-3-glucoside (*i.e.* 22,400 L·mol^{-1}·cm^{-1}); and *L* is the length of the light path (1 cm). The results were expressed as milligrams of pelargonidin-3-glucoside per liter of juice (mg Pg-3-glu·L^{-1}) since pelargonidin-3-glucoside (Pg-3-glu) is the predominant anthocyanin in the strawberry juice.

3.3.1.5. Analysis of percent polymeric color

Percent polymeric color (PPC) was determined using the method described by Giusti and Wolstad (2001). This method is based on the selected bleaching of the monomeric anthocyanins by bisulfite solution. Monomeric anthocyanins combine with bisulfite to form a colorless sulfonic acid adduct whereas polymerized colored anthocyanin-tannin complexes are resistant to bleaching by bisulfites. Polymeric color (PC) is the sum of the absorbance values at the $\lambda_{vis\text{-}max}$ and at 420 nm of the bisulfite treated sample, while color density (CD) is the sum of the absorbance values at the same wavelengths but for

the control sample (without bisulfite). A measure of percent polymeric color is obtained as the ratio between these two indexes (Giusti & Wrolstad, 2001).

Extracts were prepared by mixing the juice samples with methanol (1:2.5, v/v). After mixing, the solution was centrifuged at 14,500 *g* and 4 °C for 10 minutes (ref. 1468R, Finsen-R, Bunsen S. A., Madrid, Spain) and the supernatant collected. Sample extracts were diluted with 0.025 M potassium chloride buffer, pH 1, in order to have an absorbance reading between 0.5 and 1.0 at 510 nm when evaluated by spectrophotometry (Genesys 10S UV-Visible, Thermo Scientific, Madrid, Spain). For PC determination, 72 μL of 0.9 M potassium metabisulfite was added to 1 mL of diluted sample. For CD measurements, the 0.9 M potassium metabisulfite was replaced by 72 μL of distilled water. After equilibrating for 15 min at room temperature, samples were measured spectrophotometrically at 700, 510, and 420 nm. PPC was calculated according to the following equations:

$$\mathrm{PC} = \left[\left(A_{420}^{b} - A_{700}^{b}\right) + \left(A_{510}^{b} - A_{700}^{b}\right)\right] \times DF \tag{3.6}$$

$$\mathrm{CD} = \left[\left(A_{420} - A_{700}\right) + \left(A_{510} - A_{700}\right)\right] \times DF \tag{3.7}$$

$$\mathrm{PPC} = \frac{\mathrm{PC}}{\mathrm{CD}} \times 100 \tag{3.8}$$

where A_{420}^{b}, A_{510}^{b}, and A_{700}^{b} are the absorbance values measured at 420, 510, and 700 nm, respectively, in extracts bleached with potassium metabisulfite, A_{420}, A_{510}, and A_{700} are the absorbance values measured at 420, 510, and 700 nm in extracts with no addition of potassium metabisulfite, and *DF* is the dilution factor of the sample.

3.3.1.6. Viscosity measurement

The kinematic viscosity was determined using a Cannon-Fenske reverse-flow glass capillary viscometer (Nº 150, Fungilab S.A., Spain), immersed in a thermostatic water bath (Thermocap, Fungilab S.A., Spain) at 40 ± 0.05 °C. The efflux time was manually measured using a digital stopwatch (Oregon Scientific TR118, Oregon Scientific, Spain). The kinematic viscosity (ν), expressed in centiStokes (1 cSt = 10^{-6} $m^2 \cdot s^{-1}$), was

calculated from the efflux time (t) and the viscometer calibration constant (C_{visco}) at 40 °C, provided by the manufacturer, by using the following equation:

$$\nu = C_{visco} \times t \tag{3.9}$$

3.3.1.7. Determination of methanol released

The amount of methanol formed in the juice due to PME activity was measured by colorimetry according to the method described by Klavons and Bennett (1986). In this method, methanol is oxidized to formaldehyde with alcohol oxidase from *Pichia pastoris*, followed by condensation with 0.02 M 2,4-pentanedione in 2.0 M ammonium acetate and 0.05 M acetic acid to obtain 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine. The resultant colored product was measured spectrophotometrically at 412 nm and 25 °C (Genesys 10S UV-Visible spectrophotometer, Thermo Scientific, Madrid, Spain). The amount of methanol ($\mu g \cdot mL^{-1}$) formed was calculated using a standard curve of methanol (0 to 20 $\mu L \cdot L^{-1}$) dissolved in 0.01 M phosphate buffer, pH 7.4.

3.3.1.8. Headspace volatiles analysis

Volatile analyses in the headspace of strawberry juice samples were performed using an Agilent 6890N Series gas chromatograph coupled to an Agilent 5973 Series mass selective detector (Agilent Technologies, Heilbronn, Germany) and equipped with a TurboMatrix 40 Trap Headspace sampler (Perkin Elmer, Shelton, CT, USA).

Samples were maintained at 80 °C for 25 min to drive the volatile compounds from the strawberry juice into the headspace. Volatile compounds were then sent to the TurboMatrix trap to be concentrated. Four trap load cycles of 5 min were carried out for each vial. The trap was subsequently dried by passing helium (99.995 %) through it for 7 min to remove moisture. Finally, the analytes were thermally desorbed, through a transfer line heated at 110 °C, for 3 min and transported into the injection port of the GC column, at 240 °C and in splitless mode, for separation. Chromatographic separation was achieved on an HP-5MS capillary column (30 m x 0.25 mm i. d.; 0.25 μm film thickness, 5 % Phenyl Methyl Siloxane, Agilent Technologies, Palo Alto, CA, USA), using helium as carrier gas at a constant flow rate of 1.2 $mL \cdot min^{-1}$. The initial oven temperature was held at 40 °C for 4 min, then increased at 4 $°C \cdot min^{-1}$ to 110 °C

and at 6 °C·min^{-1} to 180 °C, maintained at 180 °C for 5 min, then again increased at 8 °C·min^{-1} to 230 °C, and finally held at this temperature for 2 min.

The outlet of the column was coupled to the Agilent 5973 mass selective detector. It operated in electron impact ionization mode at 70 eV, using full-scan acquisition mode from m/z 30 to 550. MS ion source and quadrupole temperatures were 230 °C and 150 °C, respectively.

3.3.2. Microbiological determinations

The methods used to characterize the microbial quality of the juice during and after storage are given in this section.

Of each sample, 1 mL was aseptically taken and added to 9 mL of sterilized peptone water. After homogenization, serially dilutions were made with the same diluent. Then, duplicates of dilutions were plated on the appropriate media according to the procedures detailed below.

3.3.2.1. Total aerobic mesophilic

Total aerobic mesophilic (TAM) counts at 30 °C were determined, following the standard method AFNOR NF V 08-051, by the pour plate method in plate count agar. Plates were incubated at 30 ± 1 °C for 72 ± 3 h and the colonies formed were counted. The detection limit was 1 CFU·mL^{-1}. Plate counts were expressed as logarithmic of colony forming unit (CFU) units per milliliter of strawberry juice (log CFU·mL^{-1}).

3.3.2.2. Yeasts and molds

Yeasts and moulds (YM) were enumerated on Sabouraud Chloramphenicol Agar (SCA) medium by the surface spread plate method, according to the standard method AFNOR XP V 08-059. SCA plates were incubated at 25 ± 1 °C for 5 days and the colonies of yeasts and moulds were counted. The detection limit was 10 CFU·mL^{-1}. Plate counts were expressed as logarithmic of colony forming unit (CFU) units per milliliter of strawberry juice (log CFU·mL^{-1}).

3.3.2.3. Lactic acid bacteria

Lactic acid bacteria (LAB) were quantified in agreement with ISO 15214, by the pour plate method in Agar Man Rogosa and Sharpe. Plates were incubated at 30 ± 1 °C and after 72 ± 3 h the colonies formed were counted. The detection limit was 1 $CFU \cdot mL^{-1}$. Plate counts were expressed as logarithmic of colony forming unit (CFU) units per milliliter of strawberry juice (log $CFU \cdot mL^{-1}$).

3.3.3. Enzymatic determinations

The methods employed to analyze the enzymatic contribution to color and viscosity change in the juice during and after storage are recapitulated below.

3.3.3.1. Extraction and activity assay of polyphenol oxidase and peroxidase enzymes

The extraction of polyphenol oxidase (PPO) and peroxidase (POD) enzymes from the strawberry juice samples to be analyzed was carried out as described by Terefe et al. (2009) with slight modifications. The enzyme extraction solution was 0.2 M sodium phosphate buffer (pH 6.5) consisting of 1 M sodium chloride, 1 % w/v polyvinylpolypirrolidone (PVPP), and 1 % v/v Triton X-100. 1.5 mL of juice was stirred for 10 min at 4 °C with 1.5 mL of enzyme extraction solution. The mixture was then centrifuged at 14,500 *g* and 4 °C for 10 min (ref. 1468R, Finsen-R, Bunsen S. A., Madrid, Spain) and the supernatant was collected to be used as enzymatic extract. The extraction of each juice sample was carried out in triplicate.

For PPO assay, 37.5 μL of enzymatic extract were mixed with 1.5 mL of 0.07 M 4-methyl-chatecol in 0.05 M sodium phosphate buffer (pH 6.5). For POD assay, 100 μL of enzymatic extract were mixed with 750 μL of 1 % p-phenylenediamine in 0.05 M sodium phosphate buffer (pH 6.5) and 50 μL of 1.5 % hydrogen peroxide was added. Blanks were prepared in the same way except that 0.05 M sodium phosphate buffer (pH 6.5) was used instead of the enzyme extract.

The absorbance of the assay mixture was measured either at 420 nm for 10 min (PPO) or at 485 nm for 20 min (POD) using a spectrophotometer (Genesys 10S UV-Visible, Thermo Scientific, Madrid, Spain) in the kinetic mode and recorded every 2 s. PPO and

POD activities were calculated from the slope of the linear portion of their respective plots of absorbance against time (min) and they were expressed as the change of absorbance per minute per milliliter of juice. From these data, residual activity (RA) was calculated according to the following equation:

$$\text{RA}\ (\%) = \frac{A}{A_0} \times 100 \tag{3.10}$$

where A is the enzyme activity of the sample and A_0 is the enzyme activity of the juice at day 0 (i.e. just before storage).

3.3.3.2. Extraction and activity assay of crude pectinmethylesterase extract

The crude strawberry pectinmethylesterase (PME) extract was prepared according to the method described by Houben et al. (2012) with some modifications. Strawberry juice was centrifuged at 14,400 *g* and 4 °C for 10 min. The supernatant was discarded and the pellet was mixed with 0.2 M Tris-HCl extraction buffer (1:2, w/v), pH 8.0, containing 1.0 M NaCl and 1 % w/v polyvinylpolypirrolidone (PVPP). The mixture was stirred overnight at 4 °C and, after extraction, it was centrifuged at 14,400 *g* and 4 °C for 10 min. The supernatant obtained, that is, the crude strawberry PME extract, was divided into aliquots, frozen, and stored at -20 °C until use.

PME activity of the crude extract was measured by titration of the carboxylic groups generated by the enzyme in a pectin solution at pH 7.7 and 30 °C. The reaction mixture consisted of 0.5 mL of crude extract and 20 mL of a 0.4 % apple pectin solution containing 0.117 M NaCl. During hydrolysis, pH was maintained at 7.7 by the addition of 0.01 N NaOH using an automatic pH-stat titrator (Tritando 907, Metrohm, Herisau, Switzerland). The consumption of 0.01 N NaOH, proportional to PME activity, was recorded every 15 s during the 20 min reaction period.

The PME activity unit was defined as the amount of enzyme required to release 1 μmol of carboxyl group per minute under the aforementioned assay conditions.

3.3.3.3. Activity assay of PME enzyme in strawberry juice

PME activity was measured titrimetrically. The reaction mixture consisted of 5 mL of strawberry juice and 20 mL of a 1 % apple pectin solution, pH 7.7, containing 0.117 M NaCl. During hydrolysis, pH was maintained at 7.7 by the addition of 0.05 N NaOH using an automatic pH-stat titrator (Tritando 907, Metrohm, Herisau, Switzerland). The consumption of 0.05 N NaOH, proportional to PME activity, was recorded every 15 s during the 5 min reaction period.

From the data obtained, the residual activity (RA_{PME}) after storage was calculated for each sample according to equation (3.11):

$$\mathrm{RA_{PME}}\ (\%) = \frac{A}{A_0} \times 100 \qquad (3.11)$$

where A is the PME activity of the sample after storage and A_0 is the PME activity of the juice, at day 0, before storage.

3.4. Data analysis

The GC-MS chromatograms obtained were evaluated and integrated using the ChemStation program (Agilent Technologies, Palo Alto, CA, USA).

All the multivariate analyses (Hierarchical Cluster Analysis and Partial Least Squares Discriminant Analysis (PLS-DA) were performed with The Unscrambler® X, v. 10.2 (CAMO Software AS, Oslo, Norway).

The results were statistically analyzed using IBM SPSS Statistics v. 21.0.0.0 for Windows (SPSS Inc., Somers, NY, USA). The details of each data analysis are given in the corresponding Parts.

3.5. References

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Giusti, M. M., & Wrolstad, R. E. (2001). Characterization and Measurement of Anthocyanins by UV-Visible Spectroscopy *Current Protocols in Food Analytical Chemistry*: John Wiley & Sons, Inc.

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Waterhouse, A L. (2002). Determination of total phenolics. *Current protocols in food analytical chemistry*.

Chapter 4: Effectiveness of hyperbaric storage at room temperature for strawberry juice preservation: Comparison with refrigeration

Part 4.1: Effectiveness of hyperbaric storage at room temperature for preserving the most relevant quality parameters of strawberry juice[2]

[2] Segovia-Bravo, K. A., Guignon, B., Bermejo-Prada, A., Sanz, P. D., & Otero, L. (2012). Hyperbaric storage at room temperature for food preservation: a study in strawberry juice. *Innovative Food Science & Emerging Technologies*, *15*, 14-22.

EFFECTIVENESS OF HYPERBARIC STORAGE AT ROOM TEMPERATURE FOR PRESERVING THE MOST RELEVANT QUALITY PARAMETERS OF STRAWBERRY JUICE

4.1.1. Abstract

Hyperbaric storage at room temperature was evaluated as a new food preservation method. To do so, strawberry juices maintained at different pressure levels (0.1, 25, 100, or 220 MPa) and 20 °C for 15 days were compared to raw and thermally treated samples stored at atmospheric pressure and 5 °C for the same period. Hyperbaric storage reduced the initial microbial load of the juices by more than 2 $\log_{10}$ units to levels below the limit of detection. Moreover, pressure was effective to attenuate viscosity, color and sensory deterioration in the samples stored at 20 °C. Stability of the samples after hyperbaric storage was good and microbial load, viscosity, color, and sensory quality remained stable when samples were kept under refrigeration at atmospheric pressure for 15 additional days. All these results show that hyperbaric storage could represent an interesting technology for short-term preservation of strawberry juice.

4.1.2. Introduction

Deterioration of perishable food begins immediately after harvest, fishing, or butchery by the action of microorganisms and deteriorative enzymes. Then, it progressively increases during manufacturing, transportation, retail distribution, and home storage. Low temperature, throughout the food cold chain, is the most applied strategy to avoid or, at least, to retard food spoilage.

The cold chain extends from the initial chilling or freezing of the raw ingredients to the domestic storage of the final food product. Its adequate management is a difficult, expensive, and energy-consuming operation. Moreover, the generation of this energy contributes to CO_2 production, global warming, depletion of the ozone layer, and climatic change, which nowadays are considered major threats to our planet (Tassou,

Lewis, Ge, Hadawey, & Chaer, 2010). For all these reasons, in recent decades, many efforts have been made in the agro-food industry to improve the performance of conventional refrigeration systems, to find new environmentally friendly refrigeration technologies, and also to look for new energy-saving opportunities in food preservation (Masanet, 2008; Tassou et al., 2010; Ullah, Saidur, Ping, Akikur, & Shuvo, 2013). The development of a novel technology that does not need refrigeration facilities could represent an important breakthrough in food storage in terms of energy saving and environmental protection.

In this regard, hyperbaric storage of food at room temperature could represent an important advance in the area of food preservation. As described in Chapter 1, this novel storage technology only involves energy costs during compression and no additional energy is required to maintain the product under pressure for long times. Therefore, at industrial level, it could suppose considerable savings in storage and distribution.

Despite its enormous potential, at the time of this study, no scientific researches to assess the feasibility of this storage technique had been performed. In this Chapter, the first study in the literature about hyperbaric storage of a food product at room temperature is presented. As previously commented, strawberry juice was chosen because it is a simple liquid matrix, widely employed as a food ingredient. For this initial study, the research was focused on two main aspects: (1) juice quality immediately after storage and (2) juice stability after hyperbaric storage when the product is kept under refrigeration at atmospheric pressure. Moreover, to evaluate the feasibility of hyperbaric storage, it was also necessary to compare this novel method to other well-established techniques. Therefore, the aim of this study was to compare the effectiveness of hyperbaric storage versus conventional refrigeration for preserving the quality of strawberry juice.

To do so, raw strawberry juice was stored at different pressures (0.1, 25, 100, and 220 MPa) and 20 °C. After 15 days of storage, some safety, instrumental, and sensory attributes (microbial load, viscosity, color, odor, flavor, and overall acceptance) were evaluated and compared to those of raw and pasteurized samples stored at

atmospheric pressure and 5 °C for the same period. The stability of the samples after decompression was also studied, in order to know if the juice should be immediately processed or consumed after the hyperbaric storage or if it remains stable, under refrigeration, for 15 days.

All the results obtained in this Part provide important information to evaluate the viability of hyperbaric storage at room temperature for food preservation.

4.1.3. Materials and methods

4.1.3.1. Samples

Strawberries (*Fragaria x ananassa* Duch., cv. Chandler) were purchased at commercial maturity from a local supplier. The fruits were washed with tap water and processed with a juicer (Moulinex Frutti Pro, Moulinex, France). The liquid obtained was then centrifuged at 3,500 *g* and 7 °C for 10 min (Sorvall Evolution RC Superspeed centrifuge, Thermo Scientific, Madrid, Spain) using a Fiberlite F8-6x1000y rotor (Thermo Scientific, Madrid, Spain). The supernatant was subsequently collected, filtered through a 0.1 mm pore diameter sieve, and bottled. This juice was frozen and stored at -80 °C until utilization.

4.1.3.2. Physicochemical analysis of the raw material

Before each experiment, a frozen batch of strawberry juice was thawed overnight at 5 °C. This juice was then characterized by measuring some of its physicochemical properties (see Table 4.1.1).

Soluble solids concentration (°Brix) was approximated by using a digital refractometer (Leica AR200, Leica Microsystems Inc, New York, USA) with automatic temperature compensation. pH was measured with a pH-meter (pH-Burette 24 1S equipped with a pH 50 21 electrode and a C.A.T. 55 31 temperature sensor, Crison Instruments, Barcelona, Spain). Density was determined by the vibrating tube technique with a DMA 5000 density-meter (Anton-Paar GmbH, Graz, Austria).

Microbial load, viscosity, and color were estimated as described in the next Sections. All these measurements were performed in triplicate for each thawed batch of juice employed in each experiment.

Parameter	**Mean ± Standard Error**
Soluble solids (°Brix)	7.80 ± 0.06
pH	3.33 ± 0.02
Density ($g \cdot cm^{-3}$)	1.0294 ± 0.0001
Total aerobic mesophiles (log_{10} $CFU \cdot mL^{-1}$)	2.9 ± 0.04
Yeasts and moulds (log_{10} $CFU \cdot mL^{-1}$)	< 2.6
Kinematic viscosity (cSt)	5.01 ± 0.14
L_0^*	27.43 ± 0.02
a_0^*	8.23 ± 0.10
b_0^*	3.68 ± 0.07

Table 4.1.1. *Main characteristics of the raw strawberry juice employed in the experiments.*

4.1.3.3. Storage experiments

Experiments under pressure were carried out in a pilot-plant high-pressure storage system (model SV1, Institute of High Pressure Physics, Unipress Equipment Division, Poland). The equipment was described in Chapter 3.

Strawberry juices were stored for 15 days at 20 ± 2 °C and three different pressure levels (25, 100, and 220 MPa) to obtain the samples labelled as T20_25MPa (20 °C/25 MPa), T20_100MPa (20 °C/100 MPa), and T20_220MPa (20 °C/220 MPa), respectively. After compression, temperature in the samples increased by 1-4 °C, depending on the pressure level applied due to adiabatic heat. This heat was dissipated in less than 15 min. This phenomenon was considered to have negligible effects on the studied characteristics. T20_Patm samples were stored at atmospheric pressure (0.1 MPa) and 20 °C for the same period to make clear the effect of the pressure level on juice preservation.

Cold storage experiments at atmospheric pressure were performed in a thermostatic chamber tempered at 5 ± 2 °C. Both, raw and pasteurized juices were stored for 15 days to obtain samples labelled T5_Patm and TT_T5_Patm, respectively. For pasteurization, samples were immersed in a water bath at 90 °C until the temperature at the core was

maintained at 85 °C for 90 s. Once thermally treated, the juice was immediately cooled in an ice-water bath.

All the samples were stored in 50 mL polypropylene Falcon tubes. The tubes were completely filled (no head-space) with the strawberry juice and closed with screw caps sealed by a nitrile rubber O-ring. All the storage experiments were performed in triplicate.

4.1.3.4. Stability of the strawberry juices after the hyperbaric storage

After 15 days of storage, samples maintained under pressure were decompressed and stored at 5 °C and atmospheric pressure for 15 additional days together with the yet stored T5_Patm and TT_T5_Patm samples. This additional test was to assess the stability of the product after the hyperbaric storage.

4.1.3.5. Safety and quality evaluation in the strawberry juices

Immediately after storage, some safety and quality attributes (microbial population, viscosity, color, and some sensorial parameters) were measured in all the strawberry juices.

Total aerobic mesophiles and **yeasts and molds** were determined as described in Chapter 3. Data were expressed as logarithms of the number of colony-forming units per milliliter ($\log_{10}$ CFU·mL^{-1}). The detection limits were 10 CFU·mL^{-1} for total aerobic mesophiles and 100 CFU·mL^{-1} for yeasts and molds.

The **kinematic viscosity** of the samples was determined as described in Chapter 3. The kinematic viscosity (ν) was expressed in centiStokes (1 cSt = 10^{-6}·m^2·s^{-1}).

Color of strawberry juice was characterized objectively according to the L^*, a^*, and b^* parameters in the CIELab uniform color space defined by the Commission Internationale d'Eclairage. Method is described in Chapter 3. Total color change ΔE^*, hue angle $h°$, and chroma C^* parameters were also calculated.

An **informal hedonic sensory analysis** was carried out on all the stored strawberry juices. Control samples labelled C, that is, thawed strawberry juices at day 0, were also included in the sensory evaluation.

The analysis was performed by untrained personnel of the research group at the ICTAN (CSIC). Blind samples were presented coded in small transparent glasses and they were evaluated by, at least, 17 judges. Each judge scored the sample for each term (color, aroma, flavor, and overall acceptance) according to the following nine-point hedonic scale: 9= like extremely, 7= like moderately, 5= neither like nor dislike, 4= dislike moderately, and 1= dislike extremely.

4.1.3.6. Data analysis

All the storage experiments were performed, at least, in triplicate. Microbial load, viscosity, and color analyses in each sample were also done in triplicate. From these data, means and standard errors were calculated for each storage method.

The results were statistically analyzed using IBM SPSS Statistics v. 19.0.0 for Windows (SPSS Inc., Somers, NY, USA). After a one-way analysis of variance (ANOVA), significant differences among means ($p < 0.05$) were determined by Tukey's multiple range test, when the variances were homogeneous and by Tamhane's T2 test, when it was not possible to assume homoscedasticity.

4.1.4. Results and discussion

4.1.4.1. Stability of the strawberry juices during hyperbaric storage

4.1.4.1.1. Microbial load

The mean initial loads of total aerobic mesophiles and yeasts and molds in the raw strawberry juice, before storage, were 2.9 and < 2.6 $\log_{10}$ CFU·mL^{-1}, respectively (Table 4.1.1). These values are similar to those observed by other authors in juices obtained from fresh strawberries (Keyser, Muller, Cilliers, Nel, & Gouws, 2008; Mosqueda-Melgar, Raybaudi-Massilia, & Martín-Belloso, 2008). Thermal pasteurization at 85 °C for 90 s reduced both population levels below the detection limits.

After 15 days of storage at 20 °C, microbial growth was evident in juices maintained at atmospheric pressure (0.1 MPa) as expected. Both total aerobic plate counts and yeasts and molds increased by more than 3 $\log_{10}$ units (Table 4.1.2). T20_Patm samples were spoiled and off flavors, unpleasant odors, and gas production were detected in them. On the contrary, all the samples stored under pressure (25, 100, or 220 MPa) at 20 °C reduced their natural microflora below the detection limits. Moreover, the pressure level employed during the storage (25-220 MPa) did not have a significant effect on the microbial load after 15 days.

Sample	T20_Patm	T20_25MPa	T20_100MPa	T20_220MPa	T5_Patm	TT_T5_Patm
Total aerobic mesophiles	6.0 ± 0.1 ^{a}	< 1 ^{c}	< 1 ^{c}	< 1 ^{c}	5.1 ± 0.1 ^{b}	< 1 ^{c}
Yeasts and moulds	5.8 ± 0.1 ^{a}	< 2 ^{c}	< 2 ^{c}	< 2 ^{c}	2.6 ± 0.1 ^{b}	< 2 ^{c}

***Table 4.1.2**. Microbial counts (mean values ± standard error, $\log_{10}$ CFU·mL^{-1}) in strawberry juices stored for 15 days at different conditions. Different letters within a row indicate significant differences ($p < 0.05$) between means.*

On the other hand, cold storage at 5 °C was hardly efficient to slow down the microbial growth in the raw juices stored for 15 days at atmospheric pressure. Thus, total aerobic plate counts increased by more than 2 $\log_{10}$ units in T5_Patm samples as compared to 3 $\log_{10}$ units in T20_Patm samples. Refrigeration, unlike hyperbaric storage, was not effective to avoid microbial growth in raw juices and thermal pasteurization was needed to obtain stable strawberry juices for 15 days at 5 °C (Table 4.1.2). Therefore, hyperbaric storage is more efficient than refrigeration in terms of microbial load, since it not only avoids microbial growth, but also reduces the initial population.

It is widely recognized that high hydrostatic pressures, between 10 and 100 MPa, are generally nonlethal for those microorganisms adapted to atmospheric conditions, but they exert adverse effects on them and reduce their growth (Abe, 2007; Abe & Horikoshi, 2000; Bartlett, 2002; Matsumura, Keller, & Marquis, 1974). The inhibitory effect of pressure on the growth of the natural microflora present in different food

products has been already reported by Charm, Longmaid, and Carver (1977). These authors did not find any increase of the total bacteria counts in cod fillets, beef, and chicken samples stored at 27 MPa and temperatures close to 0 °C for 10 to 60 days. Similar results were found by Deuchi and Hayashi (1992) in unfrozen ground beef stored under pressure (50-200 MPa) at subzero temperatures for a few days or weeks. Coliforms, *Enterobacteriaceae*, Gram (-) and Gram (+) psychrophiles, *Enterococci*, and lactic acid bacteria counts in beef decreased after the hyperbaric storage.

In researches posterior to our study, it have been also reported that hyperbaric storage at moderate pressures (≤ 150 MPa) and room temperature is effective to inhibit microbial growth and even to reduce the initial microbial load in melon and watermelon juices. Thus, Fidalgo et al. (2013) concluded that hyperbaric storage at 100 MPa and room temperature allows for a better preservation of watermelon juice compared to refrigeration. These authors found that, after 8 hours of hyperbaric storage, total aerobic mesophiles, *Enterobacteriaceae*, and yeasts and molds reduced by 1, 2, and 1 $\log_{10}$ units, respectively, with no further changes up to the end of storage (60 h). By contrast, yeasts and molds in juices stored at atmospheric pressure and 5 °C were above the limits admissible for consumption. Queirós et al. (2014) also showed similar findings for melon juice stored under pressure (50-150 MPa/25-37 °C) for 8 hours. However, these authors did not find microbial growth inhibition during storage at 25 MPa and 30 °C. These results contrast with our findings in strawberry juice which show significant microbial reduction after 15 days of storage at 25 MPa. But it is important to note that the storage time in melon juice was much shorter (8 h vs. 15 days) and the pH of this juice is considerably higher than that of strawberry juice (5.7 *vs.* 3.3).

The microbial inactivation detected in strawberry juice could be related not only with the pressure applied during storage, but also with the acidic nature of the juice that could be enhanced by the reversible pH shift that aqueous solutions undergo under pressure (Neuman, Kauzmann, & Zipp, 1973). It is well known that pressure effects on microorganisms depend not only on the magnitude and duration of the pressure applied, but also on other physicochemical factors like temperature, pH, or the

composition of the culture media. In this sense, Matsumura et al. (1974) showed that pressure markedly narrows the pH ranges for growth of a variety of bacteria and, therefore, the lower limit of pH required to allow for microbial growth is higher under pressure than at atmospheric conditions. Moreover, many different authors in the literature have shown that as pH is lowered, most microbes become more susceptible to high-pressure inactivation (Farkas & Hoover, 2000; Garcia-Graells, Hauben, & Michiels, 1998; Linton, McClements, & Patterson, 1999; Smelt, 1998).

4.1.4.1.2. Viscosity

Viscosity is an important physical property and quality parameter in fruit juices since it determines consumer acceptance. This parameter mainly affects the mouthfeel and the ability to hold solids in suspension for all the product shelf life.

The initial kinematic viscosity measured in the raw strawberry juice was 5.01 ± 0.14 cSt (see Table 4.1.1). The thermal treatment applied to pasteurize samples severely reduced viscosity by more than 50 % (Figure 4.1.1) probably due to the thermal degradation of pectic substances. Similar viscosity reductions were found by other authors in tomato and guava juices after thermal processing (Thakur, Singh, & Nelson, 1997; Yen & Lin, 1999).

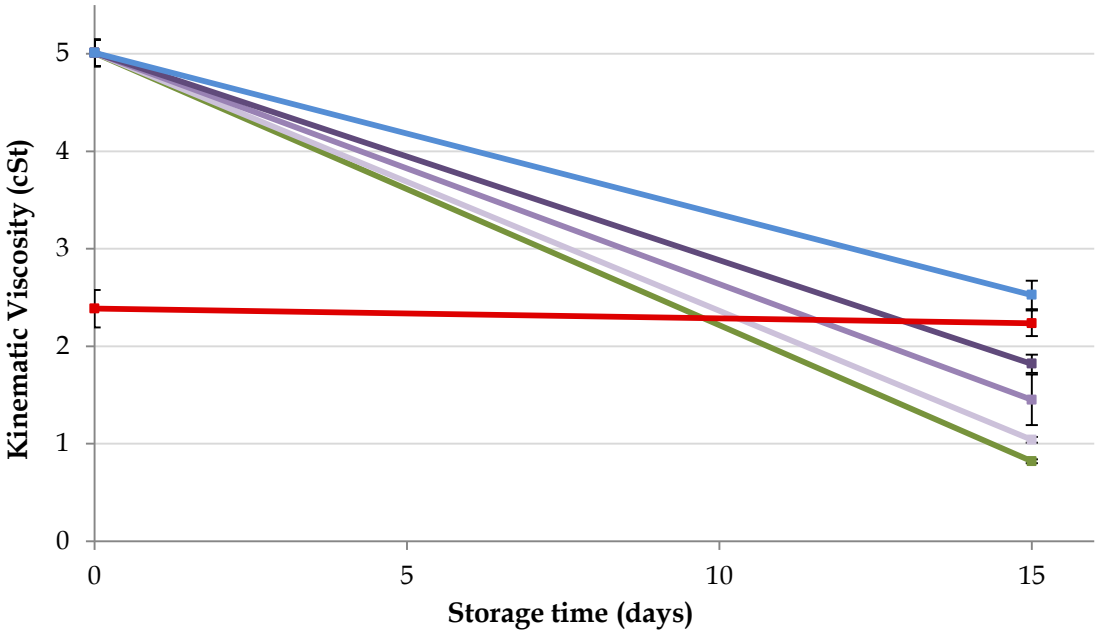

Figure 4.1.1. *Kinematic viscosity (cSt) in strawberry juices stored at different conditions. (T20_Patm (—), T20_25MPa (—), T20_100MPa (—), T20_220MPa (—), T5_Patm (—), and TT_T5_Patm (—)). Vertical bars represent standard error.*

After 15 days of storage, viscosity dropped significantly in all the samples stored at 20 °C (Figure 4.1.1). The reduction was significantly more pronounced ($p < 0.05$) in those samples maintained at atmospheric pressure (T20_Patm), where the viscosity values were less than 1 cSt. In these samples, separation of phases was clearly evident. On the contrary, neither losses of turbidity nor separation of phases were observed in samples stored under pressure, though a small amount of sediments appeared at the bottom of the sample containers.

Hyperbaric storage was effective to attenuate, to a certain extent, the viscosity decay. Thus, viscosity reduced by 79 %, 71 %, and 64 % in T20_25MPa, T20_100MPa, and T20_220MPa samples, respectively, as compared to 84 % in T20_Patm samples. Pressure level applied during storage had a significant effect ($p < 0.05$) and the higher the pressure, the lower was the decrease in the viscosity observed. Viscosity losses, during storage of fruit juices, are mainly related to the depolymerization of pectin caused by the combined action of different endogenous pectinases (Duvetter, Sila, Van Buggenhout, Jolie, Van Loey, & Hendrickx, 2009). Among them, pectin methylesterase (PME) and polygalacturonase (PG) are the most widely studied in the literature. PME

catalyzes the de-esterification of pectin, releasing methanol and low-methoxyl pectin. This de-esterified pectin is the substrate for PG which subsequently catalyzes its depolymerization and, in this way, drastic decreases in the viscosity of juices can occur during storage.

It is well known that pressure can induce structural rearrangements in enzymes which can cause their activation, especially at relatively low pressures (~ 100 MPa), or produce their partial or total inactivation, in a reversible or irreversible manner. An apparent enzyme activation can also be produced by pressure-induced disruption of intact tissues which enhances enzyme extraction and enzyme-substrate interactions (Hendrickx, Ludikhuyze, Van den Broeck, & Weemaes, 1998). The specific effect of pressure on a particular enzyme depends on several factors such as the structure of the enzyme, its origin, the medium composition, pH, or the temperature and pressure levels applied, among others. Different authors have shown that PME from different plant sources like pepper (Castro, Van Loey, Saraiva, Smout, & Hendrickx, 2006), tomato (Crelier, Robert, Claude, & Juillerat, 2001), white grapefruit (Guiavarc'h, Segovia, Hendrickx, & Van Loey, 2005), plum (Nunes, Castro, Saraiva, Coimbra, Hendrickx, & Van Loey, 2006), or carrot (Trejo Araya et al., 2007) can be regarded as a pressure resistant enzyme since pressures higher than 700 MPa are usually required to induce short-term (i.e., minutes) inactivation at room temperature. In purified strawberry PME, pressures from 850 MPa, at 10 °C and pH = 7, were needed to reach some inactivation. Moreover, the occurrence of a pressure-stable PME fraction, that contributes to about 10 % of the total activity, was detected (Ly Nguyen, Van Loey, Fachin, Verlent, & Hendrickx, 2002). Pressure effects on PG are considerably less studied and no data have been found on strawberry PG. Available data on tomato show that tomato PG is much more pressure-labile than PME. Thus, an almost complete PG inactivation has been described in cherry tomatoes after a pressure treatment at 500 MPa and room temperature (Tangwongchai, Ledward, & Ames, 2000). Similar results were found by Crelier et al. (2001) and Fachin, Van Loey, Ly Nguyen, Verlent, Indrawati, and Hendrickx (2003) in tomato juice and by Shook, Shellhammer, and Schwartz (2001) in tomato dices. Nevertheless, no significant PG inactivation has been described at pressures lower than 350 MPa (Crelier et al., 2001; Fachin et al., 2003;

Shook et al., 2001; Tangwongchai et al., 2000). All these data show that, in principle, no significant PME and PG inactivation should be expected at the pressure/temperature conditions employed in this study, although the effect of long times under pressure should not be neglected.

However, in hyperbaric storage, it is important to evaluate not only the pressure stability of enzymes, but also their catalytic activity under pressure. Pressure can induce changes in the rate of enzyme-catalyzed reactions and they can be accelerated or decelerated under pressure. These changes, as Eisenmenger and Reyes de Corcuera (2009) pointed out, can be produced by pressure-induced changes in the structure of enzymes or in the reaction mechanisms, for example, a change in the rate-limiting step. Moreover, pressure can also induce changes in the physicochemical properties (e.g. pH, density, viscosity, and phase) of the substrate and/or solvent that affect the enzyme structure or the rate-limiting step. Previous studies in the literature, most of them in model systems, showed that PME and PG activities under pressure are highly dependent on their origin, the substrate employed, the ionic environment, and the temperature and pressure levels applied (Duvetter et al., 2006; Sila, Smout, Satara, Truong, Van Loey, & Hendrickx, 2007; Van Den Broeck, Ludikhuyze, Van Loey, & Hendrickx, 2000; Verlent, Van Loey, Smout, Duvetter, & Hendrickx, 2004a). Unfortunately, studies at 20 °C and at the low pressures levels employed in this study are very scarce and no conclusive data can be extracted from them. In general terms, PME activity, at temperatures between 30 °C and 65 °C, increases with pressure up to an optimal pressure level and then decreases with increasing pressure (Duvetter et al., 2006; Sila et al., 2007; Van Den Broeck et al., 2000). But, Van Den Broeck et al. (2000) found that purified tomato PME activity, at 20 °C and neutral pH, was slightly lower at pressures up to 300 MPa than at atmospheric conditions. A subsequent study by the same research group (Verlent, Van Loey, Smout, Duvetter, Ly Nguyen, & Hendrickx, 2004b) in purified tomato PME, at pH = 4.4 and pH = 8, showed that pressure up to 450 MPa accelerates the PME catalyzed de-esterification of pectin at temperatures between 30 °C and 65 °C. This effect was clearly dependent on temperature and pH with the least response at the lowest pH and temperature conditions assayed. Regarding PG, different studies in purified tomato PG showed a reduced activity

under pressure (100-400 MPa) at temperatures between 30 °C and 50 °C (Verlent, Smout, Duvetter, Hendrickx, & Van Loey, 2005; Verlent et al., 2004a). This reduced PG activity under pressure could justify the results obtained in this study which prove that pressures up to 220 MPa are effective to slow down viscosity losses in raw strawberry juice during storage at 20 °C. But, it is important to note that all the previous results, obtained with purified PME and PG in buffer solutions, may not be representative of real tomato or strawberry products and more research work on the activity of pectolytic enzymes under pressure is needed to convincingly explain the results obtained in this study.

Refrigeration was significantly more efficient than hyperbaric storage to slow down viscosity decay in raw strawberry juices. No significant cloud losses were observed in refrigerated samples (T5_Patm or TT_T5_Patm), although a small amount of sediments, similar to that found in samples stored under pressure, was distinguished at the bottom of the sample containers.

Figure 4.1.1 shows how viscosity in T5_Patm samples is reduced by 50 % after 15 days of storage. Storage at low temperature is widely recognized as an effective method to reduce the activity of pectin-hydrolyzing enzymes (Imsabai, Ketsa, & Van Doorn, 2002), but Figure 4.1.1 shows that thermal pasteurization is needed if viscosity decay must be delayed for long times. Thermal pasteurization is able to reduce PME and PG activities and, in this way, it allows for long-term preservation of refrigerated samples. In general terms, PME can be considered a rather heat-labile enzyme while PG is very heat-resistant (Duvetter et al., 2009). Thus, in strawberry juices, Aguiló-Aguayo, Soliva-Fortuny, and Martín-Belloso (2009) found PME and PG residual activities of 22.2 % and 76.2 %, respectively, after thermal processing at 90 °C for 60 s. Therefore, the thermal treatment applied to TT_T5_Patm samples in this study is expected to cause an important decrease on PME activity, although it should hardly affect PG activity. This decrease on PME activity altogether with the cold storage applied should strongly slow down the depolymerization of pectin and it can explain the high stability found in the viscosity of TT_T5_Patm samples.

4.1.4.1.3. Color

The bright red color of strawberry juice is one of the most important quality parameters to which consumers are sensitive, but it easily degrades during processing and storage. Instrumental color parameters (L^*, a^*, and b^*) initially measured in the raw strawberry juice are shown in Table 4.1.1. The thermal treatment applied to pasteurize the samples caused a slight, but significant ($p < 0.05$), increase in L^* values ($L^* = 27.71 \pm 0.05$), but no changes were found in redness ($a^* = 7.99 \pm 0.08$) and yellowness ($b^* = 3.60 \pm 0.06$). Thus, the total color change was quite small ($\Delta E^* = 0.43 \pm 0.04$) in these samples. Similar results were found by Gössinger et al. (2009a) in strawberry nectar thermally treated at 85 °C for 10 min. Aguiló-Aguayo et al. (2009) also found an increase in the lightness of strawberry juices thermally treated at 90 °C for either 30 s or 60 s, but they reported a significant decrease in a^*/b^* probably due to the more severe thermal conditions applied.

Storage at 20 °C for 15 days produced color losses in all the samples (Table 4.1.3). The color decay was considerably more pronounced in those samples stored at atmospheric pressure (T20_Patm) as expected. In these samples, significant alterations ($p < 0.05$) in L^*, a^*, and b^* were found: lightness increased by 9.6 % and redness and yellowness decreased by 33.5 % and 64.1 %, respectively. Thus, the color of T20_Patm samples became less intense and more violet, which was indicated by a significant decrease ($p < 0.05$) in chroma and hue values (Figure 4.1.2). These color changes produced the highest $\Delta E^* = 4.5 \pm 0.4$ value.

Sample	T20_Patm	T20_25MPa	T20_100MPa	T20_220MPa	T5_Patm	TT_T5_Patm
L^*	30.05 ± 0.20 ^{c}	27.63± 0.04 ^{a}	27.48 ± 0.06 ^{a}	27.45 ± 0.05 ^{a}	27.45 ± 0.05 ^{a}	28.05 ± 0.05 ^{b}
a^*	5.47 ± 0.32 ^{c}	7.16 ± 0.06 ^{b}	7.13 ± 0.07 ^{b}	7.21 ± 0.04 ^{b}	7.91 ± 0.06 ^{a}	7.95 ± 0.09 ^{a}
b^*	1.32 ± 0.29 ^{c}	3.02 ± 0.04 ^{b}	2.94 ± 0.05 ^{b}	2.94 ± 0.03 ^{b}	3.49 ± 0.07 ^{ab}	3.64 ± 0.07 ^{a}
ΔE^*	4.5 ± 0.4 ^{a}	1.3 ± 0.1 ^{b}	1.3 ± 0.1 ^{b}	1.3 ± 0.0 ^{b}	0.4 ± 0.1 ^{c}	0.72 ± 0.0 ^{c}

Table 4.1.3. *Instrumental color parameters (L*: lightness, a*: redness, b*: yellowness, and ΔE*: total color change, mean values ± standard error) in strawberry juices stored for 15 days at different conditions. Different letters within a row indicate significant differences ($p < 0.05$) between means.*

Hyperbaric storage was effective to substantially attenuate the color degradation in samples stored at 20 °C. Thus, maximal L^* increases of 0.8 % were detected in T20_25MPa samples and maximal a^* and b^* decreases of 14 % and 20 % were found in T20_25MPa and T20_100MPa samples, respectively. This resulted in a reduced degradation of hue and chroma values as compared to T20_Patm samples (Figure 4.1.2). Pressure level applied during storage had not a significant effect ($p < 0.05$) on the color decay in the pressure range studied (up to 220 MPa) and ΔE^* was 1.3 ± 0.1 in all the samples stored under pressure (Table 4.1.3).

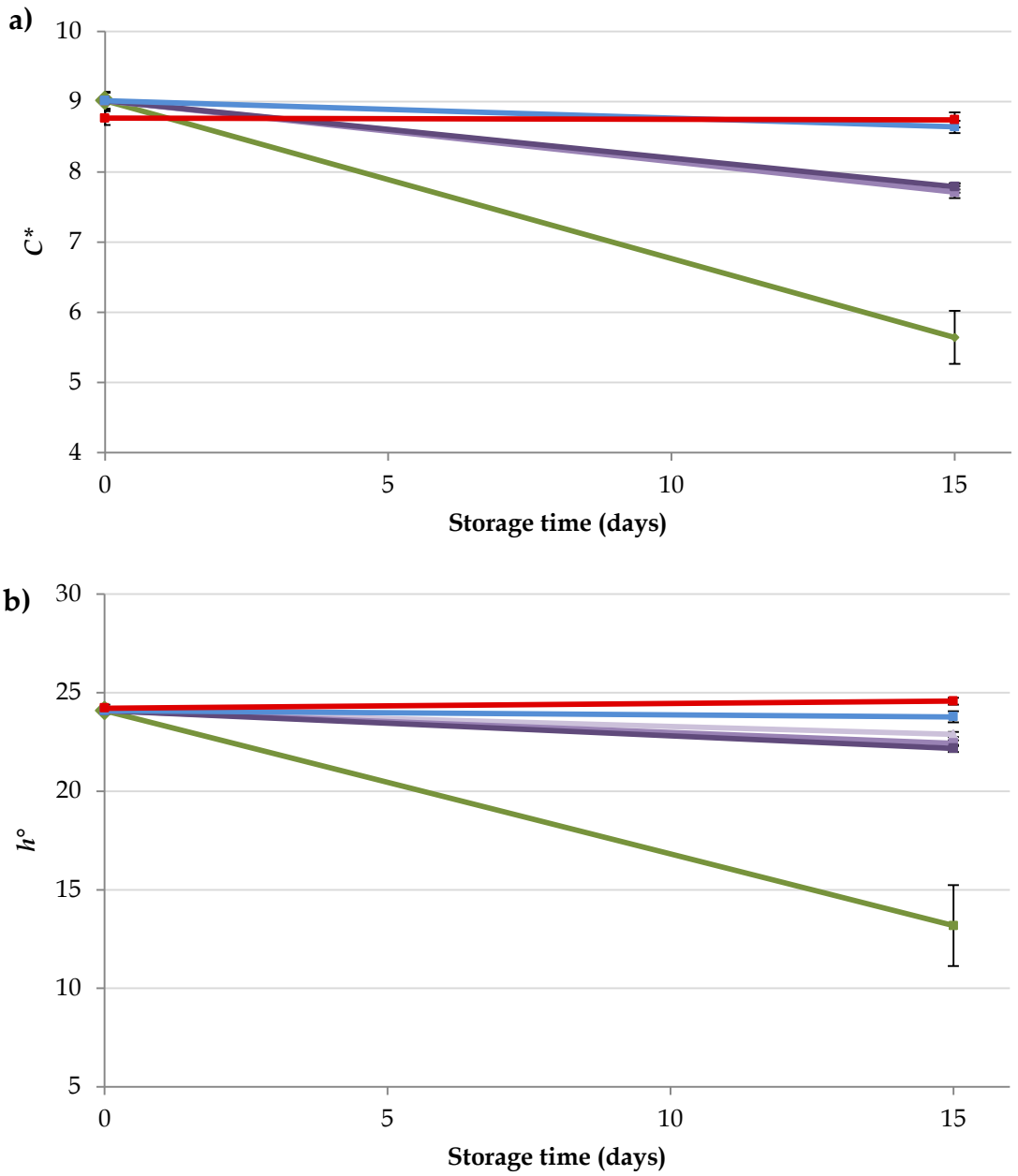

Figure 4.1.2. *a) Chroma (C*) and b) hue (h°) evolution in strawberry juices stored at different conditions. (T20_Patm (—), T20_25MPa (—), T20_100MPa (—), T20_220MPa (—), T5_Patm (—), and TT_T5_Patm (—)). Vertical bars represent standard error.*

It is well known that the attractive color of strawberry juice mainly comes from the anthocyanins present in the fruit. Pelargonidin-3-glucoside is the major anthocyanin found in cultivated strawberries, although other compounds such as pelargonidin-3-rutinoside and cyanidin-3-glucoside are also found in smaller concentrations (Aaby, Mazur, Nes, & Skrede, 2012; García-Viguera, Zafrilla, & Tomás-Barberán, 1998). Anthocyanins may degrade during storage of juices and several factors such as light,

temperature, pH, and presence of oxygen, certain metal ions, or L-ascorbic acid, among others, are implicated. All these parameters also affect the condensation of anthocyanins (self-association) and copigmentation phenomena (interaction of anthocyanin with polyphenols) which produce color changes in the juice. Nevertheless, the key role in color degradation during juice storage is commonly attributed to the presence of some enzymes such as polyphenoloxidase (PPO), peroxidase (POD), and β-glucosidase (β-Glu) which can be responsible for anthocyanin degradation (Chisari, Barbagallo, & Spagna, 2007; López-Serrano & Ros Barceló, 2002; Zabetakis, Leclerc, & Kajda, 2000).

Figure 4.1.2 and Table 4.1.3 clearly show that hyperbaric storage was efficient to attenuate, to a great extent, the color decay in samples stored at 20 °C. These results must be obviously related to the limited microbial activity detected during hyperbaric storage, but the effect of pressure on other agents responsible for color degradation must be also considered. Different authors have shown that high-pressure processing (200-800 MPa) for some minutes, at low and moderate temperatures, has a limited effect on the color and anthocyanin content of different fruits (Cao, Zhang, Zhang, Wang, Yi, & Liao, 2011; Kouniaki, Kajda, & Zabetakis, 2004; Oey, Lille, Van Loey, & Hendrickx, 2008; Patras, Brunton, Da Pieve, & Butler, 2009; Terefe, Matthies, Simons, & Versteeg, 2009; Zabetakis et al., 2000) but, the effect of long-term storage under pressure has not been studied yet. The reduced color decay found in samples stored under pressure as compared to T20_Patm samples could be indicative of a slowdown in anthocyanin degradation under pressure. It could be related to some partial PPO, POD, or β-Glu inactivation since different authors have shown that high-pressure processing for some minutes can produce a partial inactivation of these enzymes in strawberry products (Cano, Hernandez, & De Ancos, 1997; Cao et al., 2011; Dalmadi, Rapeanu, Van Loey, Smout, & Hendrickx, 2006; Garcia-Palazon, Suthanthangjai, Kajda, & Zabetakis, 2004; Terefe, Yang, Knoerzer, Buckow, & Versteeg, 2010; Zabetakis et al., 2000). On the other hand, no studies have been found in the literature about PPO, POD, or β-Glu activities under pressure but, as previously commented, pressure can also induce changes in the rate of enzyme-catalyzed reactions (Eisenmenger & Reyes de Corcuera, 2009). In this sense, the aforementioned reversible pH shift that aqueous

solutions undergo under pressure (Neuman et al., 1973) should affect the rate of PPO and POD catalyzed reactions under pressure since Chisari et al. (2007) and Dalmadi et al. (2006) have proved that strawberry PPO and POD activities are strongly affected by pH.

Therefore, the improved stability of color in samples stored under pressure could be related, not only with the inhibition produced on the microbial growth, but also to some partial PPO, POD, or β-Glu inactivation and/or some reduction in their catalytic activity under pressure. However, specific experiments should be designed to probe the hypothesis presented. Moreover, other mechanisms implied in anthocyanin degradation, apart from enzymatic browning, should not be neglected.

On the other hand, Figure 4.1.2 and Table 4.1.3 show that refrigeration was slightly more efficient than hyperbaric storage to preserve color in strawberry juice. Raw and pasteurized samples only suffered minor changes in the color parameters measured after 15 days of cold storage at 5 °C. Thus, only some significant differences ($p < 0.05$) appeared through the statistical analysis: a significant increase of 1.3 % in L^* values was detected in TT_T5_Patm samples while a significant decrease of 3.9 % in a^* values was perceived in T5_Patm samples. These color degradations were, in any case, small and they involved limited total color changes: $\Delta E^* = 0.7 \pm 0.0$ and $\Delta E^* = 0.4 \pm 0.1$ in TT_T5_Patm and T5_Patm samples, respectively. These ΔE^* values were rather close to those produced in samples stored under pressure and, therefore, no sensory differences should be noticed between cold and pressure stored samples after 15 days of storage since a threshold value of $\Delta E^* = 1$ is frequently assumed as a basis for a color perceptible difference (Gonnet, 1998; Rein & Heinonen, 2004).

The results obtained confirm previous findings in the literature which proved that storage at low temperature is an efficient method to slow down the degradation of color components and anthocyanins (García-Viguera, Zafrilla, Romero, Abellán, Artés, & Tomás-Barberán, 1999; Gössinger et al., 2009a; Wang & Xu, 2007) and to decrease the activity of PPO and POD (Chisari et al., 2007).

4.1.4.1.4. Hedonic sensory analysis

An informal hedonic sensory analysis was carried out to have a general idea about the quality of the juice immediately after hyperbaric storage. Although it was performed informally, it provides valuable information because sensory quality is one of the most relevant parameters to assess the viability of hyperbaric storage.

Table 4.1.4 presents the scores of the juices stored for 15 days at different conditions for color, odor, flavor, and overall acceptance. Control samples (C, juices at day 0) are included in the analysis to have a reference of the initial quality of the juice.

Sample	Color	Odor	Flavor	Overall Acceptance
C	6.83 ± 0.22 ^{a}	7.12 ± 0.29 ^{a}	6.76 ± 0.32 ^{a}	6.82 ± 0.29 ^{a}
T20_Patm	3.80 ± 0.36 ^{b}	2.16 ± 0.34 ^{b}	1.82 ± 0.26 ^{c}	2.06 ± 0.26 ^{c}
T20_25MPa	6.53 ± 0.25 ^{a}	5.82 ± 0.33 ^{a}	5.19 ± 0.38 ^{b}	5.25 ± 0.36 ^{b}
T20_100MPa	6.34 ± 0.29 ^{a}	6.36 ± 0.26 ^{a}	6.00 ± 0.33 ^{ab}	5.78 ± 0.34 ^{ab}
T20_220MPa	6.89 ± 0.24 ^{a}	6.26 ± 0.34 ^{a}	5.86 ± 0.47 ^{ab}	5.93 ± 0.39 ^{ab}
T5_Patm	6.96 ± 0.17 ^{a}	6.53 ± 0.33 ^{a}	6.47 ± 0.27 ^{ab}	6.35 ± 0.25 ^{ab}
TT_T5_Patm	6.58 ± 0.22 ^{a}	6.03 ± 0.31 ^{a}	5.79 ± 0.33 ^{ab}	6.04 ± 0.29 ^{ab}

Table 4.1.4. *Scores obtained by sensory evaluation (mean values ± standard error) of strawberry juices at day 0 (C samples) and juices stored for 15 days at different conditions. Different letters within a column indicate significant differences ($p < 0.05$) between means.*

At day 0, C samples obtained high scores in all the attributes tested as expected. Pasteurization did not produce perceptible changes in the strawberry juices and TT_T5_Patm samples at day 0 were not significantly different ($p > 0.05$) from C samples (data not showed). This result is in agreement with the small total color change measured in these samples ($\Delta E^* = 0.43 \pm 0.04$).

After 15 days of storage at 20 °C, samples kept at atmospheric pressure had the lowest scores in all the sensory attributes. As previously commented, these juices showed marked signs of spoilage with unpleasant odor, off flavors, and gas presence clearly

due to the microbial growth observed. Thus, T20_Patm samples differed significantly from all the other samples.

In contrast, samples stored under pressure presented significantly higher scores and all of them were between 7 (like moderately) and 5 (neither like nor dislike). Thus, hyperbaric storage was quite efficient to preserve the initial sensory characteristics of the strawberry juice. Table 4.1.4 shows that the pressure level applied during storage had a significant effect on the sensory quality and lower pressures were less effective to preserve the juice. Thus, T20_25MPa samples had significantly lower scores than C samples in flavor and overall acceptance while the juices stored at 100 and 220 MPa did not significantly differ from C samples in any of the attributes evaluated.

Cold storage was also able to maintain the sensory quality of the juice and both raw and pasteurized samples stored at 5 °C did not show differences in any of the attributes tested as compared with C samples. Moreover, none of these samples differed from those stored at 100 and 220 MPa and 20 °C. Therefore, hyperbaric storage (100-200 MPa) was as efficient as conventional refrigeration in maintaining the sensory quality of strawberry juice.

Sensory results indicate that although, in Section 4.1.4.1.2 and 4.1.4.1.3, refrigeration resulted slightly more efficient that hyperbaric storage to preserve viscosity and color, these differences, instrumentally detected, were hardly perceived by the consumer. Thus, Table 4.1.4 shows that cold and hyperbaric storage at 100 and 220 MPa/20 °C were equivalent in terms of sensory quality.

4.1.4.2. Stability of the strawberry juices after hyperbaric storage

After 15 days of hyperbaric storage at 20 °C, T20_25MPa, T20_100MPa, and T20_220MPa samples were depressurized and stored at atmospheric pressure and 5 °C for two additional weeks. Then, microbial load, viscosity, color, and sensory quality were again assessed to evaluate the stability of the product, at 0.1 MPa and refrigerated conditions, after the storage under pressure. As previously commented, T20_Patm samples, stored at 0.1 MPa and 20 °C for 15 days, were spoiled and, therefore, they were discarded in this phase of the work. Raw and pasteurized juices, stored in

refrigeration for 15 days, were maintained at 5 °C for two more weeks to make comparisons.

4.1.4.2.1. Microbial load

Microbial counts after 15 additional days of cold storage are summarized in Table 4.1.5.

Sample	T20_25MPa	T20_100MPa	T20_220MPa	T5_Patm	TT_T5_Patm
Total aerobic mesophiles	$< 1^{b}$	$< 1^{b}$	$< 1^{b}$	6 ± 0.1^{a}	$< 1^{b}$
Yeasts and molds	$< 2^{b}$	$< 2^{b}$	$< 2^{b}$	4.7 ± 0.2^{a}	$< 2^{b}$

Table 4.1.5. *Microbial counts (mean values ± standard error, log_{10} CFU·mL^{-1}) in strawberry juices stored for 15 additional days at atmospheric pressure and 5 °C. Different letters within a row indicate significant differences ($p < 0.05$) between means.*

No microbial growth was detected in T20_25MPa, T20_100MPa, and T20_220MPa samples two weeks after the hyperbaric storage. In all these samples, the microbial population remained below the detection limits. The same result was obtained in the thermally pasteurized juices after 30 days of cold storage. On the contrary, total aerobic plate counts and yeasts and molds in T5_Patm increased in more than 1 and 2 log_{10} units, respectively, during the last two weeks of cold storage.

These results reveal that, after hyperbaric storage, strawberry juices were stable in microbiological terms for, at least, 15 days at atmospheric pressure and 5 °C. But, it is important to note that pressure applied during storage could be only partly responsible for the microbial stability observed. The acidic pH of the strawberry juice and the refrigeration temperature applied after decompression could be also involved in the results obtained, since both acid pH and low temperature may hinder the capacity of the cells to repair sublethal damage (Farkas & Hoover, 2000; Garcia-Graells et al., 1998; Linton et al., 1999; Smelt, 1998), the microbial growth, and the germination of spores.

4.1.4.2.2. Viscosity

Figure 4.1.3 depicts the evolution of viscosity in strawberry juices stored at different conditions.

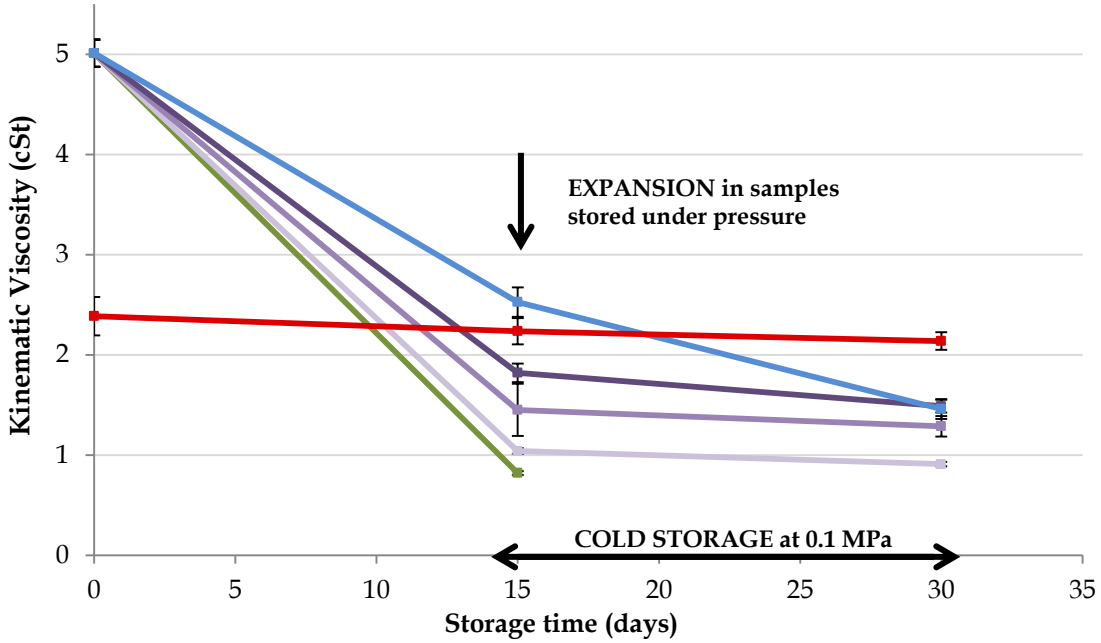

Figure 4.1.3. *Evolution of kinematic viscosity (cSt) in strawberry juices stored at different conditions. After the first 15 days samples stored under pressure were decompressed and stored at 0.1 MPa and 5 °C for 15 additional days. (T20_Patm (—), T20_25MPa (—), T20_100MPa (—), T20_220MPa (—), T5_Patm (—), and TT_T5_Patm (—)).Vertical bars represent standard error.*

All the samples stored under pressure underwent, during the cold storage after expansion, a slight but significant ($p < 0.05$) reduction in their viscosity values. This viscosity decay was considerably lower than the decline detected in T5_Patm samples during the last 15 days of storage (see Figure 4.1.3). Thus, viscosity reduced by 12.5 %, 11.0 %, and 18.7 % in T20_25MPa, T20_100MPa, and T20_220MPa, respectively, as compared to 42.5 % in T5_Patm samples. By contrast, viscosity of pasteurized juices remained stable for the complete storage period.

4.1.4.2.3. Color

Figure 4.1.4 presents the evolution of chroma and hue parameters in all the juice samples stored at different conditions.

Chroma and hue values were very stable in all the samples considered, whichever the preservation technique employed in the first 15 days of storage. This confirms the major role played by temperature in slowing color degradation in strawberry juices (Cao, Bi, Huang, Wu, Hu, & Liao, 2012; Gössinger, et al., 2009b).

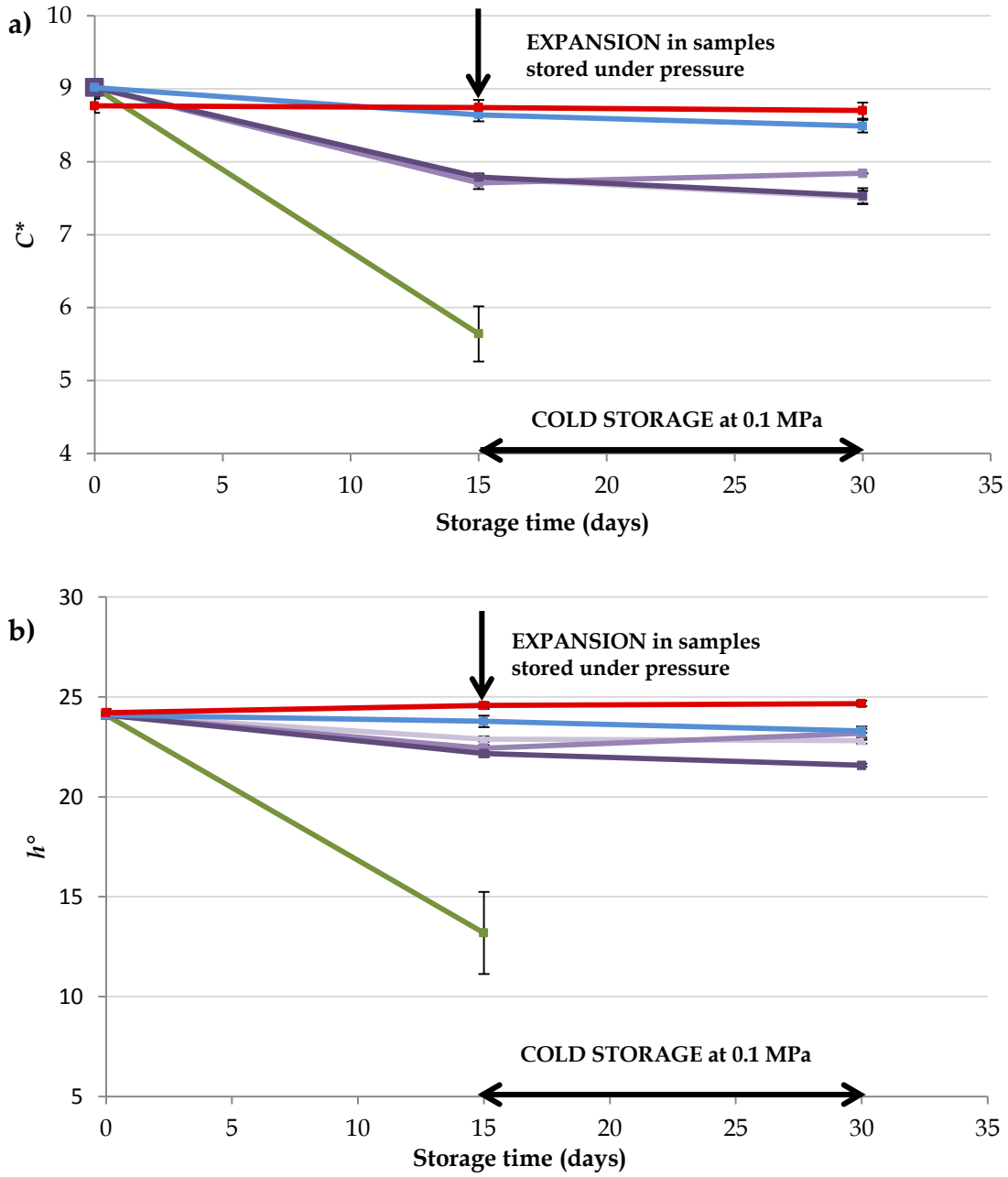

Figure 4.1.4. *(a) Chroma and (b) hue evolution in strawberry juices stored at different conditions. After the first 15 days, samples stored under pressure were decompressed and stored at 0.1 MPa and 5 °C for 15 additional days. (T20_Patm (—), T20_25MPa (—), T20_100MPa (—), T20_220MPa (—), T5_Patm (—), and TT_T5_Patm (—)).Vertical bars represent standard error.*

4.1.4.2.4. Hedonic sensory analysis

Scores for the sensory attributes evaluated in juice samples after 15 additional days of cold storage are summarized in Table 4.1.6.

Sample	Color	Odor	Flavor	Overall Acceptance
C	6.83 ± 0.22 ^{a}	7.12 ± 0.29 ^{a}	6.76 ± 0.32 ^{a}	6.82 ± 0.29 ^{a}
T20_25MPa	6.21 ± 0.28 ^{a}	5.75 ± 0.38 ^{a}	5.55 ± 0.34 ^{a}	5.43 ± 0.35 ^{b}
T20_100MPa	6.53 ± 0.30 ^{a}	6.71 ± 0.36 ^{a}	5.54 ± 0.42 ^{a}	5.80 ± 0.39 ^{ab}
T20_220MPa	6.89 ± 0.24 ^{a}	6.66 ± 0.29 ^{a}	6.43 ± 0.32 ^{a}	6.56 ± 0.26 ^{ab}
T5_Patm	7.07 ± 0.16 ^{a}	6.34 ± 0.36 ^{a}	6.05 ± 0.31 ^{a}	6.10 ± 0.30 ^{ab}
TT_T5_Patm	6.90 ± 0.19 ^{a}	6.06 ± 0.29 ^{a}	5.62 ± 0.38 ^{a}	5.74 ± 0.35 ^{ab}

Table 4.1.6. *Scores (mean values ± standard error) obtained by sensory evaluation of strawberry juices at day 0 (C samples) and juices stored for 15 days at different conditions + 15 additional days at atmospheric pressure and 5 °C. Different letters within a column indicate significant differences ($p < 0.05$) between means.*

Results are similar to those obtained after the first 15 days of storage. All samples scores remained between 7 (like moderately) and 5 (neither like nor dislike). No significant differences were found in any of the attributes tested between samples stored at 100 and 220 MPa and C samples. Moreover, these samples neither differ from raw and pasteurized juices stored at 5 °C and atmospheric pressure for 30 days. In contrast, scores of T20_25MPa samples for the overall acceptance were significantly lower than those of C samples.

These results confirm the stability of the juices stored under pressure after decompression for, at least, two more weeks at 5 °C.

4.1.5. Conclusions

Hyperbaric storage has been found to be an efficient method for reducing the microbial load and avoiding the growth of microorganisms in raw strawberry juices stored at 20 °C for 15 days. Pressure was also effective in attenuating viscosity and color losses in samples stored at 20 °C, although instrumental measures indicate that cold storage was significantly more efficacious in delaying viscosity and color decay. Moreover, both hyperbaric and cold storage were able to preserve the sensory quality of the raw juice for, at least, 15 days. Nevertheless, low temperature by itself failed in avoiding microbial growth and thermal pasteurization was needed to obtain stable strawberry juices for 15 days at 5 °C.

All these results show that hyperbaric storage at room temperature could represent an interesting technology for short-term preservation of raw strawberry juice. Long-term preservation should involve the previous enzymatic inactivation of the product.

Besides, the strawberry juice after decompression was stable, under refrigeration, at least for 15 days. This means that it is not necessary to process or consume the juice immediately after hyperbaric storage, so there is a margin of time to employ the juice.

However, more research is needed before giving categorical conclusions about the potential of this preservation method. Thus, it is necessary to study the evolution of different safety and quality attributes during hyperbaric storage and how pressure acts on the agents that cause juice deterioration (mainly microorganisms and enzymes). Only this knowledge will allow for the establishment of the adequate pressure level during storage. Once elucidated the best storage conditions, sensory acceptability of the preserved juice must be tested and, of course, the feasibility of the pressure equipment needed, the capital and operating costs, and the environmental impact have to be also evaluated. All these issues will be the subject of the following Chapters.

4.1.6. References

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Part 4.2: Effectiveness of hyperbaric storage at room temperature for preserving the volatile profile of strawberry juice[3]

[3] Bermejo-Prada, A., Vega, E., Pérez-Mateos, M., & Otero, L. (2014). Effect of hyperbaric storage at room temperature on the volatile profile of strawberry juice. *LWT-Food Science and Technology*. In Press.

EFFECTIVENESS OF HYPERBARIC STORAGE AT ROOM TEMPERATURE FOR MAINTAINING THE VOLATILE PROFILE OF STRAWBERRY JUICE

4.2.1. Abstract

The effect of hyperbaric storage at room temperature on the volatile profile of raw strawberry juice was evaluated. To do so, volatile profiles of strawberry juices maintained at 20 °C and different pressure levels (0.1, 50, and 200 MPa) for 15 days were analyzed by gas chromatography-mass spectroscopy and compared with those of control samples at day 0. Data corresponding to juices stored under refrigeration (0.1 MPa/5 °C) for the same period are also presented for comparison. Hierarchical Cluster Analysis (HCA) and Partial Least Squares Discriminant Analysis (PLS-DA) were applied to discriminate the samples according to the storage conditions. The results clearly showed that samples stored under pressure were more similar to the control juices at day 0 than samples stored under refrigeration at atmospheric pressure. Moreover, hyperbaric storage, unlike refrigeration at atmospheric pressure, was efficient in avoiding changes in all the key aroma compounds detected in the strawberry juice.

4.2.2. Introduction

Aroma is an important quality attribute in strawberry juice and it is determinant for consumer acceptance. Various authors have proved that the aroma of strawberry products changes drastically during storage (Aguiló-Aguayo, Oms-Oliu, Soliva-Fortuny, & Martín-Belloso, 2009; Golaszewski, Sims, O'Keefe, Braddock, & Littell, 1998; Siegmund, Derler, & Pfannhauser, 2001). The deterioration of the original aroma depends strongly on the storage conditions, mainly on temperature. Therefore, cold storage is strongly recommended to preserve the fresh, fruity, and typical strawberry-like aroma notes and to retard the appearance of stale, oxidized, acrid, and musty attributes (Golaszewski et al., 1998; Siegmund et al., 2001).

It is widely assumed that high pressure does not substantially alter the fresh odor of fruits and vegetables because small molecular flavor compounds are not directly affected by pressure. Thus, various authors have reported that pressure (200–600 MPa) applied for short times (1–20 min) at room temperature has no significant effect on the volatile profile of some homogenized fruit products, such as strawberry coulis (Lambert, Demazeau, Largeteau, & Bouvier, 1999) and guava (Yen & Lin, 1999) or orange (Baxter, Easton, Schneebeli, & Whitfield, 2005; Vervoort et al., 2012) juices, among others. However, there are hardly any data about the effect of longer-term pressure exposures. Pressure storage could indirectly modify the concentrations of some odor compounds by enhancing or retarding enzymatic and chemical reactions, and subsequently result in undesired changes in the overall odor (Viljanen, Lille, Heiniö, & Buchert, 2011).

The aim of this work was to study the effect of hyperbaric storage at room temperature on the volatile fraction of strawberry juice. To do so, the volatile profiles of strawberry juices stored at different pressure levels (0.1, 50, and 200 MPa) and 20 °C for 15 days were analyzed by gas chromatography-mass spectroscopy (GC-MS) and compared with those of control samples at day 0. Data corresponding to samples stored under refrigeration (0.1 MPa/5 °C) for the same period are also presented for comparison. The results obtained in this study provide important data to evaluate the effectiveness of hyperbaric storage at room temperature for food preservation.

4.2.3. Materials and methods

4.2.3.1. Samples

Strawberries (*Fragaria x ananassa* Duch., cv. Chandler) were purchased at commercial maturity from a local supplier. The fruits were washed with tap water and processed with a juicer (Moulinex Frutti Pro, Moulinex, France). The liquid obtained was then centrifuged at 3,500 *g* and 7 °C for 10 min (Sorvall Evolution RC Superspeed centrifuge, Thermo Scientific, Madrid, Spain). The supernatant was subsequently collected, filtered through a 0.1 mm pore diameter sieve, and stored at –80 °C until utilization. Before each storage experiment, a frozen batch of strawberry juice was thawed

overnight at 5 °C and transferred to 50 mL polypropylene tubes. The tubes were completely filled with strawberry juice and closed with screw caps sealed by a nitrile rubber O-ring.

Control juice at day 0 (C samples) was then characterized by measuring some of its physicochemical properties (see Table 4.2.1).

Physicochemical property	Mean ± Standard Deviation (n = 3)
Soluble solids (°Brix)	5.6 ± 0.1
pH	3.31 ± 0.03
*L**	27.20 ± 0.28
*a**	7.67 ± 0.77
*b**	3.15 ± 0.48

Table 4.2.1. *Physicochemical properties of the strawberry juice employed in the experiments (day 0).*

Soluble solids concentration (°Brix) was approximated by using a digital refractometer (Leica AR200, Leica Microsystems Inc., New York, USA) with automatic temperature compensation, pH was measured with a pH-meter (pH-Burette 24 1S equipped with a pH 50 21 electrode and a C.A.T. 55 31 temperature sensor, Crison Instruments, Barcelona, Spain), and color was determined, as *L** (lightness), *a** (redness), and *b** (yellowness), with a CM-3500d spectrophotometer (Konica Minolta, Japan) as described in Chapter 3.

4.2.3.2. Storage experiments in strawberry juice

Storage experiments under pressure were carried out in a pilot-plant high-pressure storage system (model SV1, Institute of High Pressure Physics, Unipress Equipment Division, Poland). The characteristic of the equipment are described in detail in Chapter 3.

Strawberry juices were stored for 15 days at 20 ± 2 °C and two different pressure levels (50 and 200 MPa) to obtain samples labeled as T20_50MPa (20 °C/50 MPa) and T20_200MPa (20 °C/200 MPa). Temperature and pressure were recorded every 30 s by a data acquisition system (MW100 Data Acquisition Unit, Yokogawa Electric Corporation, Tokyo, Japan). Storage experiments at atmospheric pressure for 15 days were performed in two thermostatic chambers tempered either at 20 ± 2 °C or at 5 ±

2 °C to obtain T20_Patm (20 °C/0.1 MPa) and T5_Patm (5 °C/0.1 MPa) samples, respectively. All the storage experiments were performed in triplicate.

4.2.3.3. Headspace analysis in strawberry juice

Immediately after storage, three grams of each strawberry juice sample was transferred into 22 mL glass vials. Then the vials were sealed with polytetrafluoroethylene (PTFE)/Butyl septa and crimp caps, and finally frozen at –80 °C until use.

Volatile analyses were performed using an Agilent 6890N Series gas chromatograph coupled to an Agilent 5973 Series mass selective detector (Agilent Technologies, Heilbronn, Germany) and equipped with a TurboMatrix 40 Trap Headspace sampler (Perkin Elmer, Shelton, CT, USA) as is detailed in Chapter 3. Before the analyses, all the sample vials were completely thawed at room temperature and an aliquot of 10 μL of 2-octanone (32.72 $mg \cdot L^{-1}$ in water) was added as internal standard to each vial.

4.2.3.4. Data analysis

The GC-MS chromatograms obtained were evaluated and integrated using the ChemStation program (Agilent Technologies, Palo Alto, CA, USA). Identification of peaks in the chromatograms was performed by injection of commercial standards, by spectra comparison with the Wiley Registry 7th Edition Mass Spectral Library (Wiley and Sons Inc., Germany) and the National Institute of Standards and Technology (NIST) 2005 Mass Spectral Library, and by calculation of linear retention indices (LRI) using retention time data from a series of alkane standards (C_6 – C_{20}) run under the same chromatographic conditions. The normalized peak area of each compound was then calculated as the ratio of its peak area to the area of the internal standard.

In a first step, Hierarchical Cluster Analysis (HCA), an unsupervised pattern recognition method, was applied to calculate similarities among the samples and establish whether a discriminant classification method could be developed subsequently. A hierarchical clustering procedure with complete linkage, using the Pearson correlation distance, was used to generate clusters.

After this exploratory analysis, data were subjected to Partial Least Squares Discriminant Analysis (PLS-DA) to look for potential differences in the volatile profiles of the juices in order to classify the samples according to storage conditions. In this analysis, compound abundances were considered as explanatory X-variables and the different classes of samples as categorical Y-variables or responses. All data were mean-centered and the variables were weighted by their standard deviation to give them equal variance. A PLS-DA calibration model was generated using all the samples to find the latent variables (LV) or factors in X that would best predict the latent variables in Y. Full cross-validation (leave-one-out) was then used to select the optimum number of latent variables or PLS-DA factors.

To evaluate the importance of each volatile compound in discriminating a specific sample, Variable Identification (VID) coefficients were estimated for each compound and response. VID coefficients were calculated as the correlation coefficient between each original X-variable and the Y-variables predicted by the PLS-DA model. In this study, X-variables with an absolute value of the VID coefficient higher than 0.80 were considered of interest for the response examined. Moreover, to have a global view of these discriminant variables, they were individually plotted as a function of the class of juice.

All the multivariate analyses (HCA and PLS-DA) were performed with The Unscrambler® X, v. 10.2 (CAMO Software AS, Oslo, Norway).

4.2.4. Results and discussion

4.2.4.1. Characteristics of the volatile profiles of the studied samples

Thirty-one volatile compounds, including esters, aldehydes, alcohols, terpenoids, aromatic compounds, a furanone, and a ketone, were identified in the analyzed strawberry juices (Table 4.2.2). Resolution between hexanal and ethyl butanoate was too low for a proper quantitative measurement, and therefore data for the two compounds are presented together in Table 4.2.2. All the compounds detected in the samples had previously been described in the volatile profile of strawberry and

strawberry products by many authors (Aubert, Baumann, & Arguel, 2005; Golaszewski, Sims, O'Keefe, Braddock, & Littell, 1998; Jetti, Yang, Kurnianta, Finn, & Qian, 2007; Kafkas et al., 2005; Pérez & Sanz, 2010).

Compound	RT (min)[a]	LRI (calculated)[b]	LRI (Wiley-NIST)[c]	Identification ions	Peak identification[d]
ethanol	1.36	--	427	31, 45, 29	ST, MS
methyl acetate	1.69	--	526	43, 74, 15	MS
methyl butanoate	2.96	649	724	43, 74, 71	MS
3-methyl-1-butanol	3.27	663	743	55, 42, 43	ST, MS
2-methyl-1-butanol	3.29	664	744	57, 41, 56	ST, MS
methyl 3-methylbutanoate	3.74	686	776	74, 43, 85	MS
hexanal + ethyl butanoate	4.11	803	794/800	41, 44, 56/71, 88, 43	ST, MS/MS
butyl acetate	4.40	817	812	43, 56, 61	MS
Trans-2-hexenal	5.21	856	835	41, 55, 69	MS
1-hexanol	5.61	875	876	56, 43, 55	ST, MS
3-methylbutyl acetate	5.70	879	883	43, 70, 55	MS
2-methylbutyl acetate	5.74	881	879	43, 70, 55	MS
2-heptanone	5.99	894	894	43, 58, 51	MS
2,4-hexadienal	6.42	912	916	81, 96, 39	MS
methyl hexanoate	6.79	927	927	74, 43, 87	MS
benzaldehyde	7.64	961	961	106, 105, 77	ST, MS
furan-2-methyl acetate	8.55	997	995	81, 98, 140	MS
cis-3-hexenyl acetate	8.86	1010	1005	43, 67, 82	MS
hexyl acetate	9.03	1016	1012	43, 56, 55	MS
trans-2-hexenyl acetate	9.10	1019	992	43, 67, 82	MS
d-limonene	9.43	1032	1026	68, 93, 67	MS
ocimene (trans)	9.67	1042	1048	93, 83, 55	MS
mesifurane	10.24	1065	1057	142, 43, 69	MS
linalool	11.28	1102	1098	71, 93, 41	MS
nonanal	11.38	1106	1102	57, 41, 56	MS
benzyl acetate	12.93	1166	1169	108, 91, 90	MS
terpinen-4-ol	13.28	1180	1177	71, 93, 111	ST, MS
α-terpineol	13.62	1193	1193	59, 93, 121	MS
β-farnesene	19.90	1462	1453	69, 93, 41	MS
nerolidol	22.19	1567	1565	69, 93, 41	MS

Table 4.2.2. *Main volatile compounds identified in strawberry juices.*

In control juices, trans-2-hexenal, methyl acetate, methyl butanoate, and hexanal + ethyl butanoate peaks exhibited the largest abundances (data not shown). Moreover, according to other authors (Kafkas et al., 2005; Pérez & Sanz, 2010), esters were the qualitatively and quantitatively most important class of volatiles in C samples. However, from a flavor point of view, it is well recognized that the most abundant volatile compounds are not necessarily the most important sensory compounds. Some volatile compounds, usually known as key flavor compounds, are determinant in the aroma perceived, even at very low concentrations. Among the major compounds detected in control juices, methyl and ethyl butanoates, methyl hexanoate, trans-2-hexenyl acetate, and linalool have previously been identified by sensory evaluation methods as key flavor compounds in the typical strawberry-like odor (Aubert et al., 2005; Jetti et al., 2007; Larsen, Poll, & Olsen, 1992; Schieberle & Hofmann, 1997; Siegmund, Derler, & Pfannhauser, 2001). Other compounds, found in C samples at much lower concentrations, such as 3-methylbutyl acetate, 2-heptanone, hexyl acetate, and 2,5-dimethyl-4-methoxy-3(2H)-furanone, also known as mesifurane, have also been described as playing and important role in strawberry aroma (Forney, Kalt, & Jordan, 2000; Larsen & Poll, 1992; Larsen et al., 1992; Siegmund et al., 2001). From this analysis, it is checked that the volatile profile found in this study for fresh samples is representative and typical of strawberry juice.

After 15 days of storage, samples maintained at atmospheric pressure and 20 °C were clearly spoiled and stale, and musty notes were detected in their aroma owing to considerable microbial spoilage. Volatile compounds identified in these samples were those typical for fermented fruit products (data not shown). In contrast, samples maintained at 20 °C under pressure did not show any evidence of deterioration. These results could be related with limited microbial activity during hyperbaric storage, because previous experiments in strawberry juices maintained for 15 days under pressure and at room temperature showed that pressure inhibited microbial growth (Segovia-Bravo et al., 2012).

At this point, T20_Patm samples were excluded from further testing and only C, T5_Patm, T20_50MPa, and T20_200MPa samples were included in the following analyses to focus differentiation on unspoiled samples.

4.2.4.2. Exploratory analysis

A Hierarchical Cluster Analysis of the data was performed first, as an exploratory technique, to detect groups in the samples, based on similarity or closeness measures. As Figure 4.2.1 shows, all the replicated samples were correctly grouped together.

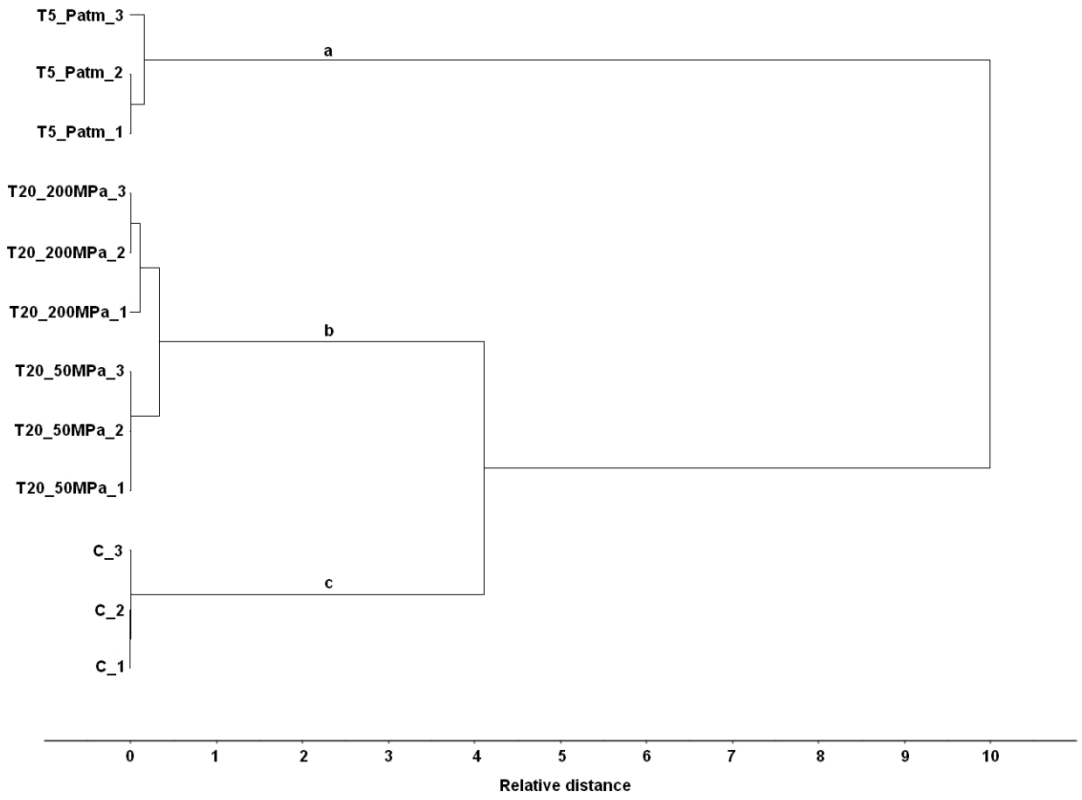

Figure 4.2.1. *Dendrogram of the strawberry juices after hierarchical cluster analysis based on complete linkage and Pearson correlation distance. C: Control juices (day 0); T5_Patm: Juices stored at 5 °C and atmospheric pressure for 15 days; T20_50MPa: Juices stored at 20 °C and 50 MPa for 15 days; T20_200MPa: Juices stored at 20 °C and 200 MPa for 15 days.*

HCA allowed subdivision of the juice samples into clusters that exhibited a high degree of both intracluster similarity and intercluster dissimilarity. At the maximal distance (relative distance = 10), that is, at the highest level of differentiation, T5_Patm

juices were separated from the rest and therefore they were classified as completely different from the other ones. At a relative distance of about 4.1, three clusters were established: cluster "a" consists of T5_Patm samples; cluster "b" comprises juices stored under high pressure (T20_50MPa and T20_200MPa samples), and cluster "c" corresponds to C samples. These results clearly showed that samples stored under pressure for 15 days were more similar to control samples at day 0 than samples stored under refrigeration at atmospheric pressure. This is a clear indicator that hyperbaric storage at 20 °C makes it possible to preserve the volatile fraction of strawberry juices better than traditional cold storage does for at least 15 days.

4.2.4.3. Discriminant analysis

A Partial Least Squares Discriminant Analysis of the compounds detected in the aroma profile of the strawberry juices gave some interesting information about the differences between them. The PLS-DA model performed consisted of seven latent variables or factors which explained 99.4 % of the Y-variance. Figure 4.2.2a presents the correlation loadings plot for the first two latent variables, which together explained 63 % of the Y-variance. It shows that the effect of storage temperature (20 °C or 5 °C) is mainly explained on the basis of the first latent variable, while the effect of pressure (atmospheric or high pressure) is mainly included in the second factor.

The correlation loadings plot indicates the correlation between the original variables and the PLS-DA factors of the model, and it is very useful for determining volatiles that characterize classes of samples. As an example, the coordinates of a given type of juice on the first and second latent variables show how well this juice is correlated with these latent variables. The inner and outer ellipses in the plot represent correlation coefficients r = 70 % and r = 100 % (or R^2 values of 50 % and 100 %), respectively. Thus, for a variable located between the two ellipses, more than 70 % of its variability is explained by the first two latent variables. Figure 4.2.2a clearly shows that C and T5_Patm samples can be characterized relatively well by these two factors. Volatile compounds located between the ellipses and close to C samples, such as furan-2-methyl acetate, 2,4-hexadienal, or trans-2-hexenal, should be characteristic of C samples, while those located at similar positions in the opposite quadrant of the plot,

such as linalool or α-terpineol, should present lower abundances in control samples than in all the other juices. Obviously, both highly negatively and highly positively correlated compounds could act as potential discriminants of C samples. Similarly, volatile compounds located close to T5_Patm juices, such as 1-hexanol, have a high positive correlation with these samples, while those located at similar positions in the opposite quadrant, such as hexanal + ethyl butanoate, trans-2-hexenyl acetate, or benzyl acetate, have a high negative correlation with T5_Patm juices.

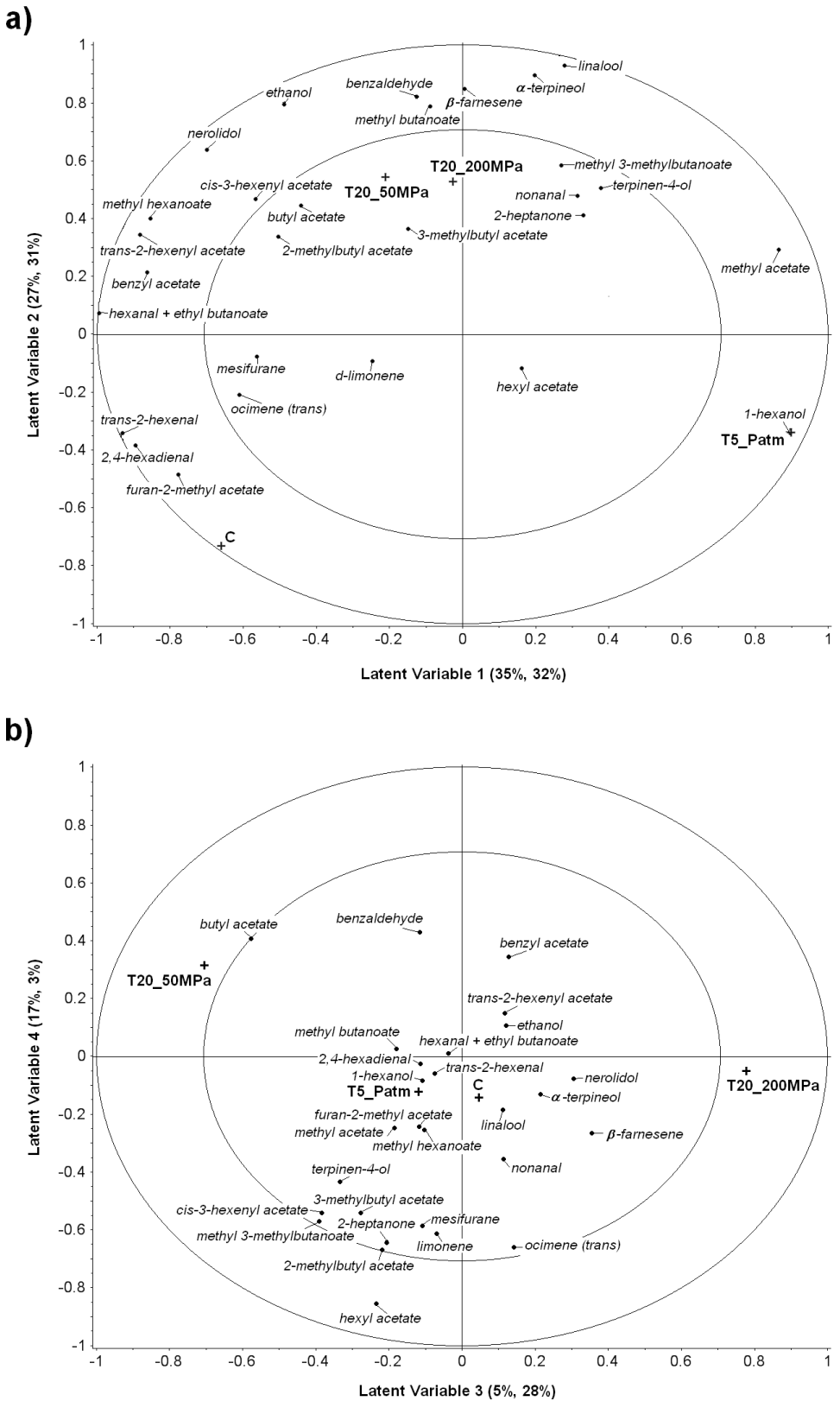

Figure 4.2.2. *Correlation loadings plots of (a) the first and second latent variables and (b) the third and fourth latent variables of the model performed after partial least squares discriminant analysis (PLS-DA) of the volatile compounds detected in strawberry juices. Sample nomenclature is the same as in Figure 4.2.1.*

Figure 4.2.2a also reveals that two latent variables are not enough to discriminate T20_50MPa and T20_200MPa samples, which are grouped close together. Therefore, more factors are needed in the model to differentiate these samples effectively. Figure 4.2.2b presents a correlation loadings plot accounting for the third and fourth latent variables of the PLS-DA model. It illustrates how discrimination between T20_50MPa and T20_200MPa samples is mainly managed through the third latent variable of the PLS-DA model. T20_200MPa juices present a large positive loading on LV3, while T20_50MPa samples exhibit negative values, just like butyl acetate.

4.2.4.4. Effect of storage conditions on the volatile profile of strawberry juice

The results obtained clearly show that the different storage conditions assayed in this study distinctly affect the volatile profile of strawberry juice, and therefore sample discrimination by PLS-DA is possible. To evaluate the importance of each volatile compound in discriminating a specific sample, VID coefficients were calculated for each volatile and response. VID coefficients identify those compounds that are highly correlated, either positively or negatively, with a given class of juice (Table 4.2.3). Thus, volatiles with a high absolute value of the VID coefficient for a class of juice present a particularly high or low abundance in that specific class as compared to all the other classes, and therefore they could act as class discriminants.

SAMPLE	VOLATILE COMPOUND	VID
C	furan-2-methyl acetate	0.90
	trans-2-hexenal	0.87
	2,4-hexadienal	0.87
	linalool	-0.84
T5_Patm	1-hexanol	1.00
	hexanal + ethyl butanoate	-0.92
	trans-2-hexenyl acetate	-0.90
	benzyl acetate	-0.89
	nerolidol	-0.88
	methyl hexanoate	0.86
T20_50MPa	butyl acetate	0.82
T20_200MPa	--	--

Table 4.2.3. *VID coefficients for each class of strawberry juice. C: Control juices (day 0); T5_Patm: Juices stored at 5 °C and atmospheric pressure for 15 days; T20_50MPa: Juices stored at 20 °C and 50 MPa for 15 days; T20_200MPa: Juices stored at 20 °C and 200 MPa for 15 days.*

Table 4.2.3 reveals that, in control juices, furan-2-methyl acetate, trans-2-hexenal, and 2,4-hexadienal have VID coefficients higher than 0.80. This means, as Figure 4.2.3 clearly shows, that the abundance of these volatiles was significantly higher in C samples than in all the stored juices. This high C_6 aldehyde content is probably due to the tissue disruption involved in juice extraction. These compounds are formed enzymatically through the action of lipoxygenase, oxygen, and linoleic and linolenic acids, and it is widely known that tissue disruption and homogenization enhance their formation (Forney et al., 2000; Sumitani, Suekane, Nakatani, & Tatsuka, 1994). During storage, these aldehydes are progressively degraded if enzymatic activities are not completely inhibited. Significant decreases in C_6 aldehyde concentration during cold storage of strawberry juices have been described previously in the literature (Aguiló-Aguayo, Oms-Oliu, Soliva-Fortuny, & Martín-Belloso, 2009).

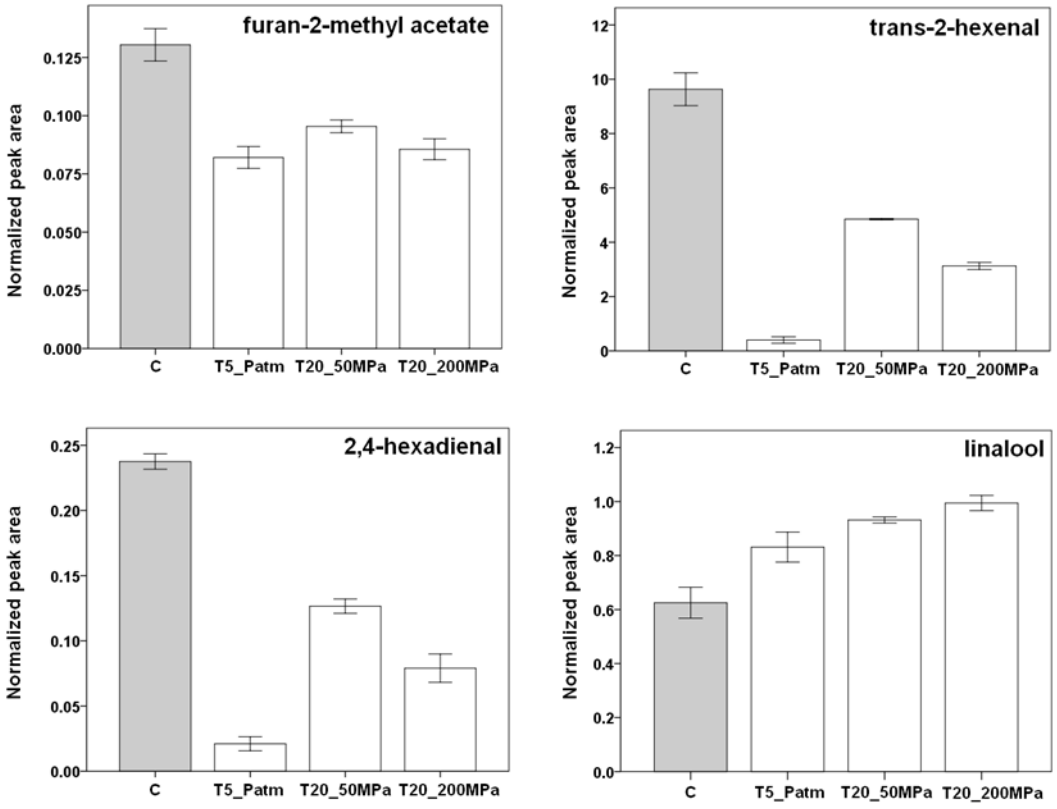

Figure 4.2.3. *Normalized peak areas (mean values ± standard error, n = 3) of the discriminant compounds of control samples, selected through the VID procedure. Sample nomenclature is the same as in Figure 5.4.1.*

The VID coefficients in Table 4.2.3 also reveal that linalool is less abundant in C samples than in the other juices. Therefore, during storage, linalool could be released from its glycosidic precursor by enzymatic hydrolysis carried out by β-glucosidase.

4.2.4.4.1. Storage at 5 °C: traditional refrigeration

After 15 days of storage at 5 °C, some changes occurred in the volatile profile of the juice, as expected. The VID coefficients in Table 4.2.3 reveal that T5_Patm samples differed substantially from all the other samples in a number of volatile compounds (Figure 4.2.4a). Thus, 1-hexanol had a large positive correlation with T5_Patm juices, while hexanal + ethyl butanoate, trans-2-hexenyl acetate, benzyl acetate, nerolidol, and methyl hexanoate had a large negative correlation.

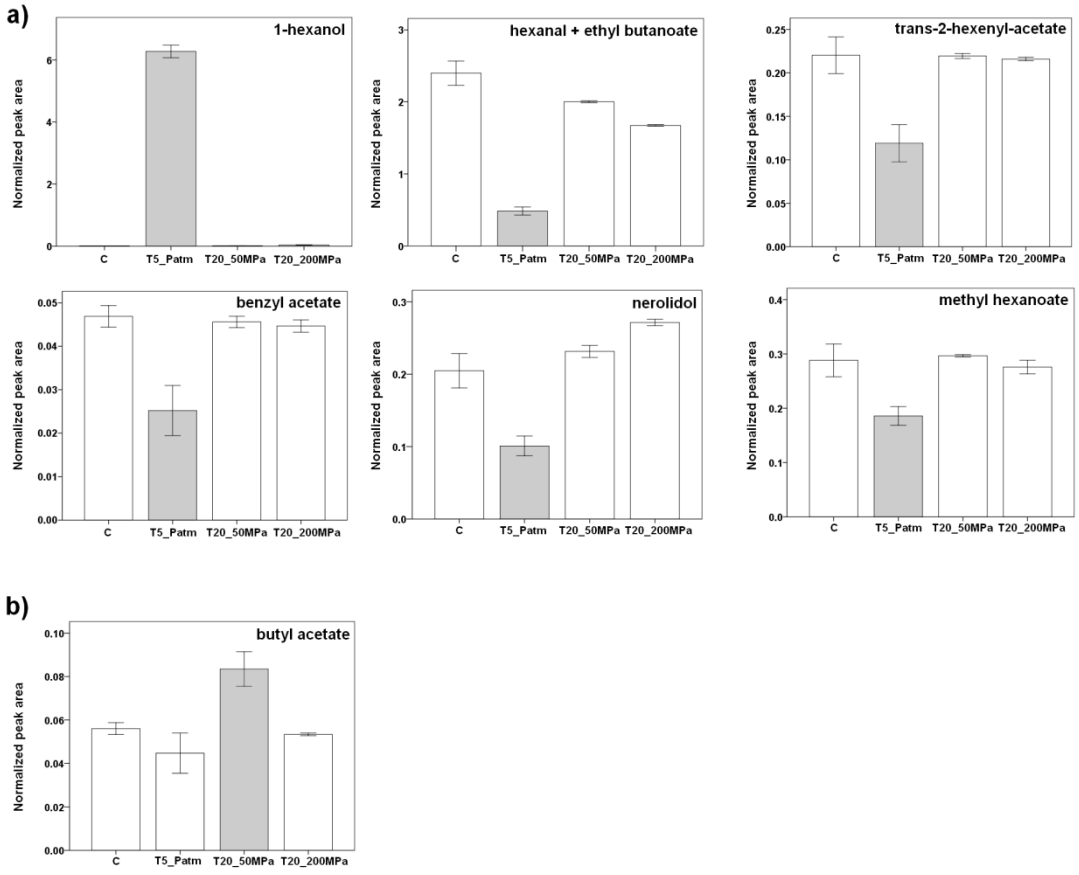

Figure 4.2.4. *Normalized peak areas (mean values ± standard error, n = 3) of the discriminant compounds of a) T5_Patm juices and b) T20_50MPa juices, selected through the VID procedure. Sample nomenclature is the same as in Figure 4.2.1.*

Significant increases in 1-hexanol concentration were reported by Navarro, Verret, Pardon, and El Moueffak (2002) in untreated strawberry puree stored at 4 °C. In this study, after 15 days of storage at 5 °C and 0.1 MPa, 1-hexanol concentration increased by more than 600 times. This increase in 1-hexanol content could be related with the activity of alcohol dehydrogenase, because a decrease was also observed in the relative abundance of hexanal + ethyl butanoate (Figure 4.2.4a). With regard to key aroma compounds, Figure 4.2.4a shows that refrigeration produced substantial drops in trans-2-hexenyl acetate and nerolidol abundances. The peak corresponding to hexanal + ethyl butanoate also decreased substantially, but degradation of ethyl butanoate cannot be justified from these data because, as commented earlier, individual contributions of hexanal and ethyl butanoate could not be differentiated. However, Aguiló-Aguayo et al. (2009) found that ethyl butanoate concentration decreased during refrigerated

storage of strawberry juices. This probable ethyl butanoate degradation together with the proved decay of trans-2-hexenyl acetate and nerolidol could significantly affect the aroma perceived in T5_Patm juices.

4.2.4.4.2. Hyperbaric storage at 20 °C

Detailed comparison of the volatile profiles of the strawberry juices showed that storage under pressure at 20 °C avoided most of the changes experienced in T5_Patm samples (Figure 4.2.4a), although a decrease in the abundance of furan-2-methyl acetate, trans-2-hexenal, and 2,4-hexadienal, potential discriminators of C samples, was still observed (Figure 4.2.3). Nevertheless, the drop in these aldehyde contents, especially in the samples stored at 50 MPa, was substantially lower than that observed in T5_Patm samples. Various authors have proved that pressure between 200 and 400 MPa, applied for 20 min at room temperature, significantly increases hexanal and trans-2-hexenal contents in strawberry products such as coulis or purees (Lambert et al., 1999; Navarro et al., 2002). Increases in C_6 aldehydes in fruit and vegetable products after pressure processing are widely reported in the literature, especially in non-homogenized products (Sumitani et al., 1994; Viljanen et al., 2011). However, it is important to note that these increases should be attributed to enhanced enzymatic oxidation of linoleic and linolenic acids induced by pressure, which produces tissue disruption and favors contact between enzymes and substrates. In this study, the C_6 aldehyde content in T20_50MPa and T20_200MPa juices after storage was considerably higher than in T5_Patm samples. This could be due either to increased formation of C_6 aldehydes induced by pressure or to limited alcohol dehydrogenase (ADH) activity during hyperbaric storage. ADH, which can convert C_6 aldehydes to their derived alcohols, could present a low activity under pressure. Thus, unlike in T5_Patm samples, no increases in 1-hexanol content were detected in T20_50MPa and T20_200MPa juices (Figure 4.2.4a).

Table 4.2.3 also reveals that, after hyperbaric storage, only butyl acetate exhibited a positive VID coefficient slightly higher than 0.80 for T20_50MPa juices, and thus this compound is more abundant in T20_50MPa samples than in the other juices (Figure 4.2.4b). No more volatiles with high VID coefficients appeared in T20_50MPa samples,

and T20_200MPa juices did not present any potential characteristic compound. Discrimination of samples stored under pressure is, therefore, more difficult, as previously mentioned, but this means that no substantial changes occurred in any compound in these samples in comparison with all the other juices.

However, the most remarkable fact was that none of the degradations observed in key flavor compounds in T5_Patm samples occurred when the storage took place under pressure. Thus, Figure 4.2.4a shows that decreases in trans-2-hexenyl acetate, methyl hexanoate, and nerolidol were not detected in T20_50MPa and T20_200MPa samples. Moreover, a significant increase in linalool concentration can be observed in samples preserved under pressure. This increase is especially noteworthy because it could be associated with relatively high levels of β-glucosidase activity during storage. β-glucosidase is involved in the release of flavor volatiles in fruits, and various authors have shown previously that its activity in strawberry is not only not affected but even increased after pressure treatments between 200 and 400 MPa for 15 min at room temperature (García-Palazon, Suthanthangjai, Kajda, & Zabetakis, 2004; Zabetakis, Koulentianos, Orruño, & Boyes, 2000).

The evolution of methyl butanoate, 3-methyl butyl acetate, 2-heptanone, hexyl acetate, and mesifurane was also studied during the hyperbaric storage (data not shown), although these volatiles were not classified as potential discriminants for any class of juice by the VID procedure. Nevertheless, they are considered of interest because they have been reported in the literature as key flavor compounds in strawberry (Jetti et al., 2007; Larsen & Poll, 1992; Larsen et al., 1992; Schieberle & Hofmann, 1997). The results revealed that the abundance of these compounds remained unaltered after 15 days of storage in juices preserved under pressure.

4.2.5. Conclusions

This study offers the first data in the literature about the effect of hyperbaric storage at room temperature on the volatile profile of a homogenized fruit product. The results obtained clearly showed that pressure avoided spoilage of samples stored at 20 °C for 15 days. Moreover, hyperbaric storage was more efficient than refrigeration in

maintaining the volatile profile of strawberry juices unaltered for 15 days, and thus samples stored under pressure were more similar to control juices at day 0 than samples stored under refrigeration at atmospheric pressure. In fact, no changes in any key aroma compound were detected after hyperbaric storage. Nevertheless, sensory analyses are needed to test whether the differences observed would be detectable by human perception.

The results obtained in this study offer encouraging new data for the characterization of hyperbaric storage of food at room temperature. This new environmentally friendly technology could provide an interesting opportunity to reduce energy costs in food preservation. However, much more research is needed (microbial behavior and enzymatic activities under pressure, stability of bioactive compounds, capital and operating costs, among other things) to establish its real potential.

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Part 4.3: Effectiveness of hyperbaric storage at room temperature for preserving the sensory attributes of strawberry juice

EFFECTIVENESS OF HYPERBARIC STORAGE AT ROOM TEMPERATURE FOR PRESERVING THE SENSORY ATTRIBUTES OF STRAWBERRY JUICE

4.3.1. Abstract

The objective of this study was to evaluate the effectiveness of hyperbaric storage at room temperature for preserving the sensory attributes of strawberry juice. Thus, several triangle tests were organized to determine whether organoleptic differences could be perceived between "fresh" samples before storage (control) and samples after storage. Storage was conducted according to two preservation strategies: cold storage of pasteurized juice, as the conventional strategy, and hyperbaric storage of "fresh" juice (25 and 50 MPa/20 °C), as the novel one, both for 15 days. Moreover, the equivalence of hyperbaric storage and refrigeration, in terms of preserving the organoleptic characteristics of pasteurized strawberry juice, was tested. In parallel to these tests, the color, total titratable acidity, total soluble solids content, viscosity, and pH were measured in an attempt to relate instrumental and sensory results. The results showed that hyperbaric storage at 25 or 50 MPa and room temperature does not maintain unaltered the initial physicochemical and organoleptic characteristics of the "fresh" juice. Discrimination between samples could be established mainly from their taste by judges and essentially from their viscosity by instrumental measurements, reflecting the complexity of relating both kinds of organoleptic characteristics evaluation. In pasteurized juices, hyperbaric storage at 25 MPa and room temperature was found to be equivalent to cold storage in terms of the perceived organoleptic quality.

4.3.2. Introduction

Hyperbaric storage at room temperature has been proposed as a novel storage method for the preservation of food products (Chapter 1). The effectiveness of this method is

just starting to be examined and it is within the objectives of this Thesis (Chapter 2) to evaluate it in the case of strawberry juice preservation. In a previous study (Part 4.1), it was shown that strawberry juice could be successfully stored under pressure at room temperature for 15 days. Hyperbaric storage inhibited microbial and slowed down quality degradation (color, viscosity). Another fundamental feature addressed in this study was the acceptance of the juice by consumers. If strange or bad flavors were developed during hyperbaric storage, the viability of the method could be jeopardized. A performed hedonic sensory analysis indicated that the overall acceptance of the juices stored under pressure at room temperature was similar to those stored under refrigeration. Besides, another study presented in Part 4.2 showed that hyperbaric storage at room temperature was more efficient than cold storage in maintaining the volatile profile of strawberry juice unaltered for 15 days. Thus, samples stored under pressure were more similar to control juices at day 0 than samples stored under refrigeration.

However, these positive results raise new questions. On the one hand, the scores obtained in the sensorial study describe in Part 4.1 were relatively low even for the control ("fresh" sample): around 6 out of 10. These low scores were explained by the lack of habit in consuming strawberry juice which is not a usual drink but rather an ingredient of other food products, at least, in Spain. Moreover, the results showed that (i) differences in scores between the acceptable[4] juice samples were small, and that (ii) the juice stored at 25 MPa received lower scores compared to juices maintained at 100 MPa or 200 MPa or even simply refrigerated. Thus, at 25 MPa, as for refrigeration, a pasteurization step may be necessary prior to storage. In this case also, the efficacy of hyperbaric storage in preserving the organoleptic characteristics of pasteurized juice should be also checked. On the other hand, unlike for refrigeration, no changes in any key aroma compound were detected from the analysis of strawberry juices volatiles after hyperbaric storage at pressures of 50 MPa and higher. A sensory analysis should be additionally suitable to test whether these results correspond to human perception.

[4] by acceptable, it is meant not visually spoiled *i.e.* control, stored under refrigeration, or high pressure samples.

For all these reasons, it appears necessary to refine these initial evaluations of the organoleptic characteristics of stored juices.

Thus, the first objective of this study was to confirm whether differences from "fresh" samples, samples stored under relatively low pressure (25 or 50 MPa) and room temperature (novel storage), and pasteurized samples stored under refrigeration (conventional storage), all of them for 15 days, are detected by human perception. The second objective was to test whether hyperbaric storage is equivalent to refrigeration in terms of preserving the organoleptic characteristics of pasteurized strawberry juice.

In this study, the sensory analyses were carried out by discrimination testing as an easy and fast tool. Discrimination testing represents one of the most useful analytical tools available to perceive differences between two products. These methods are intended to answer a simple question: "Are these products perceived as different?" Within them, the triangle test is one of the most well-known discrimination tests and it has been used to a great extent. The triangle test, as its name implies, is a three-products test in which all three products are coded and the subject's task is to determine which one is the most different from the other two (Stone & Sidel, 2004). This type of test will be used to determine if organoleptic differences between a series of strawberry juice samples stored under different conditions could be perceived by consumers. In parallel to the sensory tests, the strawberry juices were characterized by physicochemical analyses (instrumental color, viscosity, total soluble solids content (TSS), pH, and total titratable acidity (TA)). This will allow to determine if relation between organoleptic and sensory results exists.

4.3.3. Materials and methods

4.3.3.1. Strawberry juice samples

Strawberries were purchased at commercial maturity from a local supplier before each series of storage experiments (three series). Strawberry juices were always processed in the same way. The fruits were washed with tap water and processed with a blender (Royal Blender Turbo 10-Speed, Type 212004, Princess, Netherlands). The liquid

obtained was then centrifuged at 3,500 *g* and 4 °C for 10 min (Sorvall Evolution RC Superspeed centrifuge, Thermo Scientific, Madrid, Spain) with a Fiberlite F8-6x1000y rotor (Thermo Scientific, Madrid, Spain). The supernatant was subsequently collected, filtered through a 0.1 mm pore diameter sieve, and kept at -20 °C until utilization.

About 1.5 L of juice was used per storage experiment including 1.2 L for triangle test, 0.1 L for physicochemical measurements, and 0.2 L as a margin for mishap. This quantity was imposed by the capacity of the high-pressure vessels: this was the maximal volume that can be stored at the same time by using the two vessels at the same pressure for 15 days.

Samples were stored in their raw or pasteurized form. To prepare the pasteurized samples, each raw juice sample was immersed in a water bath at 90 °C until the temperature at the core was maintained at 85 °C for 90 s. Once processed, the juice was immediately cooled in an ice-water bath.

4.3.3.2. Storage experiments

A storage experiment consisted in keeping strawberry juice during 15 days either refrigerated in a cold chamber at 5 ± 2 °C or under pressure at 20 ± 2 °C. Refrigeration and hyperbaric storage experiments at a given pressure were conducted at the same time while different pressure levels for hyperbaric storage had to be tested on different periods of time due to the limited capacity of the vessels. Thus, three series of storage experiments were prepared and each one was realized on a different date.

Before each series of storage experiments, the strawberry juice was thawed by keeping it overnight at 5 °C. Then, samples were packaged in 250 mL thermo-sealed plastic bags, avoiding headspace, and placed under the chosen storage conditions (refrigerated or under a given pressure) for 15 days. Refrigeration took place in a cold chamber at the laboratory. Storage experiments under pressure were carried out in the pilot-plant high-pressure storage system (model SV1, Institute of High Pressure Physics, Unipress Equipment Division, Poland) as described in Chapter 3. Two different pressure levels were tested: 25 MPa and 50 MPa. These levels were selected

because low pressures were identified to be the most critical in the preliminary hedonic analysis.

According to their labels, the samples obtained for the physicochemical and sensory analyses were:

- C samples: frozen juice after thawing overnight at 5 °C.
- TT_R samples: pasteurized juice stored in the cold chamber at 5 °C.
- HP25 and HP50 samples: raw juice stored at 25 MPa or 50 MPa and 20 °C, respectively.
- TT_HP25 samples: pasteurized juice stored at 25 MPa and 20 °C.

Immediately after each storage experiment, samples were physicochemically analyzed and a triangle test was performed. C samples represent "fresh" juices at day 0.

4.3.3.3. Physicochemical analyses of the strawberry juice samples

The analyzed physicochemical parameters were chosen so as to reflect the main sensory characteristics of strawberry juice: instrumental color for its appearance, viscosity for its mouth feeling, and TSS and TA for their influence on the perception of flavor (Malundo, Shewfelt, Ware, & Baldwin, 2001). pH was also measured because of its effect on some physicochemical changes such as color and texture (Andrés-Bello, Barreto-Palacios, García-Segovia, Mir-Bel, & Martínez-Monzó, 2013).

Color of strawberry juice was characterized objectively according to L^*, a^*, and b^* color parameters in the CIELab uniform color space defined by the Commission Internationale d'Eclairage. The method followed is described in Chapter 3. From L^*, a^*, and b^* parameters, total color change ΔE^* was calculated.

Kinematic viscosity, ν, was determined by capillary viscosimetry as described in Chapter 3 and it was expressed in centiStokes (1 cSt = $10^{-6} m^2 \cdot s^{-1}$). Viscosity deviation ($\Delta\nu$) was calculated by Eq (4.3.1) and expressed in percentage.

$$\Delta\nu = \frac{100 \cdot (\nu_S - \nu_0)}{\nu_0} \tag{4.3.1}$$

where ν_0 and ν_S are the viscosity of the samples compared.

TSS was approximated from the refractive property of the juice in terms of equivalent content in sucrose (°Brix). This was done by using a digital refractometer (Leica AR200, Leica Microsystems Inc, New York, USA) with automatic temperature compensation.

pH was measured with a combined glass electrode (6.0280.300 iEcotrode Plus, Metrohm, Herisau, Switzerland) previously calibrated against standard buffers at pH 7.0 and 4.0.

TA was measured using an automatic titrator (Titrando 907, Metrohm, Herisau, Switzerland) as described in Chapter 3. Total titratable acidity was expressed in g citric acid·mL^{-1} of juice.

4.3.3.4. Triangle tests

Four triangle tests were performed. These were distributed over one year in three different sessions according to each series of storage experiments:

- Series 1, first session of triangle tests:
    - Triangle test 1: TT_R *vs.* C
    - Triangle test 2: HP25 *vs.* C
- Series 2, second session of triangle tests:
    - Triangle test 3: HP50 *vs.* C
- Series 3, third session of triangle tests:
    - Triangle test 4: TT_R *vs.* TT_HP25.

Panels for each triangle test comprised from 20 to 24 semi-trained judges belonging to the staff of the Institute of Food Science, Technology and Nutrition (ICTAN-CSIC). Three strawberry juice samples were presented to the judges and each one was codified by a three-digit random code. The order of presentation was randomly assigned for each judge, verifying that, whenever possible, the presentation order of the samples was balanced. Juice was served in a transparent plastic glass (30 mL per glass). Care was taken to present the three juice glasses at approximately the same

temperature. This was done by always using the same preparation procedure: after storage, the bags were opened and mixed in a bottle; if necessary, the bottle was refrigerated during at least one hour to have the same initial temperature for the two kinds of samples to taste; the three glasses were prepared at the same time some minutes before the test. No information about the aim of the study nor about juice samples was provided to the judges prior to the test. Judges were informed that two samples were identical and one sample was different and they were forced to choose one. In addition, the judges were asked about their preferred sample and invited to write the comments that they considered pertinent. An example of the scorecard used in the triangle tests can be found (Appendix 2).

4.3.3.5. Data analysis

All physicochemical measurements in each sample were carried out in triplicate, with the exception of the kinematic viscosity that was performed in duplicate. Results of color, TTS, pH, and TA were statistically analyzed by using IBM SPSS Statistics v. 19.0.0 for Windows (SPSS Inc., Somers, NY, USA). After a one-way analysis of variance (ANOVA), significant differences among means ($p < 0.05$) were determined by Tukey's multiple range test when the variances were homogeneous and by Tamhane's T2 test when it was not possible to assume homoscedasticity.

The results of triangle tests were compared with tables of minimum number of correct responses required for significance (UNE-EN ISO 4120:2008).

4.3.4. Results and discussion

4.3.4.1. Effect of storage on the physicochemical characteristics of strawberry juice

In parallel to each triangle test, strawberry juices were characterized by measuring some of their physicochemical properties. Table 4.3.1 shows the high variability of some physicochemical characteristics, namely color and viscosity, between the different batches of strawberry juice (control samples in sessions 1 and 2). But more interestingly, these analyses were performed to obtain an objective reference of how

much different or similar the samples compared in the subjective sensory test are. The absence or presence of significant differences between two types of samples may be related with the sensory test results as it will be commented later.

In agreement with the previous study (Part 4.1), both cold and hyperbaric storage had an effect on juice color and viscosity after 15 days. TSS, pH, and TA remained unchanged ($p < 0.05$) independently of the storage method. This indicates some changes in the visual aspect and texture of the juices but not in acid levels (in terms of pH and TA) and sugar concentrations (reported as TSS).

In order to quantify color and viscosity changes, the total color difference (ΔE^*) and the fractional deviation between the viscosities ($\Delta \nu$) of two samples to be compared in a given triangle test were calculated. Table 4.3.2 reveals that the color of both HP25 and HP50 samples was the most similar to that of C samples while the color of TT_R samples was the most different. Color changes in TT_R samples are probably produced during the pasteurization step prior to storage. However, differences between samples found by means of instrumental measurements do not necessarily have to be detected by judges since a threshold value of perception exists. In the case of color, a threshold value of $\Delta E^* = 1$ is frequently assumed as a basis for a color perceptible difference (Gonnet, 1998; Rein & Heinonen, 2004). On this basis, color differences would only be easily detected by judges in the case of TT_R samples compared to C ones. Regarding the viscosity fractional deviations, results were the other way around: TT_R samples showed the lowest viscosity deviations.

	Session 1			Session 2		Session 3	
	C	**TT_R**	**HP25**	**C**	**HP50**	**TT_HP25**	**TT_R**
L*	33.99 ± 0.11 [a]	37.35 ± 0.06 [b]	33.96 ± 0.04 [a]	33.21 ± 0.14 [a]	34.20 ± 0.02 [b]	35.91 ± 0.04 [a]	35.21 ± 0.02 [b]
a*	13.90 ± 0.14 [a]	17.41 ± 0.24 [b]	13.91 ± 0.24 [a]	19.10 ± 0.12 [a]	18.80 ± 0.25 [a]	16.71 ± 0.16 [a]	17.44 ± 0.06 [b]
b*	3.90 ± 0.02 [a]	6.27 ± 0.07 [c]	4.71 ± 0.05 [b]	7.47 ± 0.05 [a]	6.92 ± 0.05 [b]	6.49 ± 0.05 [a]	6.45 ± 0.03 [a]
ν [1] (cSt)	6.37 ± 0.02	3.38 ± 0.07	1.09 ± 0.00	1019.82 ± 2.03	67.78 ± 0.10	25.09 ± 0.28	28.35 ± 0.13
TSS (°BRIX)	8.7 ± 0.1 [ab]	8.6 ± 0.0 [b]	8.7 ± 0.0 [a]	not measured	not measured	7.7 ± 0.1 [a]	7.6 ± 0.06 [a]
pH	3.69 ± 0.03 [a]	3.71 ± 0.01 [a]	3.72 ± 0.01 [a]	not measured	not measured	3.70 ± 0.00 [a]	3.71 ± 0.00 [a]
TA (g of citric acid·ml^{-1} of juice)	0.71 ± 0.01 [a]	0.71 ± 0.01 [a]	0.72 ± 0.00 [a]	not measured	not measured	0.79 ± 0.01 [a]	0.79 ± 0.01 [a]

Table 4.3.1. *Physicochemical characteristics of the strawberry juices employed in the triangle tests. Different letters within a row (of the same session) indicate significant differences ($p < 0.05$) between means. [1] Kinematic viscosity was measured in duplicate and therefore the statistical analysis could not be done.*

	TT_R *vs.* C	**HP25 *vs.* C**	**HP50 *vs.* C**	**TT_R *vs.* TT_HP25**
ΔE^*	5	1	1	1
$\Delta\nu$ [1]	47 %	83 %	93 %	13 %
TSS	not significant	not significant	not measured	not significant
pH	not significant	not significant	not measured	not significant
TA	not significant	not significant	not measured	not significant

Table 4.3.2. *Total color difference (ΔE^*) and fractional deviation between the viscosities of the samples used for each triangle test. [1] Kinematic viscosity was supposed to be significantly different when the difference between the means compared exceeds 0.6% (i.e. twice the standard uncertainty of the method).*

Besides, Table 4.3.2 shows that the physicochemical properties of pasteurized samples stored both under pressure (TT_HP25) and under refrigeration (TT_R), for 15 days, were similar between them at the end of storage, regardless the storage method applied. In these samples, differences in TSS, TA, and pH before and after storage were not significant ($p > 0.05$) and those in color and viscosity were small: $\Delta E^* = 1$ and $\Delta \nu = 13$ %.

A priori, from these analyses, each pair of samples of any triangle test should be found essentially different at least in viscosity parameter (Table 4.3.2).

4.3.4.2. Effect of storage on sensory perception of strawberry juice

4.3.4.2.1. Under pressure and cold stored juices *vs* "fresh" juices

Here are first presented the results of the triangle tests 1, 2, and 3 (sessions 1 and 2) in order to confirm whether significant sensory differences exist between "fresh" juices before storage (C samples) and either pasteurized juices preserved by the conventional strategy (TT_R samples) or "fresh" juice preserved by hyperbaric storage at room temperature (HP25 or HP50 samples).

Results of the triangle tests are given in Table 4.3.3. TT_R, HP25, and HP50 samples were perceived as different from C samples with a significance level of 0.001, getting approximately the same percentage of correct responses (about 70 %) in all cases. Increasing pressure level from 25 to 50 MPa did not improve the preservation of the initial characteristics of the raw strawberry juice.

Result	Triangle test	Total responses	Correct responses	p
Perceptible differences between "fresh" and stored samples in all cases	TT_R *vs.* C	24	16	< 0.001
	HP25 *vs.* C	24	16	< 0.001
	HP50 *vs.* C	20	14	< 0.001

Table 4.3.3. *Results of the triangle tests 1, 2, and 3. n. s. = not significant differences.*

Therefore, neither the conventional preservation strategy (pasteurization + refrigeration) nor the novel one (hyperbaric storage at room temperature of "fresh" juice) were able to maintain the original organoleptic characteristics of the raw

strawberry juice. Triangle tests demonstrated that the stored juices, independently of the preservation strategy, are perceived as different from the juice before storage (C sample).

It is important to note that thermal treatment probably had a strong influence in the differences found in TT_R samples. The judges' comments reported on the scorecards revealed that TT_R samples are noticed different in flavor more than in color and viscosity, probably as a consequence of the thermal treatment. Flavor of TT_R samples was described as sweeter than C samples. This sweet flavor could be caused by the caramelization of the sugars contained in the juice during the thermal processing. Despite the differences observed in the instrumental color and viscosity measurements (Table 4.3.2), judges did not report any comment about them.

As in the case of TT_R samples, the flavor of HP25 samples was also detected as different by the judges. Some of them described the taste of these samples as a sour taste. Despite the differences observed in the instrumental viscosity measurements between HP25 and C samples (Table 4.3.2), only one judge referred the lower viscosity of the HP25 sample. Although some of the judges remarked the sour taste of the samples stored under pressure, TSS content, TA, and pH were not significantly ($p > 0.05$) different from the corresponding values of the "fresh" sample. Consequently, TSS, TA, and pH appear not to be related with the organoleptic differences found by sensory analysis. Besides, as it could be expected from the instrumental results, none of the judges did any comment about color differences.

On the contrary, HP50 samples were perceived as different more because of their lower viscosity than because of their sour flavor. This is in agreement with the large differences observed in viscosity values (Tables 4.3.1 and 4.3.2). Some of the judges pointed out the difficulty of the test 3. This is not surprising since the volatile profile of juices stored at 50 MPa and room temperature for 15 days was very close to the volatile profile of control juices (Part 4.2).

In addition to the comments, judges were asked for their preference between the two samples that they found different in the test. In order to confidently establish this

preference, only the responses from those judges who answered correctly the triangle test were taken into account (Figure 4.3.1). Appraisals show that TT_R samples were preferred to C samples while C samples were preferred to the samples stored under pressure (whatever the case, HP25 or HP50). These preferences may be related with the sweetness of the samples since TT_R samples were described as sweeter than C samples, and C samples less sour than HP25 or HP50 samples. A specific hedonic sensory test would be necessary to conclude about the importance of this factor in the choice. Besides, the preference for thermally treated samples is also often a question of habit of the consumers (Pliner, 1982). In Spain, it is not common to drink strawberry juice alone but strawberry taste is present in many processed food products of the Spanish market: flavored milk, ice-cream, yogurt, etc. All these products are sweet, and then, this may also be a reason for the found preference.

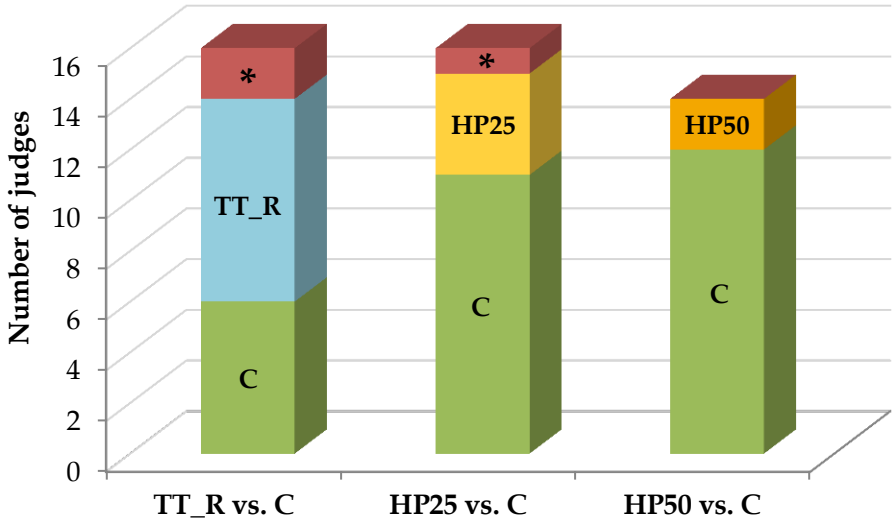

Figure 4.3.1. *Judges' preferences for a given sample. (* unreliable answers)*

4.3.4.2.2 Comparison of the storage methods for pasteurized juice preservation

From the above results, the advantage conferred by hyperbaric storage at low pressures (25 or 50 MPa) over the conventional storage method is unclear in terms of consumer preference. High pressures between 25 and 50 MPa were not enough to completely avoid the organoleptic changes which took place in raw strawberry juice during storage at room temperature. Significant differences in their sensory

characteristics were found between juices before and after hyperbaric storage. Significant differences were also found after cold storage. However, quite surprisingly, thermally treated samples were preferred over non-processed samples. All this led us to make a more direct comparison of both storage methods by focusing on pasteurized samples.

Therefore, samples of pasteurized juice were stored either at 5 °C and 0.1 MPa or at room temperature and 25 MPa. After 15 days of storage, they were submitted to triangle test and this test did not reveal any significant sensory difference between samples from any of the two storage methods. TT_HP25 and TT_R juices were perceived as indistinguishable by the judges (Table 4.3.4). This result is in agreement with the results of the corresponding physicochemical measurements. In fact, the differences were relatively small (Table 4.3.2). Since there were no significant differences between the juices, judges' answers about their preferences could not be taken into account.

Result	Triangle test	Total responses	Correct responses	*p*
No significant differences between pasteurized samples stored either under pressure or refrigerated	TT_HP25 *vs.* TT_R	24	12	n. s.

Table 4.3.4. *Results of the triangle test 4. n. s. = not significant differences.*

According to these last results, it is possible to conclude that hyperbaric storage at 25 MPa and room temperature and cold storage preserved the organoleptic characteristics of the pasteurized strawberry juice with the same efficiency.

4.3.5. Conclusion

Neither pasteurization + cold storage nor hyperbaric storage at 25 or 50 MPa and room temperature maintain unaltered the physicochemical and organoleptic characteristics of "fresh" strawberry juice after 15 days of storage. Instrumental measurements showed that pasteurization + cold storage affected color and viscosity while hyperbaric storage mainly produced viscosity losses. Sensory analyses revealed that

pasteurization + cold storage produced a sweet taste in the strawberry juice and TT_R samples were preferred by judges to C juices. In contrast, hyperbaric storage induced a sour taste in strawberry juices, especially in samples stored at 25 MPa and, therefore, control juices were preferred to those stored under pressure. The viscosity decay observed after instrumental measurements was also detected, mainly in juices kept at 50 MPa. Nevertheless, it is important to underline that these changes a not necessarily a problem. Strawberry juice is rarely consumed alone, since it is usually employed as an ingredient in sauce, jam, coulis, yogurts, etc. Therefore, deviations from the initial organoleptic characteristics could vanish or, at least, become less relevant when it is mixed and even further processed with other ingredients. This should be considered in next studies.

Moreover, with a pasteurization step prior to storage, hyperbaric storage at 25 MPa and room temperature is equivalent to cold storage in terms of the perceived organoleptic quality, at least, for 15 days. In the sensory analysis, judges were not able to detect differences and the instrumental measurements revealed that differences between them were very small. Therefore, as pasteurization step is common to both methods and hyperbaric storage does not require energy to maintain the product under pressure, considerable saving could be achieved with the hyperbaric storage.

The relation between the instrumentally measured physicochemical changes (included changes in the volatile profile from Part 4.2.) and the perceived sensory differences is not clear. The threshold from which a sensory difference is detected is different according to the kind of analysis (color, texture, or taste). For example, a change of juice color or viscosity can be significant from objective measurements and not from the consumer's point of view. Inversely, a change of juice flavor can be perceived by the consumer which is not reflected by TSS, pH, TA, or volatile profile. Therefore, in order to evaluate the impact of hyperbaric storage on the strawberry juice taste, a sensory analysis remains indispensable.

Given the lack of research in this area, this study should be considered as an exploratory investigation into the preservation of the organoleptic characteristics of the strawberry juice stored under high pressure.

In perspective of this study, it can be suggested to test additional pressure levels between 50 and 100 MPa in order to determine the lowest pressure from which the mentioned sour taste stops appearing after the hyperbaric preservation of raw juice. A descriptive sensory analysis could be also of interest to obtain more information about the potential relation between the sour taste, loss of viscosity, color change, and pressure level of storage.

4.3.6. References

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Chapter 5: Effect of pressure level and storage time on some of the most relevant quality parameters of strawberry juice

Part 5.1: Effect of hyperbaric storage at room temperature on microbial growth

EFFECT OF HYPERBARIC STORAGE AT ROOM TEMPERATURE ON MICROBIAL GROWTH

5.1.1. Abstract

The effect of hyperbaric storage at room temperature on microbial growth was evaluated in freshly-squeezed strawberry juice. To do so, strawberry juices were stored for one day at 0.1, 25, 50, 100, or 200 MPa and 20 °C and the effect of pressure level on microbial counts was assessed. For those pressures at which no microbial inactivation was detected after storage, new experiments were carried out. In these experiments, juices were stored for 10 and 15 days to evaluate the effect of storage time. In all the cases, total aerobic mesophilic, lactic acid bacteria, and yeasts and molds populations were counted just after storage and also after a 3-day recovery period at 0.1 MPa and 20 °C to assure the capability of microorganisms to grow after hyperbaric storage. Both pressure level and storage time had an effect on the microbial load. The greater the pressure and the longer the storage time, the greater the microbial damage produced. Thus, hyperbaric storage at 25-50 MPa for 1 day inhibited microbial growth in strawberry juice, but no microbial inactivation was detected. Higher pressures (100-200 MPa) or longer storage times (10-15 days) were needed to reduce the initial microbial load of the juices. After 1 day of hyperbaric storage, surviving microorganisms quickly recovered their cell proliferating capacity in juices stored at 25-50 MPa, but damage produced at higher pressures hampered microbial recovery at the acidic pH of strawberry juice. Longer storage times (15 days) at 50 MPa made also difficult microbial recovery, but not at 25 MPa.

5.1.2. Introduction

Acid fruit juices are products vulnerable to a number of spoilage yeasts and molds and a few acid-tolerant bacteria due to their substantial content in fermentable sugars. In general, the predominant spoilage microorganisms are yeasts because of their high acid tolerance and their frequent ability to grow anaerobically. Growth of yeasts is

usually accompanied by formation of ethanol and CO_2. Ethanol imparts a characteristic sweet taste and CO_2 can bulge cartons, split cans, and cause explosion of plastic bottles. Other signs of yeast spoilage include fermented flavors, turbidity, flocculation, pellicles, and clumping. Besides, the natural pectin cloud can be destroyed if pectinesterases are produced by the yeasts. On the contrary, molds are, with a few exceptions, strict aerobes and, therefore, their development in juices is restricted. Most bacteria do not grow in acidic media, but a few are able to thrive at low pH, namely some acetic acid and lactic acid bacteria and, also, some species from *Clostridium* and *Bacillus* genera. For example, lactic acid bacteria can grow in juices at pH as low as 2.8 and produce turbidity, opalescence, and sometimes, visible bubbles of gas and bursting of containers.

It is widely recognized that high pressure inhibits microbial growth in food and, in the literature, there are many studies demonstrating it. High pressure exerts many simultaneous effects on microorganisms which makes difficult to identify pressure individual action on cell growth and viability (Bartlett, 2002). Thus, depending on the level applied, pressure can produce damage in cell membrane, changes in cell morphology and in biochemical reactions, protein and key enzyme denaturations, inhibition of generic mechanisms, or disruption of ribosomes, among others (Sun, 2005).

In hyperbaric storage, pressure is intended to be used as a limiting factor for microbial growth, just like temperature in refrigeration. Nevertheless, depending on the pressure level employed, pressure can not only inhibit microbial growth, but also produce microbial inactivation. Thus, as described in Part 4.1, Segovia-Bravo, Guignon, Bermejo-Prada, Sanz, and Otero (2012) reported that the initial microbial load of strawberry juice was reduced by more than 2 $\log_{10}$ units after 15 days of hyperbaric storage (25-220 MPa) at room temperature. At the end of storage, microbial levels were below the detection limit and remained stable, at least for 15 additional days, under refrigeration at atmospheric pressure. Santos et al. (2014) found that storage pressures of 50-75 MPa, applied for 8 h, were needed to inhibit microbial growth in watermelon juice during hyperbaric storage at 20-37 °C. Hyperbaric storage at 25 MPa slightly

reduced total aerobic mesophiles growth as compared with that observed at atmospheric pressure, but *Enterobacteriaceae* and yeasts and molds counts were not significantly affected. Microbial inactivation was only detected after hyperbaric storage at pressures of 100 MPa and higher. Similar results were obtained by Queirós et al. (2014) in melon juice stored for 8 h at 25-150 MPa and 25-37 °C. On the other hand, Fidalgo et al. (2014) studied the microbial stability of watermelon juice after hyperbaric storage at 100 MPa and room temperature for 60 h. These authors found that total aerobic mesophiles, *Enterobacteriaceae*, and yeasts and molds reduced by 1, 2, and 1 $\log_{10}$ units, respectively, during hyperbaric storage, but remaining microorganisms were able to grow when the juice was decompressed and stored at 5 °C and 0.1 MPa for 7-14 days.

The different results observed in the microbiological stability of strawberry juice and melon and watermelon juices, both during and after hyperbaric storage, can be due to differences in the storage time (15 days in strawberry juice *vs.* 8-60 h in melon and watermelon juices), but also to differences in juice characteristics. As previously commented, the low pH of strawberry juice (about 3.5) provides some intrinsic microbiological stability while pH of melon and watermelon juices is substantially higher (5.7 and 5.9, respectively). Moreover, it is important to note that strawberry, melon, and watermelon juices employed for the storage experiments described were previously frozen. Different authors have shown that a freeze-thaw cycle can reduce microbial load in fruit juices, especially yeasts and molds counts (Duan & Zhao, 2009; Jeremiah, 1996; Sodeko, Izuagbe, & Ukhun, 1987). In fact, this treatment has been proposed as a relatively simple and effective way to reduce populations of foodborne pathogens and to extend the shelf life of apple juice and cider (Ingham, Schoeller, & Engel, 2006; Uljas & Ingham, 1999; Vojdani, Beuchat, & Tauxe, 2008). Freeze-thaw treatment is particularly effective in acid juices because a low pH sensitizes cells to the subsequent freeze-thaw stress (Uljas & Ingham, 1999; Yamamoto & Harris, 2001). Therefore, the microbial inactivation observed by Segovia-Bravo et al. (2012) in strawberry juices stored for 15 days at pressures as low as 25 MPa could be produced not only by pressure, but also by the combined stresses of the previous freeze-thaw treatment and the low pH of the juice.

The aim of this study was to evaluate the effect of pressure level and storage time on the microbial load of non-frozen strawberry juice during storage at room temperature. Moreover, the capability of microorganisms to grow after hyperbaric storage was also assessed. To study the effect of pressure level, strawberry juices were stored for 1 day at five different pressure levels (0.1, 25, 50, 100, and 200 MPa) and 20 °C. After storage, the microbial load (total aerobic mesophiles, lactic acid bacteria, and yeasts and molds) was quantified and compared with that initially present in the juice. For those pressures at which no microbial inactivation was detected after storage, new experiments were carried out. In these experiments, juices were stored for 10 and 15 days to evaluate the effect of storage time. In all the cases, microbial load was measured immediately after storage and, also, after a 3-day recovery period at atmospheric pressure and 20 °C.

5.1.3. Materials and methods

5.1.3.1. Preparation of the strawberry juice samples

In this study, unlike in all the others presented in this Thesis, different batches of strawberry juice were employed in each storage experiment to avoid freeze-thaw stresses.

Thus, before each storage experiment, a strawberry batch was purchased at commercial maturity from a local supplier. The fruits were washed with tap water and processed with a blender (Royal Blender Turbo 10-Speed, Type 212004, Princess, Netherlands). The liquid obtained was centrifuged at 3,500 *g* and 4 °C for 10 min (Sorvall Evolution RC Superspeed centrifuge, Thermo Scientific, Madrid, Spain) using a Fiberlite F8-6x1000y rotor (Thermo Scientific, Madrid, Spain). After that, supernatant was collected, filtered through a 0.1 mm pore diameter sieve, and packaged in 8 mL plastic bags avoiding headspace.

5.1.3.2. Storage experiments of strawberry juice

Storage experiments under pressure were carried out in a pilot-plant high-pressure storage system (model SV1, Institute of High Pressure Physics, Unipress Equipment

Division, Poland). The characteristics of the equipment are described in detail in Chapter 3.

To study the effect of pressure level, strawberry juices were stored for 1 day either at atmospheric pressure (control samples) or under high pressure (25, 50, 100, and 200 MPa) at 20 ± 2 °C. Control samples were stored in the dark and immersed in the same fluid as that used for compression in hyperbaric experiments to simulate exactly the same conditions. Temperature and/or pressure were recorded every 30 s by a data acquisition system (MW100 Data Acquisition Unit, Yokogawa Electric Corporation, Tokyo, Japan). In samples stored under high pressure, juice temperature increased by 1-4 °C during compression, depending on the pressure level applied but, in all cases, the target temperature was reached again in no more than 15 minutes.

For each storage condition, two juice bags were stored. After storage, one bag was immediately analyzed for microbial load. The other bag was kept at 20 ± 2 °C and atmospheric pressure for three additional days to evaluate the capability of microorganisms to grow during this recovery period after hyperbaric storage.

For those pressures at which no microbial inactivation was detected after storage, new experiments were carried out. In these experiments, juices were stored for 10 and 15 days to evaluate the effect of storage time. Again, microbial load was measured immediately after storage and, also, after a 3-day recovery period at atmospheric pressure and 20 °C.

All the storage experiments were performed, at least, in duplicate.

5.1.3.3. Microbial analysis

All samples were analyzed for total aerobic mesophiles (TAM), lactic acid bacteria (LAB), and yeasts and molds (YM). Of each sample, 1 mL was aseptically taken and added to 9 mL of sterilized peptone water. After homogenization, serial dilutions were made with the same diluent and duplicates of dilutions were plated on the appropriate media, according to the procedures described in Chapter 3. The detection limit was 1 $CFU \cdot mL^{-1}$ for TAM and LAB and 10 $CFU \cdot mL^{-1}$ for YM. Plate counts were expressed as

the decimal logarithm of colony forming units (CFU) per milliliter of strawberry juice (log_{10} CFU·mL^{-1}).

The evolution of the microbial load during storage in each batch of juice was expressed as the difference between counts at the end of storage (N_S) and initial counts (N_0). In the same way, the evolution of the microbial load during the 3-day recovery period was given as the difference between counts at the end of recovery period (N_R) and counts at the end of storage (N_S) for each microorganism category.

5.1.4. Results and discussion

5.1.4.1. Effect of the pressure level during storage on the microbial load of strawberry juices

Four batches of strawberry juice were employed to carry out replicated storage experiments at 0.1, 25, 50, 100, and 200 MPa and 20 °C for 1 day. Since only two hyperbaric vessels were available, a given batch of juice could only be tested at three pressure levels at the same time (at 0.1 MPa and two hyperbaric levels). Table 5.1.1 shows the microbial counts in the juices from the different batches at day 0 (N_0), after 1 day of storage (N_S) at different pressure levels and 20 °C, and after a 3-day recovery period (N_R) at 0.1 MPa and 20 °C.

Table 5.1.1 reveals that the initial microbial load was rather different in the four batches employed. Thus, TAM, LAB, and YM counts varied from 3.2 to 5.4, from 3.0 to 5.0, and from 3.6 to 5.0 log_{10} CFU·mL^{-1}, respectively. Batch 2 presented the lowest microbial load while Batch 4 was the most contaminated one.

Batch number	P (MPa)	TAM			LAB			YM		
		N_0	N_S	N_R	N_0	N_S	N_R	N_0	N_S	N_R
1	0.1	4.3	6.4	6.9	4.3	6.4	7.2	4.4	6.2	7.2
	25		4.8	5.8		4.3	6.2		4.2	6.2
	50		3.9	6.2		4.1	6.9		4.1	6.7
2	0.1	3.2	3.9	7.1	3.0	4.7	7.5	3.6	4.7	7.3
	25		3.1	6.3		3.4	6.8		3.1	6.6
	50		3.1	5.7		2.7	6.5		3.0	6.3
3	0.1	4.6	7.4	5.8	3.4	5.3	5.9	3.6	5.2	5.9
	100		2.4	1.6		2.0	1.0		2.4	3.4
	200		1.6	#		1.4	#		1.4	#
4	0.1	5.4	6.7	4.7	5.0	4.8	4.3	5.0	6.2	4.6
	100		3.9	4.9		3.8	3.2		4.1	3.6
	200		1.6	#		#	#		#	#

Table 5.1.1. *Total aerobic mesophiles (TAM), lactic acid bacteria (LAB), and yeasts and molds (YM) counts (log_{10} CFU·mL^{-1}) in strawberry juice at day 0 (N_0), after 1 day of storage (N_S) at different pressure levels and 20 °C, and after a 3-day recovery period (N_R) at 0.1 MPa and 20 °C. Symbol* **#** *indicates values under the detection limit.*

5.1.4.1.1. Microbial load just after one day of storage

Figure 5.1.1 shows the evolution of the microbial load in strawberry juice after one day of storage at 20 °C as a function of the pressure level. Positive log_{10} (N_S/N_0) values in Figure 5.1.1 mean microbial growth during storage while negative log_{10} (N_S/N_0) values mean microbial inactivation during storage.

After one day of storage at atmospheric pressure, microbial growth was detected in all the samples as expected. Mean positive increments of 1.8, 1.4, and 1.4 log_{10} units were measured for TAM, LAB, and YM populations, respectively. On the contrary, no microbial growth was observed in any of the samples stored under pressure. Thus, at 25 and 50 MPa, TAM, LAB, and YM growth was inhibited and the initial counts were maintained almost invariable during storage. These results agree with those reported by other authors. Thus, ZoBell and Johnson (1949) showed that hydrostatic pressures from 50 to 60 MPa retarded the growth of many kinds of microorganisms. Aoyama, Shigeta, Okazaki, Hagura, and Suzuki (2004) observed that pressures between 40 and 70 MPa at 30-40 °C were able to reduce the viable counts of different microorganisms (bacteria and yeasts) inoculated in two liquid culture media and in fresh fish (anchovy). Moreover, as already mentioned in Section 5.1.2, Queirós et al. (2014) and

Santos et al. (2014) also found that storage at 50-75 MPa for 8 h inhibited microbial growth in melon and watermelon juices. However, in contrast to our results, these authors found that hyperbaric storage at 25 MPa did not produce any inhibitory effect. The microbial growth inhibition observed in strawberry juice at such a low pressure could be partially caused by its acidic pH. Thus, it is well known that the susceptibility of microorganisms to pressure increases as pH deviates from neutral values (Farkas & Hoover, 2000; Linton, Patterson, & Patterson, 2000; Rendueles, Omer, Alvseike, Alonso-Calleja, Capita, & Prieto, 2011; Smelt, 1998).

Table 5.1.1 and Figure 5.1.1 also reveal that the microbial population was substantially reduced in juices stored at 100 and 200 MPa. Moreover, the greater the storage pressure, the greater the microbial inactivation produced and, thus, mean TAM, LAB, and YM reductions of 1.8, 1.3, and 1.1 $\log_{10}$ units and of 3.3, 3.5, and 3.1 $\log_{10}$ units were detected after hyperbaric storage at 100 and 200 MPa, respectively. Similar results were observed by Queirós et al. (2014), Santos et al. (2014), and Fidalgo et al. (2014) when storing melon and watermelon juices at pressures of 100-150 MPa. Moreover, Queirós et al. (2014) also observed an effect of the pressure level applied during storage and, after 8 h of hyperbaric storage, they described a log linear decrease of TAM and YM counts in melon juice as a function of the storage pressure.

Figure 5.1.1 shows that the results obtained in replicated experiments were consistent, even though different juice batches were employed. No great differences must be expected in the physicochemical characteristics (pH, water activity, titratable acidity, for example) of the different juice batches, but their microbial characteristics could be rather different (species of microorganisms present in the juice, strain, amount, state, and stage). Therefore, the consistency between results is noteworthy because it is well known that the effect of any stress on a microbial population largely depends on the characteristics of both the treatment medium and the microbial population (Linton et al., 2000).

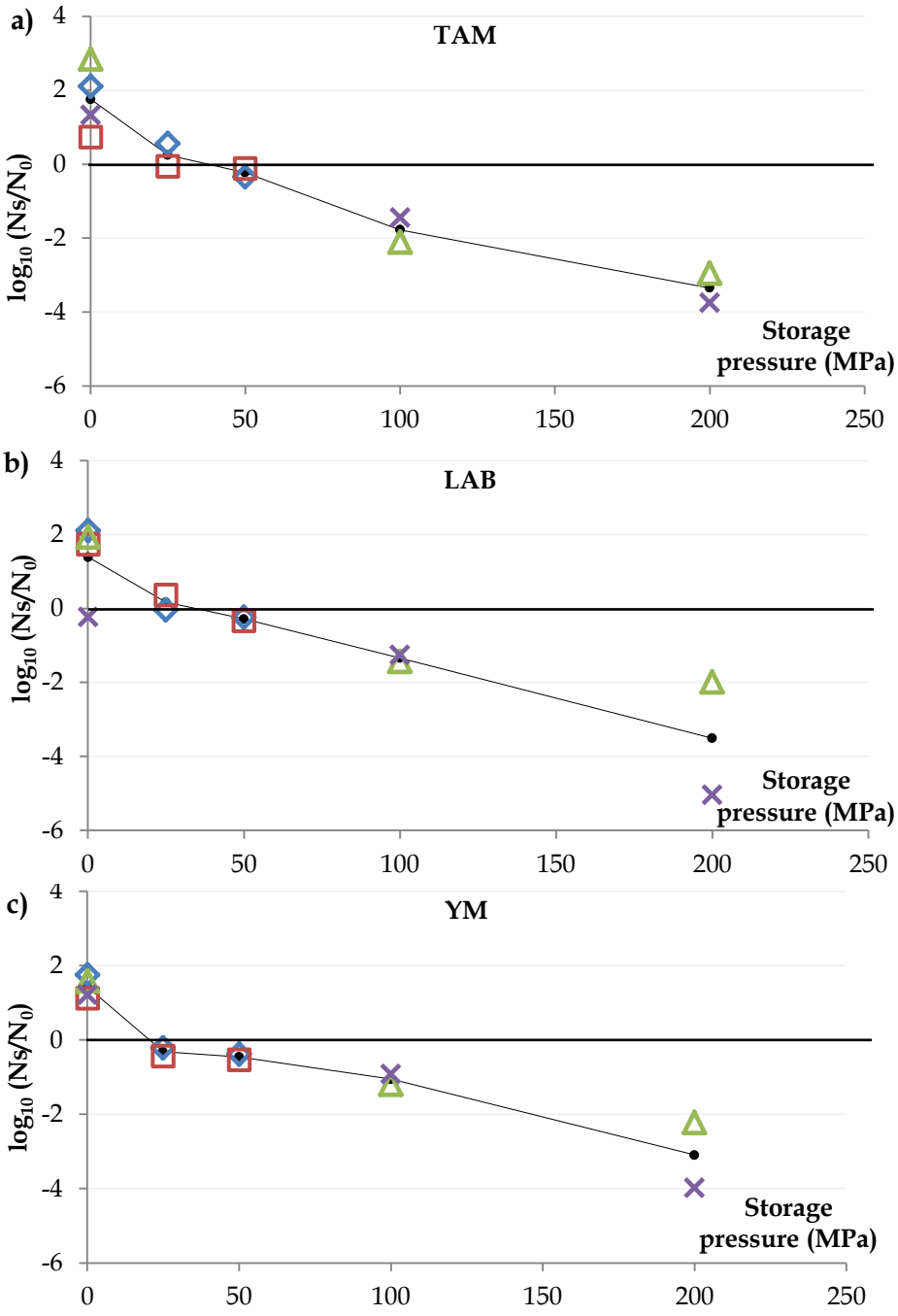

Figure 5.1.1. *Evolution of a) total aerobic mesophiles (TAM), b) lactic acid bacteria (LAB), and c) yeasts and molds (YM) in strawberry juice during storage at different pressure levels and 20 °C for 1 day. Positive $\log_{10}(N_S/N_0)$ values mean microbial growth during storage while negative $\log_{10}(N_S/N_0)$ values mean microbial inactivation during storage. Different symbols represent different batches of juice (Batch 1: ◇, Batch 2: □, Batch 3: △, and Batch 4: ×). Black lines connect mean values (●) for each pressure level.*

5.1.4.1.2. Microbial load after a 3-day recovery period

It is well known that, under moderated pressures, microorganisms are more likely to be stressed, or injured, than killed. Thus, once the high pressure is released, cells can repair the injuries and proliferate (Abe, 2007; Huang, Lung, Yang, & Wang, 2014). Therefore, the capability of microorganisms to grow in the juice after hyperbaric storage was evaluated after a 3-day recovery period, at atmospheric pressure and 20 °C.

The results, shown in Figure 5.1.2, reveal a clear effect of the storage pressure on the microbial growth after decompression. As described in the previous Section, hyperbaric storage at 25-50 MPa inhibited microbial growth. However, once pressure was released, microorganisms recovered their cell proliferating ability and they were able to grow in the strawberry juice. In fact, TAM, LAB, and YM counts after the 3-day recovery period were quite similar to those observed in juices maintained at 0.1 MPa for the same period (Table 5.1.1). On the other hand, hyperbaric storage at 100-200 MPa produced some microbial inactivation. The data in Table 5.1.1 and Figure 5.1.2 suggest that, after pressure release, surviving microorganisms presented some sublethal damage. The extent of damage depended on the pressure applied during storage. Thus, in juices maintained at 100 MPa, TAM, LAB, and YM counts after the 3-day recovery period were quite similar to those just after storage (Table 5.1.1). Therefore, surviving microorganisms hardly grow in the strawberry juice during the recovery period, but they proliferated when they were plated on the appropriate media just after storage. Sublethal damage in juices stored at 200 MPa was considerably greater, and surviving microorganisms could not overcome the acidic conditions of the strawberry juice during the recovery period. Thus, TAM, LAB, and YM counts after this period were under the limits of detection in all the cases (Table 5.1.1).

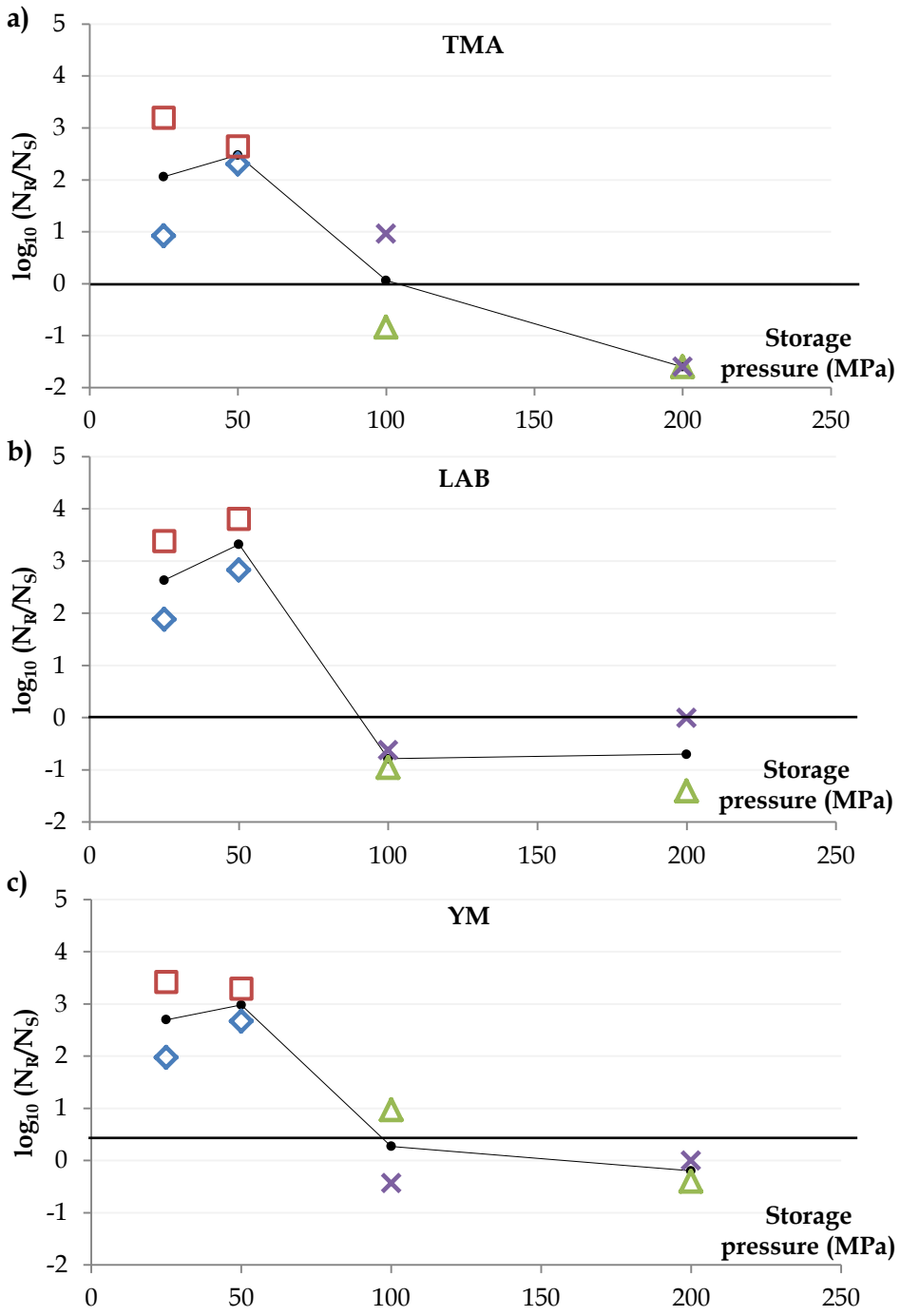

Figure 5.1.2. *Evolution of a) total aerobic mesophiles (TAM), b) lactic acid bacteria (LAB), and c) yeasts and molds (YM) in strawberry juice stored at different pressure levels for a day (25, 50, 100, and 200 MPa) during the 3-day recovery period at atmospheric pressure and 20 °C. Positive $\log_{10}(N_R/N_S)$ values mean microbial growth during the recovery period while negative $\log_{10}(N_R/N_S)$ values mean death during the recovery period. Different symbols represent different batches of juice (Batch 1: ◇, Batch 2: □, Batch 3: △, and Batch 4: ×). Black lines connect mean values (●) for each pressure level.*

Similar results were found by Fidalgo et al. (2014) in watermelon juice. This is a highly perishable food, with low acidity and high water activity, where microorganisms can

grow easily. Fidalgo et al. (2014) reported that hyperbaric storage at 100 MPa for 60 h reduced the initial microbial load in watermelon juice but, after pressure release, surviving TAM, LAB, YM, and *Enterobacteriaceae* were able to grow at atmospheric pressure and 5 °C.

All these results reveal that after hyperbaric storage at pressures lower than or equal to 100 MPa, strawberry juice should be kept refrigerated if it is not going to be immediately consumed or processed. Low temperature may hinder the capacity of the cells to repair sublethal damage and, therefore, storage temperature can play a critical role in the recovery of microorganisms after stresses (Farkas & Hoover, 2000; Linton et al., 2000; Smelt, 1998). For example, after pressure processing at 500 MPa for 10 minutes, Koseki & Yamamoto (2006) observed an apparent complete inactivation of *Escherichia coli* in phosphate buffered saline solution. However, after a 120-hour incubation period at 25 °C, *E. coli* recovered its cell proliferating ability. No recovery was observed when it was incubated at 4 °C.

5.1.4.2. Effect of the storage time on the microbial load of strawberry juices

Pressure effects on microorganisms depend not only on the pressure level applied, but also on the holding time. Previous results, described in Part 4.1 (Segovia-Bravo et al., 2012), showed that hyperbaric storage at 25 MPa and 20 °C for 15 days reduced the initial TAM and YM counts in strawberry juice by more than 2 $\log_{10}$ units. However, the samples used in that study had been frozen and thawed before the experiment. Therefore, it is unclear if these results were exclusively due to the long storage period under pressure or if the previous freeze-thaw treatment could also have contributed to that microbial inactivation. Therefore, in this study, additional storage experiments were performed at 25 and 50 MPa (pressure levels at which no microbial inactivation was detected after 1 day of storage) for 10 and 15 days to evaluate the effect of storage time.

Batch number	P (MPa)	TAM			LAB			YM		
		N_0	N_S	N_R	N_0	N_S	N_R	N_0	N_S	N_R
1 day of storage										
1	0.1	4.3	6.4	6.9	4.3	6.4	7.2	4.4	6.2	7.2
	25		4.8	5.8		4.3	6.2		4.2	6.2
	50		3.9	6.2		4.1	6.9		4.1	6.7
2	0.1	3.2	3.9	7.1	3.0	4.7	7.5	3.6	4.7	7.3
	25		3.1	6.3		3.4	6.8		3.1	6.6
	50		3.1	5.7		2.7	6.5		3.0	6.3
10 days of storage										
5	0.1	4.7	6.7	*	3.7	6.9	*	3.6	6.9	*
	25		1.9	2.9		1.0	**		2.6	6.5
	50		1.7	4.1		0.0	4.0		1.7	1.7
6	0.1	6.3	6.6	*	6.2	6.6	*	6.1	6.0	*
	25		4.3	7.9		4.6	7.5		4.2	7.5
	50		3.3	6.5		2.7	6.5		3.0	6.5
15 days of storage										
7	0.1	3.4	*	*	3.3	*	*	3.9	*	*
	25		5.4	6.2		6.0	6.1		5.8	6.0
	50		1.7	1.3		0.4	1.6		1.9	1.9
8	0.1	4.1	6.5	*	3.3	6.5	*	3.9	6.6	*
	25		3.3	5.8		1.6	3.3		2.6	3.1
	50		1.6	1.7		#	#		1.4	#
9	0.1	4.1	6.5	*	3.5	6.6	*	3.7	6.6	*
	25		4.3	6.5		4.4	6.4		4.2	6.2
	50		1.6	#		#	#		#	1.6

Table 5.1.2. *Total aerobic mesophiles (TAM), lactic acid bacteria (LAB), and yeasts and molds (YM) counts (log_{10} CFU·mL^{-1}) in strawberry juice at day 0 (N_0), after storage (N_S) at different pressure levels and 20 °C for different times, and after a 3-day recovery period (N_R) at 0.1 MPa and 20 °C. Symbol * indicates missing data due to the explosion of the package. Symbol ** indicates missing data due to molds contamination. Symbol* **#** *indicates values under the detection limit.*

As previously explained, different batches of strawberry juice had to be employed to carry out replicated storage experiments. Table 5.1.2 shows the microbial counts of the juices from the different batches at day 0 (N_0), after storage (N_S) at different pressure levels and 20 °C for different times, and after a 3-day recovery period (N_R) at 0.1 MPa and 20 °C. Again, the heterogeneity in the initial microbial load of the samples is evident. TAM, LAB, and YM counts ranged from 3.2 to 6.3, from 3.0 to 6.2, and from 3.6 to 6.1 log_{10} CFU·mL^{-1}, respectively.

5.1.4.2.1. Microbial load just after storage

Figure 5.1.3 shows the evolution of TAM, LAB, and YM loads in strawberry juice just after 1, 10, and 15 days of storage at different pressure levels and 20 °C.

During storage at atmospheric pressure, considerable microbial growth was detected in all the samples as expected. However, Figure 5.1.3 shows some dispersion in the extent of microbial growth observed in some replicated experiments, probably due to the different microbial characteristics of the juice batches employed (species of microorganisms in the juice, amount, and physiological state, among others). For example, juices of Batch 6 presented a very high initial load (see Table 5.1.2). Thus, initial TAM, LAB, and YM loads were close to 7 $\log_{10}$ CFU·mL^{-1}, that is, the maximal microbial load observed in all the experiments after storage. After 10 days of storage at atmospheric pressure, TAM, LAB, and YM loads remained almost constant: the increments were only of 0.33, 0.40, and -0.15 $\log_{10}$ units, respectively (Figure 5.1.3). In contrast, juices of Batch 5, with an initial load considerably lower, presented TAM, LAB, and YM increments of 2.1, 3.3, and 3.3 $\log_{10}$ units, respectively, after 10 days of storage. In both cases, sample packages were swollen due to the microbial gas production and juice samples presented clear signs of spoilage. After 15 days of storage at 0.1 MPa, spoilage was more evident and, even, sample packages from Batch 7 exploded.

The effect of the storage time was obvious in the samples stored under pressure. Thus, as commented in Section 5.1.4.1.1, after 1 day of storage, microbial growth was inhibited in samples held at 25 and 50 MPa, but no significant microbial inactivation was observed. When storage time was extended up to 10 days, the decrease of the initial counts was evident and slightly higher at 50 MPa. Thus, at 25 MPa, mean decreases of 2.3, 2.1, and 1.5 $\log_{10}$ units were detected in TAM, LAB, and YM counts, respectively, while at 50 MPa, these decreases were 3.0, 3.6, and 2.6 $\log_{10}$ units. Similar results were found after 15 days of hyperbaric storage at 50 MPa. The relationship between microbial damage and pressure-holding time has been shown by different authors (Aoyama, et al., 2004; Patterson, Quinn, Simpson, & Gilmour, 1995; Varela-Santos et al., 2012). However, this relationship is not linear: the effect of pressure-

holding time decreases as the pressure-holding time is prolonged (Koseki & Yamamoto, 2007). Thus, for pressure processing, Patterson et al. (1995) reported that, in general, inactivation curves tend to be exponential, with an initial rapid decrease during the first minutes followed by a "tail", representing the most pressure resistant population.

A deep insight into Figure 5.1.3 showed that juices maintained at 25 MPa presented inconsistent results. Thus, depending on the juice batch, the microbial load after storage was higher, similar, or lower than the initial counts. Nevertheless, in all the cases, microbial counts were lower than those observed after storage at 0.1 MPa (Table 5.1.2). Moreover, unlike in samples stored at atmospheric pressure, sample packages were not swollen in any case and no visual signs of spoilage were observed. These results clearly indicate that hyperbaric storage at 25 MPa slows down microbial growth in strawberry juice, but this pressure level is too low to guarantee microbial inactivation, even after relatively long storage times. Results depend on the characteristics of the initial microbial load in the product. Moreover, the elimination of pressure labile microflora can favor the growth of pressure resistant populations. On the other hand, it is important to note that sublethal stresses can induce the expression of cell repair systems (Lado & Yousef, 2002) and, therefore, an adaptation of microorganisms to stress could take place during storage at 25 MPa. In the light of these results, the microbial inactivation reported in Part 4.1 after 15 days of hyperbaric storage at 25 MPa and room temperature was likely related, at least in part, to the stress caused by the previous freeze-thaw treatment.

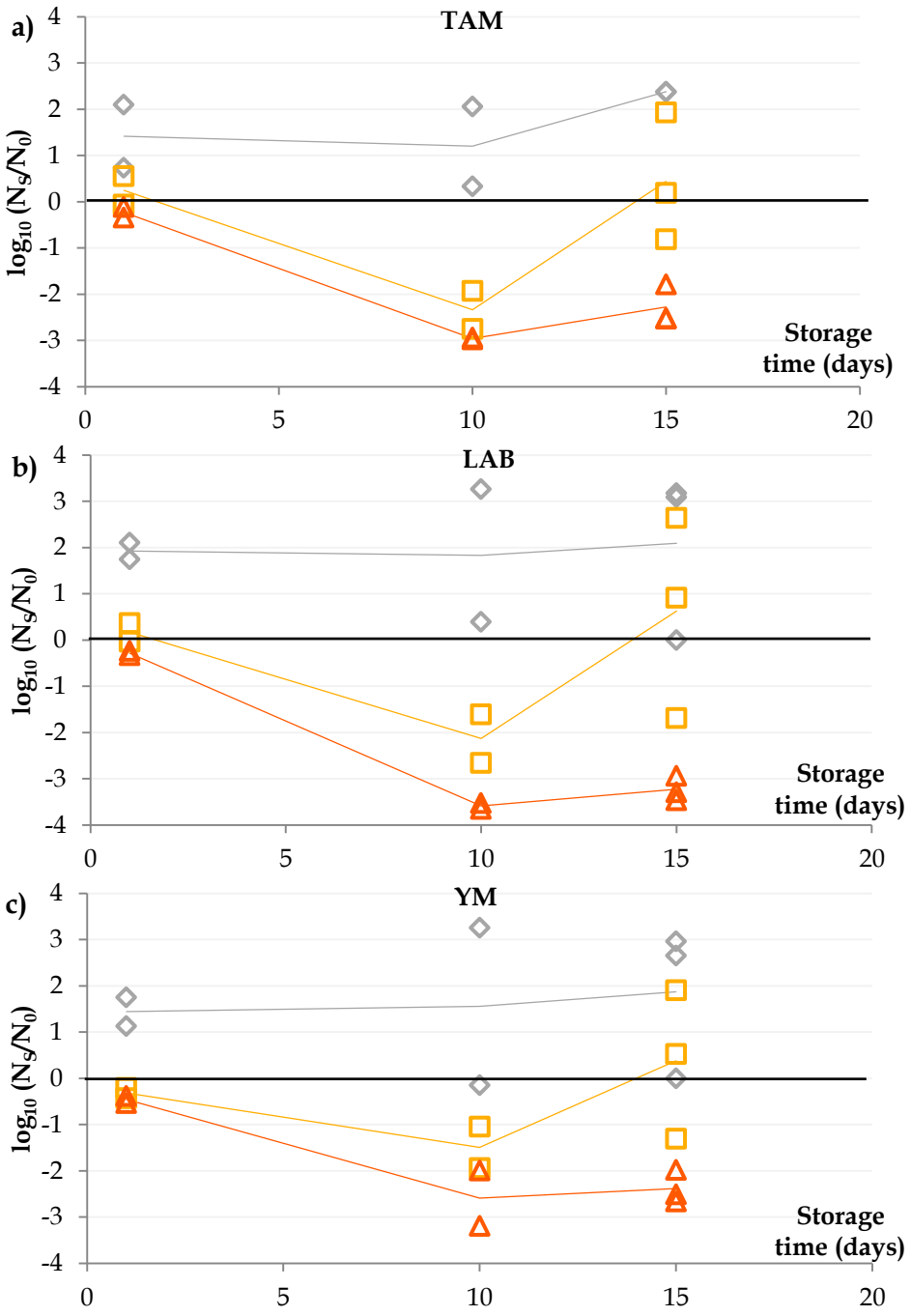

Figure 5.1.3. *Evolution of a) total aerobic mesophiles (TAM), b) lactic acid bacteria (LAB), and c) yeasts and molds (YM) in strawberry juice during storage at different pressure levels (0.1 MPa: ◇ , 25 MPa: □ , and 50 MPa: △) and 20 °C for different times (1, 10, and 15 days). Positive $log_{10}(N_S/N_0)$ values mean microbial growth during storage while negative $log_{10}(N_S/N_0)$ values mean microbial inactivation during storage Solid lines connect mean values for each pressure level.*

5.1.4.2.2. Microbial load after a 3-day recovery period

The capability of microorganisms to grow in the juice after 1, 10, and 15 days of hyperbaric storage at 25 and 50 MPa was evaluated during a 3-day recovery period at atmospheric pressure and 20 °C.

The results, shown in Figure 5.1.4, reveal a clear effect of the storage time on the microbial growth after pressure release, especially in samples stored at 50 MPa. As previously commented, after hyperbaric storage for 1 day at 25-50 MPa, microorganisms recovered their cell proliferating ability and they were able to grow in the strawberry juice. The same occurred after 10 days of hyperbaric storage. Thus, Figure 5.1.4 shows positive values of $\log_{10}(N_R/N_S)$, that means, microbial growth during the 3-day recovery period. Moreover, in general terms, the extent of microbial growth during the recovery period was similar in juices stored under pressure for 1 and 10 days and, also, in juices maintained at 25 and 50 MPa. After 15 days of hyperbaric storage, microorganisms in juices maintained at 25 MPa could proliferate when returned to atmospheric pressure, but LAB and YM growth during the recovery period seems to be lower than in juices stored for 1 or 10 days. This is more evident in juices stored at 50 MPa. In fact, in these juices, microbial population maintained almost constant during the recovery period ($\log_{10}(N_R/N_S)$ values close to zero) and, even, in some experiments microbial death was observed (see TAM counts in Batch 9, Table 5.1.2). Thus, after 15 days at 50 MPa, microorganisms were in some way damaged and they were not able to grow in the strawberry juice during the 3-day recovery period.

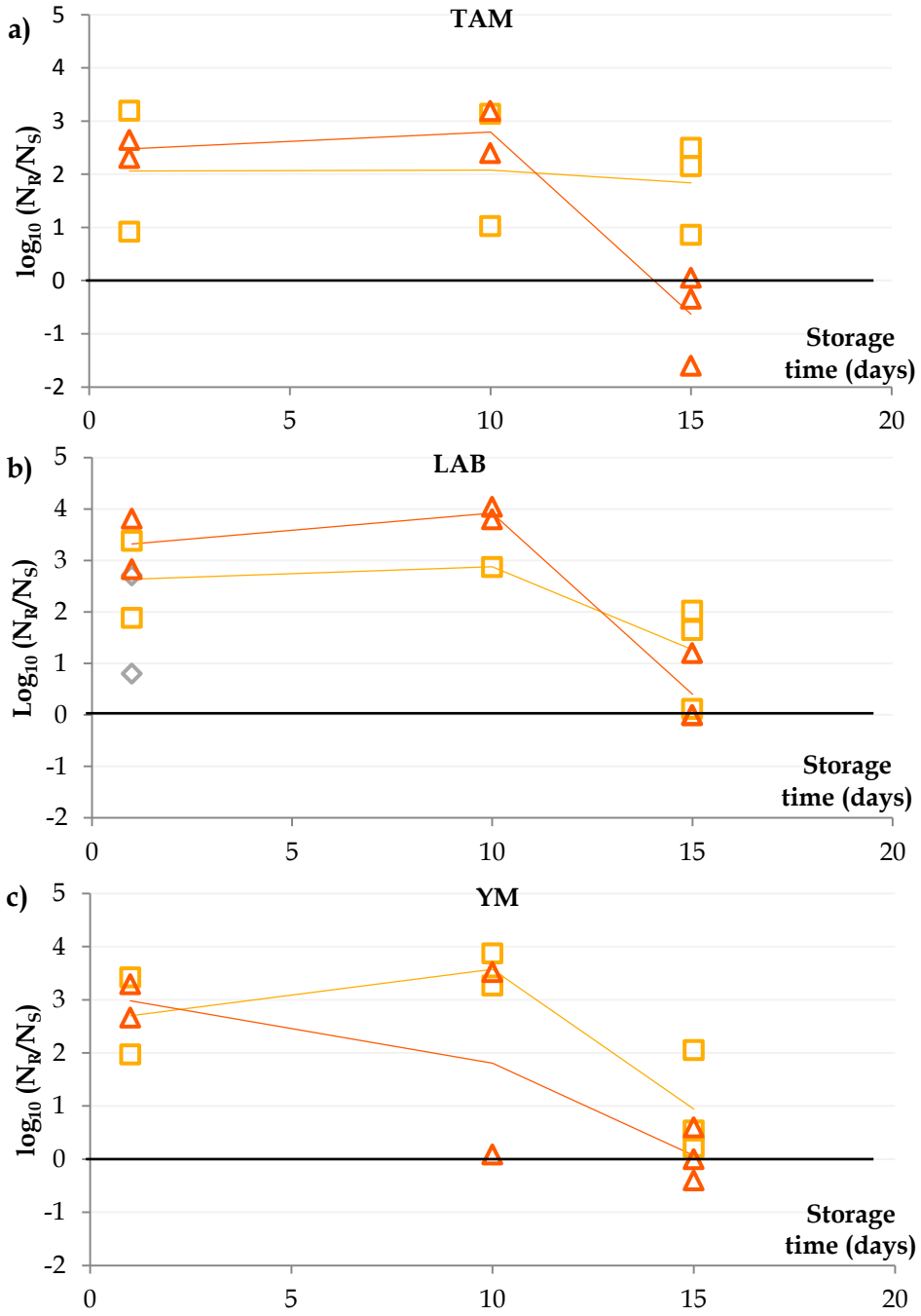

Figure 5.1.4. *Evolution of a) total aerobic mesophiles (TAM), b) lactic acid bacteria (LAB), and c) yeasts and molds (YM) in strawberry juice stored at different pressure levels (25 MPa: □ and 50 MPa: △) for different times (1, 10, and 15 days) during the 3-day recovery period at atmospheric pressure and 20 °C. Positive log10 (NR/NS) values mean microbial growth during the 3-day recovery period while negative log10 (NR/NS) values mean microbial death during the 3-day recovery period. Solid lines connect mean values for each pressure level.*

5.1.5. Conclusions

The results obtained in this study clearly showed that microbial growth during storage is affected by both storage pressure and time. The greater the pressure and the longer the storage time, the greater the microbial damage produced. Thus, hyperbaric storage at relatively low pressures (25-50 MPa) for short times (1 day) inhibited microbial growth in strawberry juice, but no microbial inactivation was detected. Pressure levels of 100-200 MPa were needed to reduce the initial microbial load of the juice after 1 day of storage. Longer storage times produced the same effect and, thus, microbial inactivation at 25-50 MPa was observed after 10-15 days of storage.

Damage observed in surviving microorganisms after pressure release also depended on the storage pressure and time. Thus, after 1 day of storage at 25-50 MPa, microorganisms recovered their cell proliferating capacity when the juice was maintained at atmospheric pressure and room temperature. In contrast, after storage at 100-200 MPa, microorganisms were seriously damaged and their recovery at the acidic pH of the strawberry juice was difficult, especially after storage at 200 MPa. Longer storage times (10-15 days) also hampered microbial recovery after storage at 50 MPa.

These results make clear the advantages of hyperbaric storage at room temperature over traditional refrigeration. Depending on the pressure level applied and the storage time, hyperbaric storage at room temperature does not only retard microbial growth as refrigeration does, but it is also able to reduce the microbial load in the food. Therefore, from a microbiological point of view, this novel method may prove more efficient than cold storage in preserving food quality.

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Part 5.2: Effect of hyperbaric storage at room temperature on color[5]

[5] Bermejo-Prada, A., & Otero, L. Effect of hyperbaric storage at room temperature on some mechanisms of color degradation in strawberry juice. *Food and bioprocess technology.* Submitted in August 2014.

EFFECT OF HYPERBARIC STORAGE AT ROOM TEMPERATURE ON COLOR

5.2.1. Abstract

The effect of hyperbaric storage at room temperature on some mechanisms of color degradation in strawberry juice, apart from microbial spoilage, was evaluated. To do so, strawberry juices, with an added antibiotic solution, were stored for 1, 2, 5, 7, 10, and 15 days at three pressure levels (0.1, 50, and 200 MPa) and 20 °C. The evolution of instrumental color parameters (L^*, a^*, and b^*), total phenolic and anthocyanin contents, polyphenol oxidase and peroxidase activities, and percent polymeric color during storage was compared in samples kept at different pressures. Color differences due to the storage under pressure were very slight to the naked eye, but instrumentally perceptible and significant ($p < 0.05$). The results clearly showed that storage pressure affected some mechanisms of color degradation and significant peroxidase inactivation and lower percent polymeric color were found in samples stored at 200 MPa as compared to samples kept at atmospheric pressure.

5.2.2. Introduction

Color is one of the most important sensory attributes in food because this visual property is the first evaluated by consumers and, most of the time, it will determine whether the food product is acceptable or not. In strawberry products, their attractive bright red color is mainly due to the presence of phenolic compounds, more specifically anthocyanins. The most common are derivatives of bright red pelargonidin and dark red cyanidin (Gössinger et al., 2009a). Among all the anthocyanins, pelargonidin 3-O-glucoside (Pg-3-glu) is the main responsible for the appealing, bright red color of strawberry products (Aaby, Wrolstad, Ekeberg, & Skrede, 2007; Garzón & Wrolstad, 2002) which represents between 76 and 95 % of total anthocyanins (Aaby et al., 2007; Cerezo, Cuevas, Winterhalter, Garcia-Parrilla, & Troncoso, 2010; da Silva,

Escribano-Bailón, Pérez Alonso, Rivas-Gonzalo, & Santos-Buelga, 2007; Holzwarth, Korhummel, Carle, & Kammerer, 2012a; Verbeyst, Hendrickx, & Loey, 2012).

However, these compounds are unstable and easily susceptible to degradation during storage, even in those products that have been previously pasteurized (Gössinger et al., 2009b; Holzwarth, Korhummel, Kammerer, & Carle, 2012b). Apart from microbial spoilage, color losses can be produced by enzymatic oxidation and non-enzymatic browning reactions. Enzymatic oxidations are catalyzed by oxidoreductases, such as polyphenol oxidase (PPO) or peroxidase (POD), among others, that degrade phenolic compounds to undesirable yellow, brown, or black pigments, responsible for color degradation. Moreover, monomeric anthocyanins are also involved in complex associations, including copigmentation, self-association, and polymerization reactions that produce derived pigments and color changes during storage (Eiro & Heinonen, 2002; Ngo, Wrolstad, & Zhao, 2007).

All these degradation mechanisms are influenced by many factors, some of them intrinsic to the product (pH, structure and concentration of anthocyanins and other phenolic compounds and flavonoids, presence of metal ions, L-ascorbic acid, enzymes) while others depend on the storage conditions (temperature, presence of oxygen, light, time, among others). The effect of pressure on these degradation reactions is not clear. Most of data in the literature refer to pressure processing for some minutes (5-25 min) and data about the effect of pressure applied for longer times are still very scarce (Fidalgo et al., 2013; Queirós et al., 2014; Segovia-Bravo, Guignon, Bermejo-Prada, Sanz, & Otero, 2012). In a previous work (Part 4.1), Segovia-Bravo et al. (2012) reported that color losses in strawberry juices stored under pressure (25-220 MPa) for 15 days at 20 °C were similar to those observed in conventionally refrigerated samples. In contrast, color of samples stored at 20 °C and atmospheric pressure was dramatically altered. In these samples, microbial load increased exponentially during storage and juices were completely spoiled after 15 days. Pressure inhibited microbial growth in juices stored at 20 °C and, in this way, it indirectly reduced color degradation. But, from these results, it is not clear if other mechanisms of color degradation, apart from microbial spoilage, are also affected by pressure.

Therefore, the purpose of this study was to evaluate the effect of pressure on color degradation of strawberry juice during storage at room temperature, without microbial interference. To achieve this goal, strawberry juices, with an added antimicrobial agent, were stored for 1, 2, 5, 7, 10, and 15 days at three pressure levels (0.1, 50, and 200 MPa) and 20 °C. Color evolution (L^*, a^*, and b^*) during storage was then compared between the samples maintained at different pressures. Moreover, the concentration of total phenols and anthocyanins, the main compounds responsible for color of strawberry juice, was measured during storage. Finally, PPO and POD activities and percent polymeric color (PPC) were also studied to have an insight on the effect of pressure on some mechanisms involved in color changes and degradation of phenolic compounds during storage.

The results obtained in this study provide relevant data to clarify the effect of pressure on the color degradation of strawberry juice during storage at room temperature. This implies important information to evaluate the viability of hyperbaric storage for food preservation.

5.2.3. Materials and methods

5.2.3.1. Samples

Strawberries (*Fragaria x ananassa* Duch., cv. Sabrina) were purchased at commercial maturity from a local supplier. The fruits were washed with tap water and processed with a blender (Royal Blender Turbo 10-Speed, Type 212004, Princess, Netherlands). The liquid obtained was then centrifuged at 3,500 *g* and 4 °C for 10 min (Sorvall Evolution RC Superspeed centrifuge, Thermo Scientific, Madrid, Spain). The supernatant was subsequently collected, filtered through a 0.1 mm pore diameter sieve and stored at -20 °C until utilization.

5.2.3.2. Physicochemical analysis in strawberry juice at day 0

Before each storage experiment, a frozen batch of strawberry juice was thawed overnight at 5 °C. Then, an antibiotic solution (Antibiotic antimycotin solution, Sigma-Aldrich, St. Louis, Ref. A5955) was added (1 %, v/v) to avoid microbial interference in

color changes. The juice was subsequently transferred into 150 mL plastic bags to be stored. Bags were thermo-sealed, avoiding headspace.

Juice at day 0 was characterized by measuring some of its physicochemical properties (see Table 5.2.1).

Parameter	**Mean ± Standard Error**
TSS (°Brix)	8.89 ± 0.08
pH	3.738 ± 0.003
TA (g citric acid·mL^{-1} of juice)	0.688 ± 0.002
*L**	33.87 ± 0.10
*a**	13.46 ± 0.08
*b**	3.80 ± 0.02
TP (mg GAE·L^{-1} of juice)	781.3 ± 28.06
TMA (mg Pg-3-glu·L^{-1} of juice)	195.07 ± 7.30
PPO (OD·min^{-1}·mL^{-1} of juice)	1.78 ± 0.09
POD (OD·min^{-1}·mL^{-1} of juice)	0.27 ± 0.00
PPC (%)	6.8 ± 0.4

Table 5.2.1. *Main characteristics of the raw strawberry juice employed in the experiments.*

Total soluble solids concentration (TSS) was approximated by using a digital refractometer (Leica AR200, Leica Microsystems Inc, New York, USA) with automatic temperature compensation. pH was measured with a pH glass electrode (6.0280.300 iEcotrode Plus, Metrohm, Herisau, Switzerland). Total titratable acidity (TA) was determined using an automatic titrator (Titrando 907, Metrohm, Herisau, Switzerland) according to the method described by Friedrich (2001). Color, total phenolic (TP) and total monomeric anthocyanin (TMA) contents, PPO and POD activities, and PPC were estimated as described in the next Sections.

All measurements were performed in triplicate for each thawed batch of juice employed in each experiment. Data in Table 5.2.1 are mean and standard error values calculated from the results obtained in all the experiments.

5.2.3.3. Storage experiments

Storage experiments under pressure were carried out in a pilot-plant high-pressure storage system (model SV1, Institute of High Pressure Physics, Unipress Equipment Division, Poland). The equipment was detailed in Chapter 3.

Strawberry juices were stored for 1, 2, 5, 7, 10, and 15 days at 20 ± 2 °C and two different pressure levels (50 and 200 MPa) to obtain samples labeled as T20_50MPa and T20_200MPa, respectively. Temperature and pressure were recorded every 30 s by a data acquisition system (MW100 Data Acquisition Unit, Yokogawa Electric Corporation, Tokyo, Japan). After compression, temperature in the samples increased by 1-4 °C, depending on the pressure level applied. In all cases, the target temperature was subsequently achieved in no more than 15 minutes.

T20_Patm samples were stored for the same periods at atmospheric pressure (0.1 MPa) in a thermostatic chamber tempered at 20 ± 2 °C.

All the storage experiments were performed in triplicate. Immediately after storage, juice samples were frozen at -30 °C till analysis.

5.2.3.4. Color measurements

L^*, a^*, and b^* color parameters were determined with a CM-3500d spectrophotometer managed by the software CM-S100w SpectraMagic™ (Konica Minolta, Japan). The corresponding method for measurements of strawberry juice samples color is described in Chapter 3.

5.2.3.5. Main compounds responsible for color

TP content was determined using the Folin-Ciocalteau method described by Waterhouse (2002) and detailed in Chapter 3. TP content was expressed as mg of gallic acid equivalent (GAE) per liter of juice (mg GAE·L^{-1}).

TMA content was determined by using the pH differential method described by Giusti and Wrolstad (2001) with slight modifications. The explanation of the method is in Chapter 3. The results were expressed as milligrams of pelargonidin-3-glucoside per

liter of juice (mg Pg-3-glu·L^{-1}) since pelargonidin-3-glucoside (Pg-3-glu) is the predominant anthocyanin in the strawberry juice.

5.2.3.6. Mechanisms involved in color changes

PPO and **POD** extraction and activity assays were carried out as is detailed in Chapter 3. Both enzymatic activities were expressed as residual activity (RA).

PPC was determined using the method described by Giusti and Wrolstad (2001). The explanation of the method appears in Chapter 3.

5.2.3.7. Data analysis

All the storage experiments were performed in triplicate and all the analyses in each sample obtained were also done in triplicate.

The results were statistically analyzed using IBM SPSS Statistics v. 21.0.0.0 for Windows (SPSS Inc., Somers, NY, USA). A two-way analysis of variance (ANOVA) was performed on the data using the General Linear Model procedure of the statistical software.

5.2.4. Results and discussion

5.2.4.1. Color changes during storage

The initial values of the chromatic parameters L^*, a^*, and b^* in the strawberry juice, at day 0, were 33.87 ± 0.10, 13.46 ± 0.08, and 3.80 ± 0.02, respectively (Table 5.2.1). During storage, color changes were very slight in all the juices and they appeared identical to the naked eye. However, instrumental measurements revealed some differences between samples.

Figure 5.2.1 summarizes the evolution of L^*, a^*, and b^* in the strawberry juices during storage at different pressure levels and 20 °C. Data corresponding to samples stored at 200 MPa for 15 days (D15/T20_200MPa samples) are not presented because, prior to analysis, these samples were completely destabilized. As mentioned in Materials and Methods Section, all the samples were frozen immediately after storage and they were

maintained at -30 °C until analysis. After thawing, the appearance of all T20_Patm and T20_50MPa samples was the same as prior to freezing, but the aspect of D15/T20_200MPa samples was markedly modified. These juice samples presented two completely separated layers: a clear layer at the top and a cloudy one at the bottom. These modifications were probably produced by the ice crystals formed that broke the weak stability of the juice cloud leading to clarification. Since this phenomenon was only observed in samples stored at 200 MPa for 15 days, both pressure level and storage time would significantly affect the structure of the juice. The effect of the pressure on the viscosity will be further discussed in the next Part of the Thesis. Therefore, no comparable and reliable color measurement could be expected from these samples and they were discarded from the analysis.

Statistical analysis of the data showed that both pressure level (P) and storage time (t) significantly affected ($p < 0.05$) all color parameters (Table 5.2.2). Moreover, a significant interaction between pressure level and storage time (P x t) was found for all of them, that means, color evolution was different in samples stored at different pressures.

	Source of variation	*fd*	*F*	*p-value*
L^*	**Pressure level (*P*)**	2	8.835	0.001
	Storage time (*t*)	5	15.410	0.000
	P* x *t	10	2.617	0.017
a^*	**Pressure level (*P*)**	2	104.928	0.000
	Storage time (*t*)	5	57.662	0.000
	P* x *t	10	5.043	0.000
b^*	**Pressure level (*P*)**	2	40.384	0.000
	Storage time (*t*)	5	35.413	0.000
	P* x *t	10	6.308	0.000

Table 5.2.2. *Results of the two-way ANOVA for the effect of pressure level and storage time on the color parameters in strawberry juices during storage (the factor has a significant effect when p < 0.05).*

During storage at atmospheric pressure, sample lightness gradually increased (Figure 5.2.1.a) and, after 15 days, T20_Patm samples presented a small, but statistically significant, 3 % rise in L^* values. Lightness of juices stored under pressure also

increased until day 5 but, from that day, a downward trend was observed in T20_50MPa and T20_200MPa samples. Therefore, at the end of storage, no significant differences were detected in L^* values of the samples preserved under pressure as compared to the juice at day 0.

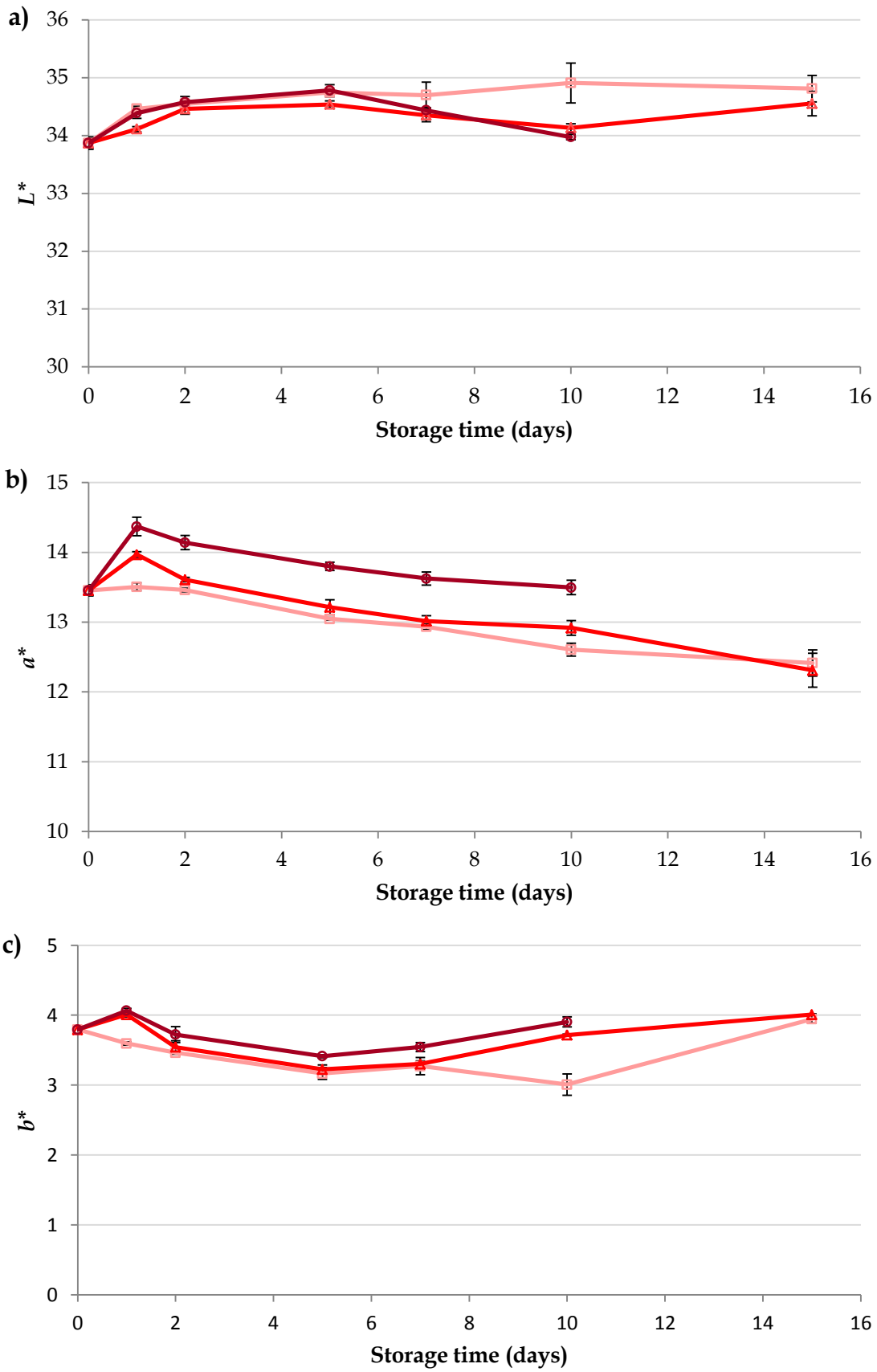

Figure 5.2.1. *(a) Lightness, (b) redness, and (c) yellowness evolution in strawberry juice during storage at different pressure levels (0.1 MPa (□), 50 MPa (△), and 200 MPa (○)) and 20 ºC. Vertical bars represent standard error.*

The evolution of redness and yellowness was also different in juices stored at atmospheric and high pressure (Figures 5.2.1.b and 5.2.1.c). The most striking differences were observed at day 1. Thus, after one day of storage, a^* increased by 4 % and 7 % and b^* increased by 6 % and 7 % in T20_50MPa and T20_200MPa samples, respectively. Therefore, at day 1, redness and yellowness were significantly higher in these samples as compared to juices stored at atmospheric pressure. After day 1, a^* and b^* evolution was quite similar in all the juices. Redness decreased gradually during the rest of storage while yellowness also decreased during the first days to reach a minimum but, after that, it increased up to the end of storage. However, it is important to note that this minimum occurred at day 5 in T20_50MPa and T20_200MPa samples and five days later, at day 10, in juices stored at atmospheric pressure.

Comparison with data from the literature is rather difficult because, as mentioned in the Introduction (section 5.2.2), color evolution in strawberry products depends not only on storage conditions, but also on factors such as pH, structure and concentration of anthocyanins, and the presence of other phenolic compounds, flavonoids, metal ions, L-ascorbic acid, and enzymes, among others. All these parameters are strongly dependent on the characteristics of the raw material (i. e., genotype, growing conditions, or maturity degree, among others) and the processing steps performed to obtain the final product (blending, filtration, clarification, deaeration, pasteurization, packaging, among others). Taking into account the enormous amount of sources of variability, apart from a generalized a^* decrease, no clear trends can be found in the literature for L^* and b^* evolution in strawberry products during storage at atmospheric pressure and room temperature. Most of papers refer to an increase of L^* and b^* during storage, but the opposite has also been reported (Cao, Bi, Huang, Wu, Hu, & Liao, 2012; García-Viguera, Zafrilla, Romero, Abellán, artés, & Tomás-Barberán, 1999; Garzón & Wrolstad, 2002; Gössinger et al., 2009b; Holzwarth et al., 2012a; Holzwarth et al., 2012b; Holzwarth, Korhummel, Siekmann, Carle, & Kammerer, 2013a; Ngo et al., 2007; Rein & Heinonen, 2004).

Concerning the effect of pressure on color, data in the literature show that pressure (100-800 MPa) applied for only some minutes, at low or moderate temperatures, can

produce some changes in strawberry products. However, there is not a clear trend in the changes observed by different authors, probably due to the big amount of factors implied in color evolution. Thus, for example, Cao et al. (2011) observed a non-significant a^* increase of 3 % in strawberry purée samples treated at 500 MPa and 20 °C for 15 min while Patras, Brunton, Da Pieve, and Butler (2009) reported a significant a^* decrease of 5 % in purée samples also treated at 500 MPa and 20 °C for 15 min. It is clear, nevertheless, that the extent of the chromatic change strongly depends on the pressure level applied and the duration of the treatment. Thus, Cao et al. (2011) detected quite different L^* decreases, 17 % and 6 %, in strawberry pulp samples treated, for 25 min, at 400 MPa and 600 MPa, respectively. These authors also reported different b^* increases, 14 % and 22 %, in the same samples treated at 400 MPa for 5 and 25 min, respectively.

In this study, the pressure applied is relatively low, but the storage time is, obviously, considerably longer than that employed for pressure processing. After one day of hyperbaric storage, significant differences have been found between juices stored at atmospheric and high pressure. Moreover, Figures 5.2.1a and 5.2.1c reveal that pressure has an effect on color evolution, not only at the beginning, but also during the complete storage. All these results suggest pressure effects on various mechanisms involved in color degradation.

5.2.4.2. Changes in chemical compounds responsible for color of strawberry juice during storage

TP and TAM contents in strawberry juice before storage were 781 ± 28 mg GAE·L^{-1} and 195 ± 7 mg Pg-3-glu·L^{-1}, respectively (Table 5.2.1). TP contents between 430 mg GAE·L^{-1} (Verbeyst et al., 2012) and 1571 mg GAE·L^{-1} (Hartmann, Patz, Andlauer, Dietrich, & Ludwig, 2008) and TMA contents between 111 mg Pg-3-glu·L^{-1} (Cao et al., 2012) and 453 mg Pg-3-glu·L^{-1} (Wang & Lin, 2000) have been reported in strawberry juice by different authors in the literature (Bakker & Bridle, 1992; Garzón & Wrolstad, 2002; Klopotek, Otto, & Bohm, 2005). Therefore, the values found here are representative of strawberry juice TP and TMA contents.

Figure 5.2.2 depicts TP and TMA evolution in strawberry juice during storage at different pressures and 20 °C.

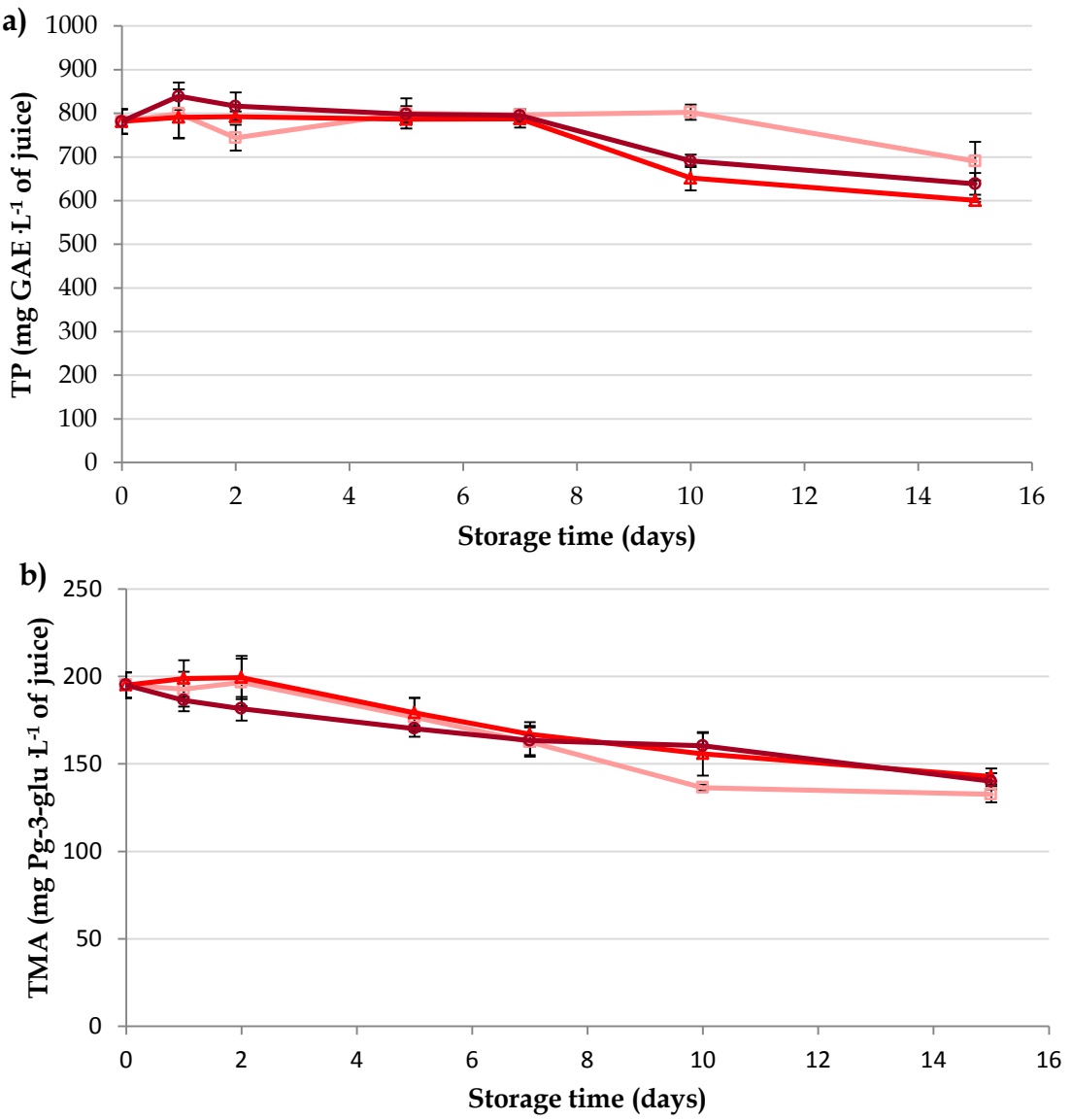

Figure 5.2.2. *(a) Total phenolic and (b) total anthocyanin content in strawberry juice during storage at different pressure levels (0.1 MPa (), 50 MPa (), and 200 MPa ()) and 20 °C. Vertical bars represent standard error.*

Statistical analysis of the data (Table 5.2.3) revealed that pressure during storage did not significantly affect these parameters and only storage time had a significant effect on them. Thus, the longer the storage time, the higher was the TP and TMA degradation.

	Source of variation	*fd*	*F*	*p-value*
TP	**Pressure level (*P*)**	2	2.369	0.106
	Storage time (*t*)	6	13.181	0.000
	P* x *t	12	1.698	0.102
TMAC	**Pressure level (*P*)**	2	1.293	0.285
	Storage time (*t*)	6	21.832	0.000
	P* x *t	12	0.622	0.812

Table 5.2.3. *Results of the two-way ANOVA for the effect of pressure level and storage time on the chemical compounds responsible for color in strawberry juices during storage (the factor has a significant effect when $p < 0.05$).*

At the beginning of storage, either at atmospheric or under pressure, TP content remained quite stable but, at day 15, 12 %, 23 %, and 18 % decreases were detected in T20_Patm, T20_50MPa, and T20_200MPa samples, respectively (Figure 5.2.2.a). Moreover, during storage, TMA decreased gradually and, at day 15, significant reductions of around 30 % ($p < 0.05$) were detected in juices maintained at 0.1 MPa, 50 MPa, and 200 MPa, respectively (Figure 5.2.2.b).

Degradation of phenols and anthocyanins during storage of strawberry juice at atmospheric pressure and room temperature is well documented in the literature (Bakker & Bridle, 1992; Cao et al., 2012; Garzón & Wrolstad, 2002; Gössinger et al., 2009a; Oszmiański & Wojdyło, 2009; Rein & Heinonen, 2004; Zabetakis, Leclerc, & Kajda, 2000). As occurred for color losses, the extent of degradation depends on several factors either related to the raw material, the processing steps to obtain the juice, or the storage conditions. Thus, for example, after one month of storage at 25 °C, Cao et al. (2012) detected quite different decreases, 6 % and 17 %, in the phenolic content of cloudy and clear strawberry juices, respectively, while losses in monomeric anthocyanins were about 37 % in both types of juice.

Regarding the effect of pressure, data in the literature are not conclusive, probably due to the high number of factors implied in TP and TMA degradation. Thus, for example, Terefe et al. (2013) observed 9-24 % and 20-28 % decreases in TP and TMA contents of different purées from three strawberry cultivars pressure treated at 600 MPa and 20 °C for 5 min. In contrast, Verbeyst et al. (2012) did not detect any difference in TP and

TMA contents of strawberry paste pressure treated at 400-800 MPa and 20 °C for 20 min and Patras et al. (2009) found a significant 10 % increase in TP content of strawberry purée processed at 600 MPa for 15 min while TMA remained unaltered. Data for longer pressure applications are not available in the literature for strawberry products, but Aaby et al. (2007) observed lower TP content in watermelon juices stored for 8-60 h at 100 MPa and room temperature as compared to juices stored at atmospheric pressure. In this study, no pressure effect has been found on the evolution of TP and TMA contents during storage although differences in color evolution were evident.

A deep insight into Figure 5.2.2.a shows that TP decrease began earlier in samples stored under pressure and, thus, at day 10, TP contents in T20_50MPa and T20_200MPa samples were significantly lower ($p < 0.05$) than in juices stored at atmospheric pressure. Nevertheless, at the end of storage, differences disappeared and TP value did not show significant differences among samples. Although not significant for TP content, the earlier TP degradation detected in samples stored under pressure could have a substantial effect on the strawberry juice color. Thus, Figure 5.2.1.c shows that b^* increases also started earlier under pressure, more specifically, after day 5 in contrast to day 10 at 0.1 MPa. This increase in yellowness could be related to an increase in degradation products of phenols.

5.2.4.3. Mechanisms involved in color changes and degradation of phenolic compounds during storage

Although no significant differences of TP and TMA retention have been found in juices stored at atmospheric and high pressure, instrumental color measurements showed significant differences between them. These differences could arise from some effect of pressure on mechanisms involved in degradation of phenolic compounds. It is well known that those reactions with a negative partial activation volume (V_a) are enhanced by pressure while those with $V_a > 0$ are hindered (Torres, Sanz, Otero, Lamela, & Saldaña, 2010). Thus, pressure can make difficult some reactions and favor some others that render different degradation products and this can justify differences found in color of T20_50MPa and T20_200MPa juices as compared to T20_Patm samples.

One of the most important mechanisms of color deterioration during storage is enzymatic browning. Initial values of PPO and POD activity in strawberry juice at day 0 were 1.78 ± 0.09 $OD \cdot min^{-1} \cdot mL^{-1}$ and 0.27 ± 0.00 $OD \cdot min^{-1} \cdot mL^{-1}$, respectively. Figure 5.2.3 shows the evolution of the residual activity of PPO and POD in strawberry juices stored at different pressure levels and 20 °C for 15 days.

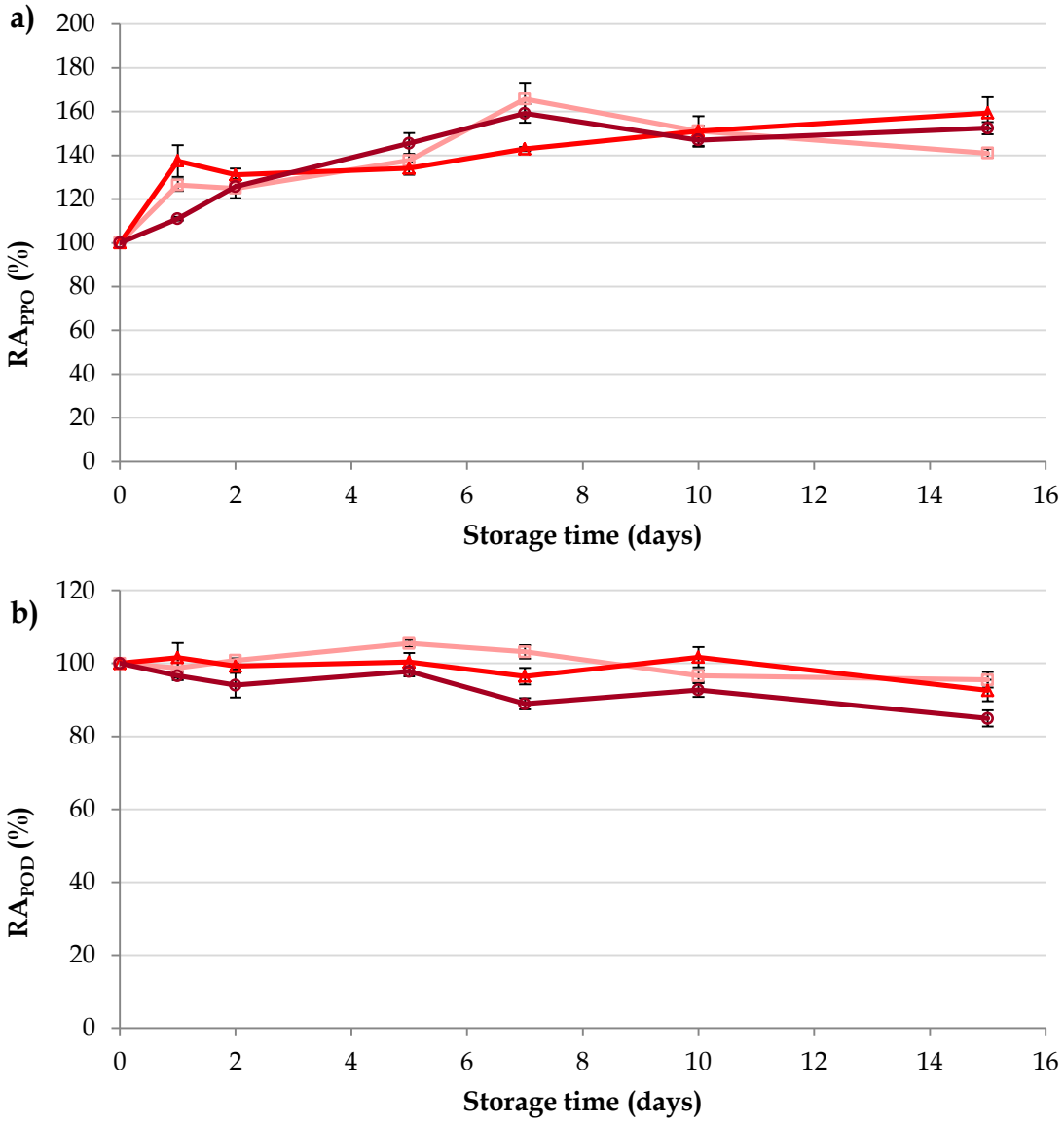

Figure 5.2.3. *Residual activities of (a) PPO and (b) POD in strawberry juice during storage at different pressure levels (0.1 MPa (), 50 MPa (), and 200 MPa ()) and 20 °C. Vertical bars represent standard error.*

Statistical analysis of the data revealed that PPO activity after storage was significantly affected by the storage time, but not by the storage pressure while POD activity was significantly affected by both factors (Table 5.2.4).

	Source of variation	*fd*	*F*	*p-value*
PPO	**Pressure level (*P*)**	2	0.518	0.599
	Storage time (*t*)	6	71.846	0.000
	P* x *t	12	4.590	0.000
POD	**Pressure level (*P*)**	2	19.699	0.000
	Storage time (*t*)	6	7.905	0.000
	P* x *t	12	2.046	0.044
PPC	**Pressure level (*P*)**	2	22.201	0.000
	Storage time (*t*)	6	118.806	0.000
	P* x *t	12	2.160	0.033

Table 5.2.4. *Results of the two-way ANOVA for the effect of pressure level and storage time on the mechanism involved in color changes and degradation of phenolic compounds in strawberry juices during storage (the factor has a significant effect when $p < 0.05$).*

Figure 5.2.3 shows that, during storage, PPO activity significantly increased in all the juices kept at different pressures. Thus, after 15 days, residual PPO activity increased by 41 %, 59 %, and 52 % in T20_Patm, T20_50MPa, and T20_200 MPa samples, respectively. In contrast, POD activity remained almost constant in samples stored at 0.1 MPa and 50 MPa, but storage at 200 MPa produced significant POD inactivation and, at day 15, its activity was reduced by 15 %.

Data in the literature about the stability of strawberry PPO during storage are scarce, but they reveal that strawberry genotype, maturity, and storage time are influent parameters (Holzwarth et al., 2012b; Tangen, 2013). Thus, Tangen (2013) found that PPO activity increased by more than 50 % in strawberry homogenate, cv. Senga sengana, stored for 2 weeks at 22 °C while it decreased by 25 % in homogenate made from cv. Sonata. Moreover, after 5 weeks of storage, PPO activity increased in all the cultivars studied.

Strawberry PPO has been found to be highly resistant to pressure processing (Cao et al., 2011; Dalmadi, Rapeanu, Van Loey, Smout, & Hendrickx, 2006; Terefe et al., 2013;

Terefe, Matthies, Simons, & Versteeg, 2009; Terefe, Yang, Knoerzer, Buckow, & Versteeg, 2010). Dalmadi et al. (2006) studied the pressure and temperature stability of purified strawberry PPO and revealed the existence of heat resistant and heat labile isoenzymes. At 25 °C, the heat labile fraction of strawberry PPO was quickly inactivated at pressures higher than 550 MPa, whereas the heat resistant isoform showed a D-value of 8.3 min at 25 °C and 750 MPa. In this study, although time under pressure was substantially longer, pressure during storage was considerably lower and, therefore, no PPO inactivation was detected after hyperbaric storage. Thus, PPO evolution was identical in samples stored at atmospheric and high pressure. On the other hand, POD seems to be more pressure labile (Cao et al., 2011; Terefe et al., 2013; Terefe et al., 2010). Terefe et al. (2009) found that the higher the pressure and the longer the processing time, the higher was POD inactivation. In this study, hyperbaric storage at 200 MPa produced significant inactivation in T20_200MPa samples and this POD inactivation could be responsible, at least in part, for the differences found in color evolution of juices stored at different pressures.

Apart from oxidation, monomeric anthocyanins are also involved in complex associations such as copigmentation and polymerization reactions, among others, that produce derived pigments and color changes during storage.

The phenomenon of copigmentation is due to molecular associations between pigments and other, usually non-colored, organic molecules called co-pigments. In strawberry juices, the anthocyanin glucosides can associate to certain co-pigments such as phenolic acids, flavonoids, alkaloids, amino acids, nucleotides, polysaccharides, or metals (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). In general, anthocyanin copigmentation results in more intense, brighter, and more stable colors than those expressed by monomeric anthocyanins (Eiro & Heinonen, 2002). Redness and yellowness increases observed in Figures 5.2.1.b and 5.2.1.c, at day 1, could be related to some effect of pressure on copigmentation reactions.

On the other hand, anthocyanins can polymerize with other juice components, and also with themselves, over time. Figure 5.2.4 shows the evolution of the percent polymeric color in the strawberry juices during storage at different pressure levels and 20 °C.

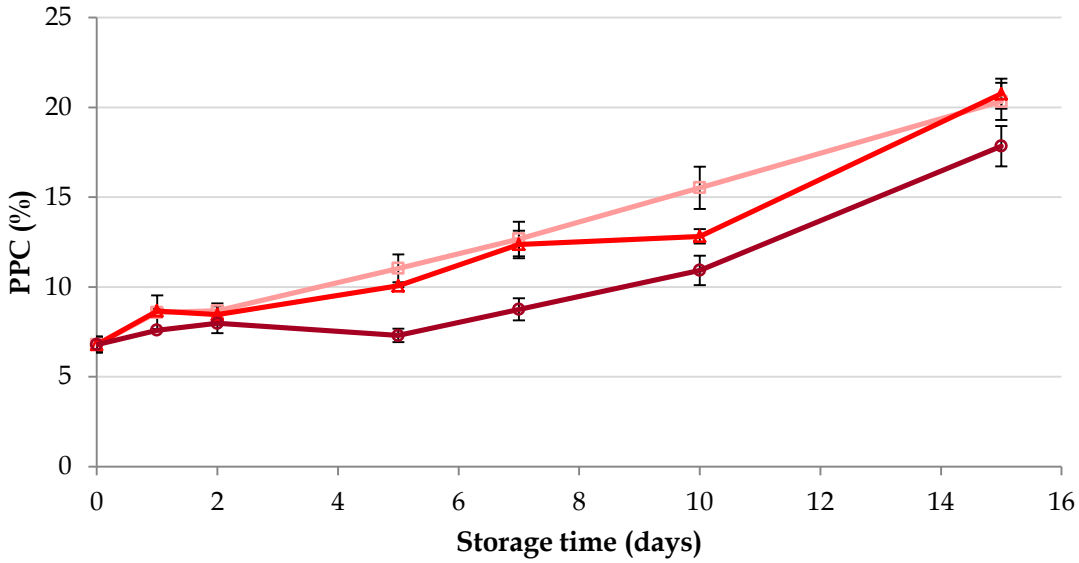

Figure 5.2.4. *Percent polymeric color (%) in strawberry juice during storage at different pressure levels (0.1 MPa (), 50 MPa (), and 200 MPa ()) and 20 °C. Vertical bars represent standard error.*

Statistical analysis of the data (Table 5.2.4) showed that both pressure level and storage time significantly affected PPC ($p < 0.05$). During storage at atmospheric pressure, PPC increased as expected. Different authors in the literature have showed that percent polymeric color of strawberry products increases during storage (Bakker & Bridle, 1992; Garzón & Wrolstad, 2002; Holzwarth et al., 2012a; Holzwarth et al., 2012b; Holzwarth, Wittig, Carle, & Kammerer, 2013b; Ngo et al., 2007; Skrede, Wrolstad, Lea, & Enersen, 1992). In samples stored under pressure, PPC also increased with time, but at 200 MPa, this increase was significantly lower ($p < 0.05$). Pressure, therefore, hampers the participation of anthocyanins in polymerization reactions.

5.2.5. Conclusions

The results obtained in this study clearly showed that some mechanisms of color degradation, apart from microbial spoilage, are affected by pressure. Thus, lower PPC and significant POD inactivation were found in samples stored at 200 MPa as

compared to samples maintained at atmospheric pressure. Moreover, these differences were reflected on the instrumental color measurements that also revealed a significant effect of the storage pressure on all the chromatic parameters.

Color differences due to the storage pressure, although instrumentally perceptible, were very slight and too subtle to be easily perceived by the naked eye. Therefore, large color differences, previously reported in strawberry juice stored for 15 days at 20 °C, should be mainly due to the inhibitory effect of pressure on microbial growth.

This study offers important new data for the characterization of hyperbaric storage of food at room temperature. This new environmentally friendly technology could provide an interesting opportunity to reduce energy costs in food preservation. However, much more research is needed (microbial behavior and enzymatic activities under pressure, stability of bioactive compounds, capital and operating costs, among other things) to establish its real potential.

5.2.6. References

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Part 5.3: Effect of hyperbaric storage at room temperature on pectinmethylesterase activity and serum viscosity[6]

[6] Bermejo-Prada, A., Segovia-Bravo, K. A., Guignon, B., & Otero, L. effect of hyperbaric storage at room temperature on pectinmethylesterase activity and serum viscosity of strawberry juice. *Food and Bioprocess Technology*. Submitted in September 2014.

EFFECT OF HYPERBARIC STORAGE AT ROOM TEMPERATURE ON PECTINMETHYLESTERASE ACTIVITY AND SERUM VISCOSITY

5.3.1. Abstract

The effect of hyperbaric storage at room temperature on pectinmethylesterase (PME) activity and serum viscosity of strawberry juice was evaluated. To achieve this goal, the catalytic activity of crude strawberry PME extract was studied first. The obtained results revealed that pressure (0.1-200 MPa) did not affect the catalytic activity of crude PME extract at the conditions assayed. PME activity and serum viscosity were then measured in strawberry juice. To do so, strawberry juices, with an added antibiotic solution, were stored for 1, 2, 5, 7, 10, and 15 days at three pressure levels (0.1, 50, and 200 MPa) and 20 °C. PME residual activity and catalytic activity during storage were determined to test the effect of pressure on the enzyme in the real food matrix. Moreover, the evolution of serum viscosity during storage was compared in samples maintained at different pressures. The results showed that residual PME was slightly higher in samples kept at 200 MPa, especially at the beginning of storage, probably due to an apparent PME activation. During the first two days of storage, PME catalytic activity was similar in samples stored at different pressures. However, in this period, serum viscosity decay was significantly higher in samples stored under high pressure. These results reveal that pressure affects some mechanism(s) inducing serum viscosity decay, apart from microbial growth and PME activity. At the end of storage, PME catalytic activity was higher in samples stored at 200 MPa probably due to a higher availability of the substrate.

5.3.2. Introduction

Juice viscosity is an important sensory attribute that limits consumer acceptability. Juices are biphasic colloidal systems consisting of a liquid phase, termed as “serum”,

and a suspended solid phase, termed as "cloud". Serum phase contains solubilized cell material, in particular solubilized pectin, sugars, salts, and organic acids while cloud phase mainly consists of a complex mixture of protein, lipids, hemicellulose, cellulose, and other minor components. The intrinsic characteristics of both serum and cloud phases determine juice viscosity. In cloud, particle properties (size, shape, composition, charge or deformability, among others) and their concentration are important for viscosity while, in serum, the amount of solubilized pectin and its characteristics, such as chain length, degree and pattern of methoxylation, degree of branching, composition and conformation, are determinant (Croak & Corredig, 2006; Moelants et al., 2013; Terefe, Buckow, & Versteeg, 2014).

It is widely known that, during storage, cloud destabilization and losses of serum viscosity occur in fruit juices (Cao et al., 2012; Igual, Contreras, Camacho, & Martínez-Navarrete, 2014; Krapfenbauer, Kinner, Gössinger, Schönlechner, & Berghofer, 2006; Schultz, Anthon, Dungan, & Barrett, 2014). These degradation phenomena are usually attributed to the activity of endogenous pectinolytic enzymes, mainly pectinmethylesterase (PME) and polygalacturonase (PG), together with microbial growth that, obviously, implies an associated enzymatic activity. Therefore, fruit juices are usually thermally treated and/or cold stored to avoid or, at least, slow down viscosity losses during storage.

In a previous work (Part 4.1), Segovia-Bravo et al. (2012) reported that hyperbaric storage at room temperature was efficient in reducing losses in serum viscosity of strawberry juices. Thus, viscosity decay in juices stored under pressure (25-220 MPa) for 15 days at 20 °C was similar to that observed in conventionally refrigerated samples. In contrast, serum viscosity of samples stored at 20 °C and atmospheric pressure was dramatically reduced. In these samples, microbial load increased exponentially during storage and juices were completely spoiled after 15 days. Pressure inhibited microbial growth in juices stored at 20 °C and, in this way, it indirectly reduced viscosity degradation. But, from these results, it is unclear if the activity of pectinolytic enzymes involved in serum viscosity degradation is also affected by pressure or not.

Among all the pectinolytic enzymes implicated in viscosity losses of juices, PME is particularly interesting because it affects not only serum viscosity, but also cloud particles stability. PME de-esterifies the methyl groups on the galacturonic acid backbone of pectin releasing methanol and low-methoxyl pectin. This de-esterified pectin is the substrate for PG that subsequently catalyzes its depolymerization. Moreover, free carboxylic acids of low-methoxyl pectin can be cross-linked by divalent cations such as Ca^{2+}, that are intrinsically present in the juice, leading to precipitation of pectin and juice clarification. PME also acts on the pectin present in cloud particles, decreasing their stability throughout not well understood mechanisms (Croak & Corredig, 2006).

To understand how PME acts during hyperbaric storage of strawberry juice, it is necessary to evaluate not only the pressure stability of the enzyme, but also its catalytic activity under pressure. Ly Nguyen, Van Loey, Fachin, Verlent, and Hendrickx (2002) found that purified strawberry PME is very barotolerant at room temperature, but its catalytic activity under pressure has not been quantified yet. Several studies in the literature, made in PME from other plant sources, reveal that, in general terms, high pressure enhances the PME-catalyzed de-esterification of pectin (Jolie et al., 2012; Terefe et al., 2014). However, some studies have also shown that, at temperatures lower than the optimal temperature for PME activity, pectinolytic activity does not increase with increasing pressure (Castro, Loey, Saraiva, Smout, & Hendrickx, 2006b; Sila et al., 2007; Van Den Broeck, Ludikhuyze, Van Loey, & Hendrickx, 2000). Most research works have been carried out with purified PME solubilized in buffer solutions and studies in real food systems are very limited (Sila et al., 2007). Environmental factors such as pH and presence of sugars, salts, or other food constituents can also affect PME activity under pressure. Therefore, the effect of pressure on PME activity in real food systems remains largely unknown in the conditions usually employed for hyperbaric storage (i.e., room temperature, pressures below 250 MPa, and relatively long periods of time).

The aim of this study was to evaluate the effect of pressure on PME activity and serum viscosity of strawberry juice during storage at room temperature, without microbial

interference. To achieve this goal, the catalytic activity of crude PME extract under pressure was studied first. Then, strawberry juices, with an added antimicrobial agent, were stored for 1, 2, 5, 7, 10, and 15 days at three pressure levels (0.1, 50, and 200 MPa) and 20 °C. PME residual activity and catalytic activity during storage were determined to test the effect of pressure on the real food matrix. Finally, the evolution of serum viscosity during storage was compared in samples maintained at different pressures.

The results obtained in this study provide relevant data to elucidate the effect of pressure on some mechanisms involved in the viscosity decay of strawberry juice during storage at room temperature. This implies important information to assess in terms of quality the viability of hyperbaric storage at room temperature for food preservation.

5.3.3. Materials and methods

5.3.3.1. Preparation of strawberry juice

Fresh strawberries (*Fragaria x ananassa* Duch., cv. Brillante) were purchased at commercial maturity from a local supplier. The fruits were washed with tap water and processed with a blender (Royal Blender Turbo 10-Speed, Type 212004, Princess, Netherlands). The liquid obtained was then centrifuged at 3,500 *g* and 4 °C for 10 min (Sorvall Evolution RC Superspeed centrifuge, Thermo Scientific, Spain). The supernatant was subsequently collected, filtered through a 0.1 mm pore diameter sieve, packaged, and stored at -20 °C until utilization.

5.3.3.2. Methodology for the study of strawberry PME activity in model system

5.3.3.2.1. Preparation of the crude PME extract

A batch of crude strawberry PME extract was prepared according to the method described in Chapter 3. The crude strawberry PME extract, was divided into aliquots, frozen, and stored at -20 °C until use.

5.3.3.2.2. Initial PME activity

Initial PME activity of the crude PME extract was measured by titration of the carboxylic groups generated by the enzyme in a pectin solution at pH 7.7 and 30 °C according to Chapter 3. The PME activity unit (U) was defined as the amount of enzyme required to release 1 μmol of carboxyl group per minute under the aforementioned assay conditions.

Titrimetric assays for PME activity in the crude strawberry extract employed for the experiments were performed at the beginning and at the end of the series of experiments to ensure that PME activity did not change during the frozen storage.

5.3.3.2.3. Experiments at isothermal-isobaric conditions

Experiments were performed in a lab-scale high-pressure equipment (U111, Institute of High Pressure Physics, Unipress Equipment Division, Poland). The characteristics of the equipment are detailed in Chapter 3. Each experiment consisted in carrying out the enzymatic reaction during a given time under a selected pressure at 37 °C according to the procedure detailed below.

The enzymatic reaction was initiated at atmospheric pressure as slowly as possible by placing the reactants in a water-ice bath. Exactly 0.5 mL of crude PME extract was added to 1mL of 0.4 % apple pectin solution (70-75 % esterification, Sigma-Aldrich, St. Louis) and mixed. The pectin solution was prepared by dissolving the pectin in 0.01 M phosphate buffer, pH 7.4, containing 0.117 M NaCl to avoid uncontrolled pH variations during the enzymatic reaction. These enzyme and substrate concentrations were previously identified as appropriate by titration to obtain a linear PME activity as a function of time for, at least, 120 min. For each assay, the enzyme-substrate solution was enclosed in 1.5 mL flexible plastic tube and placed in the pressure vessel, already equilibrated at the chosen pre-set temperature. Moreover, two samples of buffer solution, the first containing pectin alone and the other crude PME extract alone were also included as blank samples. The sample preparation and pressure vessel filling took approximately 10 minutes.

Three pressure levels (0.1, 50, and 200 MPa) were assayed to cover the complete pressure range with potential interest for hyperbaric storage at room temperature. Since PME reaction rate at room temperature is too slow but optimal near 60 °C, experiments were carried out at 37 °C in order to suitably accelerate the reaction rate (Ly-Nguyen et al., 2002). In experiments under high pressure, pre-set temperatures in the vessel were chosen for each pressure level to take advantage of the compression heating to reach the target temperature (37 °C) as quickly as possible. Reproducible compressions were achieved by fixing the hydraulic pump speed from the potentiometer of the control unit of the equipment. The pressurization step was lasting less than 45 s (pressurization rate around 4 MPa·s^{-1}).

After reaching the target pressure, an equilibration period (around 3 min) was taken into account to allow samples to reach the target temperature. This equilibration period was considered as "zero time". By starting the time course of the experiment after this equilibration period, the process could be considered as an isobaric-isothermal treatment (Van Den Broeck et al., 2000; Verlent, Van Loey, Smout, Duvetter, & Hendrickx, 2004). Samples were maintained at isothermal-isobaric conditions for pre-set time intervals of 0, 30, 60, 90, 120, 150, and 180 min from the zero point of the experiment and, then, decompressed and removed from the vessel.

After each depressurization, the reaction in the sample drawn from the vessel was immediately stopped by a heat shock (85 °C, 2 min), followed by cooling in an ice-water bath. Finally, all the samples were frozen and stored at -20 °C until analysis.

During the treatments, sample temperature and pressure were monitored and recorded every 0.5 s as described in Chapter 3.

5.3.3.2.4. Determination of methanol quantity produced from the PME catalyzed reaction during the isothermal-isobaric experiments

The yield of the reaction due to PME catalytic activity was determined by measuring the methanol released by the enzyme. The **amount of methanol** formed was measured colorimetrically according to the method described in Chapter 3. The amount of methanol formed was expressed as μg·mL^{-1}.

5.3.3.2.5. Data analysis and statistics

A complete isothermal-isobaric experiment consists of seven treatments for 0, 30, 60, 90, 120, 150, and 180 min, respectively. After these treatments, methanol content was measured spectrophotometrically in each sample in triplicate. From these data, PME activity (μg MeOH·mL^{-1} pectin solution·min^{-1}) at each storage pressure was estimated from the initial linear part of the curve obtained by linear regression of the amount of methanol produced by PME versus time.

Linear regressions were performed using IBM SPSS Statistics v.19.0.0 for Windows (SPSS Inc., Somers, NY, USA).

5.3.3.3. Methodology for the study of PME catalysis in strawberry juice during storage

5.3.3.3.1. Storage experiments at 20 °C

Before each storage experiment, a frozen batch of strawberry juice was thawed overnight at 5 °C. Then, an antibiotic solution (Antibiotic antimycotin solution, Ref. A5955, Sigma-Aldrich, USA) was added (1 %, v/v) to avoid microbial interference in the results. Juice was subsequently transferred to 150 mL plastic bags to be stored. Bags were thermo-sealed, avoiding headspace.

Storage experiments under pressure were carried out in a pilot-plant high-pressure storage system (model SV1, Institute of High Pressure Physics, Unipress Equipment Division, Poland). The equipment was described in Chapter 3.

Strawberry juices were stored for 1, 2, 5, 7, 10, and 15 days at 20 ± 2 °C and two different pressure levels, 50 and 200 MPa, to obtain samples labeled T20_50MPa and T20_200MPa, respectively. Temperature and pressure were recorded every 30 s by a data acquisition system (MW100 Data Acquisition Unit, Yokogawa Electric Corporation, Tokyo, Japan). After compression, temperature in the samples increased by 1-4 °C, depending on the pressure level applied. In all cases, the target temperature was subsequently achieved in no more than 15 minutes. At the end of the storage period, pressure was released in a few seconds and the samples were removed for subsequent analysis.

T20_Patm samples were stored for the same periods at atmospheric pressure in a thermostatic chamber at 20 ± 2 °C.

Each stored sample was divided into three parts to perform the three analyses described below.

5.3.3.3.2. Analysis of PME activity after storage

PME activity after storage was measured titrimetrically in all the samples as is described in Chapter 3. Enzymatic activities were expressed as residual activity (RA_{PME}).

5.3.3.3.3. Determination of methanol quantity produced from the PME-catalyzed reaction during storage

PME catalytic activity during storage of strawberry juice was measured through methanol content as described in Chapter 3.

5.3.3.3.4. Analysis of serum viscosity

Immediately after storage, the strawberry juice samples were centrifuged at 45,500 *g* and 4 °C for 10 min (Sorvall Evolution RC Superspeed centrifuge, Thermo Scientific, Madrid, Spain). Serum viscosity of the juices was estimated by measuring its kinematic viscosity as detailed in Chapter 3. The kinematic viscosity (ν) was expressed in centiStokes (1 cSt = 10^{-6} $m^2 \cdot s^{-1}$).

5.3.3.3.5. Data analysis and statistics

All the storage experiments were performed in triplicate and all RA_{PME}, methanol content and serum viscosity analyses in each sample obtained were also done in triplicate.

Mean ± standard error values were calculated with the software program IBM SPSS Statistics v. 19.0.0 for Windows (SPSS Inc., Somers, NY, USA). A two-way analysis of variance (ANOVA) was performed on the data, using the General Linear Model procedure of the statistical software. This was done to test the main effects of storage pressure and storage time on the parameters studied. The significance level was set at 5 %. Tukey test was applied for post-hoc comparisons.

5.3.4. Results and discussion

5.3.4.1. Catalytic activity of the crude strawberry PME extract under pressure at 37 °C

Firstly, it was checked whether the initial PME activity of the crude extracts employed in different experiments remained constant during the frozen storage. No significant differences ($p < 0.05$) were found in the initial PME activity of the crude extracts at the beginning (0.076 ± 0.005 U·mL^{-1}) and at the end (0.076 ± 0.002 U·mL^{-1}) of the series of experiments. Therefore, frozen storage did not affect PME activity of the crude extracts and normalization was not needed to process the data obtained on different days of experimentation.

The catalytic activity of the crude PME extract in model system was observed during 180 min at 0.1, 50, or 200 MPa and 37 °C. To estimate PME activity accurately, only the initial, linear part of the curve, up to 120 min, was taken into account for linear regression. No methanol release was detected in none of the blanks. Therefore, no spontaneous de-esterification of pectin occurred at the pH-pressure-temperature conditions tested (pH 7.4, 37 °C, 0.1, 50, and 200 MPa). Several authors in the literature have also reported that chemical de-esterification of pectin does not occur at pressure-temperature conditions similar to those employed in this study at acidic conditions (Castro, Loey, Saraiva, Smout, & Hendrickx, 2006a; Duvetter et al., 2006; Verlent et al., 2004). However, the reaction is accelerated with increasing pH and Verlent et al. (2004) observed chemical de-esterification of pectin at alkaline conditions (pH 8.0) and 200 MPa/30 °C.

Table 5.3.1 reveals that, at the studied conditions, pressure (0.1-200 MPa) did not affect the catalytic activity of the crude extract: the methanol release rate was the same independently of the considered pressure. Similar results can be found in the literature when PME catalytic activity from different plant sources is evaluated at pressures lower than 300 MPa and temperatures below the optimal value for PME activity. For example, Van Den Broeck et al. (2000) reported that pressure, up to 300 MPa, had almost no influence on the activity of tomato PME at 20 °C and pH 7.2 and Sila et al.

(2007) found no pressure effect (0.1-200 MPa) on the activity of purified carrot PME at 30 °C and pH 4.5. Moreover, Verlent et al. (2004) reported that, at pH 4.4 and 35 °C, catalytic activity of purified tomato PME was quite similar between 0.1 and 300 MPa. Only, Castro et al. (2006a) found that the activity of purified pepper PME in citrate buffer, pH 5.6, at 30-45 °C was even lower under 200-600 MPa than at atmospheric pressure.

Storage pressure (MPa)	**Catalytic activity Mean ± Standard error of regression ($\mu g\ MeOH \cdot mL^{-1}$ pectin solution$\cdot min^{-1}$)**
0.1	1.428 ± 0.059
50	1.526 ± 0.047
200	1.485 ± 0.067

Table 5.3.1. *Catalytic activity of the crude PME extract at 37 °C, pH 7.4, and different pressure levels.*

From all the above results, it could be expected the same PME activity at atmospheric pressure as at 50 or 200 MPa during the storage of strawberry juice. However, strawberry juice presents characteristics of pH and of sugar and salts contents obviously different from those of the model system studied here. Therefore, PME activity could be modified during the storage and this should be checked as done below.

5.3.4.2. Pectinmethylesterase activity in the strawberry juice during storage at room temperature

After characterizing the effect of pressure on the activity of crude strawberry PME extract, PME activity was evaluated in conditions as closest as possible to that encountered during storage: strawberry juice as substrate, room temperature, and sampling over 15 days. In first place, PME stability was checked and secondly, the catalytic activity was determined, both all over the period of storage.

5.3.4.2.1. PME stability during storage

Figure 5.3.1 shows the evolution of the residual PME activity in strawberry juices stored at different pressure levels and 20 °C for 15 days. During storage, PME activity significantly decreased in all the juices maintained at different pressures. Thus, after 15

days, RA_{PME} was 56 %, 52 %, and 57 % in T20_Patm, T20_50MPa, and T20_200MPa samples, respectively. Previous studies in the literature showed that PME activity in fruit juices decreases during storage (Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2009, 2010; Rodrigo et al., 2003). Thus, for example, Aguiló-Aguayo, Oms-Oliu, Soliva-Fortuny, and Martín-Belloso (2009) reported that, after 15 days of storage at atmospheric pressure and 4 °C, PME activity in fresh strawberry juice decreased by 80 %.

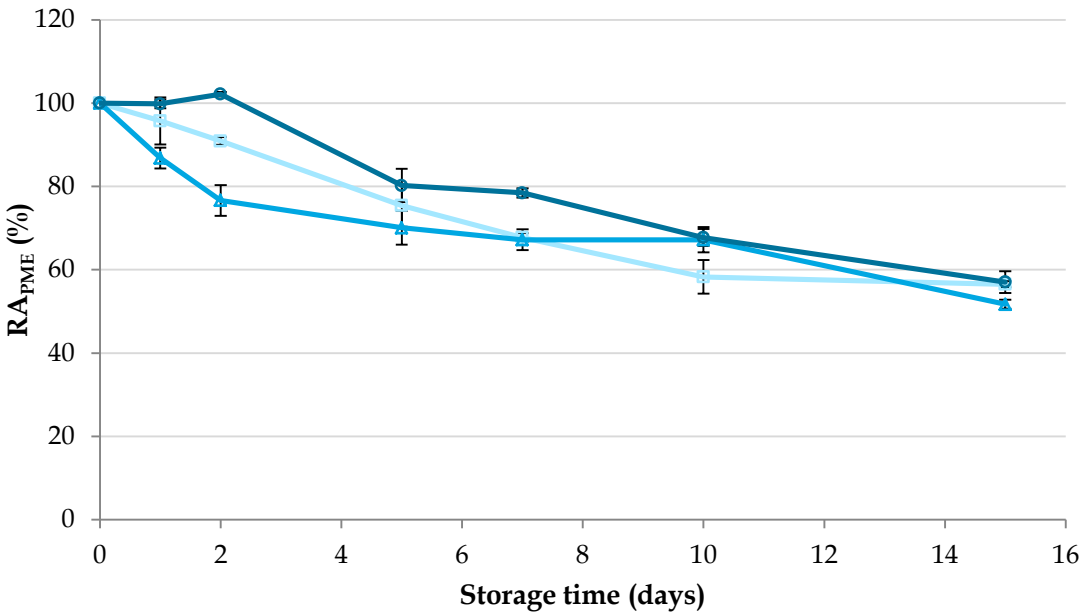

***Figure 5.3.1**. Residual PME activity (%) in strawberry juice after storage at different pressure levels (0.1 MPa ( ), 50 MPa ( ), and 200 MPa ( )) and 20 °C. Vertical bars represent standard error.*

Statistical analysis of the data revealed that PME activity after storage was significantly affected by both pressure level and storage time (Table 5.3.2). Furthermore, the evolution of the residual activity was different in samples stored at different pressures because a significant interaction between pressure level and storage time (P x t) was found. RA_{PME} evolution during storage was quite similar in T20_Patm and T20_50MPa samples, but it differed significantly ($p < 0.05$) in T20_200MPa juices. In these samples, no RA_{PME} reduction was detected during the two first days of storage and, at days 2 and 7, PME activity was significantly higher than that observed in all the other juices. These differences among samples, although significant, were very slight and, after day 7, RA_{PME} was similar in all the juices stored at different pressures.

	Source of variation	*fd*	*F*	*p-value*
RA$_{PME}$	**Pressure level (*P*)**	2	20.783	0.000
	Storage time (*t*)	6	108.742	0.000
	P* x *t	12	3.359	0.002
Methanol content	**Pressure level (*P*)**	2	53.040	0.000
	Storage time (*t*)	6	108.490	0.000
	P* x *t	12	11.215	0.000
Serum viscosity	**Pressure level (*P*)**	2	18.922	0.000
	Storage time (*t*)	6	1029.226	0.000
	P* x *t	12	9.894	0.000

***Table 5.3.2**. Results of the two-way ANOVA for the effect of pressure level and storage time on parameters related with PME activity and serum viscosity in strawberry juice during storage at 20 °C ($p < 0.05$).*

Pressure stability of PME from different plant sources, such as tomato (Van Den Broeck et al., 2000), carrot (Trejo Araya et al., 2007), banana (Ly Nguyen et al., 2002), orange (Basak & Ramaswamy, 1996; Nienaber & Shellhammer, 2001; Polydera, Galanou, Stoforos, & Taoukis, 2004; Van Den Broeck et al., 2000), or white grapefruit (Guiavarc'h, Segovia, Hendrickx, & Van Loey, 2005), among others, has been previously studied in the literature. In general terms, PME has been classified as a quite barotolerant enzyme at room temperature, although threshold pressures for inactivation largely depend on PME origin and medium used for enzyme inactivation. Among all PMEs studied, strawberry PME has been found to be very pressure resistant (Bodelón, Avizcuri, Fernández-Zurbano, Dizy, & Préstamo, 2013; Ly-Nguyen et al., 2002). Moreover, Ly-Nguyen et al. (2002) discovered a very pressure stable isoform, responsible for 10 % total activity. According to the fractional-conversion model developed by these authors, at least, 850 MPa applied for more than 3 hours, at pH 7.0 and 10 °C, are needed to reduce activity of purified strawberry PME by 85 %. This high

barotolerance observed in purified strawberry PME dissolved in buffer solution has been, in some way, corroborated in real food systems. Thus, Bodelón et al. (2013) found no PME inactivation in pressure-treated strawberry purée at 400 MPa and 20 °C for 15 min. The results obtained in our study also confirm the high pressure resistance of strawberry PME. In hyperbaric storage, the pressure applied is relatively low, but the storage time is obviously considerably longer than that usually employed in the previous studies of the literature. Even at so long times as those applied in the present work, no pressure PME inactivation was observed in strawberry juice after storage at 50 or 200 MPa.

The slightly higher RA_{PME} observed in T20_200MPa samples at the beginning of storage could be due to an apparent activation of the enzyme caused by a pressure enhanced PME release from small cell wall particles present in the juice. Thus, in the literature, several authors have described increases in PME activity after pressure treatment in different products such as cloudy apple (Baron, Dénes, & Durier, 2006) and tomato (Hsu, 2008) juices, capsicum purée (Castro, Van Loey, Saraiva, Smout, & Hendrickx, 2005), and carrots (Trejo Araya et al., 2007), among others.

5.3.4.2.2. PME activity during storage

The initial methanol content in the strawberry juice, at day 0, was 2.49 ± 1.70 $\mu g \cdot mL^{-1}$ juice. Figure 5.3.2 depicts the evolution of methanol content in the samples during storage at different pressure levels and 20 °C. Figure 5.3.2 reveals that methanol content increased progressively during storage in all the samples, as expected. Various authors in the literature have previously reported that methanol increases in fresh squeezed fruits and vegetables during storage due to PME activity on pectin (Baron et al., 2006; Hou, Lin, Tai Wang, Jiang, & Wu, 2008).

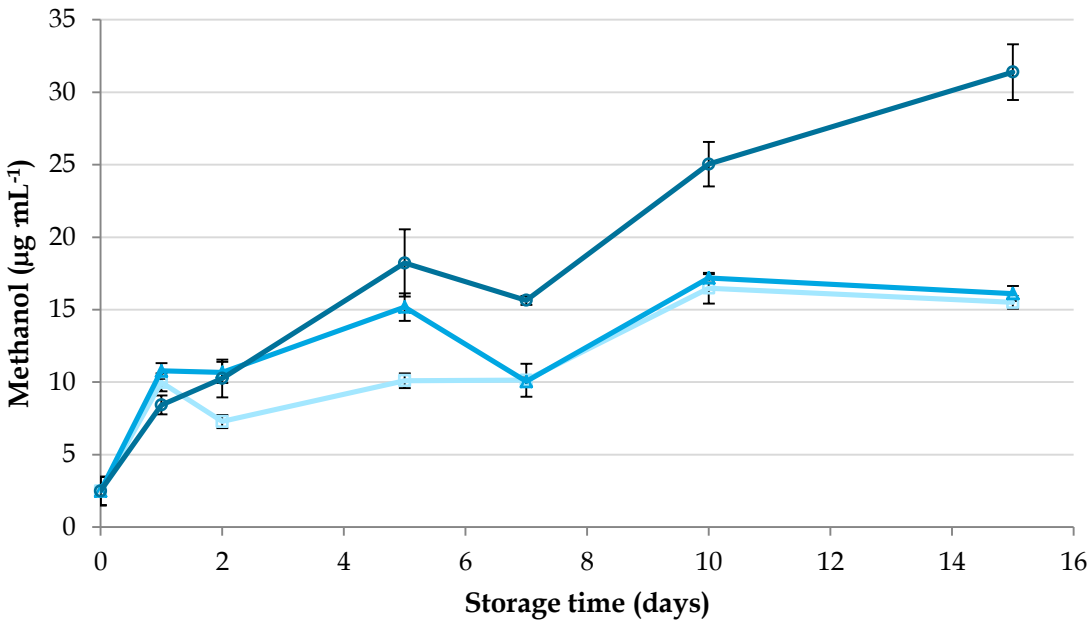

***Figure 5.3.2**. Evolution of methanol content (µg·mL^{-1} juice) in strawberry juice during storage at different pressure levels (0.1 MPa ( ), 50 MPa ( ), and 200 MPa ( )) and 20 °C. Vertical bars represent standard error.*

Statistical analysis of the data showed that both pressure level (P) and storage time (t) significantly affected ($p < 0.05$) methanol content (Table 5.3.2). Besides, as can be seen from the significant interaction between pressure level and storage time (P x t), methanol content changed differently depending on the pressure level. At day 1, a sharp increase in methanol content was detected in all the samples and, thus, methanol concentration at day 1 was about four times higher than at day 0. During the two first days of storage, no significant differences ($p < 0.05$) were found between samples stored at different pressure levels. These results agree with those reported for crude strawberry PME extract that showed no pressure effect on the PME catalytic activity. However, after day 2, methanol progression was different depending on the storage pressure. Thus, pectin demethoxylation was very slow in T20_Patm and T20_50MPa samples, probably due to the depletion of the more accessible methyl ester bonds of pectin after the intense PME activity observed at the beginning of storage. On the contrary, in T20_200MPa juices, pectin demethoxylation occurred significantly faster and methanol content increased continuously up to the end of storage. Thus, at day 15, T20_200MPa samples had twice as much methanol as T20_Patm and T20_50MPa samples.

It seems improbable that the slightly higher RA_{PME} detected in T20_200MPa juices at the beginning of storage could be responsible for their different behavior because the greatest differences in methanol content appeared at the end of storage. On the other hand, chemical de-esterification of pectin under pressure should be discarded due to the low pH of the strawberry juice (Jolie et al., 2012). A more plausible explanation points to differences in the substrate availability. During storage at 200 MPa, pressure can induce structural changes in pectin, rendering the substrate more susceptible to PME attack. However, these possible conformational changes were not evidenced in experiments made in model system with crude PME extract, although effects of pH and strawberry juice components on pectin structure could be implicated. Another more convincing hypothesis points to a pressure-enhanced activity of some endogenous pectinases, other than PME, that would reduce steric hindrance and ease the PME access to methyl ester bonds of pectin. Candidates include enzymes affecting not only the linear homogalacturonan (HG) chains of pectin, but also those acting on the rhamnogalacturonan chain (hairy region). Debranching enzymes catalyzing changes in pectin side chains such as β-galactosidase or α-arabinofuranosidase, for example, can produce changes in the pectin structure and, in this way, improve PME accessibility to its substrate (Houben et al., 2012; Van Buggenhout, Sila, Duvetter, Van Loey, & Hendrickx, 2009).

5.3.4.3. Evolution of serum viscosity during storage

The initial value of the serum viscosity of the strawberry juice, at day 0, was 35.3 ± 1.8 cSt. Figure 5.3.3 depicts the evolution of the serum viscosity in the samples during storage at different pressures and 20 °C. During storage, serum viscosity showed a downward trend in all the juices maintained at 0.1, 50, or 200 MPa. Statistical analysis of the data (Table 5.3.2) indicated that both pressure level (P) and storage time (t) significantly affected ($p < 0.05$) this parameter. Moreover, a significant interaction between pressure level and storage time ($P \times t$) was found, meaning that viscosity evolution was different in samples stored at different pressures.

Figure 5.3.3 reveals that the greatest viscosity losses occurred in the first two days of storage and, in this period, the greater the storage pressure, the greater the viscosity

decay. Thus, at day 1, viscosity drops of 43 %, 56 %, and 75 % were detected in T20_Patm, T20_50MPa, and T20_200MPa samples, respectively. After day 5, differences among juices were less obvious and viscosity values decreased very slowly up to the end of storage. At day 15, serum viscosity was extremely low, close to that of pure water, in all the juices.

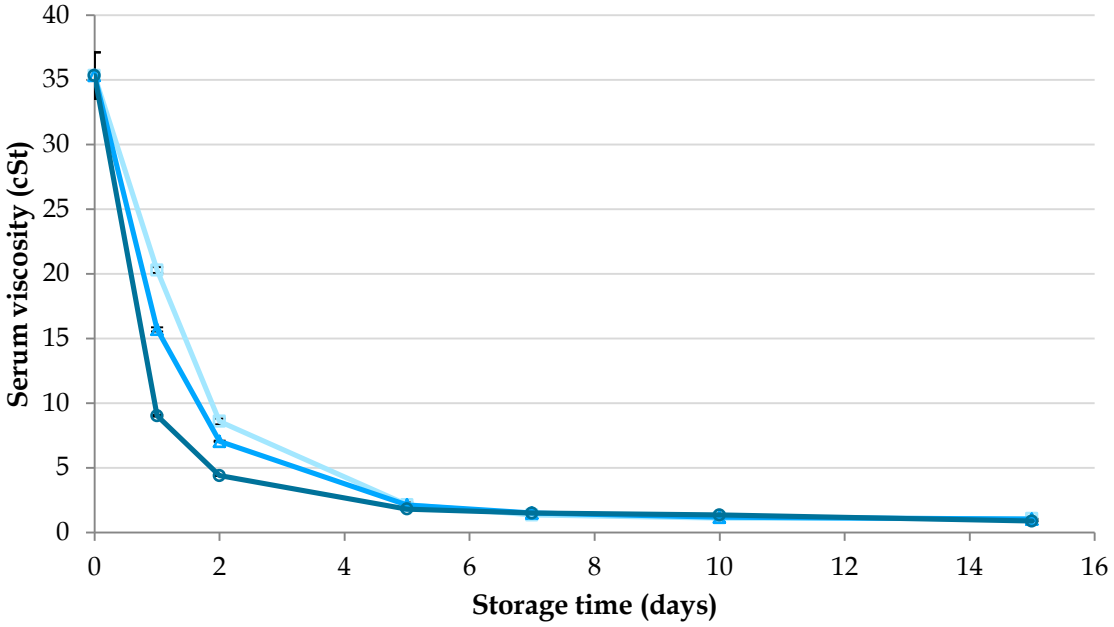

***Figure 5.3.3**. Evolution of serum viscosity (cSt) in strawberry juice during storage at different pressure levels (0.1 MPa (□), 50 MPa (△), and 200 MPa (○)) and 20 °C. Vertical bars represent standard error.*

These results diverge from data previously described by Segovia-Bravo et al. (2012) who reported that losses in serum viscosity of strawberry juice, stored for 15 days at 20 °C, were lower in samples maintained under high pressure (25-220 MPa) than in juices kept at atmospheric pressure. The results obtained in the present study reveal that differences found by Segovia-Bravo et al. (2012) must be mainly due to the inhibitory effect of pressure on microbial growth as it has been also demonstrate in Part 5.1. Serum viscosity values measured during the first 5 days of storage clearly prove that other mechanisms involved in viscosity degradation, apart from microbial growth, must be also affected by pressure.

As commented in Section 5.3.2, serum viscosity of fruit juices depends not only on the amount of solubilized pectin, but also on its characteristics (chain length, degree and

pattern of esterification, degree of branching, composition and conformation, among others). During storage, both chemical and enzyme catalyzed reactions can occur that modify these pectin characteristics and, therefore, affect serum viscosity. Chemical pectin de-esterification and pectin depolymerization seem not to be enhanced by pressure up to 200 MPa at room temperature. Thus, as commented in Section 5.3.4.1, no spontaneous pectin de-esterification was observed in this study in pectin solutions (blanks) under pressure. Moreover, pH of the strawberry juice and storage temperature are too low to expect significant chemical de-esterification during storage (Jolie et al., 2012). On the other hand, chemical pectin depolymerization is also improbable to occur at the conditions assayed in this study because Kato, Teramoto, & Fuchigami (1997) proved that high pressure (700 MPa, 1 h) does not induce β-eliminative cleavage of pectin chains at ambient temperature. However, these authors also found that the viscosity of pressurized pectin solutions was lower than that of controls at all pH levels tested (1-13) and they attributed these results to a pressure induced structural change in pectin.

As regards enzymatic reactions, given the complex pectin composition and architecture, a particularly wide range of possible conversions exist, affecting both the linear HG and hairy regions of pectin (Pedrolli, Monteiro, Gomes, & Carmona, 2009; Sila et al., 2009; Van Buggenhout et al., 2009). In the present work, PME activity during storage has been studied in depth. PME activity can indirectly reduce serum viscosity by both enhancing depolymerizing activity of some pectinases such as PG and pectate lyase (PL) and by increasing Ca^{2+} cross-linking of the pectin chains, producing their precipitation (Jolie, Duvetter, Van Loey, & Hendrickx, 2010; Jolie et al., 2012). However, PME activity cannot be responsible for the sharp viscosity drop observed in T20_50MPa and T20_200MPa in the first two days of storage because, in this period, PME catalytic activity was not significantly different in juices stored at different pressure. Therefore, other enzymatic reactions, apart from PME activity, could be enhanced by pressure. Among them, those affecting the linear HG chain of pectin are the most studied. Enzymatic depolymerization of HG can be produced by PGs and PLs, but studies on PG are much more abundant because, initially, it was thought that PLs were secreted only by plant pathogens. There is no data about the catalytic activity

of strawberry PG under pressure in the literature, but results obtained in tomato PG show that pressure slows down PG catalytic activity (Verlent, Smout, Duvetter, Hendrickx, & Van Loey, 2005; Verlent et al., 2004). On the other hand, various authors have shown that PG activity in strawberry is extremely low (Abeles & Takeda, 1990; Barnes & Patchett, 1976; Nogata, Ohta, & Voragen, 1993; Vicente, Costa, Martínez, Chaves, & Civello, 2005) and, therefore, PG depolymerizing action neither seems to be implicated in the quick viscosity decay observed in Figure 5.3.3. More research is needed to clarify which mechanisms involved in serum viscosity decay (chemical and/or enzymatic) are accelerated by pressure.

In this study, the enhanced PME activity observed in T20_200MPa samples after the first two days of storage did not produce substantial differences in serum viscosity, probably because, at day 5, serum viscosity had almost reached its minimal value. Nevertheless, it affected cloud stability and, thus, T20_200MPa juices presented, at the end of storage, a slight cloud destabilization presumably induced by PME action. This cloud destabilization was not observed in none of the other juices stored either at atmospheric pressure or at 50 MPa.

5.3.5. Conclusions

The results obtained in this study clearly showed that the catalytic activity of strawberry PME was not directly affected by pressure at the conditions tested. However, after 15 days of storage, juices maintained at 200 MPa had twice as much methanol as those kept at atmospheric pressure or at 50 MPa. Conformational changes, chemical, and/or enzymatic reactions affecting pectin could be induced and enhanced at 200 MPa and, thus, indirectly affect PME activity.

Serum viscosity decreased much more quickly in juices stored under high pressure. Therefore, the inhibitory effect of pressure on microbial growth should be the main responsible for previous results in the literature that describe better viscosity preservation in strawberry juices maintained at 25-220 MPa as compared with juices kept at atmospheric pressure. The results presented in this work prove that pressure enhances some mechanisms accelerating serum viscosity decay, apart from PME

activity. Given the complex pectin composition and architecture, a particularly wide range of mechanisms could be implicated and more research is needed to clarify the role of pressure on them.

Methanol content, serum viscosity decay, and cloud destabilization were greater in juices stored at the greatest pressure. Therefore, pressure levels as low as possible, but able to guarantee microbial growth inhibition, should be employed for hyperbaric storage at room temperature of strawberry juice. Hyperbaric storage at room temperature could represent an interesting technology for short-term preservation of fresh juices, but long-term preservation should involve the previous enzymatic inactivation of the product.

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Chapter 6: Application of hyperbaric storage at room temperature at industrial scale: Feasibility and viability study

Part 6.1: Equipment design for hyperbaric storage at industrial scale

EQUIPMENT DESIGN FOR HYPERBARIC STORAGE AT INDUSTRIAL SCALE

6.1.1. Abstract

The hyperbaric storage of foods at room temperature has recently been envisaged as an alternative to refrigeration. Its viability in terms of food safety and quality was checked at laboratory scale in the previous Chapters of this Thesis. However, its feasibility in terms of equipment, especially vessel size and logistics management at industrial scale, has not been considered yet. Since high-pressure vessels are renowned to be heavy, this feature could compromise the possibility to transfer this method from the investigation field to the food industry. Thus, the objective of this study was to address this issue defining the domain of viable designs for such a high-pressure storage vessel. The limiting factors were the vessel dimension and mass: vessel must be cylindrical with a length of no more than 2 m and a maximal mass of about 2 t. From these statements constraints, vessel dimensions and vessel mass were calculated as a function of the shape and material of the vessel, of the product mass, and of the operating pressure. Under conditions similar to those of commercial containers (capacity for 200 kg of product and shape ratio diameter/length of 0.66), several designs of vessel were found to be viable. The most suitable design was a vessel made of 15-5PH stainless steel with two hemispherical heads able to work at any pressures below 155 MPa.

6.1.2. Introduction

Hyperbaric storage at room temperature is a promising method for food preservation. Up to date, its implementation is restricted to the investigation field. There is no hyperbaric storage installation of large capacity nor its implementation at industrial scale has been envisaged yet. The hypothetical flow chart of hyperbaric storage is presented in Figure 6.1.1. In this process, the vessel is placed close to the pump and connected with it. The vessel could be filled either with the packaged product, using

water as the pressure-transmitting fluid, or directly with the product itself if it is liquid. In this last case, the product acts as the pressure-transmitting fluid. Once the vessel is pressurized and isolated, it is transported to a warehouse at room temperature. There, the vessel remains stored until its depressurization. Several high-pressure vessels could be used in an equivalent way to the containers habitually stored under refrigeration or freezing conditions. The pressurization system would be unique, being the same for each vessel.

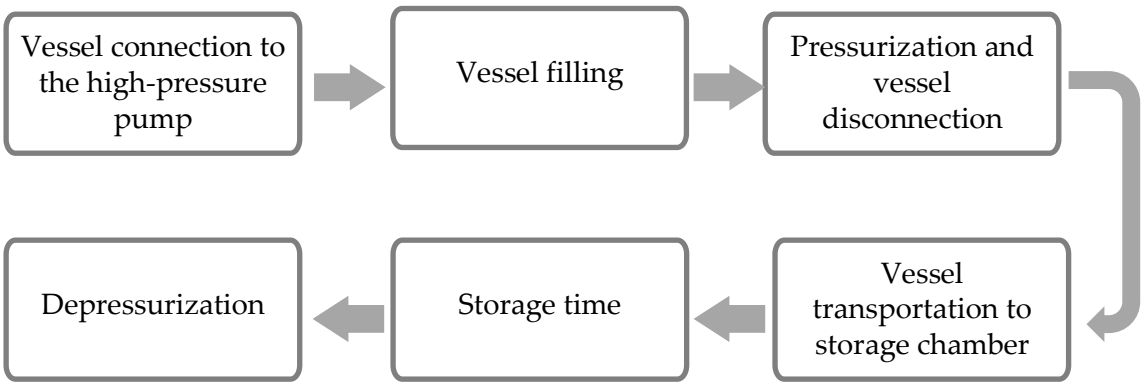

Figure 6.1.1. *Flow chart of hypothetical hyperbaric storage process for strawberry juice employed in this feasibility study.*

The question whether this method would be viable at industrial scale is a logical one since high-pressure vessels are known to be huge and expensive equipment in the food industry. Such heavy high-pressure vessels are usually employed for food high-pressure processing. The key components of high hydrostatic pressure (HHP) units are the high-pressure vessel, the pressure-generating pump, the pressure intensifier (one or more), and the yoke to ensure a secure sealing of the vessel while it is under pressure (Figure 6.1.2). The pressure vessel is the most important component of the equipment given that it is the place where the products are processed. It consists of a stainless steel cylinder which can weigh about several tens of tones with walls about 40 cm in thickness to resist pressures up to 600 MPa. All the components are connected by capillary tubing through which the pressure is transmitted via a fluid. The pressurizing fluid transmits pressure uniformly and instantaneously to the products. In the food industry, water is usually employed because of its convenience and compatibility with food materials.

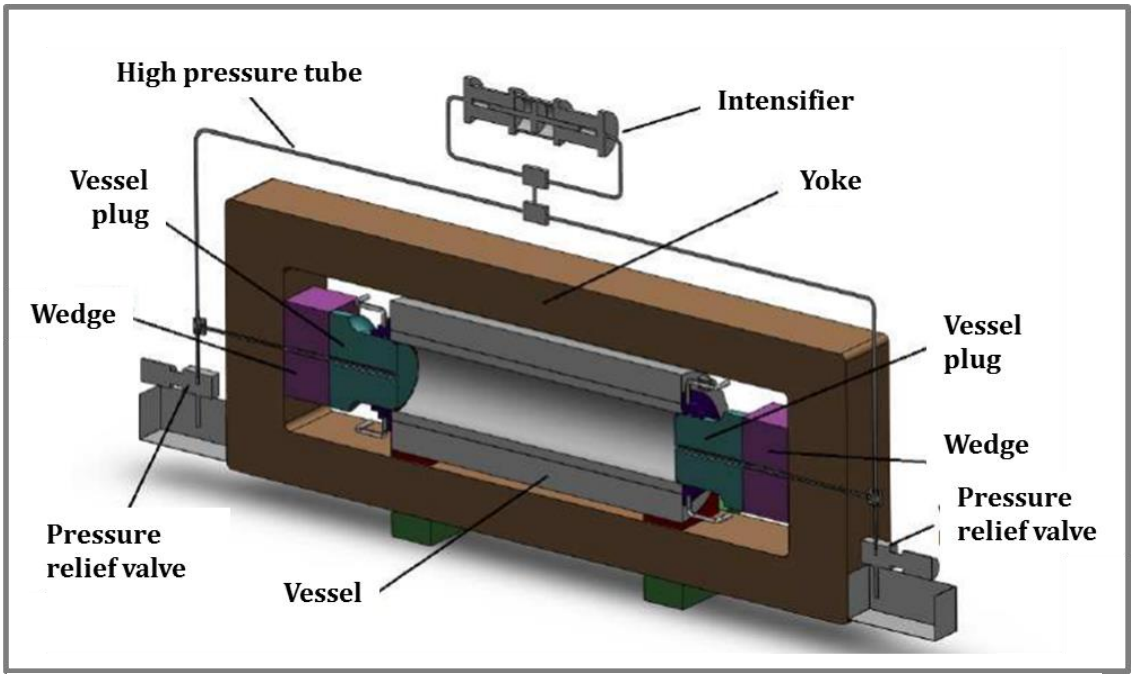

Figure 6.1.2. *Components of high pressure processing equipment. Courtesy of Hiperbaric, Burgos, Spain.*

In the case of hyperbaric storage, the high pressure installation would be very similar in that it would also include a hydraulic pump, an intensifier (unnecessary if the low pressure delivered by the pump is enough) and a high-pressure vessel. The fundamental difference between equipment for HHP processing and hyperbaric storage would be the design of the high-pressure vessel because of the extremely different pressure levels employed. For hyperbaric storage, low pressures are intended to be applied; pressure could be more than 10 times lower than for high-pressure processing. Therefore, the technical requisites are lesser, wall thickness should be smaller, and the vessels are expected to be lighter and consequently movable. Actually, to our knowledge, no data are available on the size and mass characteristics of high-pressure vessels of large capacity in the pressure range of interest for hyperbaric storage.

Thus, this study is focused on this part of the hyperbaric storage installation. The aim is to address the feasibility of food hyperbaric storage in terms of vessel size and logistics management. This will allow for an initial definition and discussion of the technical domain of hyperbaric storage industrial viability. Besides, this step is indispensable

before being able to address economic and environmental aspects of this method. Although it is not intended to enter in much technical detail, the results of this study are expected to constitute a basis for future more sophisticated developments.

6.1.3. Design of the high pressure vessel: Founding principles

As commented above, nowadays, the closest high-pressure installations existing in the industry to hyperbaric storage equipment are the HHP units for food processing. The basic technical knowledge about these existing industrial installations will serve as a starting point to set an initial design for the hyperbaric storage vessels.

Most of the high pressure processing machines installed in the food industry have horizontal vessels due to several advantages compared with the vertical design (Figure 6.1.3): loading/unloading process is more accessible and faster; the installation of the equipment is easier; sublevel construction requirements and floor load restriction are avoided; and traceability is facilitated (Balda, Aparicio, & Samson, 2012; Mújica-Paz, Valdez-Fragoso, Samson, Welti-Chanes, & Torres, 2011). In the case of hyperbaric storage, both configurations are also suitable. However, more than the configuration, the size of the vessel is an important parameter. In particular, its length in the horizontal configuration or its height in vertical position have to be taken into account because handling from the pressurization point to the warehouse should be the easiest as possible. A too long vessel or a too high one would complicate the movement within the manufacture installation. So, in this study, the maximal vessel length to be considered for the design was set to 2 m.

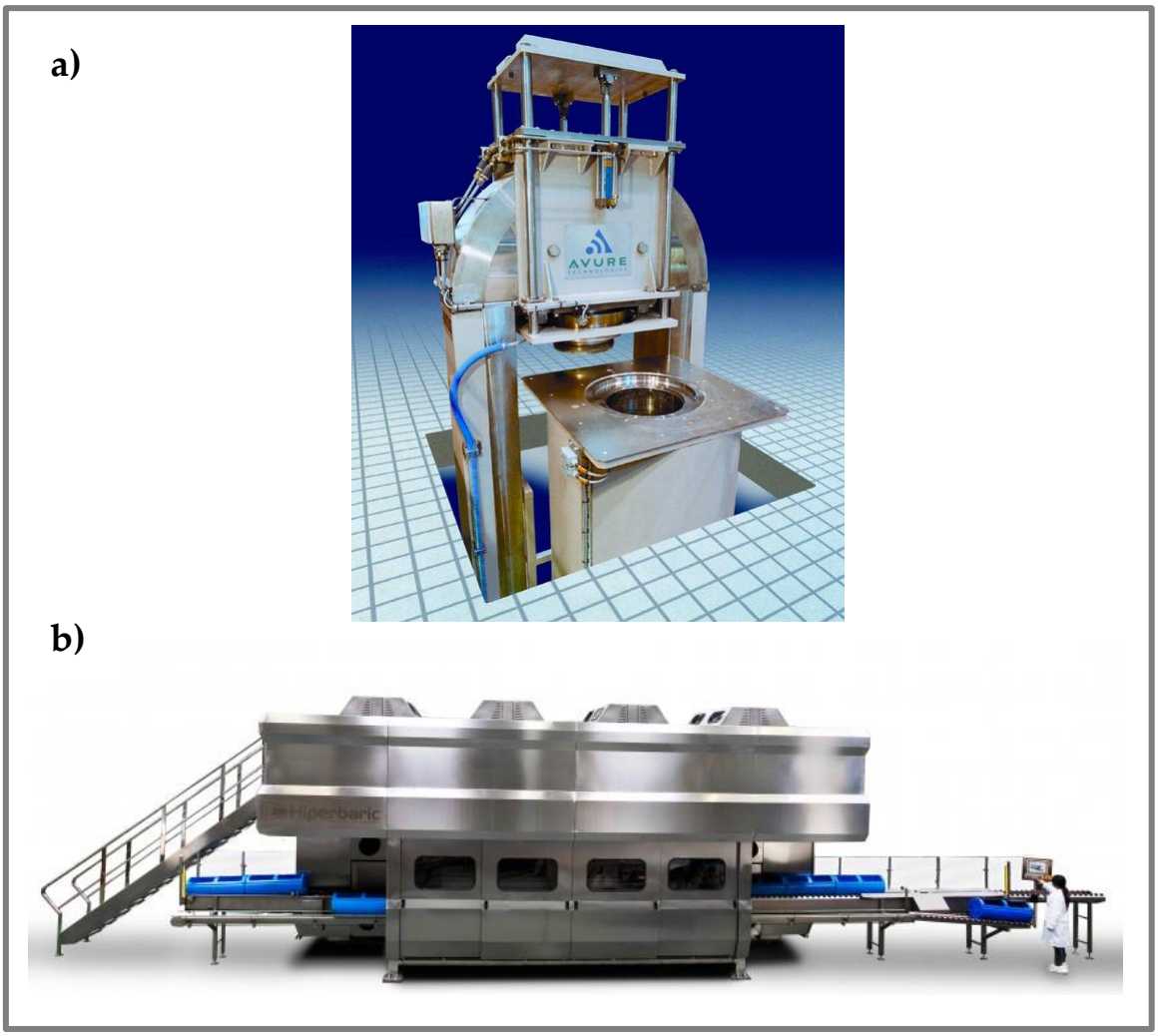

Figure 6.1.3. *Example of (a) vertical commercial pressure processing equipment (Model QFP 320L-400, courtesy of Avure (United States), http://www.avure.com), and (b) horizontal, large commercial pressure processing equipment (Model Hiperbaric 420, courtesy of Hiperbaric, Spain, http://www.hiperbaric.com).*

The vessel capacity of HHP processing machines available on the market ranges from 35 to 687 L. A hyperbaric storage vessel could have the same range of capacity. The limitation is provided by the resulting mass of the vessel plus the product inside. Since the container displacement is necessary, the total mass (vessel plus stored product, i.e. strawberry juice in this Thesis) cannot exceed that supported by a pallet truck. This mass also determines the handling of the vessel. As a reference, Toyota Company commercializes from hand pallet trucks with 2 t of capacity to electric pallet trucks with 3 t of capacity. Thus, for this study, a limiting mass of 2 t has been fixed as the maximal total mass possible for the vessel filled with juice.

From a designing point of view, one of the most critical components of HHP equipment is the high-pressure vessel since it supports many cycles of pressurization/depressurization and it is more prone to fatigue failure. Different high-pressure vessel designs have been developed in order to overcome the technological barriers (Figure 6.1.4). When vessels are constructed from a single block, their inner volume is limited to 25 L for operating pressures in excess of 400 MPa (Torres & Velazquez, 2005). With the aim of increasing the operating pressures and of improving the durability of the vessel (fatigue life), the concentration of stresses at critical points in the vessel wall, when it is under pressure, must be avoided. Thus, it is necessary to redistribute these stresses. This is possible by generating residual stresses in the inner core of the vessel when it is at room conditions. Three techniques are currently used for this: the compounding, the autofrettage, and the wire winding methods. The compounding method consists in using two (or more) cylinders placed in a concentric manner such that the external one is shrink-fitted onto the inner one by getting advantage of thermal dilatation and subsequent shrinkage (Harvey, 1985; Patil, 2013). The autofrettage technique consists in subjecting the internal core of the vessel to an over-pressure which causes a plastic deformation of the inner part of the wall: the internal radius of the cylindrical vessel gets slightly enlarged. When the internal pressure is released, at room conditions, some residual stresses persist due to the resulting permanent deformation: the inner part is in compression while the outer part is in tension (Alegre, Bravo, & Preciado 2007; Harvey, 1985; Partovi & Shamili, 2012). The winding method consists of a wire helically wound edge-to-edge in pretension in a number of turns and layers around the outside of the inner cylinder (Alegre, Bravo, Preciado, & Solaguren-Beascoa 2010). A vessel designed using the wire winding method is composed by three parts: (a) an internal liner or small thickness cylinder in contact with the internal pressure (it can be reasonably easily replaced if fatigue failure takes place), (b) a jacket or intermediate thick-walled cylinder, subjected to compressive stresses generated by the winding process, and finally (c) the winding with a flat wire of a high strength material.

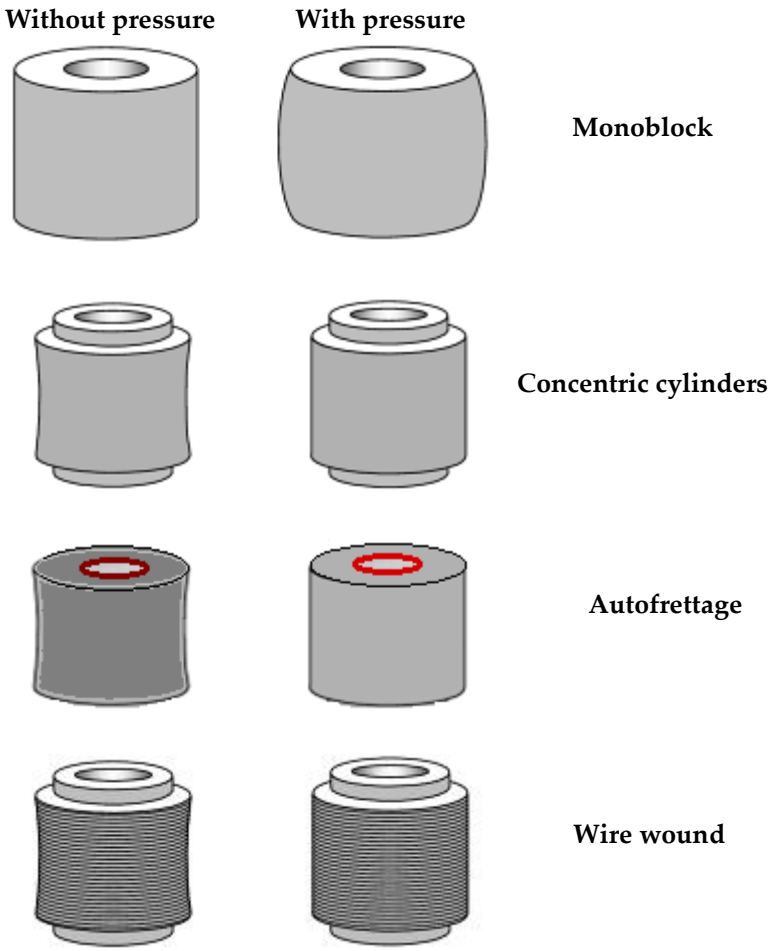

Figure 6.1.4. *High pressure technology. (Adapted from Torres, Velazquez, Jun, and Irudayaraj, 2008).*

For the design of hyperbaric storage vessels, any of these technological improvements is possible. The working pressure is considerably lower than for HHP processing vessels, ranging between 25 and 220 MPa compared to 200 to 600 MPa. Thus, stresses and fatigue are expected to be lower. In the frame of this study, to simplify the calculations, a monoblock vessel design will be considered. The wall thickness of this monoblock vessel is a function of the maximal pressure to be supported (internal pressure), vessel radius, mechanical properties of the material employed for its construction (maximal allowable stress value), and of the joint efficiency (weld joints between the different parts of the vessel). For its calculation, the American Society of Mechanical Engineers (ASME) provides standards in the form of Boiler and Pressure Vessel Code equations. This ensures a long and useful service life as well as a safety guarantee for high-pressure vessels designed according to these rules. These equations

of the ASME will be employed in this study for the design of a hyperbaric storage vessel.

From all the designing features described above, two main limiting factors can be considered as decisive for the feasibility of the storage procedure: the vessel **size** and the vessel **mass**. Both depend on a series of parameters among which the most relevant ones are: vessel shape, vessel material, mass of the product to be stored, and, of course, the operating pressure which determines vessel wall thickness. Thus, in the following Sections, vessel dimensions and vessel mass are calculated as a function of these parameters in order to cover as many options as possible. In this way, we will be able to define the domain where hyperbaric storage is feasible in terms of vessel design.

6.1.4. Methodology

6.1.4.1. Selection of design parameters

Vessel shape. Commonly, high-pressure vessels are composed of two parts: the shell and the heads (Figure 6.1.5). In the industry, the most common type of vessel shell is cylindrical because its manufacture is relatively easy and food items can be arranged inside in a more efficient way than inside a spherical vessel. However, from the physical point of view, spherical pressure vessels are more resistant since they have approximately twice the strength of a cylindrical pressure vessel with the same wall thickness. So, in this study, the vessel design was carried out for two cylindrical vessels with different shapes of head: hemispherical and 2:1 semi-elliptical. In the case of the hemispherical head, the radius of the head equals the radius the cylindrical part of the vessel while in the case of the 2:1 elliptical head, the length of the head is just a half of the cylinder radius (Figure 6.1.5). Despite the fact that the hemispherical head is the strongest, the semi-elliptical head is the most popular. The reason is that the manufacturing cost of hemispherical head is bigger and, therefore, the semi-elliptical is a more economic option. By return, the hemispherical heads need less material for their construction than the semi-elliptical heads. Hence, when an expensive material is used, the manufacturing cost can be compensated by a lower expense in quantity of material.

Besides, the ratio between diameter and length of the vessel (D/L) was fixed to avoid shapes out of proportion. A container of similar characteristics commercialized for other purposes was used as a reference. For example, the company Distrigaz offers a vessel of 760 L with 1.0 m of diameter and 1.5 m of length. The corresponding ratio (D/L) is 0.66. Therefore, this value was used for calculations in this study. Since the total length of the vessel cannot exceed 2.0 m for practical reasons, the permitted maximal diameter in this study was 1.3 m.

Vessel material. According to Regulation (EC) No 1935/2004 of the European Parliament and of the council on materials and articles intended to come into contact with food (Parliament, 2004), any material to come into contact directly or indirectly with food must be sufficiently inert to preclude substances from being transferred to food in quantities large enough to endanger human health or to bring about an unacceptable change in the composition of the food or a deterioration in its organoleptic properties. Stainless steel is attractive and highly corrosion resistant, whilst at the same time having good strength, toughness, and fatigue properties (Baddoo, 2013). This is the reason why it is one of the most used materials in the food industry.

In order to design the vessel, two stainless steels were contemplated: 15-5PH and SAF 2205. 15-5PH has been used by Alegre et al. (2007) to design a high-pressure vessel working at an internal pressure of 500 MPa. These authors pointed out that this steel presents good mechanical properties: high strength and hardness along with an excellent resistance to corrosion and a good toughness against fracture. SAF 2205 was also chosen because the high-pressure vessels of our pilot storage system are made of this stainless steel. SAF 2205 is a duplex (austenitic-ferritic) stainless steel characterized by high resistance to corrosion, high mechanical strength, good physical properties for design, and good weldability, according to the datasheet of a stainless steel supplier (Sandvik, 2014). The chemical composition and some characteristics of both stainless steels are shown in Table 6.1.1 and 6.1.2 for comparison. Main differences are in Cr, Mo, Cu, and N contents and, consequently, tensile strength and proof stress are lower for SAF 2205.

	C	Si	Mn	P	S	Cr	Ni	Mo	Cu	N
15-5 PH	≤ 0.07	≤ 1.00	≤ 1.00	≤0.03	≤0.015	14.00-15.00	3.50-5.50	≤0.50	2.50-4.50	-
SAF 2205	≤ 1.00	≤ 1.00	≤2.00	≤0.03	≤0.015	22	5	3.2	-	0.18

Table 6.1.1. *Chemical composition by (weight %) of 15-5PH and SAF 2205 stainless steesl.* (*Sandvik, 2014; Smith Metal Centers Ltd., 2007).*

	0.2% proof stress (MPa)	Tensile Strength (MPa)	Density (kg/m³)
15-5PH (condition H1025)	1,000	1,069	7,800
SAF 2205	485	680	7,800

Table 6.1.2. *Some mechanical and physical properties of 15-5PH and SAF 2205 stainless steels.* (*Sandvik, 2014; Smith Metal Centers Ltd., 2007).*

Mass of the product to be stored. Since the object of study in this Thesis is strawberry juice, that is, a liquid product usable without any specific pressure transmitting fluid, it was considered that the vessel is directly filled with juice. An amount of 200 kg of strawberry juice was established as being the target quantity to store because this quantity represents a standard batch of juice sold in the food industry (lemonconcentrate.com; www.hudisa.com; www.indulleida.com; www.quirantefruits.com). In order to evaluate the corresponding capacity of the vessel, the density of the strawberry juice measured in the Part 4.1 was used: 1,029.4 kg·m^{-3} at 20 °C. This gives a volume of 0.194 m^3. So, to store the target mass of strawberry juice, i.e. 200 kg, the internal volume of the vessel should be about 0.200 m^3. In order to determine the influence of the product mass on the vessel size and mass and to estimate the domain of the product mass that it is possible to store, it was considered a range between ten times more and ten time less of the target mass (200 kg), i.e. from 20 kg to 2,000 kg of juice.

Storage pressure. The last parameter examined which has a high influence on the vessel design is the storage pressure. It determines vessel wall thickness and, consequently, the mass of the vessel. To estimate the effect of the operating pressure on the vessel mass, different storage pressures were considered. The minimal pressure level taken into account was 25 MPa since it was demonstrated in Chapter 4 of this

Thesis that it is a pressure level effective to preserve strawberry juice for 15 days. The maximal pressure level considered was 220 MPa because it is the maximal pressure investigated in this Thesis.

6.1.4.2. Determination of the vessel dimensions

As previously commented, two different vessel shapes were selected for the design of the pressure vessel: a cylindrical shell with hemispherical heads and a cylindrical shell with 2:1 semi-elliptical heads (Figure 6.1.5).

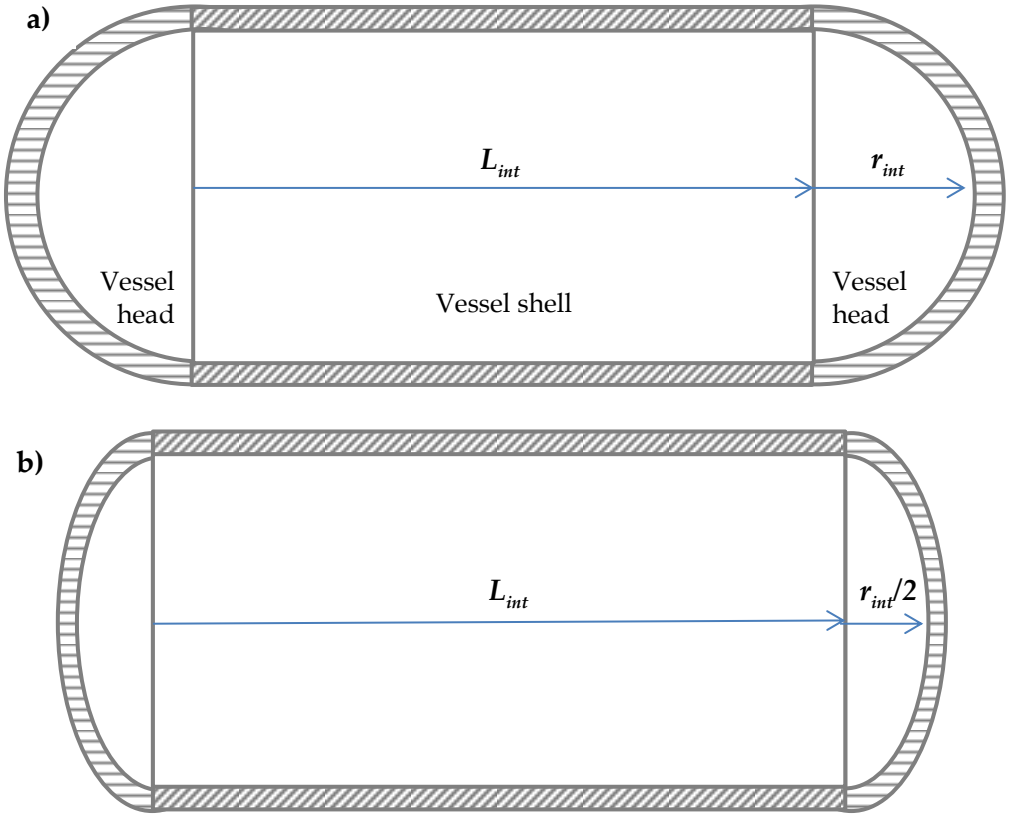

Figure 6.1.5. *Schematic representation of the selected designs for the high pressure storage vessel: a) cylindrical shell with hemispherical heads, b) cylindrical shell with 2:1 semi-elliptical heads.*

For the calculation of the volume of the vessel, the following expressions were used:

$$V_{\text{int}} = V_{shell} + 2 \times V_{head} \tag{6.1.1}$$

$$V_{shell} = \pi \times L_{\text{int}} \times r_{\text{int}}{}^{2} \tag{6.1.2}$$

$$V_{head} = \begin{cases} \left(\frac{4}{3} \times \pi \times r_{\text{int}}{}^{3}\right) \times \frac{1}{2} & \text{, if the heads are spherical} \\ \left(\frac{4}{6} \times \pi \times r_{\text{int}}{}^{3}\right) \times \frac{1}{2} & \text{, if the heads are semi-elliptical} \end{cases} \quad (6.1.3)$$

Where:

- V_{int} is the total internal volume (m^3).
- V_{head} is the volume of the head (m^3).
- V_{shell} is the cylindrical shell volume (m^3).
- L_{int} is the internal length of the shell (m).
- r_{int} is the internal radius (m).

Reciprocally, the length and the radius of the vessel can be also deduced from these formulas when expressed as a function of the volume.

6.1.4.3. Determination of the minimal thickness of the vessel wall

The vessel must withstand the pressure load and this resistance is linked to the wall thickness which depends on the mechanical characteristics of the material chosen for its manufacture. The minimal wall thickness required for the pressure vessel components was calculated by the method of design for internal pressure, according to ASME Boiler and Pressure Vessel Code equations (Eq. 3.1.4-6.1.6).

$$t_{shell} = \frac{P_d \times r_{int}}{S \times E - 0.6 \times P_d} \quad (6.1.4)$$

$$t_{spher} = \frac{P_d \times r_{int}}{2 \times S \times E - 0.2 \times P_d} \quad (6.1.5)$$

$$t_{ellip} = \frac{P_d \times r_{int}}{S \times E - 0.1 \times P_d} \quad (6.1.6)$$

where:

- t_{shell}, t_{spher} and t_{ellip} are the thickness (m) of the cylindrical shell, the hemispherical head, and the semi-elliptical head, respectively.
- P_d is the internal design pressure (MPa).
- S is the allowable stress for the material used (MPa).
- E is the joint efficiency.

To use these equations, the parameters P_d, S, and E are defined as follows:

- The design pressure (P_d) has to be higher than the operating pressure to set a security margin. In this case, the design pressure was chosen as 150 % of the maximal operating pressure.
- The maximal allowable stress values (S) to be used in the calculation of the vessel wall thickness are given in the ASME Code for many different materials. Since we have not access to this information, alternatively, it is possible to assume that the allowable stress is two thirds of the 0.2 % proof stress (Table 6.1.2) at room temperature (Ware, 1995). Therefore, $S_{15\text{-}5PH}$ = 667 MPa and $S_{SAF\ 2205}$ = 323 MPa.
- Weld Joint efficiency (E) accounts for the degree of confidence in weld quality of vessel joints and for the concentration of local stress. The value of E is based on the type of weld and its corresponding quality (categorized as A, B, C, and D according to ASME); that is checked by radiographic examination. ASME proposes the following degrees: 1.00 if the joint is seen as strong as the parent metal and fully radiographed, 0.80-0.85 when it is spot radiographed, and 0.45-0.70 if no radiography is taken. Here, it was arbitrarily considered as E = 0.85.

6.1.4.4. Estimation of the vessel mass

Once the thickness of each part of the vessel (shell and head) has been calculated, it is possible to estimate the mass of the vessel, which allows for evaluating the viability of the logistic management. The mass was estimated by multiplying the density of the stainless steel by the volume of steel employed in each vessel. The mass of the

strawberry juice was added to obtain the mass of the vessel when it is full. The valves, closures, supports, etc. were not included in the mass calculation for the sake of simplicity.

6.1.5. Results and discussion

In order to evaluate the influence of a given parameter on the vessel size and mass (i.e. on viability), all parameters were fixed in the calculations except the one whose influence was tested. The results of this sensitivity analysis are presented below.

6.1.5.1. Importance of the vessel shape on the vessel mass

To appreciate the importance of the vessel shape in the total mass, the calculations were performed using the properties of 15-5PH stainless steel (due to its better mechanical characteristics), an operating pressure of 100 MPa, as an intermediate value of the pressure range (25-220 MPa), and an internal volume of 0.2 m^3. It is recalled here that the ratio between diameter and length was set to 0.66 (Section 6.1.2.1). Results obtained are presented in Table 6.1.3.

	Hemispherical	**2:1 Semi-elliptical**
Thickness of the shell (m)	0.087	0.094
Thickness of the heads (m)	0.038	0.081
External diameter (m)	0.724	0.786
External length (m)	1.097	1.192
Steel mass of the vessel (kg)	957	1,280

Table 6.1.3. *Thickness dimensions and mass of a vessel made of 15-5PH stainless steel as a function of the shape.*

Under the studied conditions, both vessels are feasible in terms of mass since it is lower than 2 t. The lowest mass is obtained with the hemispherical heads, approximately 25 % less. This is because the resistance to pressure of the hemispherical heads is higher and, consequently, the thickness is smaller.

However, not only the shape of the heads has an influence on the vessel mass but also the overall proportion between the length and the diameter of the vessel. Thus, if instead of fixing D/L ratio to 0.66, it is varied, we obtained the dependency of the vessel mass shown in Figure 6.1.6. In these calculations, D/L was varied between 0.24 and 0.66. The lowest limit corresponds to a 2 m-long vessel (maximal external length to be able to move easily the storage vessel) while maintaining a constant volume of 0.2 m^3. Figure 6.1.6 reveals that variations in D/L ratio lead to almost linear changes in the vessel mass which are opposite for each head shape. When D/L increases (i.e. diameter increases and length decreases), the total vessel mass decreases in the case of the vessel with hemispherical heads while it increases in the case of the vessel with semi-elliptical heads. Thus, in the case of a vessel with hemispherical heads, the mass ranges from 1,082 kg to 957 kg (decrease of 11 %). In comparison, the mass of the vessel with 2:1 semi-elliptical heads ranges from 1,173 kg to 1,280 kg (increment of 9 %). As a consequence, the highest difference of mass between vessels according to the head shape considered is observed at the highest D/L values: it is around 9 % at D/L=0.24 while it reaches around 25 % at D/L=0.66.

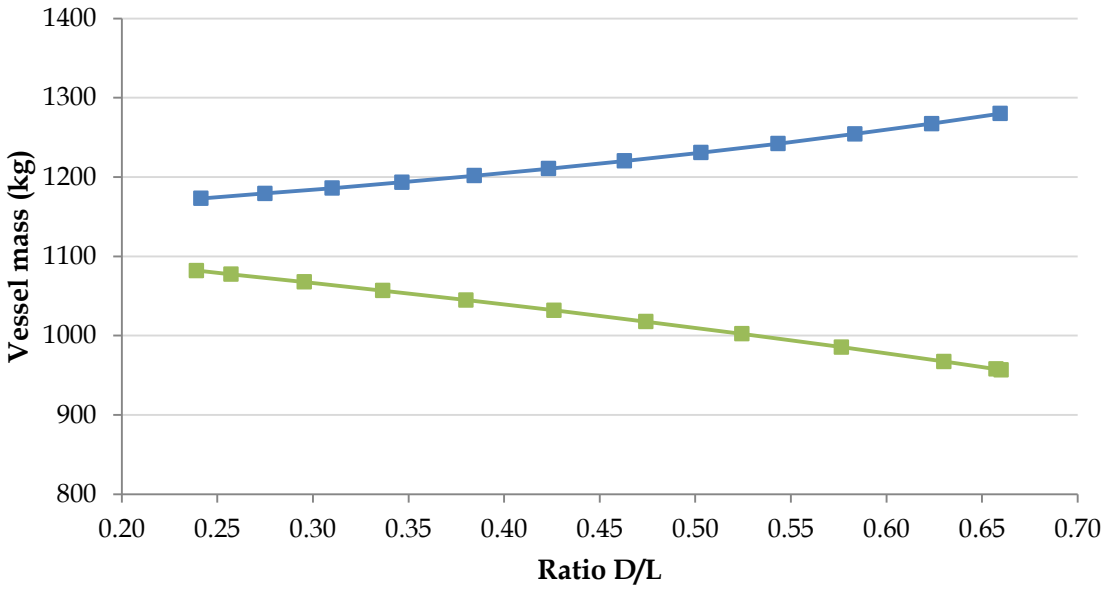

***Figure 6.1.6**. Variation of vessel mass as a function of the dimension ratio at a constant volume of 0.2 m^3. Calculations were performed using the properties of 15-5PH stainless steel and an operating pressure of 100 MPa. Design with hemispherical head: (—) and design with 2:1 semi-elliptical heads: (—).*

To sum up, Table 6.1.3 and Figure 6.1.6 reveal that the vessel shape has an influence on the vessel mass and, for a given head shape, the ratio D/L also affects the vessel mass. In the light of these results, a vessel with hemispherical heads and a maximal ratio D/L (0.66) is the design which minimizes the mass. These shape characteristics will be used in the rest of this study. The corresponding dimensions and mass of the vessel have already been given and can be consulted in Table 6.1.3.

6.1.5.2. Contribution of the vessel material to the vessel size and mass

Obviously, the vessel material has an important influence on the vessel mass since the quantity of material employed in the vessel manufacture depends on its mechanical characteristics. In order to quantify this influence, the mass of a vessel made of 15-5PH stainless steel was compared to that of an equivalent vessel made of SAF 2205. The compared vessels have the following characteristics in common: the shape chosen in Section 6.1.5.1 (hemispherical heads, D/L=0.66), an internal volume of 0.2 m^3, and an operating pressure of 100 MPa.

The results are shown in Table 6.1.4. The differences in the size and mass of each vessel clearly corroborate the importance of the material. The vessel made of SAF 2205 steel counts more than 3 times the mass of the 15-5PH steel vessel. This is because the vessel walls are almost 2.5 times thicker due to the worse tensile strength of SAF 2205 steel (Table 6.1.2). Under the studied conditions, the SAF 2205 vessel is not viable since its mass exceeds 2 t.

	15-5HP	**2205 SAF**
Thickness of the shell (m)	0.087	0.200
Thickness of the heads (m)	0.038	0.071
External diameter (m)	0.724	0.893
External length (m)	1.097	1.354
Steel mass of the vessel (kg)	957	3,000

Table 6.1.4. *Thickness, dimensions, and mass of a hemispherical vessel as a function of the vessel material.*

The mechanical characteristics of the building material of the vessel have a great importance on the vessel mass since they determine the thickness of the walls. Due to a better tensile strength, stainless steel type 15-5PH has been chosen for all the successive estimations.

6.1.5.3. Influence of the product mass on the vessel size and mass

To analyze the effect of the product mass on the vessel size and mass, a vessel made of 15-5PH with hemispherical heads and a D/L ratio of 0.66 was utilized. Besides, for calculations, an intermediate storage pressure of 100 MPa was considered. Product mass was varied between 20 and 2,000 kg in order to scan a wide spectrum of capacities. The results obtained are represented in Figure 6.1.7.

Figure 6.1.7 clearly shows that when the product mass increases, the vessel length, the diameter, and the total mass (vessel mass plus product mass) also increase as expected. There is a linear relationship between product mass and total mass of the vessel while the vessel length and diameter exhibit a power trend.

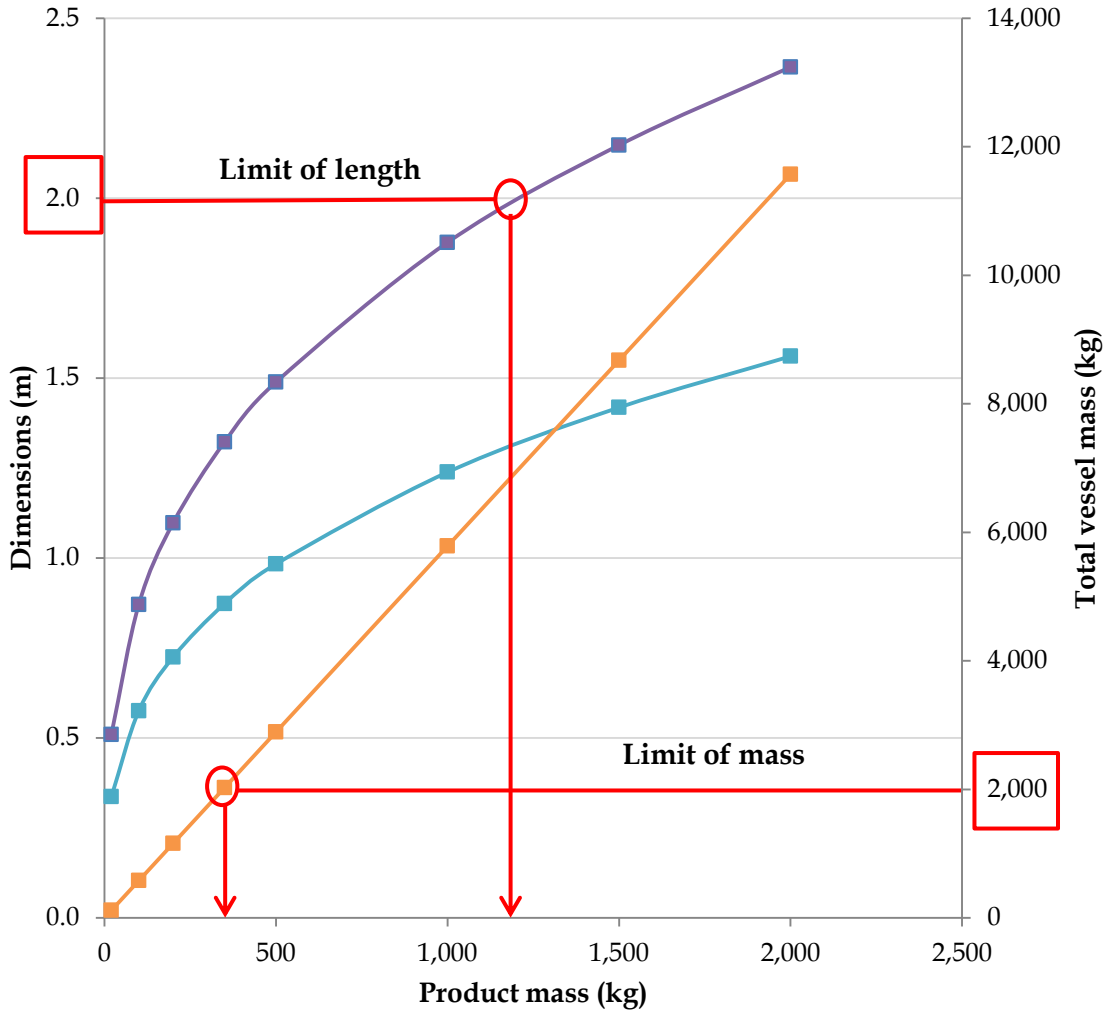

***Figure 6.1.7**. Variation of the vessel diameter (—), length (—), and total mass (—) as a function of the product mass for a hemispherical vessel made of 15-5PH steel, with a D/L=0.66, and an operating pressure of 100 MPa. The red arrows indicate the acceptable product mass considering the viability constraints on the vessel size and mass.*

The red arrows in Figure 6.1.7 indicate the acceptable product mass considering the viability constraints in terms of vessel size and mass. Figure 6.1.7 reveals that the most limiting factor is the total vessel mass that limits product mass to 345 kg.

Within the studied conditions, the limiting factor is the mass of the vessel since the permitted maximal mass is reached earlier than the permitted maximal length (Figure 6.1.7). The maximal product quantity that fulfils the mass criterion, for this particular case, is 345 kg. At this quantity, the total mass of the vessel (including the mass of the product) is 2,000 kg while the length is 1.316 m and the diameter is 0.869 m.

6.1.5.4. Effect of the target operating pressure on the vessel size and mass

Until now, all the calculations have been made considering an operating pressure of 100 MPa. This is the intermediate pressure level studied in this Thesis, but maybe the system would need to work with lower or higher pressures. Therefore, it is interesting to estimate the vessel mass at different pressures with the aim of determining the maximal operating pressure that allows a viable design for the hyperbaric storage vessels. To analyze the pressure effect, a hemispherical vessel made of 15-5PH, with a volume of 0.2 m^3, and a D/L of 0.66 was employed in the calculations. The results are shown in Figure 6.1.8.

As the operating pressure increases, the total mass of the vessel increases rapidly with a second-order polynomial trend. The thickness of the vessel walls also increases, but this increment is linear. Moreover, Figure 6.1.8 shows that the thickness of the shell increases faster than the thickness of the heads. Under the studied conditions, the maximal operating pressure for which the vessel design would be viable is 155 MPa since higher pressures would imply a vessel mass higher than 2 t. If it would be necessary to work at higher pressures, other means than a normal truck will have to be found to move the vessels.

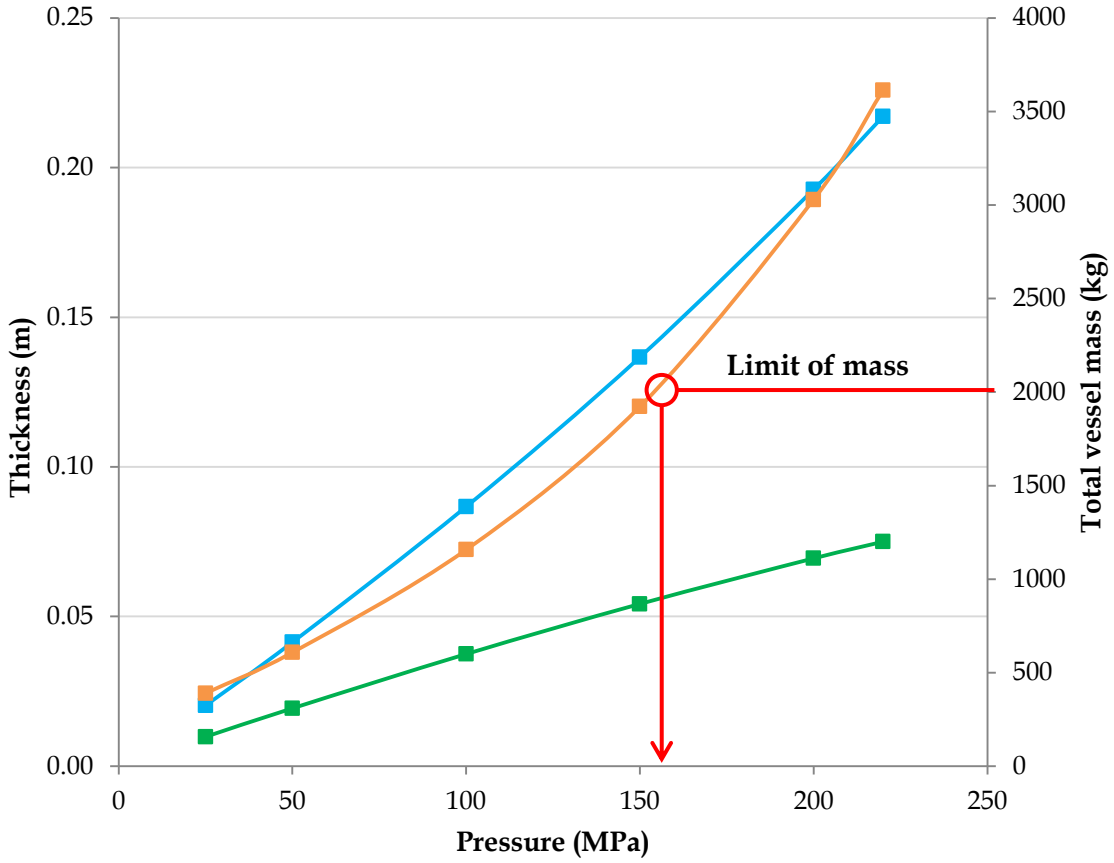

***Figure 6.1.8**. Variation of the vessel wall thickness (shell and head) and the total vessel mass ( ) as a function of the operating pressure for a hemispherical vessel made of 15-5PH steel, with an internal volume of 0.2 m³, and D/L=0.66. The red arrows indicate the acceptable product mass considering the viability constraints on the vessel size and mass.*

6.1.6. Conclusion

Hyperbaric storage is a novel food preservation method that is still at an early stage of investigation and, therefore, it has not been set up in the industry yet. Its viability from the point of view of installation size and logistics management at industrial scale is far from intuitive.

The results of this study show the great influence of the design parameters (shape, material, capacity, and pressure) on the vessel size and vessel mass, considered as the main limiting factors of viability. Both the vessel shape and vessel material can be optimized to minimize the vessel mass. The vessel capacity and the operating pressure are critical parameters to design a vessel with an acceptable mass. The vessel mass

increases rapidly when these parameters are increased. Thus, a compromise has to be found between both. In general, the operating pressure should be kept as low as possible without compromising the quality of the product.

To complete this study, the mass of the valves, frame, and other elements which were not taken into account here should be included in new calculations. Also, a more complex design, including a large opening for the entrance of packaged products (indirect pressurization) could be envisaged. The technical requirements of the other parts of the high-pressure installation (hydraulic pump, intensifier) should be established. But, even without these sophistications, it can be concluded that hyperbaric storage is likely to be a feasible method at industrial scale, at least from the design point of view.

Under the limiting factors set in this study (vessel mass ≤ 2 t and length ≤ 2 m) and with the selected conditions (capacity for 200 kg of juice and a D/L ratio of 0.66), the most suitable design is a vessel made of 15-5PH stainless steel with two hemispherical heads, capable of storing juice at any pressures up to 155 MPa. Pressure levels lower than this value have been found to be efficient in preserving juice quality in previous Chapters. In this Thesis, the subsequent studies about the economic and environmental aspects of hyperbaric storage will be based on this suitable design.

6.1.7. References

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Part 6.2: Cost analysis of hyperbaric storage in comparison with refrigeration

COST ANALYSIS OF HYPERBARIC STORAGE IN COMPARISON WITH REFRIGERATION

6.2.1. Abstract

It has been stressed that the main strength of hyperbaric storage at room temperature over traditional refrigeration is the elimination of low temperatures which are substituted by high pressures. Indeed, a priori, hyperbaric storage should allow energy and money savings. Only the high initial investment due to the expensive price of the equipment appears as a possible handicap for implementing this novel storage method at industrial scale. Hence, the objective of the present study is to analyze the cost of strawberry juice storage under hyperbaric conditions and to compare it with the traditional storage under refrigerated conditions. Amortization, maintenance, and electricity consumption costs were calculated at pilot plant scale under a series of simplifications and hypotheses. Calculations were carried out for a quantity of four batches of 200 kg of strawberry juice, stored during 15 days at 25 MPa. Under the conditions established in this study, the results reveal that the cost of hyperbaric storage at room temperature is around 3-fold higher than refrigeration cost. Nonetheless, the large dependence of the hyperbaric storage cost on the initial investment makes that, if the equipment price diminished, the hyperbaric storage cost would become more competitive.

6.2.2. Introduction

It has been pointed out that the major advantage of hyperbaric storage at room temperature over the traditional refrigeration is the elimination of low temperatures which are substituted by high pressures. This is really interesting from the economic point of view. Cold installations are huge consumers of energy and consequently are expensive over time. After cooling down the product to 4-8 °C, energy is spent to maintain this temperature constant as much as possible all along the food distribution

chain. In comparison, pressure, once created, is maintained by keeping the product container closed and tight, without any additional need of energy during storage and distribution. Thus, in theory, hyperbaric storage at room temperature should allow energy and money savings.

But, until now, this method has not been implemented at industrial scale. Before its implementation, it is mandatory to study a series of parameters to assess its viability and suitability. In a previous study (Part 6.1), it has been proved that food hyperbaric storage could be viable at industrial scale in terms of installation size and logistics management. Simplified calculations lead to the following viable design for the high-pressure container: a cylindrical vessel made of 15-5PH stainless steel with hemispherical heads, capacity for 0.2 m^3 of juice, and maximal operating pressure of 155 MPa. If the operating pressure is lower, the vessel wall thickness is smaller and the vessel is lighter. A vessel operating at about 100 MPa weighs around 1.2 tons when full of juice, a mass which sounds high but that can be relatively easily moved by a pallet truck, for example. Another extremely important factor which needs to be assessed before transferring this emerging technology to the industry is the cost. Cost analysis is compulsory in order to assist companies in the decision-making process with respect to the implantation of a new technology. The potential benefits of this new method over the traditional one must be clearly established not only from the product quality point of view but also from the economic one. Since, to the best of our knowledge, there is not any quantitative information about the cost of this storage method at the industrial scale, we propose to address here this issue at pilot plant scale. Thus, the objective of the present study is to analyze the cost of strawberry juice storage under hyperbaric conditions and to compare it with the traditional storage under refrigerated conditions.

The ambition of this comparison is not other than establishing a starting point to develop more detailed and complete economic analyses. Thus, a study at pilot plant scale is judged enough for this purpose. This means that the scope of this study is limited to the evaluation of the basic costs derived from each storage method at a scale intermediate between the laboratory and the industry. Only the amortization and maintenance costs of the system components and the energy costs will be included in

the total cost estimation. All costs prior and posterior to the storage step are not retained in this analysis (e.g. pasteurization). At this stage, the unavailability of industrial data precludes any possibility to make a more complex analysis which could take into account, for example, a cleaning step, the effect of season change, etc. In fact, a series of simplifications and hypotheses are necessary to be able to carry out the calculations even at pilot scale. These calculations are performed for a quantity of four batches of 200 kg of strawberry juice, and for an operating pressure of 25 MPa. The quantity of stored juice was chosen in relation with the mass of usual commercial juice batches in the industry (200 kg) and adapted to the space available for storage in the pilot plant facility (capacity for four batches). The pressure level was selected according to the results of the previous Chapters: high enough to preserve the quality of strawberry juice but as low as possible to minimize the vessel mass and to make handling easier. The duration of storage is set to 15 days in both cases, hyperbaric and refrigerated storages. Therefore, the comparison of costs between each storage method will be established in units of euros per juice kg per 15 days of storage.

6.2.3. Methodology

6.2.3.1. Scenarios of storage

6.2.3.1.1. Operations and hypotheses considered for refrigeration

The refrigerated storage procedure assumed for performing the economic assessment is schematized in Figure 6.2.1. Although for refrigeration, a previous pasteurization treatment of the juice is mandatory, this cost was not contemplated in this evaluation in order to stand out only the part of the cost inherent to the storage. The illustration is adapted to the case of the strawberry juice studied in this Thesis. Shortly, pasteurized strawberry juice is driven by a pump into the containers where the juice is packed for the storage. Once the containers are filled and closed, they are placed in a refrigerated chamber at 4 °C for all the storage period. The temperature of the juice before storage is room temperature (20 °C) since no cooling step prior to packaging and storage is considered. In order to simplify the calculations, it is supposed that, once the juice is

introduced in the refrigerated chamber, this one remains closed throughout all the storage period.

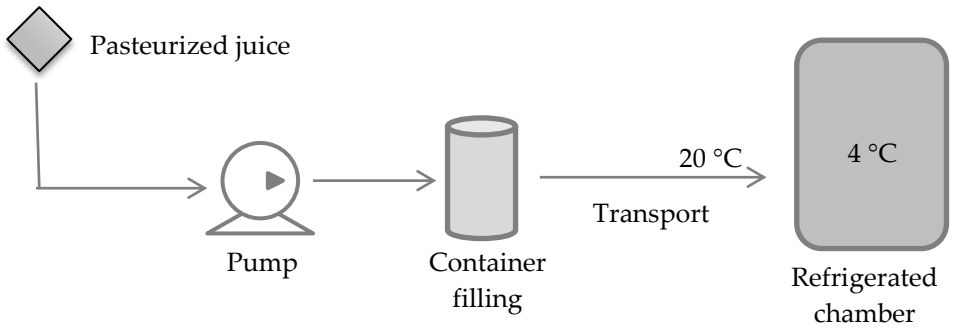

Figure 6.2.1. *Scheme of the hypothetical refrigerated storage of strawberry juice.*

For the calculations, the characteristics of the refrigeration chamber that our group has in the pilot plant at ICTAN (VIZUETE chamber, model CV9B3) were employed. The roof, walls, and door of the chamber are made of polyurethane while the ground is made of two layers of aluminum and cement, respectively. Figure 6.2.2 illustrates the dimensions of the refrigeration chamber and the characteristics of the ground. Containers of 0.2 m^3 made of stainless steel and 1 mm of thickness were chosen as being the packages where the juice is stored (Figure 6.2.2.d). Taken into account the size of the refrigeration chamber, it is possible to store up to four of these juice containers at the same time.

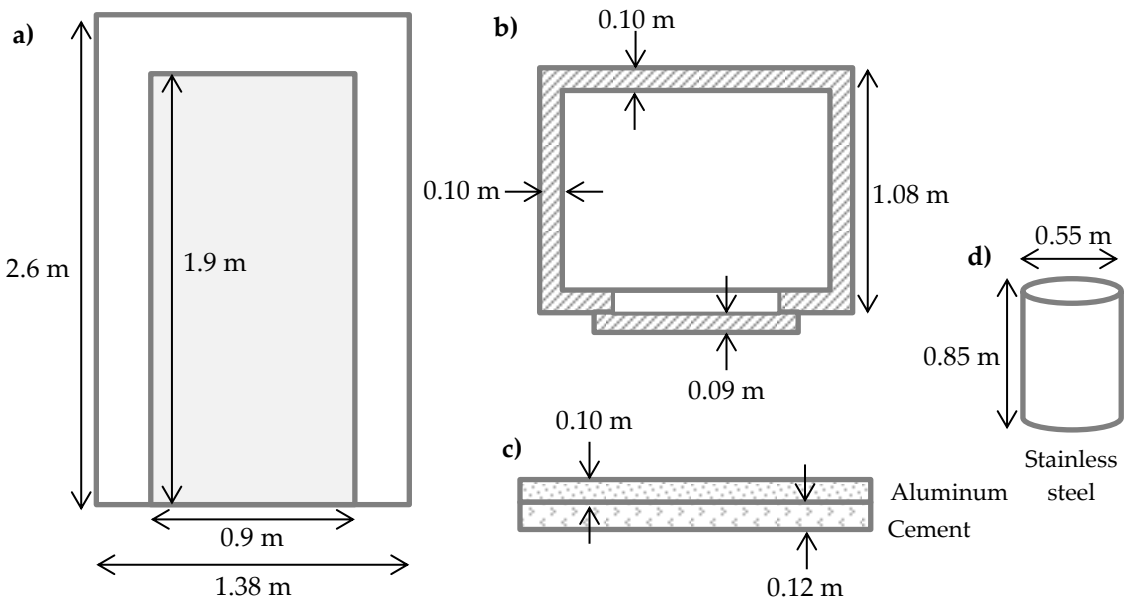

Figure 6.2.2. *Characteristics of the refrigeration chamber and the containers used in the hypothetical refrigerated storage of juice: (a) Front view of refrigeration chamber, (b) Top view of the refrigeration chamber, (c) Ground that supports the refrigeration chamber, and (c) Juice container.*

A pump with a flux of 35 $L \cdot min^{-1}$, a maximal pressure of 2.5 MPa, and a power of 0.44 kW was chosen among the commercial pumps existing on the market as a suitable pump to fill the containers with juice. Pump price and data given by the provider (ROVER POMPE, serie NOVAX) were used for the calculations.

6.2.3.1.2. Operations and hypotheses considered for hyperbaric storage

The hypothetical hyperbaric storage considered for the economic evaluation is represented in Figure 6.2.3. For hyperbaric storage, unlike for refrigeration, the previous pasteurization treatment is optional. Again this cost was not contemplated in this evaluation. As for refrigerated storage, the juice is pumped to fill the high-pressure vessel. The pressure is then built up using the strawberry juice itself: the introduction of an additional volume of juice in the vessel, already full, causes the pressure to increase (the vessel volume is constant, it cannot expand). When the desired pressure is reached, 25 MPa in this study, the vessel is isolated from the pressure generating system by means of a set of valves. Just after that, it is transported to the storage chamber at room temperature and left there until the end of storage.

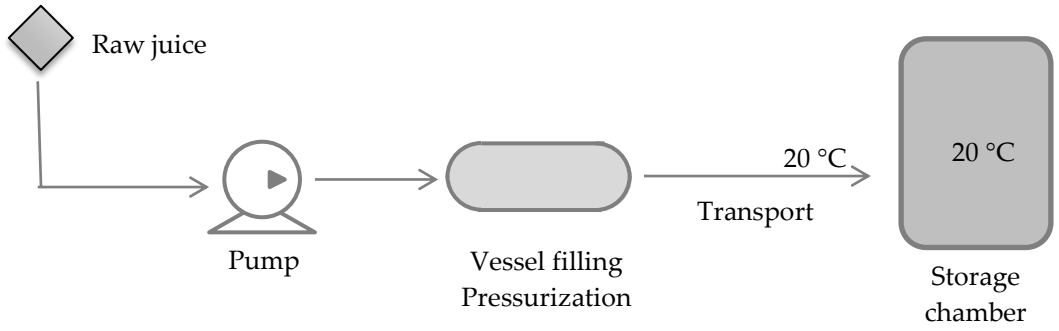

***Figure 6.2.3**. Scheme of hypothetical hyperbaric storage of strawberry juice.*

The characteristics of a high pressure vessel, operating at 25 MPa, are compiled in Table 6.2.1. To take into account the same quantity of juice as in the case of refrigerated storage, it is considered that four vessels are pressurized successively and then stored simultaneously.

Hemispherical vessel (15-5PH)	
Volume (m^3)	0.2
Thickness of the shell (m)	0.020
Thickness of the head (m)	0.010
External diameter (m)	0.629
External length (m)	0.952
Steel mass of the vessel (kg)	189.8

***Table 6.2.1.** Volume, thickness, dimensions, and mass of a hemispherical vessel made of 15-5PH stainless steel for a storage pressure of 25 MPa with a dimension ratio (D/L) of 0.66.*

Only one pump should be used to fill and pressurize each vessel. A range of high-pressure pumps exists on the market which may fulfill this requirement with no need of an intensifier, at least for pressures up to 40 MPa. The same technical characteristics of the high-pressure pump proposed by VENETO (Serie APB) were considered: a flux of 25 $L \cdot min^{-1}$, a maximal pressure of 40 MPa, and a power of 18.39 kW.

6.2.3.2. Cost analysis

To carry out the analysis of costs, it is considered that the storage cost (C) is composed by the amortization cost of the initial investment (A), the maintenance cost (M), and the electricity consumption cost (E) throughout the storage period (Eq. (6.2.1)).

$$C = A + M + E \tag{6.2.1}$$

Labor and administrative costs are not taken into account in this initial study.

6.2.3.2.1. Amortization cost

The amortization cost is estimated from the linear amortization of the initial investment as it is shown in Eq. (6.2.2). For the estimation, it is assumed that the food manufacture works 365 days a year and the useful life of the equipment is 10 years (Pardo & Zufía, 2012; Sampedro, McAloon, Yee, Fan, & Geveke, 2014). Besides, the total quantity of product stored is 800 kg corresponding to four containers of 200 kg (Section 6.2.3.1.1). The amortization cost (A) of a given storage method is calculated as follows, expressing the cost in € per kg of stored juice for 15 days:

$$A = \frac{C_i \times t}{l_u \times f \times q} \tag{6.2.2}$$

In this formula, C_i is the initial cost of the equipment (€); t is the time of storage (days); l_u is the useful life (years); f is the number of operating days per year (days·year^{-1}); and q is the quantity of stored product during 15 days (kg).

To estimate the amortization cost of refrigerated storage, the price of the refrigeration chamber, the approximated price of the containers, and the price of the pump were all added up to give the initial cost of the equipment.

As hyperbaric storage is not implemented at industrial scale yet, the estimation of the initial cost of the equipment is a complicated issue. For the pump, the price of a VENETO (Series APB) high pressure pump was taken as a reference. However, in the vessel case, the price was approximated from that of the quantity of stainless steel necessary for its manufacture. The price of the steel depends on several factors such as country, company, or level of demand, among others. An approximate price of 10 €·kg^{-1} was employed for the estimation. Moreover, in order to get closer to a real equipment, some corrective coefficients were applied:

- The steel mass of the vessel was multiplied by 1.5 to contemplate additional parts of the vessel.

- A coefficient of 3 was applied to the result of steel price in terms of manufacture cost and profit margin.
- The final price was incremented by 5 % to account for transport and installation.

6.2.3.2.2. Maintenance cost

The maintenance includes all the operations necessary to guarantee the correct work of the installation and to carry out the reparations, if ever required. It is considered that the maintenance cost per year is about 5 % of the initial cost of the equipment (Fleming, n.d.). So, the maintenance cost M (€·kg^{-1} for 15 days) is calculated according to:

$$M = \frac{0.05 \times C_i \times t}{f \times q} \tag{6.2.3}$$

where C_i is the initial cost of the equipment (€); t is the time of storage (days); f is the operating days (days·year^{-1}); and q is the quantity of product (kg).

6.2.3.2.3. Electricity consumption cost

Electricity consumption cost is calculated from the energy spent for the storage operation. This energy is determined from the power required by the pump and by the equipment during the storage period. Once the power necessary is estimated, then it is transformed into electric energy and finally, by multiplying by the unitary electricity price, the cost of the energy consumed for storage is deduced. Although the electricity prices depend on the country, the relative cost difference among processes should remain quite constant and thus comparable. According to UNESA (Asociación Española de la Industria Eléctrica), in Spain, the price of the electricity for industrial purpose in the first semester of 2012 was 0.1429 €·kWh^{-1}.

In the case of refrigerated storage, electricity is consumed by the pump during the filling of the containers and throughout all storage period in order to maintain the temperature in the chamber at 4 °C. The electricity consumption during storage was calculated from the total heat load that is necessary to remove.

For hyperbaric storage, electricity is only required by the pump during vessel filling and pressure build-up, and by the control panel during pressurization.

6.2.3.3. Sensitivity analysis

A sensitivity analysis was carried out to study the effect of the storage pressure in the range 25-100 MPa on the total cost of hyperbaric storage. Calculations were performed accounting for the variations in the vessel dimensions and mass with pressure level, but keeping constant vessel capacity and shape (Part 6.1).

6.2.4. Results

6.2.4.1. Cost of refrigerated storage

6.2.4.1.1. Amortization cost of refrigerated storage

Since refrigerated storage is a well-established technology in the industry, refrigeration chamber prices are well defined. The final price (including transport and installation) of the refrigeration chamber defined in Section 6.2.3.1.1 was 6,350 €. The estimated price of the containers where the juice is stored was 200 € per container, so, for four containers, the investment would be 800 €. Finally, the price of the pump chosen, according to the seller (electrobombas.es), is 187 €. Thus, using Eq. (6.2.2) and an initial cost of 7,337 €, the value of the amortization cost is **0.038 €·kg^{-1} of juice for 15 days of storage**.

6.2.4.1.2. Maintenance cost of refrigerated storage

In the same way as for amortization, to calculate the maintenance cost, an initial cost of 7,337 € and a product quantity of 800 kg were used. The result of Eq. (6.2.3) is **0.019 €·kg^{-1} of juice for 15 days of storage**.

6.2.4.1.3. Electricity consumption cost of refrigerated storage

Refrigerated storage consumes energy during the filling of the vessels and throughout all the storage period.

To calculate the electrical energy consumed by the pump, E_{pump} (kWh), it is necessary to know the power, P_t (kW) and the functioning time, t_p (min):

$$E_{pump} = \frac{P_t \times t_p}{60} \tag{6.2.4}$$

The electric power is given by the manufacturer (0.44 kW, Section 6.2.3.1.1). The functioning time of the pump to fill the four containers, considering a flux of 35 $L \cdot min^{-1}$ (Section 6.2.3.1.1), is 22.86 min. So, applying Eq. (6.2.4), the total energy required (E_{pump}) is 0.1676 kWh. As the price of the electricity is 0.1429 €·kWh^{-1} (Section 6.2.3.2.3), the energy consumption cost for the filling of the containers is $2.4 \cdot 10^{-2}$ €. This value is so small that it can be neglected.

To estimate the energy consumed throughout all the refrigeration period, firstly, it is necessary to calculate the refrigeration requirement of the cold chamber, and then, to transform it into energy cost. To do so, the heat load, that is, the amount of heat to be removed within a certain period of time, has to be determined. Martín (2005) reported that seven loads should be taken into consideration for the calculation of the total heat load:

1. Insulation heat leak through walls, roof, floor, and door (Q_1). Q_1 means the heat flow getting in from the outside due to the difference of temperature that has to be compensated. It is calculated from the following expression:

$$Q_1 = \left(\sum K_j \times S_j\right) \times \left(T_e - T_i\right) \times \frac{24 \times 3600}{1000} \tag{6.2.5}$$

where K_j is the heat transfer coefficient of each part j ($W \cdot m^{-2} \cdot K^{-1}$); S_j is the area of each part j (m^2); j is each part of the chamber (walls, roof, floor, and door); and T_e and T_i are the external and internal temperatures, respectively. The last factor appearing in Eq.(5) is to express Q_1 in $kJ \cdot day^{-1}$.

By definition, the heat transfer coefficient is determined as shown below:

$$\frac{1}{K_j} = \frac{1}{h_e} + \left(\sum_{k=1}^{n} \frac{e_k}{\lambda_k} \right) + \frac{1}{h_i} \tag{6.2.6}$$

where h_e and h_i are the external and internal heat transfer coefficient ($W \cdot m^{-2} \cdot K^{-1}$), respectively, and e_k and λ_k are the thickness (m) and thermal conductivity ($W \cdot m^{-1} \cdot K^{-1}$) of each material k composing a given part of the chamber, respectively. Since the values of h_e and h_i are very small in comparison with the thermal resistance of the material (e_k/λ_k), they can be neglected in the calculation. Thus, the heat transfer coefficient is:

$$\frac{1}{K_j} = \sum_{k=1}^{n} \frac{e_k}{\lambda_k} \tag{6.2.7}$$

Thermal conductivity (λ_k) is specific to each material. Table 6.2.2 shows the thermal conductivity values of the materials employed in the refrigeration chamber of this study. From the chamber specifications (Figure 6.2.2) and considering an external and internal temperature of 20 °C and 4 °C, respectively, Q_1 was calculated according to Eq. (6.2.7) and (6.2.5). The value obtained Q_1 = 29,225.5 $kJ \cdot day^{-1}$.

	Aluminum	**Cement**	**Polyurethane**
λ_k ($W \cdot m^{-1} \cdot K^{-1}$)	237[(a)]	1.73[(b)]	0.022[(c)]

Table 6.2.2. *Thermal conductivity values of the materials composing the studied refrigeration chamber. [(a)]Termodinamica.us.es; [(2)]The engineering tool box; [(3)]Shawyer & Pizzali (2003).*

2. Product load (Q_2). It refers to heating due to entry of goods when they are at a higher temperature than the chamber temperature. Here, it is necessary to determine the energy required to cool them. In the present case, it is considered that the four containers enter inside the refrigeration chamber at the same time and at an initial temperature of 20 °C. Only the strawberry juice was considered in the calculation since the contribution of the containers is so small that it can be neglected. Therefore, the heat load to remove when cooling the strawberry juice from 20 °C down to 4 °C is determined by Eq. (6.2.8):

$$Q_2 = m \times c_p \times (t_e - t_i) \tag{6.2.8}$$

where m is the mass of product (800 kg), c_p is the specific heat of juice (3.368 kJ·kg^{-1}·°C^{-1}, datum found in Dubrovic, Herceg, Režek Jambrak, Badanjak, and Dragovic-Uzelac, 2011), t_e is the temperature of the product before storage (°C), and t_i is the temperature of the refrigeration chamber (°C).

The value obtained for Q_2 is 43,110 kJ. As the introduction of the product only takes place on the first day of storage, and all the calculations are expressed in kJ per day, this result is divided by the 15 days of storage. Thus Q_2 = 2,874 kJ·day^{-1}.

3. Physiological and chemical activity load (Q_3). When live or fresh products are stored, the respiration and other chemical reactions are exothermic and generate heat. In the case of strawberry juice, the product is already processed and packed. Therefore Q_3 = 0 kJ·day^{-1}.

4. Air changes load (Q_4). In case of physiological and chemical activity of the products, the renewal of the air in the chamber is necessary. The renewal of the air together with the opening the doors increase the chamber temperature. In this case of study, as the product is packaged, this action is unnecessary. Besides, if it is considered that the door is always closed during the 15 days of storage, then Q_4 = 0 kJ·day^{-1}.

5. Motors, fans, and lights heat load (Q_5). The functioning of motors, fans, and lights provides heat. In this chamber, the heat supply only comes from the fan motor, and not from the lights, since the door is closed during all the storage period. Then, with a fan of P = 0.1 kW of power and an operating time t of 16 h·day^{-1}, Q_5=5,760 kJ·day^{-1} (Eq. (6.2.9)).

$$Q_5 = P \times t \times 3600 \tag{6.2.9}$$

6. Entry of personnel load (Q_6).

Entry of personnel into the refrigeration chamber contributes to a heat increase, since the body temperature (36.5 °C) is higher than the chamber temperature (4 °C). As it is

considered that nobody accesses the chamber during the refrigeration storage, the value of Q_6 is 0 kJ·day^{-1}.

7. Other possible loads and safety factor (Q_7). Other heat sources that contribute to heat load are the condensation and freezing of water on the evaporator. Besides, a safety factor should be included because the energy conversion efficiency is never 100 %. Tomasula, Yee, McAloon, Nutter, and Bonnaillie (2013) have considered an efficiency of 75 %. Here, Q_7 is estimated by applying Eq. (6.2.10) (Martín, 2005):

$$Q_7 = 0.25 \times Q_1 \tag{6.2.10}$$

Then, the value of Q_7 is 7,306 kJ·day^{-1}.

Finally, after adding all the calculated heat loads, the total heat load (Q_t) is 45,166 kJ·day^{-1} which is the cold store refrigeration requirement per day. Since the storage period is 15 days, the requirement of the refrigerated storage is 677,489 kJ for 800 kg of strawberry juice.

To convert this energy in kilojoules into power in kilowatt hour per kilogram of juice, it is necessary to divide by 3600 s and by 800 kg, thus the power is 0.24 kWh·kg^{-1} for 15 days of refrigeration. Considering that the price of electricity is 0.1429 €·kWh^{-1} (Section 6.2.3.2.3), the energy cost is **0.034 €·kg^{-1} of juice for 15 days of storage**.

The economic cost of refrigerated storage, after adding amortization (0.038 €·kg^{-1}), maintenance cost (0.019 €·kg^{-1}), and energy cost (0.034 €·kg^{-1}), is finally **0.091 €·kg^{-1} of juice for 15 days of storage.**

6.2.4.2. Cost of hyperbaric storage

6.2.4.2.1. Amortization cost of hyperbaric storage

Taking into account a steel vessel mass of 189.8 kg (Table 6.2.1) and a stainless steel 15-5PH price of 10 €·kg^{-1}, after applying all coefficients considered (Section 6.2.3.2.1), the price of the vessel is **8,968 €**. Hence, the investment for four vessels would be **35,872 €**. Besides, the price of the pump chosen as reference, as reported by the seller

(electrobombas.es), is 4,050 €. According to Eq. (6.2.2), the amortization cost for hyperbaric storage is then **0.205 €·kg^{-1} of juice for 15 days of storage**.

6.2.4.2.2. Maintenance cost of hyperbaric storage

The maintenance cost corresponds to 5 % of initial cost of the equipment (pump + four vessels), so, the maintenance cost determined from Eq. (6.2.3) is **0.103 €·kg^{-1} of juice for 15 days of storage.**

6.2.4.2.3. Electricity consumption cost of hyperbaric storage

The main energy advantage of hyperbaric storage is that it only requires energy during compression and no additional energy is needed to maintain the product under pressure throughout the storage period.

The required power of the pump is supplied by the manufacturer (18.39 kW, Section 6.2.3.1.2). Regarding the control panel, the electric power is independent of the operating pressure. Consequently, the same electric power as the control panel of our pilot plant equipment has been employed for the calculations: 2 kW. Therefore, by combining the electric power consumed by the pump and by the control panel, a total electric power of 20.39 kW is obtained.

The time of filling and pressurization has to be determined so as to transform this calculated power into consumed electrical energy. Both in refrigerated and hyperbaric storage, the pump drives the juice into the vessel. The difference between them is that, in hyperbaric storage, once the vessel is full, an additional juice volume has to be introduced to provoke the increase of pressure up to the target value of 25 MPa (direct pressurization).

To estimate this additional volume, it is considered that: the internal volume of the vessel is constant, the compression is adiabatic, and the strawberry juice behaves as water with pressure. The compression can be seen as adiabatic provided that it occurs in a period of time much smaller than the time needed by the generated heat to be dissipated out of the system. This is usually the case in high-pressure vessels because of the thick walls of the equipment. Compression produces an increment of temperature around 2-3 °C per 100 MPa in aqueous solutions (Balasubramanian &

Balasubramaniam, 2003). If the temperature of the juice before pressurization is set at 20 °C, it is estimated that, at the end of pressurization to 25 MPa, the temperature would be around 20.75 °C. Based on the density of water at different pressure and temperatures, and on the density of strawberry juice at atmospheric pressure, it is possible to approximate the density of the juice at 25 MPa and 20.75 °C from a compositional additive model (Guignon et al., 2012). The density of water employed in the calculation was taken from the web page of the National Institute of Standards and Technology (NIST). Thus, the density of strawberry juice increases from 1029.4 $kg \cdot m^{-3}$ at atmospheric pressure to approximately 1040.1 $kg \cdot m^{-3}$ at 25 MPa. This implies pumping a juice mass of 2.14 kg in the constant volume (0.2 m^3) of the vessel. Converted into juice volume, these 2.14 kg of juice represent an additional volume of 2.07 L necessary to reach the operating pressure of 25 MPa. So, the time the pump has to work to introduce a volume of 202.07 L in each vessel is 8.08 min with a flux of 25 $L \cdot min^{-1}$.

Knowing the total electric power (P_t=20.39 kW) and the functioning time (t_p=8.08 min), the energy required is 2.747 kWh per each vessel (Eq. (6.2.4)). Since the price of the electricity is 0.1429 $€ \cdot kWh^{-1}$ (Section 6.2.3.2.3), the energy consumption cost for the hyperbaric storage of one vessel (200 kg) of strawberry juice is 0.393 €. Taking into account that four vessels are stored in total, the cost is multiplied by four, so the energy consumption cost is 1.570 €.

If the energy cost (1.570 €/800 kg) is calculated per kg of juice, the result is **0.002 $€ \cdot kg^{-1}$ of strawberry juice** which is very small compared with the amortization and maintenance costs.

Considering the amortization (0.205 $€ \cdot kg^{-1}$), the maintenance (0.103 $€ \cdot kg^{-1}$), and the energy cost (0.002 $€ \cdot kg^{-1}$), the economic cost of the hyperbaric storage during 15 days is finally **0.31 $€ \cdot kg^{-1}$ of strawberry juice**.

6.2.4.3. Effect of pressure level on the cost of hyperbaric storage

A sensitivity analysis was conducted to estimate the impact of the pressure level on the total cost of storage. To perform this analysis, only the amortization and maintenance

cost of the hyperbaric equipment were taken into account since energy cost contribution is negligible (less than 1 % of the total cost). Figure 6.2.4 illustrates the importance of the storage pressure on the storage cost. By doubling the pressure, the storage cost is increased by more than twice.

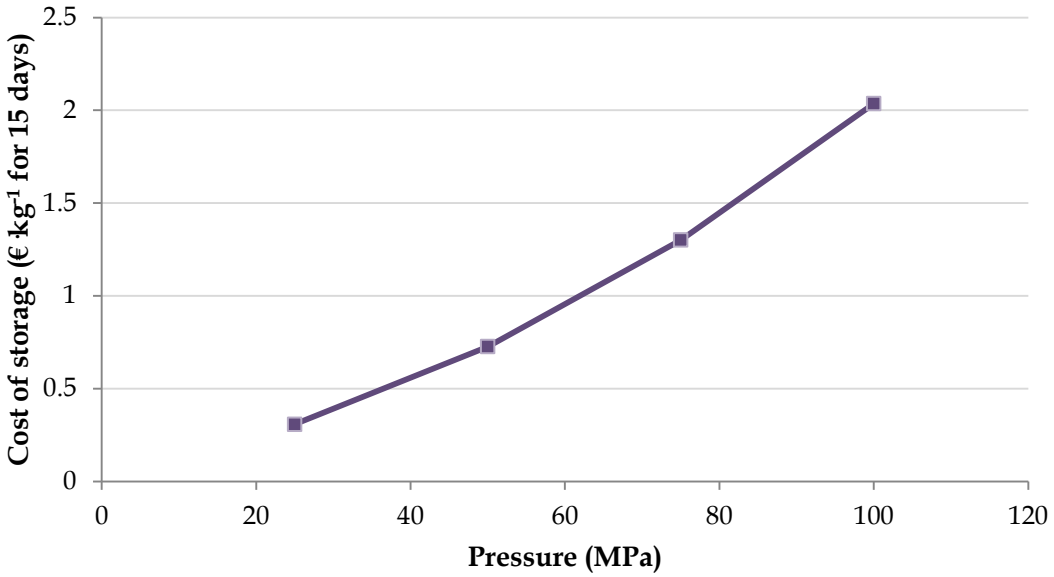

Figure 6.2.4. *Sensitivity of hyperbaric storage cost to the storage pressure.*

6.2.5. Discussion

Table 6.2.3 summarizes the cost of refrigeration and hyperbaric storage. The total cost of refrigerated storage for 800 kg of strawberry juice during 15 days was **0.09 €·kg^{-1} of juice**, whereas it was of **0.31 €·kg^{-1} of juice** for 15 days in the case of hyperbaric storage. Therefore, hyperbaric storage cost is about 3-fold higher than refrigeration cost. Although the study has been performed under a series of assumptions with the aim of facilitating the estimation, these results are valuable as a first approach of the hyperbaric storage cost.

	Refrigeration	**Hyperbaric storage**
Amortization (€·kg^{-1})	0.038	0.205
Maintenance (€·kg^{-1})	0.019	0.103
Electricity consumption (€·kg^{-1})	0.034	0.002
Total (€·kg^{-1})	0.091	0.310

Table 6.2.3. *Storage costs of 1 kg of strawberry juice for 15 days.*

Results show that, although the energy cost of hyperbaric storage is almost negligible, the initial cost of the equipment is so high that the cost of the storage results higher than refrigeration. The cost of hyperbaric storage highly depends on the initial investment and, therefore, its value is completely influenced by the estimation of the equipment price. In order to prove if the equipment price here estimated is within the order of the prices of other high pressure equipment commercially available, attempts to compile information about the market prices were made. However, detailed information about the prices is hardly available due to confidentiality concerns of the companies.

High-pressure equipment is used in different industrial areas. In the food industry, high-pressure equipment employed for the non-thermal pasteurization of food are huger and heavier because operating pressures are higher (~600 MPa) than those required for hyperbaric storage equipment. The most popular manufacturers of high pressure processing (HPP) equipment for the food industry are Hiperbaric and Avure. Information about the prices of their machines is not available on Internet. According to Balasubramaniam, Farkas, and Turek (2008), a commercial scale high-pressure processing system could cost from 371,000 € to 1,854,000 €, depending on equipment capacity and extent of automation. Unipress, another manufacturer of high-pressure equipment, gave us an indicative budget of 700,000 € for an equipment of 50 L and an operating pressure of 600 MPa. This same manufacturer sold us for 25,000 € a high-pressure equipment with 1 L of capacity and maximal allowable pressure of 220 MPa for hyperbaric storage. It is important to point out that it is a laboratory equipment and a prototype specially designed for us, so the price is significantly higher than under commercial conditions. Other similar designs of high-pressure vessels are those used by gases suppliers. These cylindrical vessels used for gas storage (e.g. propane) differ greatly from the high-pressure vessels used for non-thermal pasteurization. The supported pressures are markedly lower (0.1-20 MPa) but, at the same time, closer to those suitable for hyperbaric storage. Prices are extremely variable: from 1,000 to 10,000 € for volumes from 50 to 3,000 L and pressures up to 30 MPa.

After collecting these data, attempts were made to correlate the price of the equipment with the capacity and the operating pressure in order to compare such estimation to our case of study. The collected data were very disparate and it was difficult to establish a correlation (data not shown). The homoscedasticity hypothesis was not respected and, as a result, the obtained model lacks of physical meaning so the result was invalid.

The high price of high-pressure equipment has been already pointed out by other authors as the main limitation of their commercial application (Balda, Aparicio, & Samson, 2012; Bermúdez-Aguirre & Barbosa-Cánovas, 2011; Hogan, Kelly, & Sun, 2005; Mújica-Paz, Valdez-Fragoso, Samson, Welti-Chanes, & Torres, 2011). However, this barrier has not stopped its implantation in the food industry (Balda et al., 2012). Besides, reductions in equipment cost are expected if the demand follows on its climbing tendency. This should stimulate further the expansion of high-pressure processing in the industry and boost future innovations in this field. In fact, innovations performed during the last years in equipment design have made possible a decreasing trend in equipment costs from 1996 to now. The identification of new high-pressure applications - such as hyperbaric storage - will also contribute to this aim (Balasubramaniam et al., 2008; Hogan et al., 2005).

In contrast to the case of hyperbaric storage, electrical consumption has a special relevancy in total cost of refrigeration. Besides, if pasteurization treatment had been included in the calculations, the electrical consumption would have been higher, and consequently the cost would have been even higher too. Figure 6.2.5 shows that the electricity price more than doubled in ten years and, based on this tendency, it is expected that the price will continue to rise. The energy dependence of refrigeration makes that its cost is linked to the electricity price variations. In comparison, hyperbaric storage is almost energetically independent, which is a certain advantage. Consequently, it is possible to predict that the cost of refrigeration could increase in the following years, contrasting with the decreasing cost of hyperbaric storage.

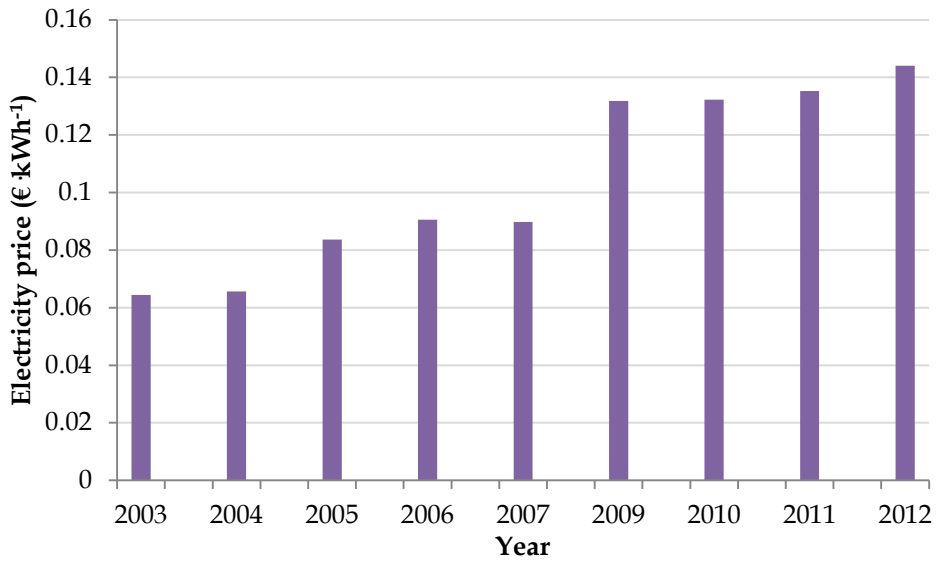

***Figure 6.2.5**. Evolution of the electricity prices for industrial consumers in Spain (taxies and levies are included in the price). Data from the database of EUROSTAT.*

Another aspect of interest is that the useful life of the high pressure vessel depends on the number of pressurization-depressurization cycles that it has to support (Alegre, Bravo, & Cuesta, 2010). To have an idea of magnitude, Sampedro et al. (2014) considered an useful life of the high-pressure equipment of 10 years with a number of 12 cycles per hour (66,000 cycles·year^{-1}). In the hyperbaric storage, the number of fatigue cycles is considerably lower; concretely, in this study, 1 cycle each 15 days corresponds to 24 cycles·year^{-1}, the number of cycles per year is divided by almost 3000! Hence, the useful life should be higher than 10 years and, at the same time, the maintenance cost should be also significantly lower. Consequently, the cost of hyperbaric storage calculated in this study is likely overestimated.

The cleanliness issue of containers and vessels has not been taken into account in the cost evaluation. Its relevancy is shortly discussed here. In the case of hyperbaric storage, if the juice is stored directly in the high-pressure vessels, the cleaning may be more difficult and expensive than the cleaning of the containers employed in refrigeration. If the food product is stored in individual bottles or packages, pressurization is performed indirectly and cleaning would be easier. In return, a lesser product quantity can be stored in the same vessel for geometrical reasons (vessel filling ratio). Different industrial strategies could be developed in order to deal with this aspect. One strategy, hybrid between direct and indirect pressurization cases, would be

the use of a unique resistant flexible envelop fitting with the inner dimensions of the vessel. This packaging would be filled with the juice and closed for indirect pressurization. This would maximize the filling vessel ratio while simplifying the cleaning operation, thus saving costs. Even, the utilization of single-use plastic films might be economically and hygienically more interesting. Thus, the hyperbaric storage method has to be developed more thoroughly before being able to calculate properly the cleaning cost but this is indubitably a relevant question.

As illustrated in Figure 6.2.4, the total cost of hyperbaric storage is highly dependent on the storage pressure. Such a huge dependency is the consequence of the equipment cost. Since the estimation is a direct function of the equipment mass, a higher pressure means more mass and, as a result, the vessel is more expensive. This approximation may not be in exact agreement with reality because pressure and equipment price are not necessarily linearly correlated (as it has been considered here). Furthermore, the equipment cost could be lower if the ordered number of units is higher (better price for raw matter) and depending on the pump provider. For instance, the pump provider Hawk International proposes a high-pressure pump working up to 25 MPa at a flow rate of 26 $L \cdot min^{-1}$ for 770 €, that is, five times lower than considered in our calculations budgeted by 4,050 €. Therefore, the storage cost shown in Figure 6.2.4, already overestimated as explained above, should also increase at a lower rate with the operating pressure.

6.2.6. Conclusion

The cost of hyperbaric storage has been estimated in comparison to refrigeration, a well-established method, at semi-industrial scale. In spite of the lack of information and the possible inaccuracies that it could originate, the economic viability of hyperbaric storage could be discussed. This founds the basis for subsequent studies.

The results of this first cost analysis reveal that the cost (€·kg^{-1} of juice) of hyperbaric storage at 25 MPa and room temperature for 15 days is around 3-fold higher than refrigeration cost (4 °C/15 days). The higher cost of hyperbaric storage is a consequence

of the initial investment since the energy consumption is practically negligible. Oppositely, the electricity consumption plays a major role in the refrigeration cost. Therefore, these differences between hyperbaric and refrigerated storage could be attenuated as far as the price of hyperbaric storage vessels diminishes or the electricity price increases.

Although the limitations of this study are clear (the price of the hyperbaric equipment remains largely unknown), this study provides preliminary information about the cost of hyperbaric storage at room temperature and its difference with respect to refrigeration. Moreover, it reveals that hyperbaric storage is an attractive alternative to refrigeration, especially, when the access to electricity supply is complicated. For instance, during long transports by ship that can take up to two weeks, hyperbaric storage offers opportunities for innovation.

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Part 6.3: Evaluation of the environmental impact of hyperbaric storage in comparison with refrigeration

EVALUATION OF THE ENVIRONMENTAL IMPACT OF HYPERBARIC STORAGE IN COMPARISON WITH REFRIGERATION

6.3.1. Abstract

Nowadays, there is a growing concern about the environment and, therefore, global warming and climate change are an increasing focus of attention. In this respect, hyperbaric storage at room temperature is presented as a clean and environmentally friendly technology. The main advantage of this novel method is that no cold is required, which contributes to diminish greenhouse gas emissions. However, this theoretical benefit has not been formally demonstrated neither quantified yet. Therefore, the aim of this study is to evaluate the environmental impact of hyperbaric storage at room temperature and to compare it with that of refrigeration. For this purpose, the carbon footprint of each storage method was calculated for a quantity of four batches of 200 kg of strawberry juice, stored during 15 days (semi-industrial scale). The results showed that the equivalent mass of CO_2 emitted during storage by refrigeration is divided by a factor 26 when this same storage takes place at 25 MPa and room temperature. Therefore, under the conditions assumed, our findings support the generally accepted idea that pressure is an environmentally friendly technology as compared with conventional technologies.

6.3.2. List of abbreviations

- AE: assembling emission.
- CF: carbon footprint.
- CFCs: chlorofluorocarbons.
- DE: disposal emission.
- EF: emission factor.
- EPLCA: European Platform on Life Cycle Assessment.
- EPA: Environmental Protection Agency of the United States.

- FU: functional unit.
- GHGs: greenhouse gases.
- GWP: global warming potential.
- HCFCs: hydrochlorofluorocarbons.
- HFCs: hydrofluorocarbons.
- IPCC: Intergovernmental Panel on Climate Change.
- LCA: life cycle assessment.
- OE: operating emission.
- UNESA: Unidad Eléctrica Sociedad Anónima.

6.3.3. Introduction

Nowadays, there is a growing concern about global warming and climate change issues. Climate change is caused by the high concentration of greenhouse gases (GHGs) in the atmosphere which affect the absorption, scattering, and emission of radiation within the atmosphere and at the Earth's surface (see the report of the Intergovernmental Panel on Climate Change (IPCC, 2007)). Among all GHGs, carbon dioxide (CO_2) is the most important anthropogenic GHG. According to the Environmental Protection Agency of the United States (EPA, 2014), 82 % of the total GHGs were CO_2 emissions.

It is estimated that the contribution of the food and drink sectors to the environmental impact is about 20-30 % in the EU (Kolokotroni, Tassou, & Gowreesunker, 2014; Pardo & Zufía, 2012). Within the food industry, it is believed that the cold chain is responsible for approximately 2.5 % of global GHG emissions through direct and indirect (energy consumption) effects (Evans et al., 2014).

Within the direct emissions, refrigerants are the most significant source. According to Debotta et al. (2005), the annualized refrigerant emission rate from the refrigeration sector was 23 % in 2002. For commercial refrigeration, the 60 % of the total emission of GHGs come from system operation, and the rest are due to indirect emissions from power consumption. Besides, the chlorofluorocarbons (CFCs) and

hydrochlorofluorocarbon (HCFCs) refrigerants do not only cause GHGs emission, but also split and release ozone destructive chlorine atoms, leading to an increase in harmful ultraviolet radiation reaching the ground (Wu, Hu, & Mo, 2013). Hence, these refrigerants are being phased out under the Montreal Protocol and are being replaced by hydrofluorocarbons (HFCs). Moreover, the contribution of the electric power necessities is not negligible. Cold storage rooms consume considerable amounts of energy, within cold storage facilities 60-70 % of the electrical energy can be used for refrigeration (Evans et al., 2014).

For all these reasons, there is a strong motivation to reduce refrigerant emissions, in addition to diminish energy consumption of cold storage installations. The European Technology Platform "Food for Life" defines sustainable food production as the most important challenge that will be faced by the European food industry (Pardo & Zufía, 2012). Innovation based on "environmental quality" is starting to be seen as an opportunity for market satisfaction or competitive advantage (Kumar, Lee, & Malhotra, 2001).

It is important to consider new technologies, changes within existing technologies, or alternative processes that could contribute to a substantial reduction of the emissions (James & James, 2010). The current trends in commercial refrigeration aim at reducing the synthetic refrigerant charge, either by minimizing the internal volume of the circuit or by utilizing natural refrigerants with lower global warm potential (GWP) (Cecchinato, Corradi, & Minetto, 2012). Other manner to reduce emissions could be the use of higher temperatures in the supply chain that would reduce energy consumption, resulting in environmental and economic benefits. In this way, Wills, Harris, Spohr, and Golding (2014) propose to minimize or eliminate refrigeration during the transport of bananas - such as it is currently practiced - by means of a suitable control of ethylene level.

In this regard, the development of a novel technology that does not need refrigeration facilities, such as **hyperbaric storage at room temperature**, could represent an important breakthrough in food storage in terms of energy saving, refrigerant elimination, and environmental protection. After studying the feasibility of hyperbaric

storage in terms of installation size and logistics management, and after performing the cost assessment, the next step is to evaluate the environmental impact. As it has been pointed out by Hospido, Davis, Berlin, and Sonesson (2010), the environmental assessment of novel products and processes is important for food producers since, lately, many of them have introduced sustainability as a core company goal.

One of the methodologies most widespread and powerful to investigate the environmental performance of a product or service is life cycle assessment (LCA) (Bala, Raugei, Benveniste, Gazulla, & Fullana-i-Palmer, 2010; Pardo & Zufía, 2012). This method measures the potential environmental impacts of a product, process, or service all along its life cycle, from "the cradle to the grave". In addition to GHGs, the LCA takes into account all other material and energy inputs and environmental releases, and it assesses their potential impact on the environment. The spectrum of impact categories is broad and includes: human health, ecosystem degradation, climate change, and natural resource depletion. LCA is therefore a "multicriteria" analysis that assesses multiple impacts (Empreinte Carbone Quebec, n.d).

However, LCA is complex due to the required exhaustive inventory and the large scope of impact assessment. Moreover, the access to Ecoinvent database and to specific LCA software (e.g. SIMAPRO) is necessary.

Carbon footprint (CF) analysis can be considered as a subset of LCA. It is limited to emissions that have an effect on climate change. CF is the overall amount of carbon dioxide (CO_2) and other GHGs emissions associated with a product (EPLCA, 2009). Therefore, CF is essentially a "monocriteria" analysis. Nonetheless, it allows to address the impact of a process on the environment in a relatively simple manner before performing a more complex analysis.

In the light of the importance of the environmental evaluation of food processes, the aim of this study is to estimate the CF of hyperbaric storage at room temperature and to compare it with that of refrigeration. For these estimations, it has been considered, as for cost assessment in Part 6.2, that four containers of 200 kg of strawberry juice are stored for 15 days.

6.3.4. Materials and methods

6.3.4.1. Simplification

In order to facilitate the estimation of the CF, some simplifications were made. The CO_2 emissions from the equipment were estimated from the mass of the equipment material and from the emission factor of the material. Neither energy consumption for production nor energy for recycling were taken into account. This is because, on the one hand, high pressure is an emerging technology and data are hardly available and, on the other hand, it was not possible to access to the data needed to perform the corresponding calculation (Ecoinvent database).

6.3.4.2. Functional unit

The functional unit (FU) provides a reference to which the data can be normalized. This makes possible the comparison between both storage methods. In this study, the FU was defined as the storage of 1 kg of strawberry juice during 15 days.

6.3.4.3. System boundaries, hypotheses, and input data

The scenarios of the storage procedures assumed in order to set the systems boundaries are the same as those assumed to perform the economic assessment (Part 6.2). Briefly, strawberry juice is packed in four containers of 0.2 m^3 (800 kg of juice in total) which are either placed at 4 °C in a cold chamber (refrigeration) or pressurized to 25 MPa, and stored at room temperature (hyperbaric storage).

6.3.4.3.1. Inventory of CO_2 emission sources for refrigeration

CF boundaries and CO_2 emissions associated to refrigeration are represented in Figure 6.3.1. The boundaries of the study only contemplate the storage stage. As in Part 6.2, all steps prior and posterior to the storage stage are not retained in this analysis. The system includes: (i) direct emissions associated with the container material, cold chamber material, and refrigerant leakage; (ii) indirect emissions derived from the energy consumption during operation.

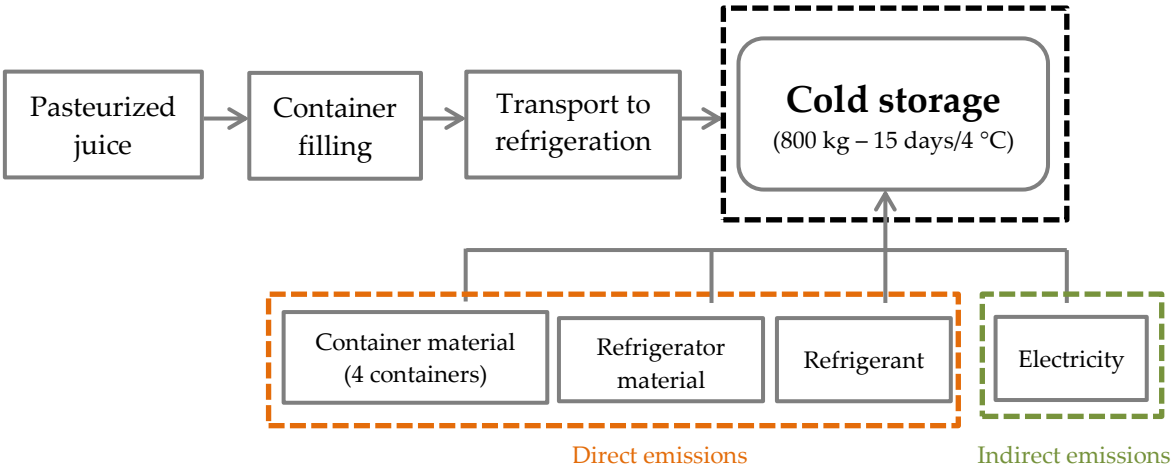

Figure 6.3.1. *System boundaries and emissions associated to refrigeration.*

The characteristics of the cold chamber (VIZUETE chamber, model CV9B3) where the juice is stored were obtained from the manufacturer. The roof, walls, and door of the chamber are made of polyurethane while the ground is made of two layers: aluminum and cement. The four juice containers are cylindrical and made of stainless steel with a wall thickness of 1 mm. The corresponding parameters relevant to the CF calculation are given in Table 6.3.1.

Material	Volume (m^3)	Density ($kg \cdot m^3$)	Mass (kg)
Polyurethane	1.24	30	37.30
Aluminum	0.13	2,699	341.26
Cement	0.15	2,200	333.80
Stainless steel	0.01	7,800	241.59

Table 6.3.1. *Input parameters corresponding to materials for refrigeration CF calculations.*

The refrigerant type of the cold chamber is R-404a and the charge of refrigerant is 0.8 kg. It is considered that at the end of the useful life of the refrigerator, the recycling rate of the refrigerant is 0.9 (Wu et al., 2013).

As it was calculated in Part 6.2, the electric energy expended during refrigerated storage of strawberry juice for 15 days is 0.24 $kWh \cdot kg^{-1}$ juice.

6.3.4.3.2. Inventory of CO_2 emission sources for hyperbaric storage

Figure 6.3.2 illustrates the CF boundaries and CO_2 emission sources associated to hyperbaric storage. As for refrigeration, only the storage stage is contemplated. The

system includes: (i) direct emissions associated with the vessel material and (ii) indirect emissions derived from the energy consumption during operation.

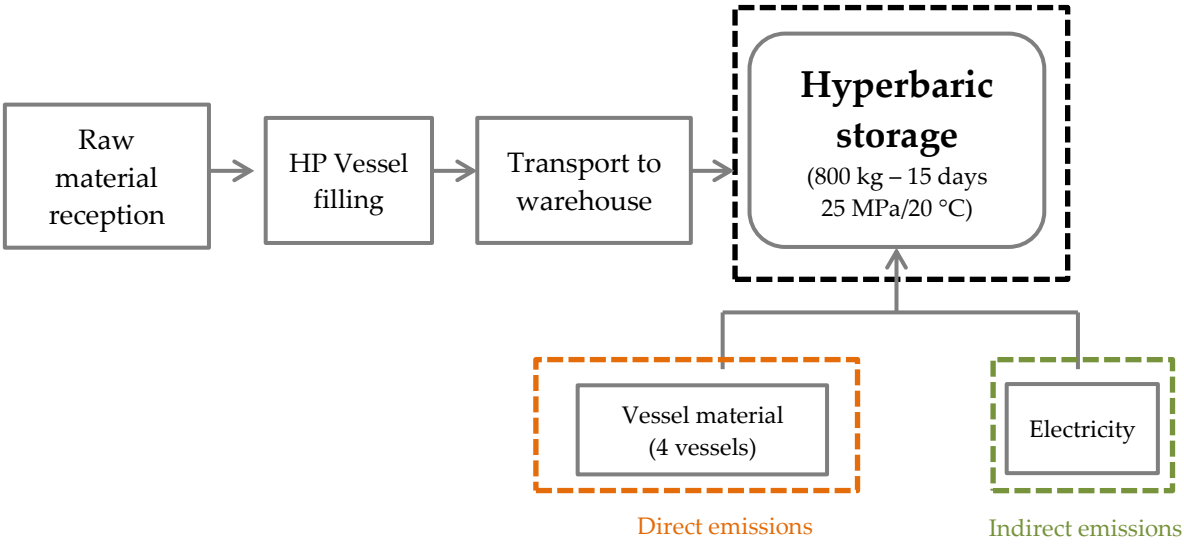

***Figure 6.3.2**. System boundaries and emissions associated to hyperbaric storage.*

The four vessels employed for storage (4x200 kg of juice) are made of 15-5PH stainless steel. As calculate in Part 6.1, each empty vessel weighs 189.8 kg (cylindrical shell with two hemispherical heads able to operate at 25 MPa). According to the estimation made in Part 6.2, the electric energy required for pressurization was $1.4 \cdot 10^{-4}$ kWh·kg^{-1} juice.

6.3.4.4. Methodology of carbon footprint

The CF of each storage method is simply calculated by adding the CFs of the inventoried sources of CO_2 emissions in each case. The calculated CF includes direct emissions and indirect emissions. Emissions of CO_2 are determined from the emission factors (EF) showed in Table 6.3.2. They are calculated as detailed below for each type of emission source: materials used in the electricity, equipment manufacturing, and refrigerant.

	EF	Unit	Reference
Electricity	0.231	kg eq. $CO_2 \cdot kWh^{-1}$	UNESA (2010)
Stainless steel	1.06	kg eq. $CO_2 \cdot kg^{-1}$	IPCC (2006)
Aluminum	1.7	kg eq. $CO_2 \cdot kg^{-1}$	IPCC (2006)
Cement	0.52	kg eq. $CO_2 \cdot kg^{-1}$	IPCC (2006)
Polyurethane	3.4	kg eq. $CO_2 \cdot kg^{-1}$	Boustead (2005)
R-404a	3,800	kg eq. $CO_2 \cdot kg^{-1}$	Devotta (2005)
Assembly	1.75	% of initial charge$\cdot yr^{-1}$	GHG Protocol (2005)
Annual leakage rate	20	% of initial charge$\cdot yr^{-1}$	GHG Protocol (2005)
Recycling efficiency	85 (of remainder)	% of initial charge$\cdot yr^{-1}$	GHG Protocol (2005)

Table 6.3.2. *Emission factors (EF) used in the carbon footprint determinations.*

In the case of the **equipment** CF, the environmental impact has to be distributed all over their useful life, hence, the following equation is employed for its determination:

$$CF_{equipment} = \frac{\sum (EF_m \times W_m) \times t}{l_u \times f \times q} \quad (6.3.1)$$

where $CF_{equipment}$ is the CF of the equipment (kg eq. $CO_2 \cdot kg^{-1}$), EF_m is the emission factor of each material m used in the manufacture of the equipment (kg eq. $CO_2 \cdot kg^{-1}$), W_m is the mass of the material m (kg), t is the time of storage (days), l_u is the useful life (years), f is the operating days (days$\cdot$year^{-1}), and q is the quantity of juice (kg).

As in the cost assessment (Part 6.2), it is assumed that the food industry works 365 days a year and the useful life of the equipment is 10 years (Pardo & Zufía, 2012; Sampedro, McAloon, Yee, Fan, & Geveke, 2014).

Refrigerant emissions from refrigerant storage result from the manufacturing, servicing, and disposal operation. A screening method proposed by GHG protocol (2005) has been employed to determine these emissions. According to this method, the refrigerant CF (kg eq. $CO_2 \cdot kg^{-1}$) is:

$$CF_{refrigerant} = AE + OE + DE \quad (6.3.2)$$

where *AE*, *OE*, and *DE* correspond to emissions (kg eq. $CO_2 \cdot kg^{-1}$) during assembling, operating, and disposal, respectively. These emissions are in turn calculated as follows:

$$AE = \frac{C \times AEF \times EF_r \times t}{f \times q} \tag{6.3.3}$$

$$OE = \frac{C \times ALR \times EF_r \times t}{f \times q} \tag{6.3.4}$$

$$DE = \frac{C \times (1 - R) \times RE \times EF_r \times t}{f \times q} \tag{6.3.5}$$

where *C* is the refrigerant charge (kg), *AEF* is the assembling emission factor (% of initial charge·$year^{-1}$), EF_r is the emission factor of the R-404a (kg eq. $CO_2 \cdot kg^{-1}$), *ALR* is the emission factor of the annual leakage rate (% of initial charge·$year^{-1}$), *R* is the refrigerant recycling rate, *RE* is the emission factor of the recycling efficiency (% of initial charge·$year^{-1}$), *t* is the time of storage (days), *f* is the number of operating days (days·$year^{-1}$), and *q* is the quantity of juice (kg).

Indirect emissions due to the **electricity** consumption have been estimated by:

$$CF_{electricity} = E \times EF_e \tag{6}$$

where *E* is the electricity consumed during the storage period ($kWh \cdot kg^{-1}$) and EF_e is the emissions factor of the electricity production (kg eq. $CO_2 \cdot kWh^{-1}$).

6.3.5. Results and discussion

Table 6.3.3 shows the contribution of each type of emission and the total CF associated with refrigeration and hyperbaric storage of 1 kg of strawberry juice during 15 days under the assumptions showed in Section 6.3.4.3. Results show that the CF of hyperbaric storage is 0.0042 kg $CO_2 \cdot kg^{-1}$ juice whereas it is 0.1085 kg $CO_2 \cdot kg^{-1}$ juice for refrigeration. This means that the CF of hyperbaric storage is about 26 times lower than that of refrigerated storage.

Although we are conscious of the limitations of this study, in the light of this finding, it is likely that hyperbaric storage at room temperature can offer real environmental advantages compared with refrigeration. This would even be more apparent if we had included the pasteurization treatment in the system boundaries of refrigerated storage since it would have also contributed to CO_2 emissions.

Inventory	Refrigeration	Hyperbaric storage
Direct emissions (kg $CO_2 \cdot kg^{-1}$ juice)		
cold chamber materials	0.0045	
container material	0.0013	0.0041
refrigerant leakage	0.0472	
Indirect emissions (kg $CO_2 \cdot kg^{-1}$ juice)		
energy consumption	0.0554	$3 \cdot 10^{-5}$
CF of 1 kg of strawberry juice for 15 days	**0.1085**	**0.0042**

Table 6.3.3*. Results of the calculation of CO_2 emissions for each storage method.*

Figure 6.3.3 represents the contribution of each source of CO_2 emissions to the CF. In the case of refrigeration, the two main sources of emissions are the electricity consumed and the refrigerant leakage, representing almost 95 % of the CO_2 total emissions. Other authors have reported the significant role of the energy consumption of cold storage in CO_2 emissions, as well as, the significant emissions due to refrigerants (Wu et al., 2013). For instance, Amienyo, Gujba, Stichnothe, and Azapagic (2013) found that refrigerated storage adds 33 % to the total GWP of drinks in the case of cans, and that 75 % of the total GWP from refrigeration is attributable to electricity and 25 % to refrigerant leakage. In their study, although the refrigerant employed is the same than ours (R-404a), they did not take into account the refrigerant emissions due to assembling and disposal, which can explain the difference from our results (25 % *versus* 44 %).

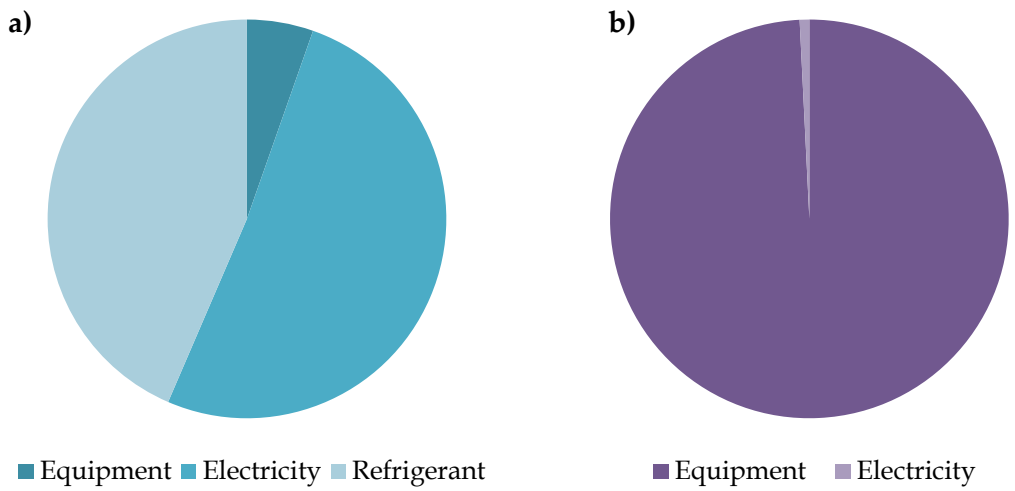

Figure 6.3.3. *Contribution of different sources to CO_2 emissions in the case of a) refrigerated storage and b) hyperbaric storage.*

The refrigerant R-404a used by the refrigerating system of this study presents one of the highest EF (3,800 kg $CO_2 \cdot kg^{-1}$). Its contribution to the CF could be lower if the refrigerant had a lower EF. For instance, the EF of R-410a (1,725 kg $CO_2 \cdot kg^{-1}$) is less than half of the EF value of R-404a (Devotta, 2005).

In contrast, in the case of hyperbaric storage, the emissions associated with vessel material correspond to almost 100 % of the emissions, being the emissions derived from electricity consumption practically negligible (Figure 6.3.3). The information about the environmental impact of high-pressure technology in the literature is scarce due to its recent appearance in the food industry. It is only possible to refer to the environmental study performed by Pardo and Zufía (2012). Even though it is related to high-pressure processing and not to hyperbaric storage, these authors already pointed out the relevance of the manufacturing stage for this technology. This is in agreement with our results.

6.3.6. Conclusion

The CF estimated for hyperbaric storage is considerably lower than for refrigeration. This reinforces the generally accepted idea that pressure is an environmentally friendly technology. This also confirms the hypothesis generally formulated about hyperbaric storage that its major advantage over refrigeration resides in its much lower

environmental impact. This result is mainly a consequence of the low energy requirements of hyperbaric storage at room temperature since electricity is only consumed during compression and no additional energy is required for pressure holding nor temperature control.

As far as we know, this is the first study which compares the environmental impact of refrigerated storage with that of hyperbaric storage. Although a more detailed environmental assessment should be convenient, the information contained in this study is valuable to have a first overview of the environmental advantages that hyperbaric storage can offer. The results obtained should serve as a starting point for future more complete and accurate evaluations.

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Chapter 7: General discussion

GENERAL DISCUSSION

7.1. General discussion

In the present Thesis, hyperbaric storage at room temperature has been characterized as a novel storage method for food preservation. Strawberry juice was chosen as object of study and the characterization was addressed from the point of view of the Total Quality concept, trying to cover as many aspects as possible from safety and quality to equipment design, economic, and environmental issues.

Hyperbaric storage at 25-220 MPa and room temperature has been found to be an efficient method to inhibit microbial growth and to preserve sensory quality of raw strawberry juice for, at least, 15 days. Although refrigeration was significantly more effective in delaying viscosity and color decay, microbial growth could be detected during cold storage and, therefore, a previous pasteurization step is indispensable to ensure the microbiological safety of the juice.

It is well known that the effect of any stress on **microorganisms** largely depends on the characteristics of both the treatment medium (pH, water activity, or composition, for example) and the microbial population (species of microorganisms present in the product, strain, amount, physiological state, among others). Thus, when assessing the ability of hyperbaric storage to inhibit microbial growth, it is important to consider not only the storage conditions (pressure level and storage time), but also the physicochemical and microbiological characteristics of the product. For example, in Chapter 5 (Part 5.1) of this Thesis, we found that hyperbaric storage at 25 MPa for 1 day inhibited microbial growth in strawberry juice. In contrast, Santos et al. (2014) and Queirós et al. (2014) reported no inhibitory effect of hyperbaric storage at 25 MPa for 8 h on microbial growth in melon and watermelon juices. As commented in Chapter 5 (Part 5.1), these differences can be partially due to the different pH of the juices (3.3-3.7 in strawberry juice vs. 5.7 and 5.9 in melon and watermelon juices, respectively) because it is recognized that the sensitivity of microorganisms to pressure increases at low pH. Obviously, the physiological state of microorganisms must also have a

relevant influence on the effects of hyperbaric storage. Thus, replicated experiments reported in Chapter 4 (Part 4.1) of this Thesis showed that hyperbaric storage at 25 MPa for 15 days reduced initial TAM and YM counts of frozen-thawed strawberry juices by more than 2 $\log_{10}$ units to levels under the detection limits. However, the results described in Chapter 5 (Part 5.1) revealed that, in fresh juices (not previously frozen and thawed), TAM and YM counts could increase, keep constant, or decrease during storage at 25 MPa for 15 days, depending on the juice batch employed.

Different authors have proved that a freeze-thaw treatment can reduce the microbial load in fruit juices, especially in those of low pH because acidic media sensitize cells to the freeze-thaw stress (Jeremiah, 1996; Uljas & Ingham, 1999; Yamamoto & Harris, 2001). Therefore, large microbial inactivation observed in Chapter 4 in strawberry juices stored for 15 days at pressures as low as 25 MPa could be influenced by the cell stress produced by the previous freeze-thaw treatment.

To assess the effect of freezing on the microbial growth after thawing, some preliminary experiments (not previously described) were carried out. Thus, samples of frozen-thawed strawberry juice were stored at atmospheric pressure and 20 °C for different periods of time (1-13 days). After storage, TAM, LAB, and YM were quantified.

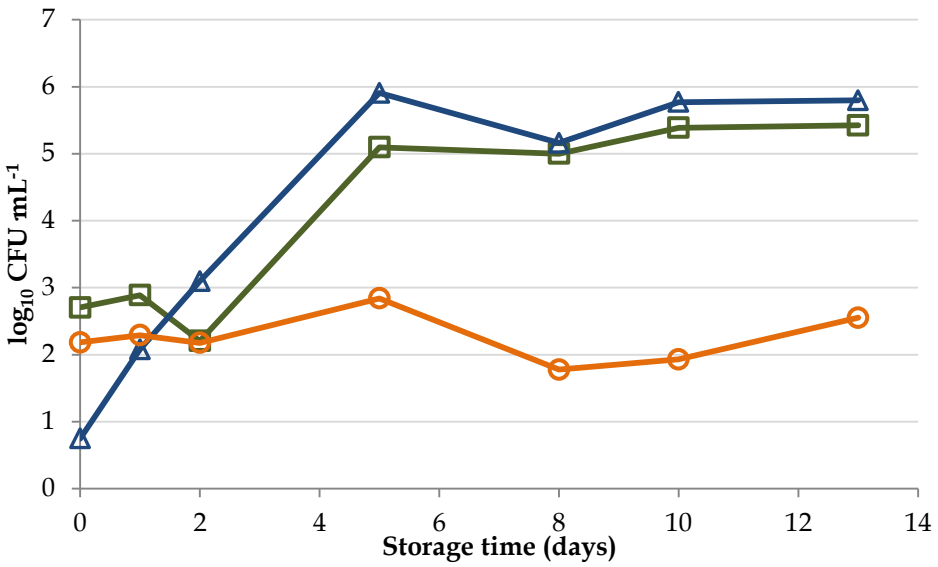

Figure 7.1. *Total aerobic mesophiles (), lactic acid bacteria (), and yeasts and molds () counts in frozen-thawed strawberry juice after storage at atmospheric pressure and 20 °C for different storage times.*

Figure 7.1 reveals that TAM presented a lag phase of about 2 days before exponential growth occurred. In contrast, LAB did not exhibit any growth retardation and cell proliferation was detected from the first day of storage. YM showed the most evident effect since the initial load remained stable through all the storage period. These results corroborate previous findings in the literature that prove that freeze-thaw treatments in fruit juices especially affect YM counts (Duan & Zhao, 2009; Jeremiah, 1996; Sodeko, Izuagbe, & Ukhun, 1987).

These microbial growth data in frozen-thawed juices strongly contrast with those corresponding to freshly-squeezed strawberry juice. Thus, no lag phases were detected in non-frozen juices and, after 1 day of storage at atmospheric pressure and 20 °C, TAM, LAB, and YM counts increased by more than 1 $\log_{10}$ unit. It is evident from the above that freezing and thawing the strawberry juice prior to storage induces significant stress in microorganisms that is clearly reflected in their growth pattern. If immediately after a freeze-thaw treatment, microorganisms are subjected to a new stress, for example, hyperbaric storage, it is evident that their resistance will be strongly weakened. Therefore, to avoid added stresses to hyperbaric storage, non-frozen strawberry juices were employed in Chapter 5 to evaluate the effect of pressure

level and storage time on the microbial load during storage at room temperature. However, this methodology implied using different batches of juice in replicated experiments. This introduced considerable variability in the study because, as previously commented, many different factors influence microbial responses to stresses (pH and composition of the juice, species of microorganisms present, amount, for example). In spite of this inconvenience, the results obtained in Chapter 5 were consistent and prove that hyperbaric storage at room temperature not only inhibits microbial growth but it is also able to produce some inactivation, depending on the pressure level and the storage time applied. Thus, the greater the pressure and the longer the storage time, the greater the microbial damage produced. Therefore, pasteurization prior to hyperbaric storage is not necessarily required if pressure level-storage time combination is correctly chosen. However, it is important to note that, when optimizing these two parameters, not only microbial safety but also other quality characteristics must be considered. Moreover, the end use of the strawberry juice can be also decisive in determining storage conditions.

After pressure release, the recovery of the surviving microorganisms in the juice also depends on the pressure level-storage time conditions applied during hyperbaric storage. For example, we found, in Chapter 5 (Part 5.1), that microorganisms quickly recovered their cell proliferating ability after 24 h of storage at 25-50 MPa. Damage produced at higher pressures (100-200 MPa) or longer times (15 days at 50 MPa) hampered microbial recovery in strawberry juice. Therefore, after hyperbaric storage, juice should be immediately consumed or processed to guarantee its microbiological stability. Otherwise, surviving microorganisms could proliferate, after pressure release if the juice is kept at room temperature for long times, especially if it has previously been stored at relatively low pressures (25-50 MPa).

Much more research is still needed to completely characterize hyperbaric storage at room temperature from the microbiological point of view. Some issues such as the proliferation of pressure resistant populations or the development of microbial adaptations to pressure, for example, should be investigated in depth. Moreover, in this Thesis, microbial studies have been made on the natural microflora of the

strawberry juice, but the effect of hyperbaric storage at room temperature should be also tested on foodborne pathogens. Although the purpose of hyperbaric storage is not food sanitization, but only preservation, it would be interesting to know its effect on some pathogens. In the past, high-acid foods were considered of minimal concern with regard to pathogenic bacteria. However, different enterohaemorrhagic *Escherichia coli* O157:H7 and *Salmonella* outbreaks in apple and orange juices, some of them with fatal consequences, have proved that fruit juices can be a vehicle for transmitting pathogens. Thus, some *E. coli*, *Shigella*, or *Salmonella* strains can survive for several days or even weeks in acidic foods (Weagant, Bryant, & Bark, 1994; Conner & Kotrola, 1995; Leyer, Wang, & Johnson, 1995). Juice may become contaminated with pathogens by several routes, for example, by using fruit that has come into contact with soil, water, sewage, or manure that contains bacteria, viruses, and parasites capable of causing illness (Vojdani, Beuchat, & Tauxe, 2008). Target microorganisms should be selected to evaluate the effect of pressure level and time during hyperbaric storage at room temperature. Among them, *E. coli* and *Listeria monocytogenes* could be good candidates. Thus, *E. coli,* a facultative anaerobe able to grow at low temperature, is considered an indicator of contamination of fecal origin and/or inefficient cleaning practices and, as commented, *Escherichia coli* O157:H7 outbreaks are not rare in fruit juices (García-Graells, Hauben, & Michiels, 1998). On the other hand, *Listeria monocytogenes,* another facultative anaerobe able to survive in acidic media at low temperature, is one of the most dangerous foodborne pathogens, since it is responsible for the development of listeriosis, a disease with high mortality, mainly among vulnerable populations (Cox, 1989). In the next future, we expect to have some results. At present, only some very initial and unsuccessful attempts to select *E. coli* and *Listeria innocua* strains able to grow at the low pH of the strawberry juice have been performed.

Hyperbaric storage at room temperature is also an efficient method to attenuate **color** decay mainly because pressure inhibits microbial growth and, in this way, it indirectly reduces color degradation. The experiments shown in Chapter 5 (Part 5.2), carried out in juice with an antimicrobial agent added to avoid microbial interference, proved that pressure during storage also affects some other mechanisms involved in color degradation, apart from microbial spoilage. However, the effect of pressure on these

mechanisms is very slight. Thus, color differences, instrumentally measured, between juices stored at different pressure levels, although statistically significant, were very small and too subtle to be perceived by the naked eye. These results agree with those obtained in the sensory analyses. Thus, in Chapter 4 (Part 4.1), judges did not perceive any color difference in juices stored at different pressures (25-220 MPa) and room temperature for 15 days. On the other hand, it is remarkable to note that color changes during hyperbaric storage at room temperature are very slow. Thus, judges in triangle tests did not report any comment about color differences when compared control juices at day 0 with juices stored either at 25 MPa or at 50 MPa for 15 days (Chapter 4, Part 4.3). It follows from the above that color is not a critical parameter for hyperbaric storage of strawberry juice at room temperature.

In contrast, **viscosity** appeared to be the most critical parameter for hyperbaric storage of strawberry juice. The results described in Chapter 4 (Part 4.1) revealed that hyperbaric storage was effective in attenuating serum viscosity losses in samples stored at 20 °C, although cold storage was much more efficient. However, this delay in serum viscosity decay under pressure is closely linked, as occurred for color, to the inhibitory effect of pressure on microbial growth. Thus, experiments with no microbial interference in Chapter 5 (Part 5.3) demonstrated that pressure *per se* accelerates viscosity decay. So, the greater the storage pressure, the quicker the viscosity decay.

The mechanisms involved in these results are not clear. Degradation phenomena in fruit juices during storage, such as serum viscosity decay and cloud destabilization, are usually attributed to the activity of endogenous pectinolytic enzymes, mainly pectinmethylesterase (PME) and polygalacturonase (PG). In this Thesis, no pressure effect in the catalytic activity of crude strawberry PME extract was found at 0.1-200 MPa and room temperature. Moreover, storage experiments in strawberry juice confirmed that catalytic PME activity during the first two days of storage was independent of the storage pressure. However, it was exactly in this period when the greatest viscosity decay occurred, especially in the samples stored under pressure. Therefore, PME activity could not be responsible for the sharper viscosity decay observed in the samples kept under pressure. On the other hand, many authors in the

literature have shown that PG activity in strawberry is extremely low (Abeles & Takeda, 1990; Barnes & Patchett, 1976; Nogata, Ohta, & Voragen, 1993; Vicente, Costa, Martínez, Chaves, & Civello, 2005). Therefore, PG depolymerizing action neither seems to be involved in the quick viscosity decay observed. In spite of that, some attempts were made to measure PG activity in strawberry juice. Our results confirmed that PG activity in strawberry juice is too low to be measured by conventional techniques. Therefore, other mechanisms must be implicated in the great viscosity decay observed under pressure.

A hypothesis, mentioned in Chapter 5 (Part 5.3), points to a pressure-enhanced activity of some endogenous pectinases, other than PME and PG, acting not only on the linear homogalacturonan chains of pectin, but also on the rhamnogalacturonan chains (hairy region). Candidates include pectin- and pectate- lyases, and, also, debranching enzymes that catalyze changes in pectin side chains such as β-galactosidase (β-Gal) or α-arabinofuranosidase, for example. In this connection, we tested the effect of pressure on the catalytic activity of crude strawberry β-Gal extract (data not previously shown). Figure 7.2 reveals that β-Gal activity is significantly lower under pressure and, therefore, this enzyme cannot be directly implicated in the pressure-enhanced viscosity decay detected in strawberry juice.

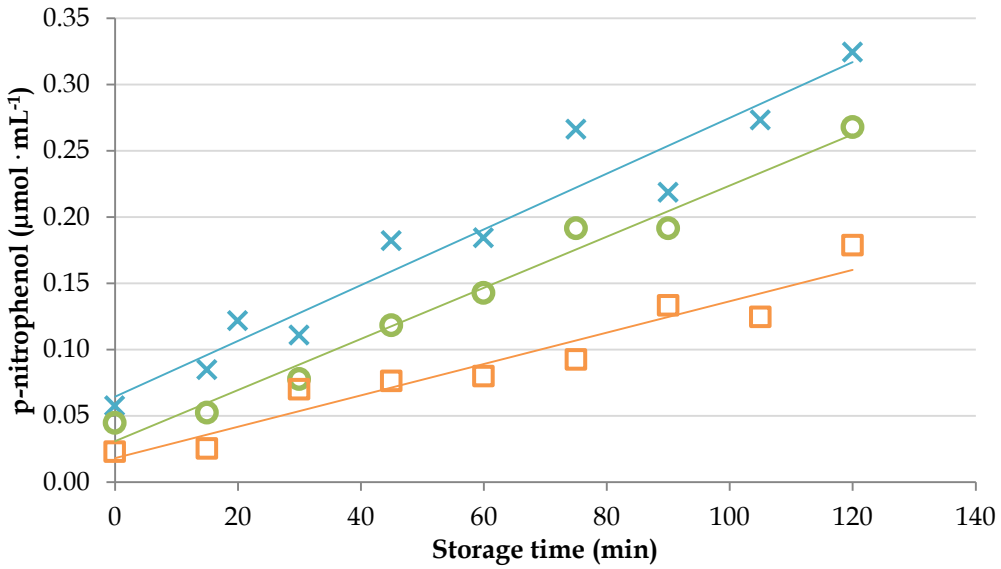

Figure 7.2. *Evolution of p-nitrophenol released by the crude strawberry β-Gal extract (µmol·mL⁻¹) during storage at different pressure levels (0.1 MPa: ×, 50 MPa: ○, and 200 MPa: □) and 37 °C.*

Given the complex pectin composition and architecture, a particularly wide range of mechanisms could be involved. Hence, it was not possible to cover all of them in the context of this Thesis. Much more research is still needed.

Although a significant viscosity decay was systematically observed after hyperbaric storage, only very slight **cloud destabilization**, hardly perceptible, was detected and only in the samples held at 200 MPa for 15 days. This pressure effect on juice stability was also revealed when performing the color studies described in Chapter 5 (Part 5.2). Thus, immediately after hyperbaric storage, no cloud destabilization was perceived in any juice (Figure 7.3.a), but when samples were frozen and thawed prior to color analysis, juices maintained at 200 MPa were completely destabilized (Figure 7.3.b). Ice crystals probably broke the weak stability of the juice cloud leading to clarification.

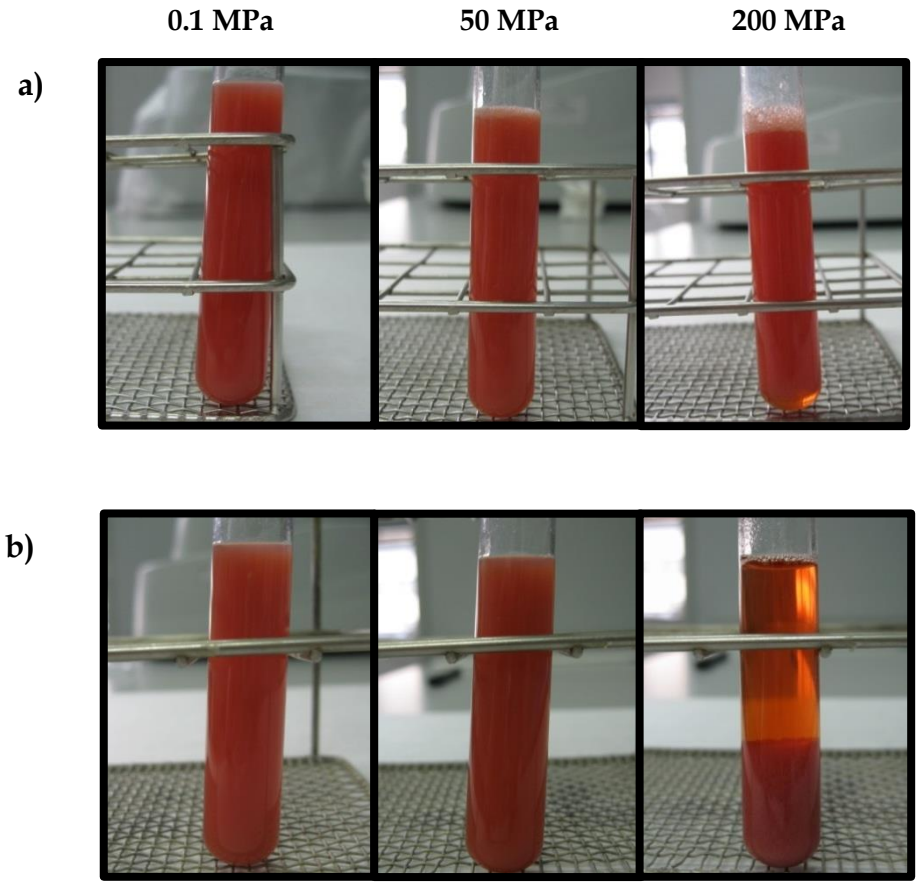

Figure 7.3. *Appearance of the strawberry juice after 15 days of storage at different pressure levels (0.1 MPa, 50 MPa, and 200 MPa) and 20 °C. (a) Immediately after storage and (b) after freezing and thawing prior to color analysis.*

Cloud destabilization is frequently associated to PME activity. Although, as above mentioned, strawberry PME was not directly affected by pressure at the conditions tested, experiments performed in Chapter 5 (Part 5.3) revealed that catalytic PME activity in samples held at 200 MPa increased considerably as storage time increased. Conformational changes, chemical, and/or enzymatic reactions affecting pectin could be enhanced by pressure and, thus, indirectly modify PME activity. Therefore, pressure levels as low as possible, but enough to ensure microbiological quality, should be employed for hyperbaric storage of strawberry juice at room temperature.

This pressure-enhanced cloud destabilization could also limit storage time. For storage times of 15 days and shorter, we never observed cloud destabilization in any hyperbaric experiment whatever the storage conditions employed. However, cloud destabilization could pose a problem for longer storage times. To assess this possibility, juices from four different strawberry batches were stored at 25 MPa and room temperature for 30 days (data not previously shown). Figure 7.4 shows the appearance of the juices at the end of storage. The juices from Batch 1, Batch 3, and Batch 4 appeared completely destabilized while the aspect of the juice from Batch 2 was good.

Figure 7.4. *Appearance of strawberry juices from four different batches after storage at 25 MPa and room temperature for 30 days.*

Differences observed between juices after storage are probably due to differences in the strawberries employed for their preparation. The processing steps to obtain the juices were identical in all cases, but strawberries from different cultivars were employed. Moreover, other characteristics of the raw material such as the growing conditions and

the maturity degree were probably not exactly the same. It is well known that all these parameters affect the strawberries composition and, therefore, the amount and characteristics of pectin and, also, the amount and relative proportion of the different pectinolytic enzymes present in the fruits can be quite different.

Figure 7.4 shows that most of the juices presented two completely separated layers, a clear layer at the top and a cloudy layer at the bottom, after 30 days of storage at 25 MPa and room temperature. Therefore, cloud destabilization clearly limits storage time when raw strawberry juices are preserved under pressure at room temperature.

Up to date, none of the few studies published about hyperbaric storage at room temperature (Fidalgo et al., 2013; Queirós et al., 2014; Santos et al., 2014) has focused on **aroma** and **flavor** attributes. However, these attributes are determinant for consumer acceptance and can jeopardize the viability of the method. Chapter 4 of this Thesis presents the first ever results about the effect of hyperbaric storage at room temperature on the volatile profile (Part 4.2) and the consumer acceptance (Parts 4.1 and 4.3) of strawberry juice.

Hyperbaric storage is more efficient than refrigeration in maintaining the volatile profile of strawberry juice unaltered for 15 days. Furthermore, in contrast to refrigeration, no changes in any key aroma compounds are detected after hyperbaric storage. However, sensory analyses are indispensable to test if the differences observed by gas chromatography-mass spectroscopy would be also detectable by human perception.

Triangle tests described in Chapter 4 (Part 4.3) showed that neither hyperbaric storage (25 and 50 MPa) at room temperature, nor conventional storage (pasteurization + refrigeration) for 15 days maintain unaltered the organoleptic characteristics of fresh strawberry juice. It was clear from the comments of the judges on the scorecards that taste and viscosity were the organoleptic characteristics more affected. Thus, some judges found the juices stored at 25 MPa for 15 days sourer than fresh juices at day 0 while the pasteurized + refrigerated juices were described as sweeter. The metabolic activity of microorganisms during storage at 25 MPa could be responsible for this

acidic taste. This hypothesis is reinforced by the fact that juices stored at 50 MPa were not described as sourer than control juices by any judge. As reported in Chapter 5 (Part 5.1), microbial counts in juices stored at 50 MPa for 15 days were considerably lower than those in juices maintained at 25 MPa. However, some judges found that the viscosity of juices stored at 50 MPa was lower than that of control juices. This agrees with the results presented in Chapter 5 (Part 5.3) that show larger viscosity decay in juices stored under higher pressure.

All these results point out that hyperbaric storage at room temperature is a promising food storage method. Furthermore, these results obtained at pilot-plant scale can be easily extrapolated to the industrial scale because pressure is applied in an instantaneous and uniform fashion, independently of juice quantity (Pascal's principle). A barrier for its industrial development may rather come from the investors' skepticism. High pressure technology usually involves expensive and heavy equipment. Therefore, there is the preconceived idea that, with hyperbaric storage, limited volumes of merchandises would be immobilized in expensive and heavy tanks. This sounds highly non-lucrative from the economic point of view and might explain why, in spite of being patented since 1997, there is still neither industrial application of this kind of storage nor much published researches in this field (Hirsch, 1997; 2000). In fact, there are currently no high-pressure vessels of large capacity specifically adapted to the pressure range of interest for hyperbaric storage available on the market. Therefore, it appeared convenient in this Thesis to address the industrial feasibility and viability issues.

Against all odds, a relatively large domain of viable designs was found covering pressures up to near 160 MPa (Part 6.1). This pressure was tentatively set as a feasibility limit for logistic management. However, other initial data and hypotheses, more sophisticated calculations, the arrival of technological progresses, and high-performance materials will certainly move this frontier. Also, several parameters (e.g. shape, capacity) can be optimized offering some flexibility for designing the equipment. In any cases, the pressure level remains the most critical parameter for the feasibility of the method so it should be as low as possible while ensuring the product

quality. This evidences the importance of determining the most suitable pressure level for each application. In this Thesis, 25 MPa was the lowest studied pressure and it was enough high to provide a safe product with an acceptable sensory quality after 15 days of storage at room temperature (with or without a previous pasteurization step). If other products are intended to be stored under pressure, the feasibility of the method should be newly examined taking into account the pressure requirements for safety and sensory quality.

Another extremely important factor that influences the industrial viability of the hyperbaric storage is the cost. The cost analysis of strawberry juice storage under pressure at room temperature showed that, for a quantity of four batches of 200 kg of strawberry juice stored during 15 days, the cost of hyperbaric storage (25 MPa/20 °C) for 15 days was higher (around 3-fold) than the refrigeration cost (Part 6.2). This leads to a price difference between the storage methods of about 22 centimes of euros per strawberry juice liter when stored for 15 days. This price increment has to be passed on the final price of the product. In the case of strawberry juice, nectars can be found at prices around 10 euros per liter. The cost of hyperbaric storage would then mean a price increment of about 2 %, which should not be excessive for the final consumer. As it can be deduced from this remark, the type of stored product has its importance too. The research of this Thesis focuses on strawberry juice, but of course, this method could be employed for a wide range of products even outside of the food area such as the medicine, pharmaceutical or cosmetics ones. Therefore, in addition to the decreasing trend of high pressure equipment price and the increasing trend of electricity price which can boost the competitiveness of hyperbaric storage cost over that of refrigeration in the future, other many factors have to be considered such as, like commented here, the product added value. Another example of factor which has not been discussed yet but may influence cost is the unique advantage provided by the energy independence of the hyperbaric storage at room temperature. This is especially useful when the access to energy supply is complicated like during long transports (from days up to weeks) by ship, train, or road, or in areas with difficult access to the mains or in case of insufficient electric power. In those cases, the additional cost of hyperbaric storage would be fully justified. To conclude on cost analysis, a case by case

study seems appropriated. The number of elements taken into account can be as complicated as one wants including, for instances, all peripherals and external devices of the installation, indirect pressurization of packaged products with different vessel filling ratios, and the pasteurization step which is mandatory for refrigeration but optional for hyperbaric storage. Up to date, the main limitation for such analysis is still the estimation of equipment price. This last almost totally accounts for the hyperbaric storage cost but, unfortunately, manufacturers were not disposed to communicate a possible budget for an installation of such unusual characteristics. Thus, it will have to be awaited for their interest and then for cost confirmation. Future works should be able to contemplate a full industrial scenario with all costs prior and posterior to the storage, that is to say, costs associated to pasteurization, cleanliness, labor, or transport of the product inside the installation, among others.

The final viability criteria addressed in this Thesis was the environmental impact of this method. Its CF was evaluated and compared with that of refrigeration (Part 6.3). Results showed that, for a quantity of four batches of 200 kg of strawberry juice stored during 15 days, the CF of hyperbaric storage (25 MPa/20 °C) is 26 times lower than that of refrigeration. Thank to this calculation, for first time, the overall perception that hyperbaric storage is an environmentally friendly technology is supported. The low CF value of hyperbaric storage is mainly a consequence of its low energy requirements. However, further studies are desirable in complement to this first approach. In this study the environmental impact is assessed by CF analysis taking a simplified and relatively "optimal" scenario as a starting point. It is likely that more complex methodologies such as LCA can also provide additional evidences on the "green" feature of this storage method. Since it takes into account all material and energy inputs and environmental releases within a broad range of categories (human health, ecosystem degradation, climate change, and natural resource depletion), useful information can be expected from such a complete analysis.

It is clear from all the above that hyperbaric storage at room temperature can represent a competitive alternative for strawberry juice preservation. The optimal storage conditions will depend on the specific application. There are many different examples

of imaginary situations in which hyperbaric storage could be applied: fruit processors that store and sell strawberry juice to the food industry, ship or truck transport for long distances, school or hospital kitchens, restaurants or, even, home applications. Moreover, the end use of the juice is also decisive. The adequate preservation of some specific quality parameters will be more or less relevant if the juice is going to be directly consumed or if it is going to be processed or used as a food ingredient without any additional processing. Depending on the context, some storage conditions will be given, but others can be optimized. In general, short-term storage (storage times up to 15 days) does not pose any problem for the product quality, either microbiological or organoleptic, but long-term storage could present some disadvantages. Thus, during storage at relatively low pressures (25 MPa), microbial growth may happen. Increasing storage pressure would reduce the microbial risk, but viscosity decay, cloud destabilization and, obviously vessel size and cost, would also increase. Therefore, depending on the specific application, a pasteurization step prior to hyperbaric storage could be recommended. This step would not diminish the advantages of hyperbaric storage over refrigeration because pasteurization usually precedes cold storage of most foods. Figure 7.5 shows the appearance of pasteurized juices (90 °C/60 s), from four different strawberry batches, after hyperbaric storage at 25 MPa and room temperature for 30 days (data not previously shown).

Figure 7.5. *Appearance of pasteurized strawberry juices from four different batches after storage at 25 MPa and room temperature for 30 days.*

Figure 7.5 clearly proves that pasteurization solves the problem of cloud destabilization observed in Figure 7.4 for long storage times. On the other hand, no sensory problems due to hyperbaric storage must be expected because triangle tests,

presented in Chapter 4 (Part 4.3), revealed that judges were not able to distinguish between pasteurized juices either stored at refrigeration or at 25 MPa and room temperature for 15 days.

On the basis of the results obtained, it is possible to conclude that hyperbaric storage at room temperature is efficient in preserving the quality (microbial load, color, viscosity, aroma, and flavor) of fresh strawberry juice, at least for 15 days. Depending on the specific application, longer storage times could require a pasteurization step prior to hyperbaric storage. In any case, the implementation of hyperbaric storage at industrial scale (equipment design, cost analysis, and environmental impact) would be viable.

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Chapter 8: Conclusions

CONCLUSIONS

1. Hyperbaric storage at 25-220 MPa and room temperature is efficient in preserving the quality of raw strawberry juice for, at least, 15 days. Moreover, after pressure release, strawberry juice remains stable under refrigeration for, at least, 15 additional days.
2. Hyperbaric storage at 25-220 MPa and room temperature is more efficient than refrigeration in avoiding microbial growth in strawberry juice. The greater the pressure and the longer the storage time, the greater the microbial damage produced in strawberry juice. After pressure release, surviving microorganism can recover their cell proliferating capacity, especially after short storage times at 25-50 MPa.
3. Hyperbaric storage at 25-220 MPa and room temperature is effective in attenuating color and viscosity losses in strawberry juice for, at least, 15 days. However, cold storage is significantly more efficacious. Pressure acts on several mechanisms involved in color and viscosity degradation, but the inhibitory action of pressure on microbial growth seems to be the most relevant mechanism delaying color and viscosity decay.
4. Pressure enhances viscosity decay and cloud destabilization and these are limiting factors for hyperbaric storage of strawberry juice. Therefore, for storage times longer than 15 days and depending on the pressure level applied, a pasteurization step prior to storage could be necessary to inactivate pectolytic enzymes.
5. Hyperbaric storage at 50-200 MPa and room temperature is more efficient than refrigeration in preserving the volatile profile of strawberry juice. Moreover, hyperbaric storage, unlike refrigeration, does not affect any aroma compound of the juice.
6. Hyperbaric storage at 25-220 MPa and room temperature, like refrigeration, is effective in preserving the sensory quality of strawberry juice for, at least, 15 days. However, some organoleptic characteristics of the fresh juice, in particular taste and viscosity, can be slightly modified after storage.

7. Hyperbaric storage at room temperature is feasible at industrial scale in terms of installation size and logistics management. For a given vessel capacity, there is a close relationship between the vessel mass and the operating pressure. Therefore, a compromise has to be found between both parameters.
8. The cost of hyperbaric storage at 25 MPa and room temperature is estimated to be around 3-fold higher than cold storage cost when pasteurization is not included in the analysis. The large dependence of the hyperbaric storage cost on the initial investment together with the low energy consumption make that, if the equipment price diminishes and the electricity price increases, hyperbaric storage cost would become more competitive.
9. Hyperbaric storage at room temperature is an environmentally friendly technology as compared with refrigeration since the carbon footprint estimated for hyperbaric storage at 25 MPa and room temperature is about 26 times lower than for refrigeration.

General conclusion:

Hyperbaric storage at room temperature is a novel storage method valid for the preservation of raw strawberry juice for, at least, 15 days. Depending on the specific application, longer storage times could require a pasteurization step prior to hyperbaric storage. In any case, the implementation of hyperbaric storage at industrial scale (equipment design, cost analysis, and environmental impact) would be viable.

Chapter 9: Resumen Ampliado/ Extended abstract[7]

[7] Este resumen ampliado se presenta en cumplimiento de las directrices de la normativa de desarrollo del Real Decreto 99/2011, de 28 de enero, que regula los estudios de doctorado de la Universidad Complutense de Madrid (UCM) (BOUC nº 14, de 21 de diciembre de 2012) y de acuerdo con las especificaciones establecidas por la Comisión de Doctorado de la UCM.

This extended abstract is included in fulfilment of the directives of the regulation of development of the Real Decreto 99/2011, 28th of January, which regulates the studies of doctorate at the Universidad Complutese de Madrid (UCM) (BOUC nº 14, 21st of December 2012) and in agreement with the specifications established by the Commission of Doctorate of the UCM.

9.1. Resumen ampliado

RESUMEN AMPLIADO

9.1.1. Introducción

Los alimentos necesitan condiciones específicas de almacenamiento para conservar su calidad y alargar su vida útil lo máximo posible. Las bajas temperaturas ralentizan los cambios microbiológicos, fisiológicos, bioquímicos y/o físicos indeseables que se producen en los alimentos. Por lo tanto, una de las estrategias más empleadas en los países desarrollados para la conservación de alimentos es la refrigeración mediante la denominada cadena de frío. Sin embargo, el manejo adecuado de la cadena de frío es complejo, consume gran cantidad de energía, es caro y, además, plantea serios problemas medioambientales. Por estas razones, en las últimas décadas, se están llevando a cabo numerosos esfuerzos para mejorar el funcionamiento de los sistemas convencionales de refrigeración, para encontrar nuevas tecnologías de frío más respetuosas con el medio ambiente y para buscar nuevas oportunidades de ahorro energético en la conservación de alimentos (Masanet, 2008; Tassou et al., 2010; Ullah, Saidur, Ping, Akikur, & Shuvo, 2013).

Una de estas nuevas oportunidades podría ser el almacenamiento hiperbárico a temperatura ambiente. Esta estrategia consiste en almacenar los alimentos a presiones superiores a la atmosférica, durante semanas o meses, sin necesidad de controlar la temperatura. El gasto de energía se produce únicamente al inicio del almacenamiento, durante la fase de compresión y, como no se requieren bajas temperaturas, todos los problemas asociados a la refrigeración desaparecen. Estas son ventajas importantes a la hora de reducir el impacto medioambiental de la actividad de las industrias alimentarias. Sin embargo, hoy en día, este método de almacenamiento sólo se ha estudiado a escala de laboratorio y las investigaciones en este campo apenas acaban de comenzar.

Así, en los últimos años, se ha evaluado la efectividad del almacenamiento hiperbárico como método para alargar la vida post-cosecha de frutas y verduras frescas (tejidos vivos). En esta aplicación, que es bastante diferente de la propuesta en esta Tesis, el

producto se almacena en una atmósfera de aire comprimido a presiones relativamente bajas, de hasta 1 MPa, para evitar daños en la estructura celular de los tejidos vivos. Se ha demostrado que la presión es capaz de influir en la fisiología post-cosecha y en la calidad de la fruta y verdura almacenada (Baba & Ikeda, 2003; Liplap, Boutin, LeBlanc, Vigneault, & Vijaya, 2014; Liplap, Vigneault, Toivonen, Charles, & Raghavan, 2013).

Cuando el almacenamiento hiperbárico se lleva a cabo en alimentos procesados (tejidos no vivos), es posible elevar considerablemente la presión, especialmente en productos homogeneizados. En este caso, los alimentos se presurizan, bien directamente si son líquidos o bien envasados y sumergidos en un medio líquido, a presiones normalmente comprendidas entre 25 y 220 MPa. Con ello, se pretende que la presión ralentice su deterioro. Al comienzo de esta Tesis, la información disponible acerca del almacenamiento hiperbárico de alimentos a temperatura ambiente era particularmente escasa. De hecho, se reducía a un estudio en filetes de pescado mantenidos bajo presión, a 25 °C, durante 12 horas (Ko & Hsu, 2001) y a dos patentes (Hirsch, 1997; 2000). Estas patentes incluían ejemplos de distintos tipos de alimentos almacenados durante un máximo de 8 días a presiones de hasta 250 MPa y temperaturas de 18-23 °C, pero no aportaban información alguna sobre la metodología seguida, ni daban justificación científica de los resultados obtenidos.

De estos estudios previos se desprende que el almacenamiento hiperbárico es potencialmente interesante para conservar alimentos cumpliendo con los criterios de sostenibilidad energética y medioambiental actuales de la industria. Sin embargo, antes de evaluar el verdadero potencial de este método, es necesario recoger suficientes evidencias científicas de su efectividad, conocer sobre qué mecanismos concretos de deterioro actúa y tener datos acerca de su viabilidad industrial. Por lo tanto, es necesario llevar a cabo un estudio sistemático del almacenamiento hiperbárico a temperatura ambiente. Para ello, en esta Tesis, se decidió abordar muchos y distintos aspectos del almacenamiento hiperbárico tomando como objeto de estudio un único producto. Se eligió zumo de fresa por ser una matriz líquida relativamente sencilla y, además, un producto altamente perecedero y relevante en la producción frutícola española, muy empleado como ingrediente en la industria alimentaria.

9.1.2. Objetivo

El objetivo principal de esta Tesis fue **caracterizar el almacenamiento hiperbárico a temperatura ambiente, eligiendo como aplicación concreta la conservación de zumo de fresa.** Para la consecución de este objetivo, se plantearon los siguientes objetivos parciales:

1. Estudiar la eficacia del almacenamiento hiperbárico a temperatura ambiente, como técnica innovadora de conservación de alimentos, y compararla con la de la refrigeración, el método más empleado hasta el momento. Dentro de este primer objetivo, las características evaluadas fueron: la calidad del producto, su estabilidad tras el almacenamiento y su aceptabilidad.
2. Analizar el efecto de la presión y el tiempo de almacenamiento en los parámetros de calidad más relevantes del zumo de fresa. Dentro de este segundo objetivo, los parámetros de calidad estudiados fueron: la carga microbiana, el color y la viscosidad.
3. Evaluar la viabilidad del almacenamiento hiperbárico a temperatura ambiente para la conservación de zumo de fresa a escala industrial. Dentro de este tercer objetivo, los factores estudiados fueron: el diseño del equipo, los costes de almacenamiento y el impacto medioambiental.

9.1.3. Resultados

9.1.3.1. Eficacia del almacenamiento hiperbárico a temperatura ambiente en la conservación de zumo de fresa: Comparación con la refrigeración.

Con objeto de resaltar la eficacia y las ventajas del almacenamiento hiperbárico sobre la refrigeración, se llevaron a cabo experimentos de almacenamiento de zumo de fresa a diferentes niveles de presión y temperatura ambiente durante 15 días. Tras el almacenamiento, se midieron los principales parámetros que reflejan la calidad del zumo (carga microbiana, viscosidad, color, aroma y sabor) y se compararon con los obtenidos en muestras almacenadas a presión atmosférica y 5 °C. Se estudió además la estabilidad de los zumos tras el almacenamiento hiperbárico.

El almacenamiento hiperbárico (25-220 MPa/20 °C) resultó eficaz tanto para inhibir el crecimiento microbiano como para atenuar las pérdidas de color y viscosidad en el zumo de fresa. Por su parte, la refrigeración fue significativamente más efectiva para ralentizar el deterioro del color y la viscosidad, pero no consiguió evitar el crecimiento microbiano. Así, se constató que, a diferencia de la refrigeración, el almacenamiento hiperbárico no requiere una pasteurización previa del producto para garantizar su seguridad microbiológica.

Además, el almacenamiento hiperbárico, a 50 y 200 MPa/20 °C, resultó más eficaz que la refrigeración para preservar el perfil de volátiles del zumo de fresa durante 15 días. Así, las muestras almacenadas bajo presión fueron más similares a los zumos control en el día 0 que las muestras refrigeradas.

Por otra parte, el análisis hedónico reveló que tanto el almacenamiento hiperbárico (25-220 MPa/20 °C) como la refrigeración son eficientes para conservar la calidad sensorial del zumo durante, al menos, 15 días. A pesar de ello, las pruebas triangulares indicaron que algunas de las características organolépticas del zumo original, concretamente el sabor y la viscosidad, se modificaron ligeramente tras 15 días de almacenamiento a 25 y 50 MPa, respectivamente. Por otra parte, cuando se compararon en una prueba triangular zumos pasteurizados almacenados, bien a 25 MPa o bien refrigerados, los jueces no fueron capaces de detectar diferencias entre ellos. Se deduce, por tanto, que el almacenamiento hiperbárico a 25 MPa y temperatura ambiente y la refrigeración conservan las características organolépticas del zumo de fresa pasteurizado con la misma eficacia.

Finalmente, se comprobó también que el zumo de fresa no pasteurizado se mantiene estable tras el almacenamiento hiperbárico durante, al menos, 15 días adicionales en refrigeración.

9.1.3.2. Efecto del nivel de presión y del tiempo de almacenamiento en el crecimiento microbiano, el color y la viscosidad

Una vez demostrada la eficacia del almacenamiento hiperbárico a temperatura ambiente, se estudió en detalle el efecto del nivel de presión (0.1-200 MPa) y del tiempo

de almacenamiento (0-15 días) en la carga microbiana, el color y la viscosidad del zumo.

Dado que el crecimiento microbiano puede producir la degradación del color y la viscosidad, se añadió una solución antibiótica al zumo para el análisis de estos parámetros. De esta manera, se pudo evaluar el efecto de la presión sin interferencia de la carga microbiana.

Los resultados obtenidos demostraron que el crecimiento microbiano, el color y la viscosidad se ven afectados tanto por el nivel de presión como por el tiempo de almacenamiento.

Los análisis microbiológicos revelaron que cuanto mayor es la presión y más largo el tiempo de almacenamiento, mayor es el daño causado en los microorganismos. Así, el almacenamiento hiperbárico durante tiempos cortos (1 día) a presiones relativamente bajas (25-50 MPa) inhibió el crecimiento microbiano en el zumo de fresa. Tiempos de almacenamiento más prolongados (10-15 días) o presiones más elevadas (100-200 MPa) no sólo inhibieron el crecimiento microbiano, sino que, también, produjeron cierto grado de inactivación microbiana. Tras la descompresión, los microorganismos pudieron recuperar rápidamente su capacidad de multiplicación, especialmente tras almacenamientos cortos a 25-50 MPa. Los daños sufridos a presiones más elevadas o durante tiempos de almacenamiento más prolongados dificultaron su recuperación al pH ácido del zumo de fresa.

Los resultados de color demostraron que la presión afecta a distintos mecanismos implicados en la degradación del color. Aparte del deterioro microbiano, se observó un efecto de la presión en el pardeamiento enzimático y en las reacciones de polimerización de las antocianinas con otros compuestos del zumo. Así, las muestras almacenadas a 200 MPa sufrieron una inactivación significativa de la enzima peroxidasa y presentaron un menor porcentaje de color polimérico en comparación con las muestras almacenadas a presión atmosférica. Cuando se evitó la interferencia microbiana, las diferencias de color debidas a la presión de almacenamiento, aunque instrumentalmente perceptibles, fueron muy leves y demasiado sutiles para poder ser

apreciadas a simple vista. Por lo tanto, las grandes diferencias de color descritas entre zumos almacenados bajo presión y a presión atmosférica (sin solución antibiótica) deben atribuirse principalmente al efecto inhibitorio de la presión sobre el crecimiento microbiano.

En relación a la viscosidad, los resultados obtenidos revelaron que la viscosidad de la fracción suero del zumo disminuyó muy rápidamente durante los primeros días de almacenamiento a temperatura ambiente, sobre todo en las muestras almacenadas bajo presión. Así, cuanto mayor es la presión de almacenamiento, mayor es la degradación de la viscosidad. En un intento de buscar los mecanismos responsables del mayor descenso de la viscosidad bajo presión, se estudió la actividad catalítica de la pectinmetilesterasa (PME), enzima directamente relacionada con las pérdidas de turbidez y viscosidad en los zumos de frutas. No se observó un efecto directo de la presión sobre la actividad PME, y así, durante los primeros días de almacenamiento, la actividad PME en el zumo de fresa fue independiente de la presión. Sin embargo, al final del almacenamiento, sí se detectó una mayor actividad PME y también una ligera pérdida de turbidez en las muestras almacenadas a 200 MPa. Estos resultados sugieren que la presión potencia ciertos mecanismos que aceleran tanto el deterioro de la viscosidad como la actividad PME. Debido a la complejidad de la composición y estructura de la pectina, se requiere mucha más investigación.

Todos los resultados comentados anteriormente demuestran que la presión y el tiempo de almacenamiento afectan significativamente a muchos de los mecanismos responsables del deterioro del zumo de fresa. Desde el punto de vista de calidad del producto, los factores que limitan el almacenamiento hiperbárico del zumo de fresa son las pérdidas de viscosidad y turbidez, ya que, ambas se aceleran bajo presión. Por lo tanto, el nivel de presión empleado durante el almacenamiento hiperbárico debe ser lo más bajo posible para ralentizar estos procesos de deterioro, pero suficiente para garantizar la inhibición del crecimiento microbiano.

9.1.3.3. Viabilidad del almacenamiento hiperbárico a temperatura ambiente para su implementación a escala industrial

Una vez demostrada la eficacia del almacenamiento hiperbárico a temperatura ambiente, y examinado el efecto del nivel de presión y del tiempo de almacenamiento en la calidad del zumo, fue necesario definir y analizar la viabilidad industrial a través de distintos criterios de aplicabilidad. Para ello, los criterios seleccionados fueron el diseño del equipo, los costes de almacenamiento y el impacto medioambiental.

En primer lugar, fue necesario definir qué diseños de vasijas de almacenamiento a alta presión pueden ser viables. Se demostró que todos los parámetros de diseño (forma, material, capacidad y presión de trabajo) tienen una gran influencia en el tamaño y en el peso de la vasija, considerados como los principales factores que limitan la viabilidad. Para minimizar el peso de la vasija, tanto la forma como el material de construcción se pueden optimizar. En general, la presión de almacenamiento debería ser la más baja posible sin comprometer la calidad del producto. Conforme a las restricciones establecidas en este estudio (peso de la vasija ≤ 2 t y longitud ≤ 2 m) y con las condiciones elegidas (capacidad para 200 kg de zumo y coeficiente D/L de 0.66), el diseño más apropiado resultó el de una vasija fabricada de acero inoxidable, tipo 15-5PH, con dos cabezas semiesféricas, capaz de almacenar zumo a presiones de hasta 155 MPa. Este diseño fue el que se utilizó para realizar los estudios posteriores sobre los aspectos económicos y medioambientales del almacenamiento hiperbárico.

Los resultados del análisis económico revelaron que el coste del almacenamiento hiperbárico (25 MPa/20 °C/15 días) es aproximadamente 3 veces mayor que el coste de la refrigeración. Si se hubieran tenido en cuenta los costes asociados a la pasteurización, estas diferencias serían menores. El almacenamiento refrigerado normalmente implica una etapa previa de pasteurización, pero ésta, dependiendo de la presión y del tiempo de almacenamiento, no siempre es necesaria en el almacenamiento hiperbárico.

El alto coste del almacenamiento hiperbárico se debe a la gran inversión inicial, ya que, el consumo energético es prácticamente despreciable. Por el contrario, el consumo energético constituye la mayor contribución a los costes de la refrigeración. Por lo

tanto, las diferencias de coste entre el almacenamiento hiperbárico y la refrigeración pueden disminuir en tanto el precio de las vasijas del almacenamiento hiperbárico disminuya o el precio de la electricidad aumente.

Por otra parte, el almacenamiento hiperbárico presenta una huella de carbono considerablemente menor que la correspondiente a la refrigeración. Esto refuerza la idea extendida de que la alta presión es una tecnología respetuosa con el medio ambiente. Los beneficios del almacenamiento hiperbárico se deben principalmente a su bajo requerimiento energético, ya que, el gasto de energía se produce únicamente durante la compresión y no se requiere energía adicional ni para mantener el producto bajo presión en el tiempo ni para controlar la temperatura.

9.1.4. Conclusiones

1. El almacenamiento hiperbárico a 25-220 MPa y temperatura ambiente es eficaz para preservar la calidad del zumo de fresa fresco durante, al menos, 15 días. Además, tras la descompresión, el zumo se mantiene estable en refrigeración durante, al menos, 15 días adicionales.
2. El almacenamiento hiperbárico a 25-220 MPa y temperatura ambiente es más eficaz que la refrigeración para evitar el crecimiento microbiano en el zumo de fresa. Cuanto mayor es la presión y/o el tiempo de almacenamiento, mayor es el daño producido en los microorganismos. Tras la descompresión, los microorganismos que han sobrevivido pueden recuperar su capacidad de multiplicación celular, especialmente tras tiempos cortos de almacenamiento a 25-50 MPa.
3. El almacenamiento hiperbárico a 25-220 MPa y temperatura ambiente es eficaz para atenuar el deterioro del color y la viscosidad en zumo de fresa, aunque la refrigeración es significativamente más efectiva. La presión afecta a distintos mecanismos involucrados en el deterioro de ambos parámetros, pero es su efecto inhibitorio en la actividad metabólica de los microorganismos el principal responsable de las mejoras observadas.

4. La presión acelera las pérdidas de viscosidad y turbidez en el zumo de fresa y esto constituye uno de los principales factores limitantes para el almacenamiento hiperbárico del zumo. Por ello, para tiempos de almacenamiento superiores a 15 días y dependiendo del nivel de presión empleado, podría ser necesario llevar a cabo una pasteurización del producto previa a su almacenamiento que permita la inactivación de enzimas pectolíticas.
5. El almacenamiento hiperbárico a 25-220 MPa y temperatura ambiente es más efectivo que la refrigeración para preservar el perfil de volátiles en el zumo de fresa. Además, el almacenamiento hiperbárico, a diferencia de la refrigeración, no afecta a ningún constituyente clave del aroma del zumo.
6. El almacenamiento hiperbárico a 25-220 MPa y temperatura ambiente, al igual que la refrigeración, es efectivo para preservar la calidad sensorial del zumo de fresa durante, al menos, 15 días. Sin embargo, algunas de las características organolépticas del zumo original, tales como el sabor y la viscosidad, pueden verse ligeramente modificadas.
7. El almacenamiento hiperbárico a temperatura ambiente es factible a escala industrial en términos de tamaño de la instalación y manejo logístico. Para una capacidad dada, existe una estrecha relación entre el peso de la vasija y la presión de trabajo. Por lo tanto, se debe llegar a una solución de compromiso para optimizar ambos parámetros.
8. El coste estimado del almacenamiento hiperbárico a 25 MPa y temperatura ambiente es aproximadamente 3 veces mayor que el de la refrigeración, si no se tienen en cuenta los costes de pasteurización. Dado que los costes del almacenamiento hiperbárico dependen, en su mayoría, de la inversión inicial siendo el consumo energético casi despreciable, en el momento en el que el precio del equipo disminuya y/o el precio de la electricidad aumente, el coste del almacenamiento hiperbárico resultará más competitivo.
9. El almacenamiento hiperbárico a temperatura ambiente es una tecnología respetuosa con el medio ambiente en comparación con la refrigeración. Así, se estimó que la huella de carbono del almacenamiento hiperbárico a 25 MPa es aproximadamente 26 veces menor que la de la refrigeración.

Conclusión general:

El almacenamiento hiperbárico a temperatura ambiente es un método innovador apto para la conservación del zumo de fresa durante, al menos, 15 días. Para tiempos más largos de almacenamiento y dependiendo del nivel de presión, podría ser necesaria una pasteurización previa del producto. En cualquier caso, la implementación del almacenamiento hiperbárico a escala industrial (diseño de equipo, análisis de costes e impacto ambiental) sería viable.

9.1.5. Aportaciones fundamentales de la Tesis doctoral

Esta es la primera Tesis que aborda la caracterización del almacenamiento hiperbárico a temperatura ambiente desde el punto de vista del concepto de Calidad Total. Así, para su desarrollo, se estudió no sólo la calidad microbiológica y organoléptica del producto almacenado, sino también el diseño del equipo, los costes económicos y el impacto medioambiental con el fin de poder establecer el potencial real de este método.

Esta Tesis ofrece los primeros datos sobre la eficacia del almacenamiento hiperbárico a temperatura ambiente para preservar la calidad de un alimento durante periodos de tiempo relativamente largos (15 días). Hasta el momento, el tiempo máximo de almacenamiento estudiado en artículos científicos de la literatura era de 60 horas.

Esta Tesis aporta nuevos datos importantes para la caracterización del almacenamiento hiperbárico a temperatura ambiente en zumos, concretamente en zumo de fresa. A pesar de que, hoy en día, ya existen algunos estudios hechos en zumos, estos son los primeros datos relativos al zumo de fresa. Así, la Tesis compara por primera vez la efectividad del almacenamiento hiperbárico y de la refrigeración para preservar la calidad (carga microbiana, viscosidad, color, aroma y sabor) del zumo de fresa. Además, la Tesis presenta también datos acerca de la estabilidad del zumo tras el almacenamiento hiperbárico durante 15 días adicionales en refrigeración.

Con respecto a los atributos organolépticos, esta Tesis proporciona, por primera vez en la literatura, datos acerca del efecto del almacenamiento hiperbárico a temperatura ambiente en el perfil de volátiles de un producto homogenizado de fruta. Otra

aportación importante es la evaluación de la eficacia del almacenamiento hiperbárico mediante el empleo del análisis sensorial. Hasta el momento, en los estudios publicados, la evaluación de la calidad de los productos tras el almacenamiento hiperbárico se basa únicamente en medidas instrumentales, sin tener cuenta las opiniones de los consumidores.

Esta Tesis ofrece, además, los primeros datos acerca del efecto de nivel de presión y del tiempo de almacenamiento en la carga microbiana del zumo de fresa. Esto es especialmente interesante porque, aunque a día de hoy, ya existen datos para otros zumos, éstos en ningún caso tienen un pH tan bajo como el del zumo de fresa. Además, como aportación novedosa, los estudios microbiológicos se realizaron en zumos sin congelar para, de esta manera, evitar que los microorganismos sufrieran algún tipo de estrés antes del almacenamiento. Se presentan, también por primera vez, datos acerca de la recuperación de los microorganismos a presión atmosférica y temperatura ambiente tras el almacenamiento hiperbárico.

Otra aportación fundamental de esta Tesis es el estudio del efecto de la presión y del tiempo de almacenamiento en el color y la viscosidad del zumo de fresa. Además, por primera vez, se estudió el efecto de la presión en algunos de los mecanismos implicados en la degradación de estos parámetros de calidad.

Una contribución singular de esta Tesis es la inclusión de un capítulo dedicado íntegramente a la aplicación a nivel industrial del almacenamiento hiperbárico a temperatura ambiente. Por primera vez, se aborda la viabilidad industrial de este método de almacenamiento de forma cuantitativa. Para ello, se ha verificado la viabilidad desde el punto de vista del tamaño de la instalación y del manejo logístico y se ha propuesto un posible diseño de vasija de almacenamiento. Además, se han estimado los costes del almacenamiento hiperbárico, principal obstáculo de este método, para reflejar las debilidades y fortalezas de este método y compararlas con las de la refrigeración. Por último, se ha demostrado la percepción generalizada de que este método es más respetuoso con el medio ambiente que la refrigeración.

9.1.6. Bibliografía

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9.2. Extended abstract

EXTENDED ABSTRACT

9.2.1. Introduction

Foods need specific conditions of storage to preserve their quality and to extend their shelf life as much as possible. Low temperatures retard microbiological, physiological, biochemical, and/or physical detrimental changes in foods. Therefore, cold storage through the cold chain is one of the most applied strategies in developed countries to preserve food. However, the adequate management of the cold chain is logistically complex, energy consuming, expensive, and pose serious environmental concerns. For all these reasons, in the last decades, many efforts have been made in the agro-food industry to improve the performance of conventional refrigeration systems, to find new environmentally friendly refrigeration technologies, and also to look for new energy saving opportunities in food preservation (Masanet, 2008; Tassou et al., 2010; Ullah, Saidur, Ping, Akikur, & Shuvo, 2013).

Hyperbaric storage at room temperature could be one of these opportunities for food preservation. This storage strategy consists in maintaining food under pressures higher than atmospheric one, during weeks or months, with no temperature control. Energy is only required at the beginning of storage for product compression. No refrigeration is additionally necessary, so all problems associated to it are eliminated. These are attractive advantages for reducing the impact of food industry activities on the environment. However, this storage method has only been implemented at laboratory scale and researches in this field have just started.

Recently, the feasibility of this technology as a postharvest life extension method for fresh fruits and vegetables (living tissues) has been assessed. In this application, quite different to that proposed in this Thesis, the product is subjected to a pressure environment built up by means of compressed air. In this case, pressure level is relatively low (up to 1 MPa) to avoid damage to the cell structure of living tissues. It was shown that pressure was able to influence the postharvest physiology and quality

of the stored fruit and vegetables (Baba & Ikeda, 2003; Liplap, Boutin, LeBlanc, Vigneault, & Vijaya, 2014; Liplap, Vigneault, Toivonen, Charles, & Raghavan, 2013).

In processed food (non-living tissues), pressure is transmitted by a liquid medium and it can be increased considerably (25–220 MPa), especially in homogenized products. At the beginning of this Thesis, the available information about hyperbaric storage of food at room temperature was particularly scarce. In fact, there were only one scientific article about fish fillets stored under pressure, at 25 °C, for up to 12 h (Ko & Hsu, 2001) and two patents (Hirsch, 1997; 2000). These patents reported some examples of several foods stored under pressure up to 250 MPa, at 18-23 °C, for up to 8 days, but the methodology and scientific justification were missing.

From the bibliographic study, it stands out that this storage strategy is potentially interesting for preserving food while fulfilling the current industrial criteria about energy and environment. However, more scientific evidences of its effectiveness, working mechanisms, and industrial viability are needed before assessing its real potential. Therefore, it appears indispensable to carry out a systematic study of hyperbaric storage at room temperature. One product was chosen as the object of study and many features of hyperbaric storage were decided to be addressed. Strawberry juice was taken on since it is a relatively simple liquid matrix, highly perishable, meaningful in Spanish fruit production, and widely employed as an ingredient in the food industry.

9.2.2. Objective

The main objective of this Thesis was **to characterize hyperbaric storage at room temperature in the frame of the preservation of strawberry juice**. To this end, the following partial objectives were considered:

1. To evaluate the effectiveness of hyperbaric storage at room temperature, as an innovative technique for preserving strawberry juice, compared with cold storage, as the most common method used up to date. The hyperbaric storage

characteristics explored under this first objective were: quality, stability after hyperbaric storage, and acceptability of the stored juice.

2. To analyze the effect of pressure level and storage time on some of the most relevant quality parameters of strawberry juice. The quality parameters examined under this second objective were: microbial growth, color, and viscosity.
3. To assess the viability of hyperbaric storage at room temperature of preserving strawberry juice at industrial scale. The hyperbaric storage characteristics evaluated under this third objective were: equipment design, storage costs, and environmental impact.

9.2.3. Results

9.2.3.1. Effectiveness of hyperbaric storage at room temperature for strawberry juice preservation in comparison with refrigeration

With the aim of standing out the effectiveness and advantages of hyperbaric storage over refrigeration, storage experiments were carried out for 15 days at different pressures at room temperature. After the storage period, the main safety and quality parameters (microbial load, viscosity, color, aroma, and flavor) were measured and compared with those of juices stored at atmospheric pressure and 5 °C. Besides, the stability of the juices after decompression was also studied.

Hyperbaric storage (25-220 MPa/20 °C) was found to be an efficient method to inhibit the growth of microorganisms as well as to attenuate viscosity and color losses in raw strawberry juices stored for 15 days. Although cold storage was significantly more efficacious in delaying viscosity and color decay, it failed in inhibiting microbial growth and a previous pasteurization step was necessary to ensure the microbiological safety of the juice.

A detailed study of the volatile fraction of strawberry juice showed that hyperbaric storage (at 50 and 200 MPa/20 °C) was more efficient than cold storage in maintaining

the volatile profile of strawberry juice unaltered for 15 days. Thus, samples stored under pressure were more similar to control juices at day 0 than cold stored samples.

A hedonic sensory analysis revealed that hyperbaric storage (25-220 MPa/20 °C), as well as cold storage, was able to preserve the sensory quality of the raw juice, for at least 15 days. However, triangle tests indicated that some organoleptic characteristics of the fresh juice, in particular taste and viscosity, were modified after 15 days of storage (20 °C) at 25 and 50 MPa, respectively. When pasteurized juices, either stored at 25 MPa or refrigerated for 15 days, were compared in a triangle test, judges were not able to distinguish between them. Thus, hyperbaric storage at 25 MPa and room temperature and cold storage preserved the organoleptic characteristics of the pasteurized strawberry juice with the same efficiency.

Finally, it has been proved that raw strawberry juice after decompression was stable, under refrigeration for, at least, 15 days.

9.2.3.2. Effect of pressure level and storage time on microbial growth, color, and viscosity

Once the effectiveness of hyperbaric storage at room temperature was demonstrated, the effect of pressure level (0.1-200 MPa) and storage time (0-15 days) on microbial growth, color, and viscosity was analyzed in detail.

Since microbial growth can produce color and viscosity degradation, an antibiotic solution was added to the juice when studying these parameters. In this way, the effect of pressure on color and viscosity could be evaluated without microbial interference.

Results showed that microbial growth, color, and viscosity were all affected by both pressure level and storage time.

Microbial results proved that the greater the pressure and the longer the storage time, the greater the microbial damage produced. Hyperbaric storage at relatively low pressures (25-50 MPa) for short times (1 day) inhibited microbial growth in strawberry juice. Longer storage times (10-15 days) or higher pressures (100-200 MPa) not only inhibited microbial growth but also produced some microbial inactivation during

storage. After pressure release, microorganisms could quickly recover their cell proliferating capacity, especially after short storage times at 25-50 MPa. Damage produced at higher pressures or longer times hampered microbial recovery at the acidic pH of the strawberry juice.

Color analyses revealed a significant effect of the storage pressure on all the chromatic parameters. Moreover, the results showed that some mechanisms of color degradation, apart from microbial spoilage, were affected by pressure. In particular, pressure acted on both enzymatic browning and polymerization reactions of anthocyanins with other juice components. Thus, significant peroxidase inactivation and lower percent polymeric color were found in samples stored at 200 MPa as compared with samples maintained at atmospheric pressure. When microbial interference was avoided, color differences due to the storage pressure, although instrumentally perceptible, were very slight and too subtle to be easily perceived by the naked eye. Therefore, large color differences, previously reported, between juices stored under high and atmospheric pressure with no antibiotic solution added must be mainly due to the inhibitory effect of pressure on microbial growth.

Regarding viscosity, the results showed that serum viscosity decreased very quickly during the first days of storage at room temperature, especially in samples stored under high pressure. Thus, the greater the pressure during storage, the greater the viscosity decay. In an attempt to search the mechanisms responsible for this pressure dependence, the catalytic activity of pectinmethylesterase (PME), an enzyme directly related to cloud destabilization and losses of serum viscosity in fruit juices, was also evaluated. At the beginning of storage, PME activity was independent of the storage pressure, but later, catalytic PME activity was significantly higher in samples stored at 200 MPa. Consequently, a slight cloud destabilization was observed in these samples at the end of storage. These results suggest that PME was not directly related to the large viscosity decay observed during the first days of storage. Therefore, other mechanisms accelerating both viscosity decay and PME activity must be enhanced by pressure. Given the complex pectin composition and architecture, much more research is needed.

All the results above commented reveal that storage pressure and time significantly affect the quality of strawberry juice. Viscosity decay and cloud destabilization seem to be the main factors limiting hyperbaric storage of strawberry juice. Therefore, pressure levels employed during hyperbaric storage at room temperature should be as low as possible to slow down these deteriorative processes, but high enough to guarantee microbial growth inhibition.

9.2.3.3. Viability of hyperbaric storage at room temperature for its implantation at industrial scale

Once the effectiveness of hyperbaric storage at room temperature in preserving strawberry juice was proved and the effects of pressure level and storage time on juice quality were examined, it was necessary to define and discuss the industrial viability through several applicability criteria. The equipment design, the cost analysis, and the environmental impact were then studied.

Firstly, the domain of viable designs for high-pressure storage vessels was defined. The results showed that the design parameters (shape, material, capacity, and pressure) have a great influence on vessel size and vessel mass, considered as the main limiting factors of feasibility. To minimize vessel mass, shape and construction material have to be optimized. In general, the operating pressure should be kept as low as possible without compromising the quality of the product. Under the limiting factors set in this study (vessel mass ≤ 2 t and length ≤ 2 m) and with the selected conditions (capacity for 200 kg of juice and D/L ratio of 0.66), the most suitable design was a vessel made of 15-5PH stainless steel with two hemispherical heads, capable of storing juice at any pressure up to 155 MPa. The subsequent studies about the economic and environmental aspects of hyperbaric storage were based on this suitable design.

The results of the cost analysis revealed that the cost of hyperbaric storage (25 MPa/20 °C) for 15 days was around 3-fold higher than the refrigeration cost. This difference would be lower if pasteurization costs had been included in the analysis. Pasteurization is a mandatory step prior to refrigeration but, depending on the storage pressure and time, it is not always a requisite for hyperbaric storage.

The higher cost of hyperbaric storage was the consequence of the huge initial investment since the energetic consumption was practically negligible. On the contrary, the electricity consumption represented the main contribution to the refrigeration cost. Therefore, these differences between hyperbaric and refrigerated storage could be attenuated as far as the price of hyperbaric storage vessels diminishes or the electricity price increases.

The carbon footprint estimated for hyperbaric storage was considerably lower than that for refrigeration. This reinforces the generally accepted idea that pressure is an environmentally friendly technology. This result is mainly a consequence of the low energetic requirements of the hyperbaric storage at room temperature since electricity is only consumed during compression and no additional energy is required for neither pressure holding nor temperature control.

9.2.4. Conclusions

1. Hyperbaric storage at 25-220 MPa and room temperature is efficient in preserving the quality of raw strawberry juice for, at least, 15 days. Moreover, after pressure release, strawberry juice remains stable under refrigeration for, at least, 15 additional days.
2. Hyperbaric storage at 25-220 MPa and room temperature is more efficient than refrigeration in avoiding microbial growth in strawberry juice. The greater the pressure and the longer the storage time, the greater the microbial damage produced in strawberry juice. After pressure release, surviving microorganism can recover their cell proliferating capacity, especially after short storage times at 25-50 MPa.
3. Hyperbaric storage at 25-220 MPa and room temperature is effective in attenuating color and viscosity losses in strawberry juice for, at least, 15 days. However, cold storage is significantly more efficacious. Pressure acts on several mechanisms involved in color and viscosity degradation, but the inhibitory action of pressure on microbial growth seems to be the most relevant mechanism delaying color and viscosity decay.

4. Pressure enhances viscosity decay and cloud destabilization and these are limiting factors for hyperbaric storage of strawberry juice. Therefore, for storage times longer than 15 days and depending on the pressure level applied, a pasteurization step prior to storage could be necessary to inactivate pectolytic enzymes.
5. Hyperbaric storage at 50-200 MPa and room temperature is more efficient than refrigeration in preserving the volatile profile of strawberry juice. Moreover, hyperbaric storage, unlike refrigeration, does not affect any aroma compound of the juice.
6. Hyperbaric storage at 25-220 MPa and room temperature, like refrigeration, is effective in preserving the sensorial quality of strawberry juice for, at least, 15 days. However, some organoleptic characteristics of the fresh juice, in particular taste and viscosity, can be slightly modified after storage.
7. Hyperbaric storage at room temperature is feasible at industrial scale in terms of installation size and logistics management. For a given vessel capacity, there is a close relationship between the vessel mass and the operating pressure. Therefore, a compromise has to be found between both parameters.
8. The cost of hyperbaric storage at 25 MPa and room temperature is estimated to be around 3-fold higher than cold storage cost when pasteurization is not included in the analysis. The large dependence of the hyperbaric storage cost on the initial investment together with the low energy consumption make that, if the equipment price diminishes and the electricity price increases, hyperbaric storage cost would become more competitive.
9. Hyperbaric storage at room temperature is an environmentally friendly technology as compared with refrigeration since the carbon footprint estimated for hyperbaric storage at 25 MPa and room temperature is about 26 times lower than for refrigeration.

General conclusion:

Hyperbaric storage at room temperature is a novel storage method valid for the preservation of raw strawberry juice for, at least, 15 days. Depending on the

specific application, longer storage times could require a pasteurization step prior to hyperbaric storage. In any case, the implementation of hyperbaric storage at industrial scale (equipment design, cost analysis, and environmental impact) would be viable.

9.2.5. Fundamental contributions of the doctoral Thesis

This is the first ever Thesis to address the characterization of hyperbaric storage at room temperature from the point of view of the Total Quality concept. Thus, not only the microbiological and organoleptic qualities of the stored product were studied, but also the equipment design, the economic cost, and the environmental impact were evaluated to establish the real potential of this method.

This Thesis offers the first data about the effectiveness of hyperbaric storage at room temperature in preserving food quality for relatively long times (up to 15 days). Up to date, 60 hours was the longest period of hyperbaric storage time reported in the scientific literature.

This Thesis provides important new data for the characterization of hyperbaric storage of fruit juices at room temperature, in particular strawberry juice. Although, nowadays, there are some studies in fruit juices, these are the first data about strawberry juice. Thus, this Thesis first ever compares the effectiveness of hyperbaric storage and refrigeration in preserving strawberry juice quality (microbial load, color, viscosity, aroma, and taste). Moreover, this Thesis also reports some data about juice stability after hyperbaric storage, when the juice is maintained in refrigeration for 15 additional days.

Regarding the organoleptic attributes, this Thesis reports the first data in the literature about the effect of hyperbaric storage at room temperature on the volatile profile of a homogenized fruit product. Another significant contribution is the employment of sensory analysis to evaluate the efficacy of hyperbaric storage. In the literature, up to date, quality assessment after hyperbaric storage is based only on instrumental measurements and the perception of consumers is not taken into account.

Moreover, this Thesis presents the first data about the effect of pressure level and storage time on the microbial load of a high acidic juice. There are some data in the literature for juices, but not so acidic as strawberry juice. As an additional innovation, microbial studies were performed in non-frozen juices to avoid any stress to the microorganisms prior to storage. Moreover, microbial recovery after hyperbaric storage was also studied at atmospheric pressure and room temperature for the first time in the literature.

Another fundamental contribution of this Thesis is the study of the effect of storage pressure and time on color and viscosity of strawberry juice. Moreover, the effect of pressure on some mechanisms involved in the degradation of these quality parameters is studied in detail for first time too.

An original contribution of this Thesis is the inclusion of a chapter entirely dedicated to industrial implementation aspects of hyperbaric storage at room temperature. For the first time, the feasibility and viability of this storage method are approached in a quantitative way. The feasibility from the point of view of installation size and logistics management has been verified and an optimal design has been proposed. Besides, hyperbaric storage cost - the usual handicap of this technology - has been analyzed in order to discuss the strengths and weaknesses of this method compared with conventional refrigeration. And lastly, the idea that this method is an environmentally friendly technology as compared with refrigeration has been demonstrated.

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Appendix

Appendix 1. *Example of temperature and pressure evolution during hyperbaric storage for 15 days.*

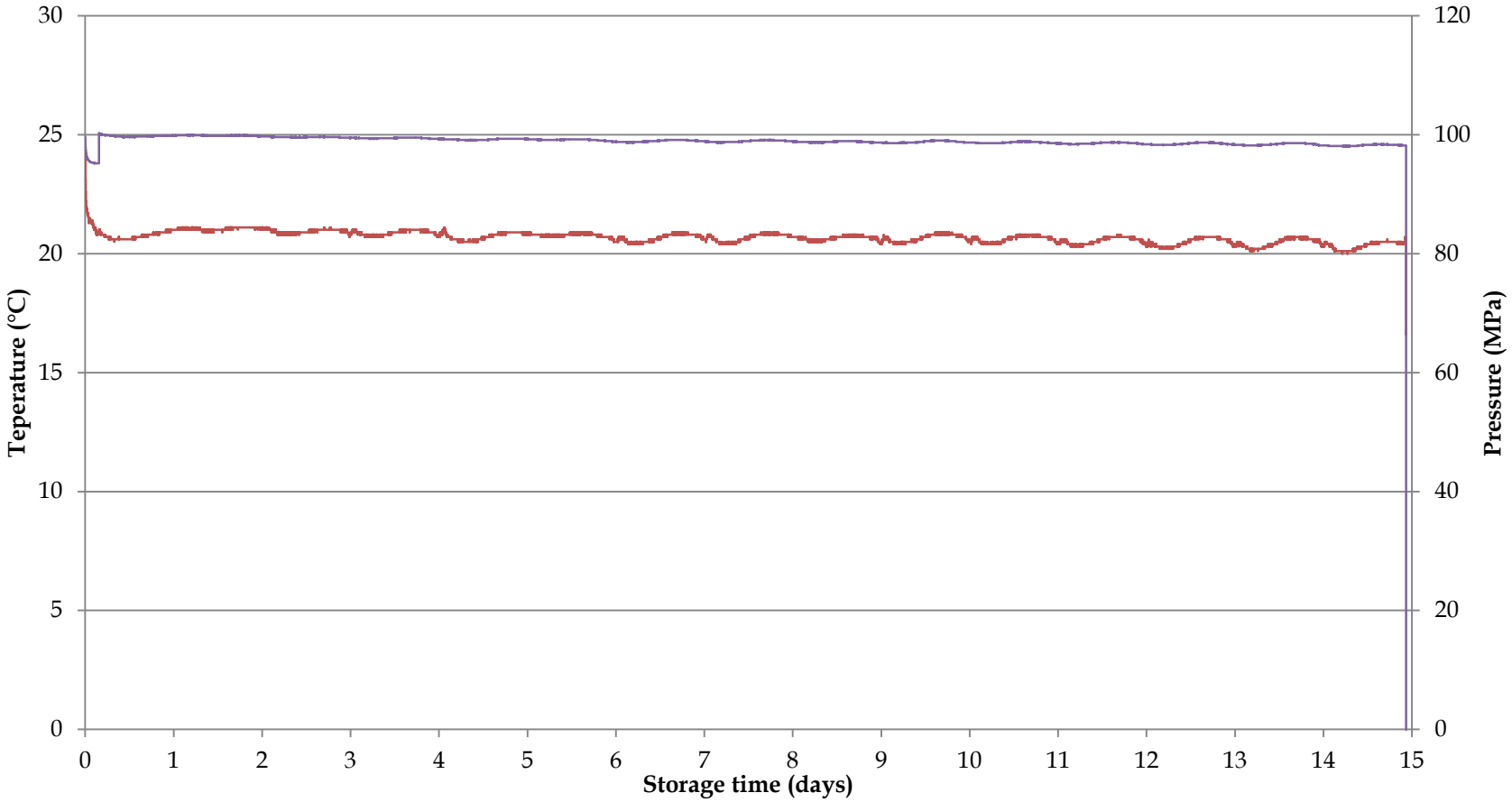


Figure 9.1. *Evolution of temperature (——) and pressure (——) during hyperbaric storage at 100 MPa and 20 ºC for 15 days. Data were recorded by the data acquisition system every 30 seconds.*

Appendix 2. *Example of the scorecard used in the triangle tests.*

ENSAYO DE ANÁLISIS SENSORIAL DE ZUMO DE FRESA

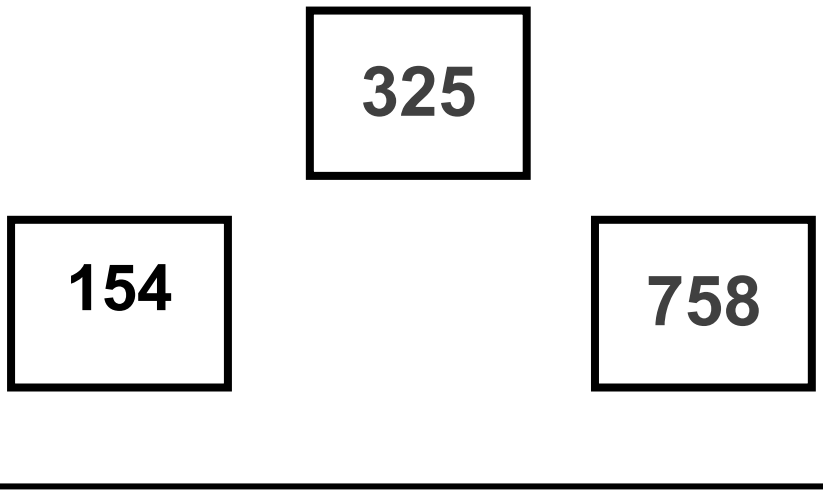
NOMBRE Y APELLIDO.....FECHA.....

CATADOR Nº:

INSTRUCCIONES:

En las siguientes pruebas se presentan tres muestras codificadas.
Dos de las muestras son iguales y una es diferente, indique la muestra que considere distinta. Es indispensable que señale una de las tres.

PRUEBA TRIANGULAR 1



De las tres muestras anteriores, ¿qué muestra prefiere usted? ☐

COMENTARIOS: Si quiere hacer alguna observación, hágalo a continuación:
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