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Study of the Ultra High Pressure Homogenization (UHPH) technology for producing high quality soymilk

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Abstract

Soymilk consumption is experiencing a noticeable increase due to it being considered as a healthy product. Soymilk has often been used as an alternative to dairy milk for people who have intolerance to dairy products. Nowadays, it is known for its important health benefits that can contribute to the reduction of chronic illness commonly prevalent in the modern style life. This is due, primarily, to characteristics of protein fraction and minor components rich in antioxidant activity (flavonoids, tocopherols and poliamines) taking into account the excellent nutritional profile of soymilk.

This thesis project was focused on the application of an emerging technology, Ultra High Pressure Homogenization (UHPH), in the production of soy vegetable milk. This non-thermal technology consists of a high pressure machine capable of applying pressures of up to 400 MPa using a special homogenizing system designed to produce a conserving effect, improving the colloidal stability while maintaining good nutritional and sensory qualities. Considering this hypothesis, UHPH could be an alternative technology to those commonly applied in the food industries. For that, a comparative study of UHPH with thermal treatments (pasteurization and UHT) was carried out in this work.

In the first part of this thesis, different UHPH conditions (200 and 300 MPa at 55, 65 and 75ºC of inlet temperature) were performed on soymilk in order to select optimal treatment conditions for producing a good quality product whether intended for refrigeration or long-term storage at room temperature. In this first step, two independent evaluations were performed. On one hand, quality parameters related to chemical, enzymatic, microbiological and colloidal characteristics were evaluated and on the other hand, an inoculation study with different strain spores was carried out in order to determine the inactivation kinetic of the UHPH treatment. Results indicated that treatments at 300 MPa were able to produce soymilk with high chemical and colloidal stability. It is also worth noting that an excellent reduction of bacterial spores was reached applying inlet temperature of 85ºC at the same pressure.

The second part consisted in the shelf-life evaluation of soymilk treated by UHPH using the selected optimal conditions determined in the previous step. As a result, soymilk was obtained with similar characteristics to those produced by pasteurization and with extended shelf-life similar to those obtained by UHT treatments. To achieve this purpose, microbiological aspects, colloidal stability, color changes, chemical parameters and sensory quality were applied to evaluate the overall quality of soymilk and its acceptance by the consumers. Refrigerated soymilk and that produced for an extended shelf-life respectively reached 1 and 6 months of storage in good conditions for consumption and with better quality than those obtained by thermal treatments.

Resumen

El consumo de licuado de soja está experimentado un notable incremento debido a su consideración de producto saludable. El licuado de soja, además de ser una alternativa a la leche de vaca, sobre todo para las personas que poseen alguna intolerancia a los productos derivado de la leche, tiene componentes bioactivos (flavonoides, vitamina E y poliaminas) que pueden contribuir a prevenir algunas dolencias crónicas prevalentes en la sociedad actual.

En este estudio se planteó la utilización de una tecnología emergente, la ultra alta presión de homogenización (UHPH) para la obtención de licuado de soja. Esta tecnología no térmica consiste en la aplicación de presiones de hasta 400 MPa utilizando un sistema de homogenización, especialmente diseñado para producir un efecto conservador, al mismo tiempo que se mejora la estabilidad coloidal y se mantiene la calidad nutricional y sensorial. Con esta hipótesis de partida, la UHPH podría ser una tecnología alternativa a las comúnmente aplicadas a nivel industrial. Por ello, en el planteamiento de este trabajo se incluyó el estudio comparativo de la UHPH con los tratamientos térmicos de pasteurización y UHT.

En la primera parte de esta tesis se llevaron a cabo diferentes tratamientos UHPH (200 y 300 MPa con temperaturas de entrada de 55, 65 y 75ºC) con la finalidad de seleccionar las condiciones óptimas para obtener productos de buena calidad, tanto de almacenamiento en refrigeración, como de larga duración de almacenamiento a temperatura ambiente. El estudio se realizó a dos niveles independientes. Por una parte se evaluaron parámetros característicos de la calidad química, coloidal, enzimática y microbiológica de licuados de consumo habitual, y por la otra, se realizó un estudio con licuados inoculados con diversas cepas microbianas para conocer su cinética de destrucción frente a tratamientos UHPH. De estos estudios se concluyó que los tratamientos a 300 MPa produjeron licuado de soja con muy buena estabilidad coloidal y química y que, aplicando una temperatura de entrada de 85ºC en combinación con dicha presión, se alcanzó una excelente reducción de las esporas bacterianas.

La segunda parte del trabajo consistió en el estudio de la evolución durante el almacenamiento de los licuados UHPH tratados en las condiciones óptimas seleccionadas del estudio previo. De este modo, se obtuvieron tanto licuados frescos similares a los pasteurizados, como licuados de larga duración, similares a los tratados por UHT. Para lograr este propósito, se evaluaron una serie de aspectos, tales como microbiológicos, estabilidad coloidal, cambios de color, parámetros químicos y sensoriales que permitieron evaluar la calidad global de los productos de soja, así como su aceptación por los consumidores. Los licuados de soja fresco y de larga duración alcanzaron respectivamente 1 y 6 meses de caducidad con mejor calidad que aquellos tratados térmicamente.

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Abbreviations key

ANS	8-Anilino-1-naphtalene sulfonic acid
ANOVA	Analysis of variance
cm	centimeters
cfu	Colony forming unit
et al.	et alter (and others)
GLM	Generalized linear model
g	gram
g	Centrifugal force
GC	Gas chromatography
h	hour
kV	kilovolts
L*	Luminosity
L	Litter
LOX	Lipoxygenase
M	Molarity
m	metter
meq	milliequivalent
min	minute
mg	milligram
mL	milliliter
mm	millimeter
mM	millimolar
MPa	Megapascal
MS	Mass spectra
nm	nanometer
o/w	oil-in-water
Pa	Pascal
PCA	Principal component analysis
PCR	Polymerase chain reaction
psi	Pound per square inch
RHHTC	Rapid hydration hydrothermal cooking
s	second

SD	Standard deviation
SPME	Solid phase microextraction
TEM	Transmission electron microscopy
TI	Trypsin Inhibitor
UHPH	Ultra high-pressure homogenization
UHT	Ultra high temperature
w/w	weight/weight
ΔB	Backscattering difference
ΔE	Color difference
μg	microgram
μL	microlliter
μm	micrometer

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Chapter 1

Background, objectives and working plan

1

Background, objectives and working plan

1.1 Background

For more than 2000 years people throughout East Asia have consumed soybeans in the form of traditional foods, such as nimame (cooked whole soy), soy sauce, tofu and soymilk (Fukushima, 2001). Historical and geographical evidence indicates that the soybean first emerged as a domestic crop in the eastern half of North China (Hymowitz, 1970; Liu, 1999).

The soybean (*Glicine max* L.*)* remained exclusive to the Orient for many centuries. Its first introduction into Europe was about 1712. However, due to poor climate and soil conditions, soybean production has been limited in Europe (Liu, 1999). In 2008, the European Union produced less than 1% of total world production (USDA, 2012).

In western countries, the soybean first drew attention in the 1960s as an economical and high quality vegetable protein source for humans. The part of the seed with greatest interest is formed by cotyledons (90%) with around 20% of lipids and 40% of protein in dry matter (Liu, 1999).

World production of soybeans has increased substantially in the last 50 years; from 13 million tons in 1939 to 265 million tons in 2011 (Kwok & Niranjan 1995; USDA, 2012). Nowadays, western countries have become the main soybean producer and exporter, whereas eastern countries have become the main soybean importer. Data on world production and trade are shown in Table 1-1.

Table 1-1. Soybeans trade and production (millions tons), 2010 – 2011[1].

	Production	Export	Import
World	264.69	88.85	92.64
United States	90.61	40.86	0.39
Brazil	75.50	29.95	0.04
Argentina	49.00	9.21	0.01
China	15.10	0.19	52.34
European Union	1.04	0.06	12.48
Japan	0.22	0.00	2.92

[1] World imports and exports may not balance due to differences in local marketing years and to time lags between reported exports and imports (USDA, 2012).

Of the total soybean importations of European Union in 2010, 12.7% correspond to France, 13.0% to Spain, 14.2% to the United Kingdom, 21.0% to the Netherland and 35.4% to Portugal. Total production of Spain in 2009 was 2767 tons, with Extremadura as the greatest producer with 68% of the total and Catalonia was responsible for 1.5% (MAPA, 2010).

In 2009, consumption of soy foods was around 30.1 million tons and predicted to reach around 33.3 million tons in 2012 (USDA, 2012). The increased soy consumption in the world and in Europe is probably related to the high content of lipids and proteins. These components in combination with others of minor content (lecithin for example) give soybeans a great applicability in the food industry. The interest in soybeans is not only based on nutritional and biological quality but also on the functional properties of components such as gel formation, emulsification, thermal stability, water and fat absorption and sparkling (Utsumi et al., 1997; Fukushima, 2001). Table 1-2 shows a complete list of these and other properties.

In addition to the industrial applications, there is an extensive bibliography about nutritive or health properties of soy components. The most relevant effects are, reduction in cholesterol levels (Potter et al., 1993; Carroll & Kurowska 1995), mitigation of menopause and osteoporosis symptoms, and reduction in risk of heart diseases and cancer (Khatib et

al., 2002; Huang et al., 2006; Rochfort & Panozzo, 2007). However, there is a controversial opinion in the scientific community. The possible health benefits have not been evidenced according to reports published by EFSA (2010) and EFSA (2011).

Table 1-2. Functional properties of soybeans in food systems (Wolf, 1970).

Functional property	Food systems
Emulsification	
Formation	Sausages, frankfurters, bologna, breads, cakes and soups
Stabilization	Frozen desserts, sausages, frankfurter, bologna and soups
Fat absorption	
Promotion	Sausages, frankfurter, bologna and meat patties
Prevention	Doughnuts, pancakes
Water absorption	
Uptake	Breads, cakes, macaroni and confections
Retention	Breads and cakes
Texture	
Viscosity and gelation	Soups, gravies, chili and simulated ground meats
Shred and fiber formation	Simulated meats
Dough and film formation	Baked goods, frankfurter and bologna
Adhesion	Sausages, lunch meats, meat loaves, hams, meats dehydrated
Cohesion	Baked goods, macaroni and simulated meats
Elasticity	Baked goods and simulated meats
Color control	
Bleaching	Brads
Browning	Breads, pancakes, waffles
Aeration	Confections

According to FAO, of all soy products soymilk was the product which has most grown in consumption (FAO, 2002). Despite the consumption rise in western countries in the last decade, soymilk has been somewhat restricted mainly because of its typical beany flavor (Yuan & Chang, 2007; Achouri et al., 2008), and finding alternative processing methods that can reduce soymilk off-flavors has become a challenge to the food industry.

Consumer opinion is a key element for the development and modernization of the industrial process, and taste is fundamental for the acceptance of products introduced into market, especially culturally different products, such as soymilk.

Consumers demand for safe food products, which are environmental friendly and which exhibit high nutritional quality has abruptly increased in recent decades. Thus, it is a challenge for the food industry to adapt the industrial processes, and to search and test for alternative technologies which improve the organoleptic quality of the products while preserving nutritional properties and reducing losses and energy costs.

This tendency impacts directly on traditional technologies, such as heat treatments, so that lately non-thermal technologies, such as pulsed electric field and oscillating magnetic field (Deeth & Datta, 2002), irradiation, ultrasonication, centrifugation, microfiltration and hydrostatic high pressure (HHP) (Datta & Deeth, 2002ab; Diels et al., 2005) have been investigated and developed.

In the case of soymilk, heat treatments cause undesirable chemical changes which may lead to the destruction of amino acids and vitamins, browning reaction, development of cooked flavor (Kwok & Niranjan, 1995) and negative effects on solubility and water absorption (Zhang et al., 2005).

Several studies applying non-thermal technologies, such as hydrostatic high pressure (HHP) were carried out on soymilk. For instance, Zhang et al. (2005) studied HHP effects on soymilk proteins and Jung et al. (2008) investigated on isoflavone profiles. However, HHP technology is a discontinuous process which is not fully adapted to the needs of soymilk processing. In the last decade, ultra-high pressure homogenization (UHPH) has been atracting certain interest for application in liquid foods since it is a non-thermal continuous process which may improve several aspects of the overall quality of such product.

The *Centre Especial de Recerca Planta de Tecnologia dels Aliments* (CERPTA) of the *Universitat Autònoma de Barcelona*, has been working with UHPH technology since 2000 and has participated in several Spanish and European projects. Some results for soymilk showed that UHPH treatment was efficient in reducing microbial populations, improved

physical stability and presented high digestibility, similar to heat treatment (Cruz, 2008). These preliminary results raised expectations for UHPH as a possible alternative to the conventional heating processing in soymilk production. UHT has some detrimental effects on the nutritive and sensory quality of soymilk, in addition to loss of colloidal stability during storage, and UHPH has a great potential to produce commercial soymilk with improved overall characteristics. According to this hypothesis, the following objectives were proposed.

1.2 Objectives

This thesis is framed into the national project (AGL2008-05430) named "Application of ultra high pressure homogenization on the elaboration of high quality vegetables milks (soymilk and almond milk)". In addition, part of this study was included in the European project (FP7/2007-2013-SME-232603) called "Study of functionality, nutritional and safety aspects of liquid foods, liquid food preparations and cosmetics processed by ultra high pressure homogenization".

General objective

The general objective of this thesis was to study the effects of applying UHPH technology to soymilk production as an alternative to conventional heat treatments such as pasteurization and UHT, in order to obtain a high quality soymilk with an extended shelf-life.

Specific objectives

- To develop an optimized method at pilot plant level for soymilk elaboration with high standards of chemical composition and good production yield.
- To study the influence of combining temperature and pressure at different levels for UHPH treatments and their influence on soymilk quality parameters. Then, selecting the best conditions for obtaining pasteurized soymilk (to be stored in refrigeration) and long storage soymilk (room temperature).
- To identify the potential spoilage-related microbiota in soymilk that may be resistant to UHPH treatments and to study the influence of combining temperature and pressure in the kinetic of inactivation.

- To study the evolution during storage of soymilks selected previously: stored at cold temperatures and long term aseptically packaged stored at ambient temperature.
- To study the aroma profile of untreated and treated soymilks (pasteurized, UHT and UHPH), and its evolution during storage.

1.3 Working plan

A preliminary study was carried out in order to obtain a standard method of soymilk elaboration with high quality characteristics and yield, using the infrastructure at the UAB pilot plant (Figure 1-1).

The next step was to apply UHPH treatments on soymilk base product (BP). Samples were subject to 200 MPa and 300 MPa of pressure combined with 55ºC, 65ºC and 75ºC of inlet temperature (Figure 1-1). Microbiological, physico-chemical and biochemical quality parameters were evaluated.

The third step included the inactivation study of spores of *Bacillus.* After isolation and identification of some spore-forming bacteria from original soymilk treated at 300 MPa and 65ºC of inlet temperature, two bacterial strains were selected (*Bacillus cereus* and *Paenibacillus taichungensis*) to study the kinetic of inactivation of inoculated soymilk. UHPH conditions applied were 300 MPa at 55ºC, 65ºC, 75ºC and 85ºC inlet temperatures (Figure 1-2).

For the fourth step, and taking into account the previous results, UHPH conditions of 200 MPa at 55ºC and 75ºC of inlet temperature were selected to produce a soymilk to be stored under refrigeration conditions, for achieving similar or better quality characteristics than pasteurized soymilk. Microbiological, chemical and physical changes were studied for 28 days of storage at 4ºC (Figure 1-2). Moreover, volatile profile at day 1 and day 28 and sensory analysis at day 15 were also evaluated.

Finally, the last step was performed in order to obtain a product with similar or better characteristics than UHT soymilk. Soymilk BP was treated by UHPH (300 MPa, 80ºC) and UHT with subsequent aseptic packaging in both treatments. Microbiological, chemical and physical changes were studied for 180 days of storage at room temperature (Figure 1-2).

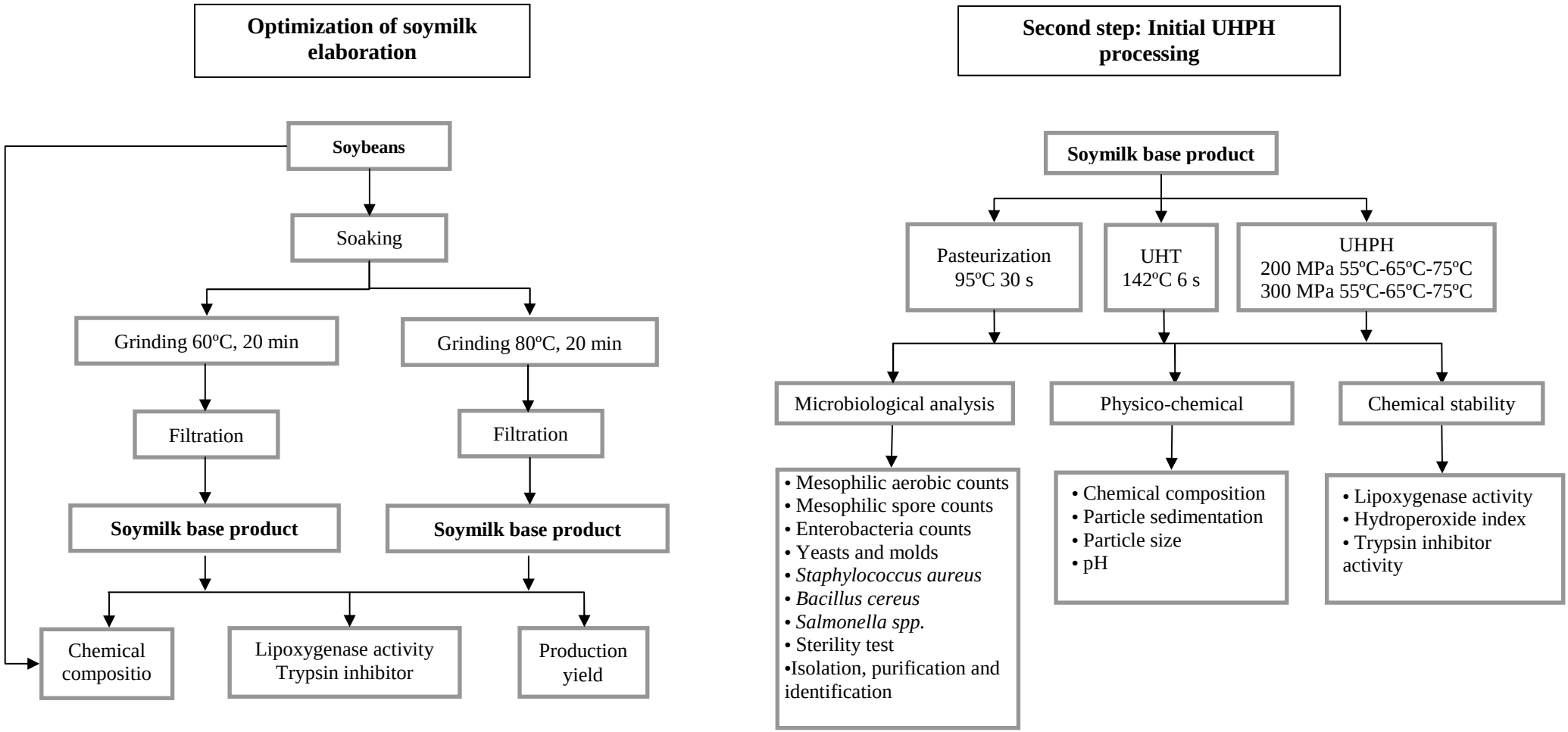

Figure 1-1. Working plan: previous study and second step.

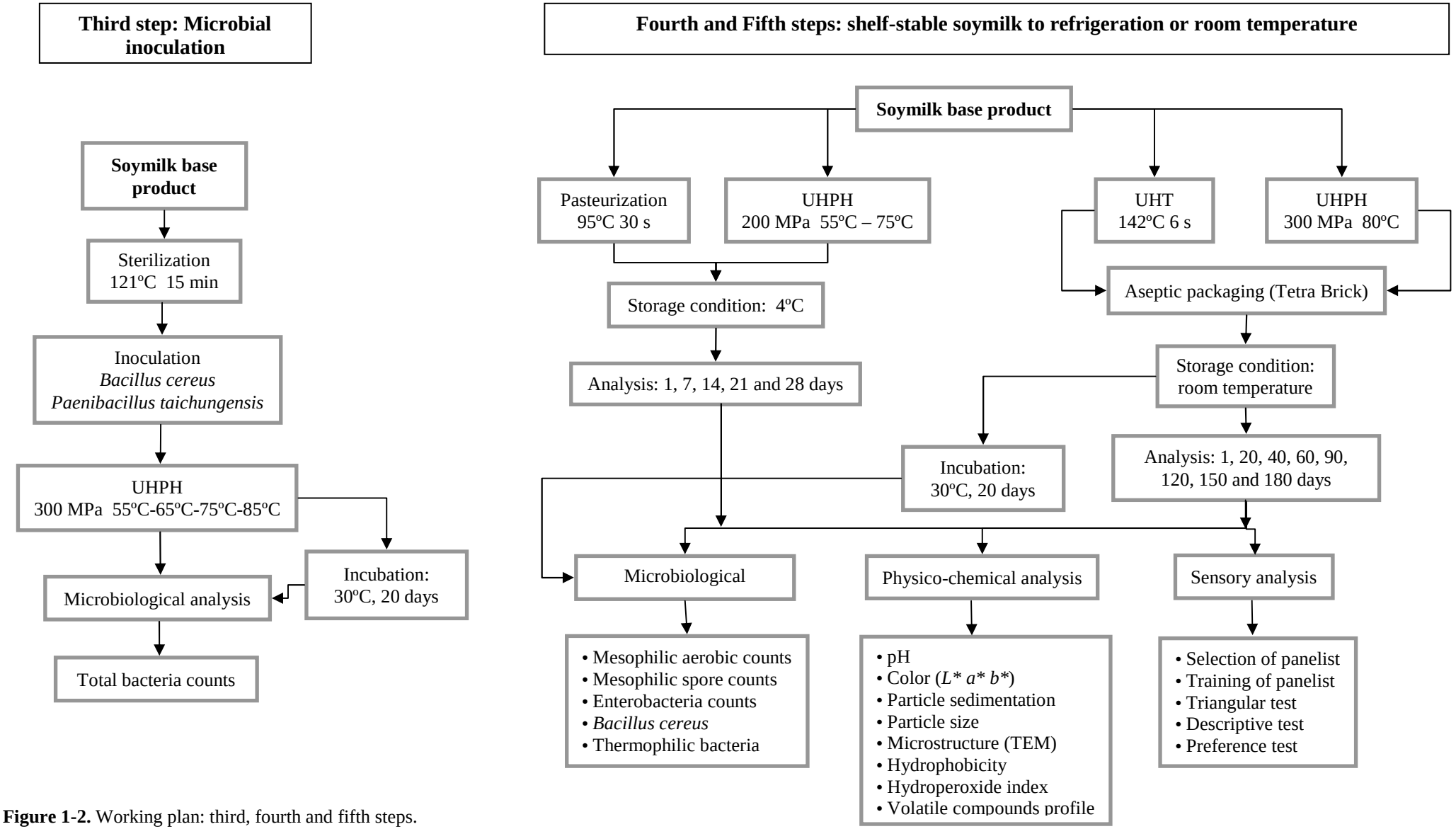

Figure 1-2. Working plan: third, fourth and fifth steps.

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Chapter 2

Introduction

2

Introduction

2.1 Soybeans

2.1.1 Definition

Soy plant is botanically denominated as *Glicyne max* L. *Glicyne* is a greek word meaning "sweet" and it applies to all the groundnut species of legumes. The word *max* means "large", referring to the large nodules of the soybean plant (Liu, 1999). Mature soybeans are nearly spherical in shape, but may change considerably according to cultivar and growing conditions. Soybean seed consists in three major parts: seed coat or *hull*, *cotyledons*, and *germ* or *hypocotyl*. The seed coat contains *hilum*, which is the point of attachment to the pod (Perkins, 1995).

2.1.2 Proximate composition

Protein and fat content of whole soybeans are about 40% and 20% (dry basis), respectively. Remaining dry matter is composed mainly of carbohydrates (35%) and ash (about 5%). Water content of stored mature soybean is usually about 13% (Liu, 1999). In addition, important components such as isoflavones and saponins that have apparently valuable health effects are present in the seed (Perkins, 1995; Huang et al., 2006a; Rochfort & Panozzo, 2007; Jung et al., 2008).

The most abundant components in soybeans are proteins. About 40 to 90% of them are present as storage proteins. Because of this, some researchers have suggested that soybeans should be called protein seeds rather than oilseeds (Liu, 1999). Due to the great content of proteins, soybean is used in many industrial processes that include protein isolation and concentration, such as infant formulas and soy foods (tofu and soymilk for example) (Friedman & Brandon, 2001).

According to biological functions, soy proteins can be characterized as metabolic proteins or storage proteins. Based on solubility, proteins are divided into albumins and globulins (Liu, 1999). Globulins are the major and the most important soy protein, being soluble in water or diluted salt solutions at pH values above or below isoelectric point (Wolf, 1970).

A precise technique to identify protein is based in approximate sedimentation coefficients by using ultracentrifugation to separate proteins of the seed (Thanh et al., 1975; Howard et al., 1983). After ultracentrifugation and under appropriate conditions, soy globulin exhibits four fractions, named 2S, 7S, 11S and 15S. Analysis of these fractions has shown that 11S and 15S fractions are pure proteins. In particular, 11S fraction corresponds to soybean glycinin and accounts for at least 33% of extractable protein, whereas 15S fraction is thought to be a polymer of glycinin and accounts for 10%. 2S fraction represents 20% of the extractable protein and includes trypsin inhibitor and cytochrome C, whereas 7S fraction represents an additional third of the extractable protein and consists of β-conglycinin, α-amylase, lipoxygenase, and hemagglutinin (Nielsen, 1985).

An important chemical property of soy protein which determines protein nutritional value is its amino acid composition. Table 2-1 lists essential amino acid composition of soybean.

Table 2-1. Soybean amino acid composition**.**

Amino acid	mg/g protein (dry matter basis)		
	(Fukushima, 1991)	(Friedman, 1996)	(USDA, 2003)
histidine	27.0	25.4	27.51
isoleucine	48.0	47.1	49.42
leucine	78.0	85.1	82.97
lysine	61.0	63.4	67.85
methionine + cysteine	65.0	68.1	-
phenylalanine + tyrosine	90.0	96.6	91.80
tryptophan	13.0	11.4	14.82
threonine	35.0	38.4	44.28
valine	48.0	49.1	50.88

The amino acids above mentioned are considered essential amino acids, and cysteine and tyrosine are considered conditionally essentials. These amino acids are responsible for promoting growth in young people, preventing diseases and maintaining a positive nitrogen balance during youth and old age (Laidlaw & Kopple, 1987). In general, soybeans

contain all essential amino acids required for human or animal nutrition, paying attention to lysine by its valuable content compared to other cereal proteins and by its important role in the growth of young children (Liu, 1999).

Lipid composition of soybean is formed by 90% of triglycerides, 7% of phospholipids and 3% of glico-lipids. Fatty acids are distributed in saturated (15%) and unsaturated (85%) (USDA, 2003). Linoleic acid is the most abundant fatty acid and represents about half of the total amount, although oleic and linolenic acids are also in a considerable quantity (Sangwan et al., 1986; Liu, 1999). Table 2-2 shows fatty acid composition of soybeans from three different sources. Among of soy lipids, phospholipids, commonly known as "lecithin" have significant commercial value as emulsifying agent in food industry. Additionally, wetting and colloidal and antioxidant are also important properties of soy lecithin.

Table 2-2. Main soybean fatty acids composition.

Fatty acid	Percent (%)		
	(Sangwan et al., 1986)	(Liu, 1999)	(USDA, 2003)
Saturated (Total)			
Palmitic (C16:0)	9.3–17.4	8–17	11.5
Stearic (C18:0)	2.2–7.0	3–30	3.90
Unsaturated (total)			
Oleic (C18:1)	15.2–29.6	25–60	23.6
Linoleic (C18:2)	33.8–59.6	25–60	53.9
Linolenic (C18:3)	4.3–15.0	2–15	7.20

Carbohydrate content in soybeans is about 35% on dry basis, making them the second largest component in the seeds. However, the economical value of soy carbohydrates is considered much less important than soy protein and oil (Liu, 1999). They are divided in monosaccharides, such as glucose and arabinose, and oligosaccharides known also as α-galactosides of sucrose, including raffinose and stachyose. These saccharides are included in the category of soluble carbohydrates and the composition of some of them ranges from 2.5 to 8.2% for sucrose, from 1.4 to 4.1% for stachyose and from 0.1 to 0.9% for raffinose.

In the category of insoluble carbohydrates include hemicelluloses, pectin and cellulose, containing respectively 50, 30 and 20% in the soy cell walls (Liu, 1999). Among of soluble carbohydrates, raffinose and stachyose receive more attention because of their flatulence and abdominal discomfort associated with consumption of soybeans and soy products. The lack of enzymes in human intestinal tract to hydrolyze galactosidic linkages of raffinose and stachyose into simple sugars, allows natural bacteria to metabolize intact glucide molecules and thus generate unpleasant flatus feeling (Calloway et al., 1971; Hymowitz et al., 1972; Knudsen & Li, 1991; Perkins, 1995; Wilcox & Shibles, 2001).

Despite the presence of oligosaccharides in soybeans and soy products, generally considered undesirable in terms of flatus activity, some studies have shown beneficial effects of oligosaccharides inclusion in the human diet. The main benefit reported was the growth stimulation of *Bifidobacteria* population in the intestinal tract preventing constipation problems and helping in the production of vitamins (Gibson & Roberfroid, 1995; Martínez et al., 2005).

Major minerals present in total ash (about 5%) of soybeans are potassium (1800 mg/100g), phosphorus (700 mg/100g), magnesium (280 mg/100g) and calcium (275 mg/100g). Minor minerals include iron, zinc, arsenic and selenium. Water-soluble vitamins content include thiamin, riboflavin, niacin, pantothenic acid and folic acid and on the other hand, oil-soluble vitamins are A, E and K (USDA, 2003).

Another minor component, ranged from 0.3 to 0.8% (dry matter basis) and has converted soybean extensively known are isoflavones (Jung et al., 2008). These components are a subclass of flavonoids and belong to the group of phytochemicals (Craig, 1997) and seem to act as phytoestrogens in human metabolism. According to data from several studies, isoflavones are believed to potentiate the decrease of cholesterol levels, to prevent both prostate and breast cancers, to attenuate bone loss in postmenopausal women and to alleviate menopausal symptoms (Jenkins et al., 2002; Achouri et al., 2005; Huang et al., 2006a; Jung et al., 2008; Aparicio et al., 2008). On the other hand, these health benefits have not been established by the scientific community of European Union (EFSA, 2010; EFSA, 2011).

In spite of apparent health benefits of soybeans consume, substances with antinutritional properties known as trypsin inhibitors are included in the composition. These substances have a proteolytic activity which reduces the availability of trypsin, an important protease in the animal digestive function (Friedman & Brandon, 2001). Two types of trypsin

inhibitors are present in soybeans: Kunitz trypsin inhibitor and Bowman-Birk (BB) inhibitor. These protein inhibitors (TI) have strong affinity for human digestive enzymes; interfere in the digestion and absorption of proteins and may cause pancreatic enlargement involving hypertrophy followed by hyperplasia of the exocrine cells by the production of trypsin, chymotrypsin, elastase, amylase and serine proteases (Gallaher & Schneeman, 1986; Weder, 1986; Liener et al., 1988; Kwok & Niranjan, 1995). On the other hand, some authors have reported that soybean BB inhibitor may contribute to the prevention of cancer because of its anticarcinogenic and cancer chemopreventive properties (Kennedy, 1998; Sessa & Wolf, 2001; Akoum et al., 2006).

To avoid potential deficiency in nutrient absorption, both inhibitors should reach high levels of inactivation. However they are rather heat stable due to the presence of disulphide bonds in their molecular structure. Heat treatment during long time, such as 60 to 70 min at 93ºC or 5 to 10 min at 121ºC are required to reach 90% of inactivation. However, overheating in order to remove completely trypsin inhibitor activity reduces nutritive value of soybeans and results in amino acid degradation and other deteriorative reactions (Kwok et al., 2002).

Additionally to the inactivation of trypsin inhibitors, lipoxygenase (LOX), an important enzyme which act as catalyst in lipid oxidation, should be inactivated as much as possible in all soy foods (Van der Ven et al., 2005; Min et al., 2005). LOX (linoleate oxygen redutase; EC 1.13.11.12) is a non-heme and non-sulfur iron containing dioxygenase which catalyses the oxidation of 1,4-*cis,cis*-pentadiene to pentadienyl, which upon abstraction of hydrogen, results in a pentadienyl radical intermediate. Pentadienyl radical may react with oxygen to form peroxyl radical isomers and fatty acid hydroperoxides. Hydroperoxides as well as decomposed products are potentially reactive substances that may cause deterioration of food proteins and formation of volatile compounds, such as aldehydes and ketones in presence of oxygen, light, enzymes and high temperatures (Kumar et al., 2003; Huang et al., 2006b; Ying-Qiu et al., 2008). For soy food applications, soymilk for example, LOX inactivation is commonly achieved by thermal treatments. Studies showed that heat treatment during 10 min from 80 to 100ºC were effective in its inactivation (Kwok & Niranjan,1995; Wang et al., 2008).

2.2 Soymilk

2.2.1 Definition and composition

Soymilk has been consumed in China since 2000 years ago. Since then, the soymilk consume was higher in China and Asian countries than milk in western countries. Furthermore, soymilk is an intermediate for preparing soy foods, such as tofu (Kwok et al., 1993; Liu, 1999; Wang et al., 2001).

Soymilk is an aqueous extract of soybeans. It is a fine emulsion closely resembling to milk in appearance and composition, in addition to be lactose free. Table 2-3 shows chemical composition of typical soymilk from different authors.

Table 2-3. Chemical composition of soymilk[1]

Reference	Moisture	Protein	Fat	Carbohydrate	Ash
Iwuoha & Umunnakwe (1997)	91.6	3.1	1.82	2.33	0.94
Liu (1999)	90.8	3.6	2.00	2.90	0.50
Wang et al. (2001)[2]	85.7	2.2	1.60	10.50	
Cruz et al. (2007)	91.7	3.8	1.86	0.68	0.68

[1] Mean values expressed as g/100g (w/w).

[2] Carbohydrates + ash.

In addition to chemical composition, quality parameters of fresh soymilk are: pH 6.60, titratable acidity about 2.7%, viscosity 38.0 Pa·s and specific gravity of 1.055 according to Iwuoha and Umunnakwe (1997).

Soymilk is a good source of vitamins. Contains about 7.36 mg/100mL of riboflavin (B_2), 0.33 mg/100mL of thiamin (B_1) (dry basis) (Hou et al., 2000) and pyridoxide (B_6) and folic acid (Kwok & Niranjan, 1995). Soymilk minerals are potassium (131.32 mg/100mL), magnesium (22.43 mg/100mL), sodium (2.86 mg/100mL) (Achouri et al., 2007), phosphorus (38.87 mg/100mL) and calcium (18.34 mg/100mL) (Achouri et al., 2008). Thanks to rich composition, soymilk compares favorably to human milk (Liu, 1999).

2.2.2 Soymilk processing

Traditional method of soymilk processing consists basically of the following steps: whole soybeans are soaked in water for 8-12 hours or overnight, then are washed and ground with water at a water:bean ratio between 8:1 and 10:1, depending on the desired final concentration. Slurry is then filtered through a cloth to remove the insoluble residue, known as “soy pulp” or “okara”, and then the filtrate is cooked for about 30 minutes (Johnson et al., 1983; Liu, 1999; Prawiradjaja, 2003).

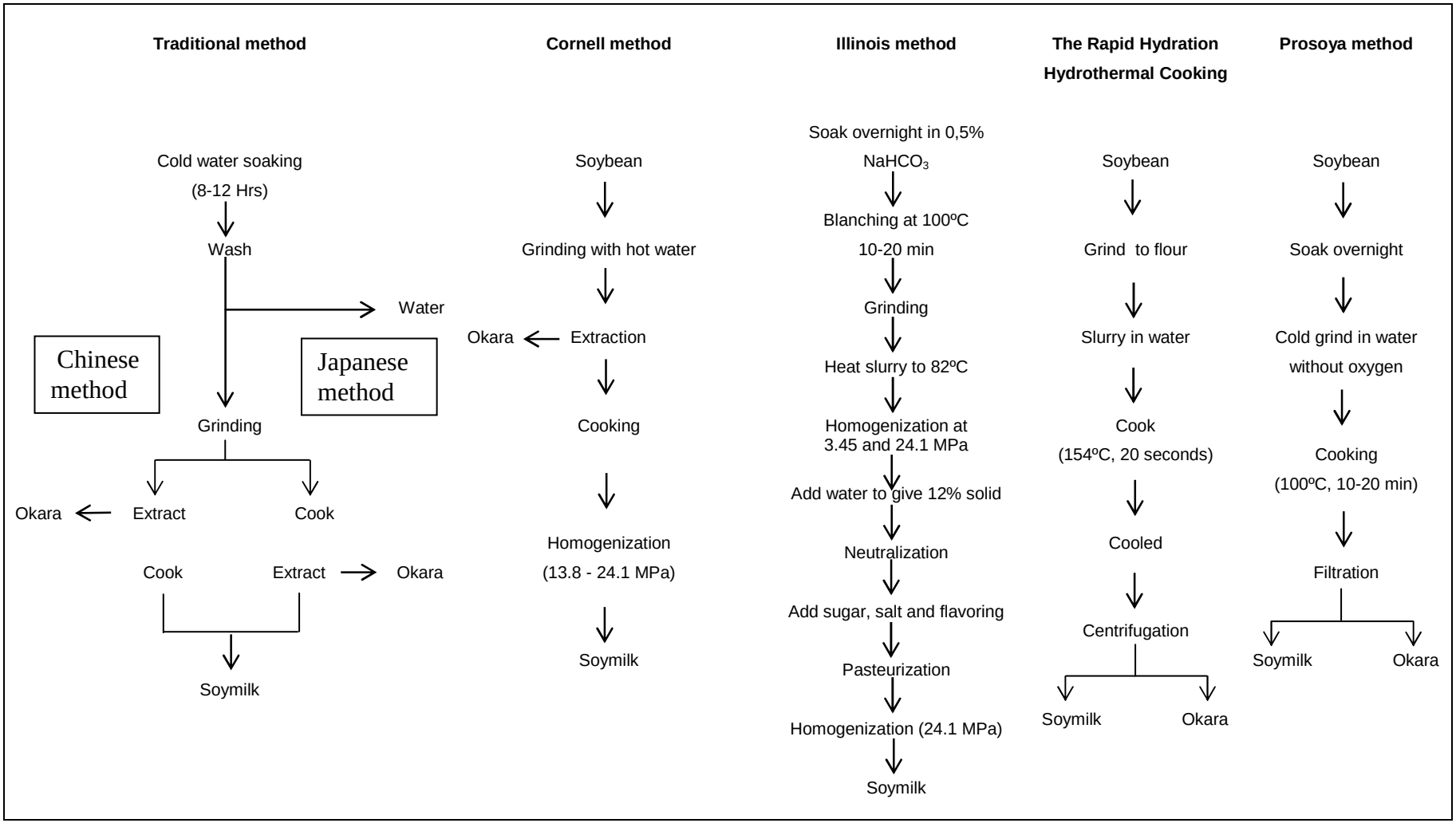
There are different industrial methods for soymilk production. The following processes showed in Figure 2-1, represent the traditional method and important developments and improvements in soymilk processing. Examples of these methods are Cornell, Illinois, Rapid Hydration Hydrothermal Cooking (RHHTC), cold-grind under vacuum (Prosoya), deodorization, and antioxidant and alkali treatment methods. Soymilk process can use a combination of these methods to produce a nutritive soymilk with high yields in solids and protein recovery (Golbitz, 1995; Liu, 1999; Prawiradjaja, 2003).

Both Illinois and RHHTC methods incorporate all of the soybean parts into the soymilk due to high shear generated in the process. This particularity results in a soymilk with high percentage of solids (86-89% w/w) and protein recovery (90-93% w/w). Prosoya and Cornell methods are modifications of the traditional methods. Both methods reach low percentage of solids (55-65% w/w) and protein recovery (70-80% w/w) (Golbitz, 1995; Kwok & Niranjan, 1995).

Even though some process modifications have improved soymilk quality, each method has its own advantages and disadvantages. RHHTC for example, applies temperature around 154ºC during soymilk processing (Figure 2-1). At this temperature chemical changes may occurs favoring formation of volatile compounds, which are responsible for undesirable off-flavors and color changes. The rest of the methods, applies moderate temperatures (about 100ºC), which may ensure microbial quality but at short shelf-life. In addition, controlling process parameters such as time, temperature and water-to-bean ratio have been suggested to improve off-flavors of soymilk (Achouri et al., 2008). On the other hand, choosing the right genetic variety of soybeans may have an important role affecting quality of finished product (Deman et al., 1975).

Three basic heat treatments are carried out to extend shelf-life of soymilk: pasteurization, sterilization and ultra high temperature (UHT). Pasteurization refers to heat treatment, normally below 100ºC, which is adequate to destroy pathogenic microorganisms. This process also inactivates some enzymes and reduces total microbial counts. Sterilization is a severe heat treatment that requires heating at 121ºC for 15-20 min to achieve sterility. UHT process involves high temperatures (135-150ºC) for short time (a few seconds) to achieve a commercially sterile product (Kwok & Niranjan, 1995).

Figure 2-1. Soymilk processes.



Source. (Johnson et al., 1983; Gupta & Gupta, 1988; Golbitz ,1995; Kwok & Niranjan, 1995; Liu, 1999; Prawiradjaja, 2003).

Consumption of soymilk in western countries has been limited partially by natural soymilk off-flavors. These unpleasant soymilk characteristics are primarily derived from enzymatic action which catalyzes the oxidation of polyunsaturated fatty acids, such as linoleic and linolenic acids. Oxidation reactions initiate mainly in the soaking and grinding steps of soymilk processing, when lipoxygenase is still active (Mizutani & Hashimoto, 2004). To avoid potential deterioration problems in soymilk as a consequence of oxidation reactions, studies applying heat treatments have reached great levels of lipoxygenase inactivation. For instance, Gupta and Gupta (1988) achieved complete LOX inactivation applying 80ºC for 10 min in the grinding step of soymilk elaboration. Other studies suggested that adding extra ingredients such as antioxidants, sodium bicarbonate or masking agents would improve soymilk flavor (Prawiradjaja, 2003).

As described above, oxidation reactions that take place during soymilk elaboration involve the formation of hydroperoxides as primary reaction. These reactive substances originate a high variety of both non-volatile and volatile final products, such as aldehydes, ketones, alcohols, furans and acids. Most compounds of these chemical families are responsible for off-flavors development, such as beany, grassy and rancid flavors, which adversely affect organoleptic quality of soymilk (Torres-Penaranda & Reitmeier, 2001; N'Kouka et al., 2004). In addition, oxidation products can be related to heart diseases, cancer and aging problems. Although thermal treatments inactivate effectively LOX and high inactivation levels of antinutritional factors (trypsin inhibitor activity), they also denature soy proteins resulting in loss of stability and amino acid degradation. Other deteriorative reactions may take place during treatment at high time-temperature, such as color changes and development of cooked flavor. Moreover, nutrients such as vitamins may also be affected by heat treatment (Kwok & Niranjan, 1995; Ying-Qiu et al., 2008). Generally, solids recovery in soymilk extracts decreases as a function of extent and intensity of heat treatment (Johnson et al., 1983).

Soymilk is an excellent growth medium for many microorganisms. High moisture, neutral pH, high amount of nitrogenous compounds, fat, carbohydrates, minerals and vitamins, make soymilk similar to microbial milk spoilage patterns. Microbial patterns of untreated soymilk include mesophilic spores, enterobacteria, total mesophilic bacteria and *Bacillus* gender (Kwok & Niranjan, 1995). At room temperature, untreated soymilk undergoes acid curdling with a rapid drop in pH accompanied by separation of curds and whey. This spoilage usually occurs after standing for 24h out of refrigeration. Proteolytic spoilage may take place within week at refrigeration temperatures of 1ºC (Kwok & Niranjan, 1995; Bai

et al., 1998). Soymilk treated by heat treatments, normally presents great reduction of microbial counts. In general counts are below the detection limit (< 10 cfu/mL) and no coliforms or *Escherichia coli* are found in soymilk products (Bai et al., 1998; Achouri et al., 2007). Bouno et al. (1989) investigated the effect of different heat treatments on the destruction of indigenous *Bacillus* spores in soymilk. Results showed that microbial load was reduced from 3.34 to 1.52 log cfu/mL for boiling soymilk (1 min) in microwave oven and to 1.40 log cfu/mL for steam heated soymilk at 110°C for 20 min. No microorganisms were detected in autoclaved soymilk (121ºC, 15 min). Evidently, sterilization requires a severe heat treatment which practically destroys all microorganisms, including spores.

In addition to mesophilic spore bacteria, thermophilic spore bacteria are extremely heat resistant and could compromises soymilk quality. Processes designed to destroy thermophilic spore bacteria may result in a product overheated obtaining sensory and nutritive characteristics degraded. Thermophilic spores may survive conventional treatments, such as UHT, but their growth and spoilage are affected under room temperature storage conditions (Kwok & Niranjan, 1995).

2.3 Ultra high pressure homogenization

2.3.1 Concept and history

Emulsions are dispersions of phases that consist of two or more liquids largely immiscible. In order to produce emulsions, disperse phase should be distributed in fine divided droplets through the continuous phase, but a surface active of molecules should also be in the interface of the droplet to prevent instantaneous coalescence (Floury et al., 2000).

Homogenization was first presented by August Gaulin at Paris in 1900 and was since then largely used in the industries to disperse non-miscible phases, stabilize emulsion, or prepare products with appropriate rheological properties (Thiebaud et al., 2003; Hayes & Kelly, 2003b). The basic homogenizer design consists of positive-displacement pump coupled to a pressure intensifier forcing the fluid to pass through the homogenization valve (Middelberg, 1995). In any type of homogenizer valve, the fluid flow under pressure through a convergent section called the homogenization gap and then expands (Figure 2-2). As a result, a combination of mechanical forces takes place producing disruption of the droplets. The operating pressure is controlled adjusting the distance between valve and seat (Floury et al., 2004b).

The best known food application is probably the homogenization of milk. Usually, the process is performed between 60 and 70ºC and involves breaking milk fat globules into fine fat droplets, preventing cream separation, thereby increasing stability and shelf-life of milk (Diels et al., 2005; Zamora et al., 2007). In the classical design (Figure 2-2), the fluid is fed axially into the valve seat, and then accelerated radially into the small gap between valve and seat. Once the fluid leaves the gap, it becomes a radial jet that stagnates on an impact ring before leaving the homogenizer at atmospheric pressure (Kleinig & Middelberg, 1996; Kelly & Muske, 2004). APV-Gaulin design is often used in dairy industry, where at large-scale processes two homogenization valves (two-stage) are used under moderate pressures (70-100 MPa) (Thiebaud et al., 2003; Pereda et al., 2007).

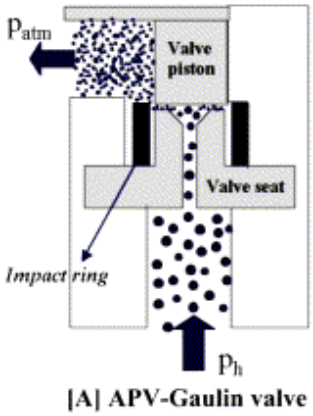


Figure 2-2. Valve design of a conventional homogenizer (APV-Gaulin) obtained from Floury et al. (2004b).

In the last 10 years, studies about homogenization technology have evolved primarily by the increase demand of products with nutritive quality, long shelf-life and high colloidal stability. Thanks to the advances in material science, improvements in the homogenizer designs allowed increasing the pressure level of working, leading to products of high quality. Especial designs, very small dimensions and material changes are required to withstand very high stresses during treatment (Pandolf & Kinney, 1998). Since then, UHPH is largely used in the chemical, pharmaceutical and biotechnological industries to emulsify, disperse, mix and process products.

2.3.2 High-pressure homogenization equipments

There are different types of high pressure homogenization equipments, from prototypes to industrial scale: MICROFLUIDIZER (Microfluidics International Corporation, USA), NANOJET (Haskel, USA), EMULSIFLEX (Avestin, Canada) and STANSTED (Stansted Fluid Power Ltd., UK) are equipments which achieve pressures higher than 200 MPa, being known as UHPH equipments.

Microfluidizer™ (Microfluidics International Corporation)

This technology reported by (Paquin & Giasson, 1989) was introduced in the food industry in 1987. The system is divided into two micro-channels and then recombined in a reacting chamber where jets of fluid collide at high velocity (up to 400 m/s), dissipating energy instantaneously at the point of impact (Figure 2-3). The reaction chamber is static and contains no moving parts. The limited aspect of this technology is the pressure delivered by the equipment, which is linked to the flow and equipment design (Middelberg, 1995; Paquin, 1999; Geciova et al., 2002). There are industrial scale equipments (2000 L/h) using this technology able to work at 270 MPa pressure.

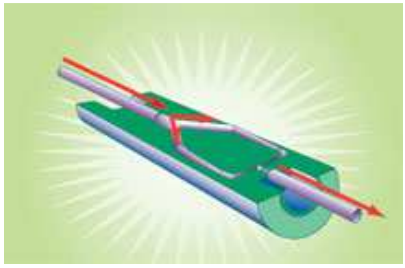


Figure 2-3. Schematic view of the Microfluidics International Corporation, obtained from http://www.iesmat.com/Productos-MFL-LAB-M-110P.htm

Emulsiflex™ (Avestin)

This technology has been applied for pharmaceutical emulsions, liposomes and dispersions. The reaction chambers are coated with ceramic material reaching pressures up to 220 MPa. Valve adjustment is achieved using a micrometer screw allowing a fine adjustment of the gap between valve seat and head. This mechanism is very critical at high

pressures (Paquin, 1999). The process has a constant flow rate of 3 L/h and is able to work at small sample volume (10 mL).

Stansted technology (Stansted Fluid Power Ltd.)

The design of Stansted homogenization valve is made from ceramic material that allows to withstand ultra high pressure levels. Moreover, geometry of the valve has been modified compared to classical design of APV-Gaulin (Figure 2-2). In the Stansted valve (Figure 2-4) the flow directions through the valve are reversed, the fluid is first fed axially at high pressure along the mobile part of the valve and flows with high velocity through the narrow gap between the valve and valve seat. The size of the gap and the resulting velocity of the fluid generated by the high pressure depend on the force acting in the valve piston, which can be adjusted to regulate the homogenizing intensity. The pressure drop of the fluid in the valve is called the homogenization pressure (Floury et al., 2004ab). The maximum pressures reached by Stansted valve (400 MPa) are due to the narrow gap (2-5 µm *vs* 10-30 µm) (Floury et al., 2004a).

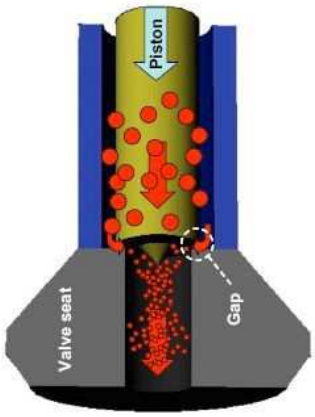


Figure 2-4. Schematic view of the Stansted high pressure homogenization valve obtained from Donsì et al. (2009a).

Although this configuration may look simple, the fluid dynamics involved is quite complex. According to Floury et al. (2000), intense energy changes occur in the homogenization valve, causing strong turbulent flow, cavitation and shear phenomena.

Studies provide some evidence supporting that shear phenomenon is one of the primary mechanisms of cell or particle disruption during UHPH treatment (Middelberg, 1995; Kleinig & Middelberg, 1998). Disruption is in fact obtained in the narrow valve gap when deformation beyond a critical level is induced by the intense shear forces and elongational flow caused by the restriction between the piston and the seat of the valve (Figure 2-4). Cavitation is the formation of cavities due to the local vaporization of the fluid under conditions of pressure lower than its vapor pressure. When cavities flowing within the liquid through the system find a region of high pressure, they collapse violently, causing vibrations with a disruption effect (Diels et al., 2005). Turbulence occurs when a fluid flows at high speed over a surface. Due to surface roughness, above a certain velocity the fluid streamlines no longer follow the shape of the surface, but deviate from the surface, resulting in the formation of vortices which interfere one another, causing a disorderly movement of the fluid particles (Doulah et al., 1975) This fluid movement leads to the break-up of the dispersed phase into small droplets which can collide among then, leading sometimes to coalescence. Usually a dynamic equilibrium between breakage and coalescence is achieved.

A schematic view of UHPH equipment (FPG11300) used in the present study is shown in Figure 2-5.

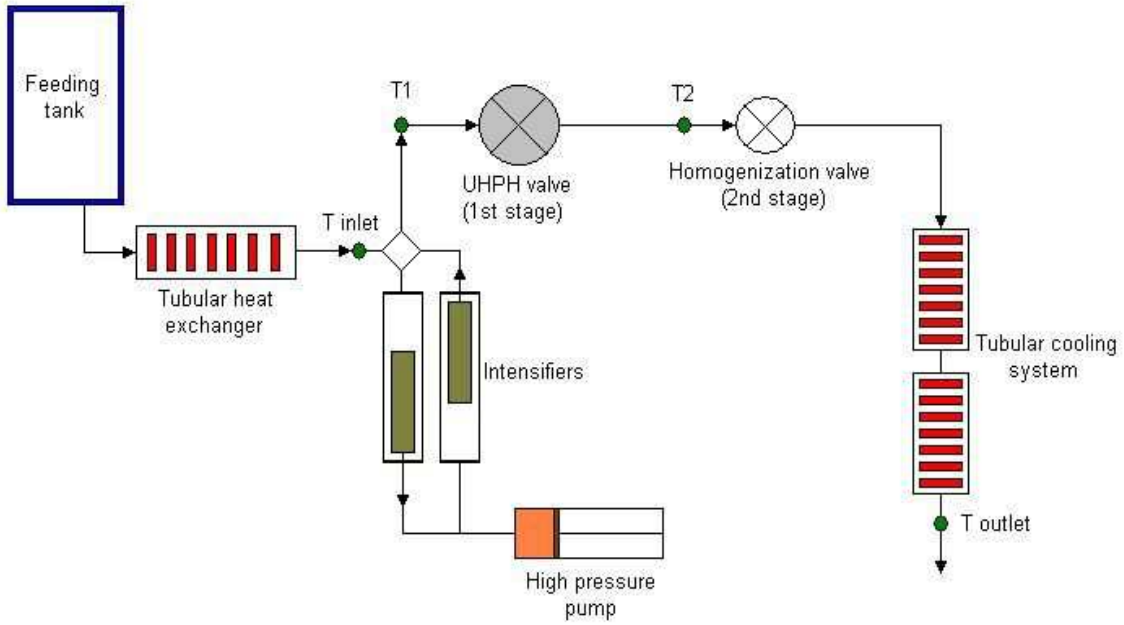


Figure 2-5. Scheme of the UHPH equipment (FPG11300:400) used in the present study, Stansted Fluid Power Ltda., Essex, UK.

2.3.3 Temperature increase during the UHPH treatment

Generally UHPH technology is considered as an alternative to heat treatments. However during high pressure process, a linear temperature increase occurs with the increase of homogenization pressure at constant inlet temperature (Floury et al., 2003; Sandra & Dalgleish 2005; Serra et al., 2008b). Temperature increase is due to the adiabatic heating generated by the viscous stress when the fluid is impinged at high velocity on the homogenization valve during treatment (Hayes & Kelly, 2003b; Bouaouina et al., 2006). Thus, kinetic energy is converted into thermal dissipation by the phenomena described above, generating fluid heating (Thiebaud et al., 2003; Floury et al., 2004b). Pereda et al. (2007) described an increase of 19.5ºC per 100 MPa from 100 to 300 MPa at 40ºC of inlet temperature, using Stansted FPG11300 equipment. These results are in accordance with Thiebaud et al. (2003) who found an increase of 18.5ºC in the same range of pressure using 4, 14 and 24ºC of inlet temperatures. Donsì et al. (2009b) reported a linear increase of 18ºC per 100 MPa, applying pressures of 250 MPa and inlet temperature of 2ºC, whereas Serra (2008c) described an increase of 19.5ºC per 100 MPa applying pressures of 100, 200 and 300 MPa at 30ºC of inlet temperature. All these groups carried out their studies using Stansted Fluid Power equipment. The resultant temperature increase in the high pressure

valve according to inlet temperature and pressure applied, may reach temperatures higher than 120ºC. The holding time at high temperature was estimated being lower than 1 second (Picart et al., 2006). Therefore, product treated by UHPH technology undergoes a combined effect between pressure and temperature at low holding time, minimizing the thermal damage in the overall quality of the product.

2.3.4 UHPH applications

The first high-pressure homogenization application in industry was intended to prepare or stabilize, disperse and mix emulsions. To reach these objectives pressure around 20 MPa was applied in different types of processes, such as pharmaceutical, chemical, cosmetics and ceramic industries (Kielczewska et al., 2003; Floury et al., 2004a; Diels et al., 2005). During the last decade, UHPH has been investigated for its potential application in the food industry. Several research groups have published studies focused on UHPH food applications using different pressures and inlet temperature combinations, either single or double homogenization stage. Table 2-4 lists the main UHPH applications.

Nowadays, it have been designed equipments able to reach pressures of 400 MPa at 500 L/h flow rate, however these equipments are still prototypes.

Table 2-4. UHPH effect on different food applications

Food	Pressure (MPa)	Inlet temperature (ºC)	Main UHPH effect	Reference
Oil-in-water emulsions	20, 150 and 300	4	Results showed significant modifications in the structure and texture with increasing pressure. Drolpet size was reduced with increasing pressure	Floury et al. (2000)
Soy protein emulsions	20 to 350	5 and 20	Droplet sizes of emulsions were greatly reduced and treatments. Pressure of 350 MPa produced highly viscous and stable emulsions.	Floury et al. (2002)
Milk	100 and 300	4, 14 and 24	At high pressure and high inlet temperature, the inactivation of endogenous flora increases. High reduction of fat globule size was reached.	Thiebaud et al. (2003)
Milk	150, 200 and 250	45	Great reduction of fat globule size and complete inactivation of psychrotropic bacteria. Reductions of mesophilic bacteria were 1.3, 1.83 and 3.06 log cfu/mL at 150, 200 and 300 MPa, respectively.	Hayes, et al. (2005)
Milk for cheese making	179 and 100 to 300+30 (first and double stage)	25 and 30	Improvement of coagulation properties and aggregation of casein micelles. Increasing of the wet yield of curd and moisture content.	Sandra and Dalgleish (2007) and Zamora et al. (2007).
Soymilk for producing soy yogurt	200 and 300	40 and 50	Considerably reduction of microbial load and highly physical stability of soymilk. Disruption of colloidal particles and aggregates at 300 MPa. High homogeneity and compact network structure of soy yogurt.	Cruz et al. (2007) and Ferragut et al. (2009)
Milk for producing yogurt	100 to 300	30	UHPH was capable of reducing particle size leading to the formation of fine dispersions. Desnsity of gel, aggregation rate and water retention are improved as increased pressure conditions.	Serra et al. (2007) and Serra et al. (2008a)
Apple juice	100, 200 and 300	4 and 20	Successful reduction of microbial load at 200 and 300 MPa (comparable to pasteurization). During 60 days of storage (4ºC) mesophilic counts did not change.	Suárez-Jacobo et al. (2010) and Suárez-Jacobo (2011)

2.3.5 UHPH effect on microbial inactivation

Traditionally UHPH has been used in the pharmaceutical and biotechnological industry for the disruption of microbial cells for obtaining intracellular extracts for several applications (Bury et al., 2001; Geciova et al., 2002). However, for the UHPH application in liquid food, the challenge relies on finding the right conditions for microbial inactivation without compromising food characteristics.

Several studies have been published the UHPH effect in the native microbiota inactivation of different food products, such as Hayes et al. (2005) in milk samples, Cruz et al. (2007) in soymilk and Suárez-Jacobo et al. (2010) in apple juice (Table 2-4). On the other hand, the UHPH effect on specific pathogen bacteria has been published by several groups. Briñez et al. (2007) applied pressure of 300+30 MPa (first and second stage) at 6 and 20ºC of inlet temperatures in milk and orange juice inoculated with *Staphylococcus aureus* and *Staphylococcus carnosus*. They found that inlet temperature, food matrix and bacterial strain influenced the lethality level, being higher for *S. aureus* in whole milk at 20ºC of inlet temperature. Velazquez-Estrada et al. (2008) treated whole egg inoculated with *Salmonella enteric* serovar Senftenberg 775W at 150, 200 and 250 MPa and 6ºC of inlet temperature. They found that level of pressure influenced significantly the lethality, obtaining a reduction of 3.8 log units at 250 MPa. Vachon et al. (2002) who applied pressures of 100, 200 and 300 MPa (1, 2 and 3 passes) at 25ºC of inlet temperature on milk inoculated with *Listeria monocytogenes* and *E. coli* O157:H7, found significant reduction of both microorganisms as increased pressure and number of passes. Results indicated that *E. coli* was much more sensitive to the treatment than *L. monocytogenes*. Other groups have shown that increasing the passes number or cycles increase microbial inactivation (Hayes & Kelly 2003b; Picart et al., 2006; Smiddy et al., 2007). Donsì et al. (2009b) who worked with *Escherichia coli* and *Saccharomyces cerevisae* inactivation at 250 MPa, observed that inactivation increased as the pressure level increased, reaching in some cases, a complete inactivation. Similar conclusions were found by Thiebaud et al. (2003), who reported that microbial inactivation was a function of homogenization pressure and inlet temperature. They concluded that high inlet temperature combined to high pressure produced an increase of the inactivation. Therefore, parameters of UHPH treatment such as pressure level, temperature, multi-pass homogenization play an important role in the microbial inactivation.

The exact cause of cell disruption is controversial in the literature. In fact, it is not possible to specify a single overall disruption mechanism, without taking into account the product parameters (e.g. viscosity), operating parameters (e.g. flow rate, temperature), and device parameters (e.g. valve geometry) (Donsì et al., 2009a). Therefore, homogenization valve and machine design could be one of the most important factors, although temperature increase produced by UHPH valve can affect microbial inactivation (Pereda et al., 2007). In this way, temperature noticeably affects membrane lipid composition and physical state of bacterial cell. At low inlet temperature (2 to 10ºC), crystallization of phospholipids occurs and cell membranes become more rigid and consequently more sensitive to pressure (Vachon et al., 2002). Microbial cells experience a non-specific tearing apart of the cell wall (Middelberg, 1995), which is determined by the physical interaction of the cells with the small-gap homogenization valve, in a co-operative action between the destructive stresses originated from the fluid condition and physical strength of the cells (Shamlou et al., 1995).

Regarding bacterial spores, little information is available. However, using mild temperatures and repeated treatment cycles, may increase significantly the inactivation of *Bacillus* spores, although high resistance to UHPH treatment is expected (Feijoo et al., 1997; Chaves-López et al., 2009).

Microbial strain and cell concentration

Some studies have shown changes in cell morphology as well as splits in the cytoplasmic membrane of bacteria submitted to UHPH treatments (Kheadr et al., 2002). According to Earnshaw (1992), cellular membrane is the main site affected by pressure. In this sense, studies indicated that Gram-negative bacteria, characterized by a thinner cell wall membrane, are more sensitive to high pressure homogenization than Gram-positive bacteria. This suggests a correlation between cell wall structure and UHPH resistance, which indicates that high pressure homogenization, destroys vegetative bacteria mainly through mechanical disruption of the cell integrity during the pass through UHPH equipment (Vachon et al., 2002; Wuytack et al., 2002).

On the other hand, initial cell concentration can have an important role in the UHPH efficacy. Studies carried out with this purpose indicated a correlation between the initial cell concentration and high pressure effect. For instance, Moroni et al. (2002) observed that UHPH treatment became less effective at greater initial load of lactococcal bacteriophages.

Similar results were found by Tahiri et al. (2006) and Donsì et al. (2006) who worked with different microbial strains and demonstrated that UHPH effectiveness increased at low initial bacterial concentration.

2.3.6 UHPH effects on physico-chemical properties

Additionally to UHPH effects on microbial inactivation, fat globules, particle size, proteins, enzymes and other colloidal components are affected by high pressure homogenization. Due to the high number of publications applying UHPH treatments in milk and milk products, most of the effects reported up to now have been on dairy products. Some of them are reported in this section.

Effect on particle and fat globule size

The different phenomena that a fluid experience thought the pass of high-pressure valve, impact strongly in the particle and protein and fat globule distribution in the continuous phase of an emulsion. For whole milk, the fat droplets diameter in non-homogenized sample is usually between 0.1 and 20 μm, having an average between 3 and 5 μm (Pereda, 2008). Generally, milk homogenized by conventional Gaulin valve achieved particle diameter of 1 μm compared to non-homogenized milk (Dalgleih et al., 1996).

Several studies carried out in milk (Hayes & Kelly 2003b; Thiebaud et al., 2003; Hayes et al., 2005; Zamora et al., 2007; Pereda et al., 2007) demonstrated that fat globule size was dramatically reduced applying pressures of 200 MPa, compared to conventional homogenized milk. Picart et al. (2006) obtained fat globule diameter lower than 0.36 μm representing around 78 and 93% of the total fat volume in UHPH-treated milk at 200 and 300 MPa respectively. For soymilk samples, few studies have investigated soymilk treated by UHPH technology at the present moment. For instance, Cruz et al. (2007) obtained evidences that fat droplets size of soymilk decreased after UHPH treatment, although no apparent differences were observed between 200 and 300 MPa conditions. Nevertheless, they observed an increasing of aggregates as pressure increased. Authors such as Thiebaud et al. (2003), Hayes et al. (2005), Pereda et al. (2007) and Zamora et al. (2007) also observed an increase in fat globule aggregates of milk treated at 250 and 300 MPa. The formation of aggregates could take place by different reasons. According to Floury et al. (2002), aggregates are formed by coalescence phenomenon of freshly-disrupted oil droplets that occurs directly after the homogenization valve. This phenomenon depends on

the flow rate in the homogenizing device, where turbulence zones and velocity gradients take place at the exit of the valve gap. In this sense, Desrumaux and Marcand (2002) suggested that, flow rate, high shear rates and heat dissipation by the pressure drop inside the high pressure valve increase the probability of collision between fat droplets and coalescence. On the other hand, some authors observed that proteins, such as milk-caseins may affect the fat globule size. Dalgleish et al. (1996) suggested that the reduction of fat globule size by high pressure homogenization treatment, cause a strong increase of surface-active of the globule in which a great proportion of casein may be adsorbed into exposed interface. However, at higher pressures, the amount of available casein may become limited, which would account an increase in fat globule size after treatment by partial agglomeration of very small globules insufficiently covered by surface-active material (Datta et al., 2005; Hayes et al., 2005).

Inlet temperature used during UHPH treatment of milk also affects the fat globule size. Datta et al. (2005) reported that fat state (liquid or solid, or part-liquid/part-solid) has a significant influence in the extent of globule size reduction. They concluded that most of the milk fat needs to be in liquid state prior to homogenization valve to ensure the treatment effectiveness (Hayes & Kelly, 2003b; Thiebaud et al., 2003).

Color

Due to the new state of particle distribution caused by the UHPH treatment on the food fluid, the reflection and transmission of the light may change and thus affecting color parameters (L^*, a^* and b^* in the CIELAb scale). For milk samples for example, Pereda et al. (2007) and Hayes and Kelly (2003b) observed an increase of lightness (L^*) compared to untreated milk, due to the increase of light reflected produced by the fat globules in homogenized milks. However, (Serra et al., 2008a) found a decrease of lightness in skim milk treated at pressures between 200 and 300 MPa. They attribute the results to the casein micelles aggregation decreasing the surface of reflection. For soymilk samples, Cruz et al. (2007) observed a decrease of lightness which was related to the protein and lipid-protein aggregates causing a decrease of light reflection. For apple juice samples, Saldo et al. (2009) observed values of lightness slightly lower for treatments at 100 and 300 MPa compared to untreated and pasteurized samples. Therefore, particle distribution, state of aggregation of food matrix and the intensity of pressure applied, define the response of quality color.

Viscosity

The high homogenization obtained after UHPH treatment may decrease the viscosity of the food fluid by the reduction of particle size and better protein dispersion in the interface o/w (oil-in-water). Some studies carried out viscosity analysis in order to determine the effect of UHPH on raw material products. For instance, Cruz et al. (2007) reported that soymilk treated at 200 and 300 MPa present lower viscosity values than untreated soymilk. In that case, untreated soymilk was submitted to the action of a colloidal mill to obtain a coarse emulsion. However, UHT-treated soymilk showed lower viscosity than those UHPH soymilks. This result was attributed to the increase in effective/volume of disperse phase due to the decrease of particle size in UHPH treatment that increased the internal friction leading to the detection of high viscosity. On the contrary, Floury et al. (2002) studied UHPH treatment on globulin 11S fraction. They reported an increase of viscosity as homogenization pressure increases in protein emulsions treated by UHPH. This result was attributed to the formation of large aggregates by intermolecular interactions among denatured protein molecules. They concluded that soy-proteins emulsion treated at 350 MPa produced highly viscous and stable emulsion.

For milk samples, Serra et al. (2008a) did not observe differences between milks treated at 100 and 300 MPa. On the other hand, Pereda et al. (2007) reported lower viscosity values of milks treated at 200 MPa instead of 300 MPa. They attributed these differences to large particles or fat aggregates formed in those samples treated at 300 MPa.

2.3.7 UHPH effects on proteins and enzymes

The UHPH effect observed in the particle distribution as well as fat globules, protein being macromolecular components may undergoes important changes in the structure which affects its solubility and functionality. The most studied proteins and enzymes are reported in this section.

Whey protein and casein micelles

High pressure homogenizer treatment causes different effects on different types of whey protein. According to Paulsson and Dejmek (1990), the major whey proteins present in the milk are β-lactoglobulin and α-lactoalbumin. Because of this, they may experience the most UHPH effects. Protein denaturation is one of the effects that take place in UHPH

treatments. For instance, Hayes et al. (2005), Datta et al. (2005), Zamora et al. (2007) and Pereda et al. (2008) reported some degree of whey protein denaturation induced by UHPH treatment. Similar conclusions were obtained by Serra et al. (2008a) who found an increase of β-lactoglobulin denaturation degree (from 14 to 26%) from 100 to 300 MPa. β-lactoglobulin was more sensible than α-lactoalbumin in both heat and UHPH treatments. Denaturation process of α-lactoalbumin is around 80-90% reversible after short-time heating (Ruegg et al., 1977), converting α-lactoalbumin more resistant than β-lactoglobulin to thermal denaturation. On the contrary, Hayes and Kelly (2003b) did not observe whey protein denaturation at pressures between 50 and 200 MPa (single stage or double stage) using Stansted equipment. This result is in agreement with those obtained by Sandra and Dalgleish (2005) and Bouaouina et al. (2006).

Hayes and Kelly (2003b) reported that casein was not affected in milk treated at pressures lower than 150 MPa. On the other hand, at 200 MPa a decrease of 5% was found in the size of casein micelle (from 180.75 nm to 170.65 nm). In this way, Roach and Hart (2008) observed a linear decrease of 30% of casein micelle size at pressures from 0 to 200 MPa reducing from 278 nm to 171 nm. These results suggested that UHPH partially remove parts of the casein micelle surface, leaving it still active (Sandra & Dalgleish 2005, 2007).

Soy protein

As reported in the section 2.1.2, the major soy protein is formed by globulins. The UHPH effect on globulins solubility was carried out by Floury et al. (2002). In that study an aqueous solution of soy globulin 11S was treated at pressures between 20 and 350 MPa. They observed that protein solubility was preserved at moderate pressures (≤150 MPa). However, increasing the homogenization pressure above 200 MPa, led to a quite strong decrease in the globulin solubility with a wide variation of 40%. The loss of globulin solubility (above 200 MPa) was caused by the protein denaturation due to the homogenization process. The mechanical forces that take place during UHPH process could have affected the macromolecular conformation of soy globulin. Changes in macromolecular structures lead to interactions among proteins which may induce denaturation and aggregation. The strength increase of hydrophobic effect causes improvement of protein-protein interactions at high pressure of homogenization. These effects produce highly viscous and stable emulsions. Cruz (2008) achieved also high

colloidal stability of UHPH-treated soymilk, despite of complete globulin denaturation reached at 300 MPa.

Trypsin inhibitor activity

Trypsin inhibitors are substances that adversely affect nutritional properties of soy products, including soymilk (see 2.1.2). Therefore, a maximum inactivation should be achieved as much as possible. Few studies have reported the UHPH effect on TI inactivation. For instance, Cruz (2008) observed that the increase of inactivation was linked to the pressure increase in UHPH-treated soymilk. Poliseli-Scopel et al. (2012) reached for UHPH-treated soymilk at 300 MPa similar degree of inactivation to pasteurization treatment (37% of initial activity). On the other hand, nowadays there is not a European regulation or recommendation about TI inactivation. Similarly, at the present time it has not reported health problems due to the consumption of soymilk in humans.

Enzymes

Regarding to enzymes, several studies have published the UHPH effect on native enzymes of different foodstuffs. Hayes and Kelly (2003a) studied the high-pressure homogenization on alkaline phosphatase and plasmin activities in raw whole milk. They observed that inactivation of plasmin increased as pressure increased. Two stage treatments were more effective than single stage treatment. However, UHPH treatment was not effective in the inactivation of alkaline phosphatase. On the other hand, Datta et al. (2005) observed important reduction of alkaline phosphatase, plasmin and lactoperoxidase activity in milk treated at 200 MPa and 45ºC of inlet temperature. Complete lactoperoxidase inactivation was achieved at 200 and 300 MPa by Pereda et al. (2007). However, they observed that psychrotrophic counts remained below the legal limit established for 21 days in samples at 200 MPa at 30ºC, since the lactoperoxidase was only partially inactivated. At pressures of 150, 200 and 250 MPa, Hayes et al. (2005) observed residual activity of 91 and 34% for 150 and 200 MPa respectively and complete inactivation at 250 MPa.

Pectin methylesterase is an enzyme heat resistant and responsible for the loss of turbidity in orange juice during storage. Lacroix et al. (2005) obtained a reduction of 20% in UHPH-treated orange juice without pre-warming. Nevertheless, a combination of UHPH and pre-

warming (50ºC, 10 min) increased significantly the effectiveness of pectin methylesterase inactivation compared to only pre-warming. On the other hand, Velazquez-Estrada et al. (2008) observed a drastic reduction (> 90%) under 200 and 300 MPa of pressure at 10 and 20ºC of inlet temperatures in orange juice compared with conventional pasteurization. Another enzyme which plays an important role in the quality of fruit juice is polyphenoloxidase. This enzyme often catalyzes reactions of color degradation, undesirable flavor formation and lost of nutrient impacting negatively in the product quality. Suárez-Jacobo, (2011) reported complete inactivation of polyphenoloxidase in apple juice treated at 300 MPa and 4ºC of inlet temperature.

Finally, lipoxygenase is commonly present in cereal foodstuffs and primarily in soy products. In soymilk it has a particular interest by its implication in flavor quality (see 2.1.2). There are few studies about the effect of UHPH on lipoxygenase activity. Cruz (2008) obtained complete inactivation at pressures of 200 and 300 MPa and inlet temperature of 40 and 50ºC for soymilk samples. Probably, physical phenomena produced by homogenization valve in combination with rising temperature, induced structural modifications on enzyme conformation causing loss of activity.

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Chapter 3

Material and methods

3

Material and methods

3.1 Soymilk elaboration

Method A. Soybean (Majesta variety) used in this study was provided by Liquats Vegetals, S.A. (Girona, Spain). The methodology used to produce soymilk was based in the method described by Liu (1999) and Yuan and Chang (2007). Whole soybeans were soaked (3:1 water-to-soybean ratio) during 15 h at room temperature. The volume increase was expressed as: (W2/W1)x100, where W2 is the mass of wet soybeans and W1 is the mass of dry soybeans.

Soaked soybeans immersed in 75% of the total water used in the soymilk elaboration and then ground in a crushing machine (9:1 water-to-hydrated soybean ratio) with heating control (adapted from Frigomat, Italy) for 20 minutes at 60ºC and 80ºC with recirculation in a colloidal mill (E. Bachiller B. S.A., Spain). After that, pulp was separated by filtration using a 0.2 mm steel sieve (model: CE98, Mejisa – Mectufry, Spain). For the second extraction, the pulp was immersed in the 25% remaining water and then the mixture was heated at 60ºC or 80ºC at continuous agitation. After that, the mixture was filtered and then mixed with the first fraction of soymilk. Soymilk and pulp were finally weighted. This mixture was considered the soymilk BP.

Method B. Using the same soybeans variety described in method A, soymilk elaboration was the same with the following modifications. After soaking soybeans, only an extraction at 80ºC for 20 min was used. Weighting control in each step of elaboration was not performed. This method was applied in all chapters, except chapter 4.

3.2 Soymilk treatments: UHPH, pasteurization and UHT

UHPH A. UHPH treatments were conducted with an ultra high-pressure homogenizer (model: FPG11300, Stansted Fluid Power Ltd., UK). This device (flow rate of 120 L/h) is provided with two intensifiers, driven by a hydraulic pump and a high-pressure ceramic valve able to support 400 MPa. Inlet and outlet temperatures of soymilk were controlled by two heat exchangers (Garvía, Spain) located before the machine entrance and after the high-pressure valve, respectively. During treatments inlet temperature, temperature after homogenization valve, outlet temperature and pressure of treatment were monitored.

UHPH B. Benchtop ultra high pressure homogenizer (model: FPG12500 Stansted Fluid Power Ltd., UK) was used for the assays. This device (flow rate of 15 L/h) is provided with two intensifiers, driven by a hydraulic pump and a high pressure ceramic valve able to support 400 MPa. Inlet and outlet temperature of soymilk were controlled and monitored by hot bath located before the machine entrance and by cold interchange located after the high-pressure valve. The pressure of treatment and the temperature after homogenization vale were also monitored. This UHPH system was applied to carry out the third step described in working plan (Figure 1-2). The spores (10^5–10^6 spores/mL) suspended in 500 mL of sterilized soymilk (sterilization at 121ºC for 10 min) were subjected to UHPH treatment of 300 MPa and 55, 65, 75 and 85 ºC of inlet temperature.

Pasteurization. Soymilk BP was homogenized at 18 MPa (LAB type: 22.51, Rannie, Denmark) and subsequently pasteurized using a tubular heat exchanger (ATI, Spain) at 95ºC for 30 s.

UHT. BP samples were subjected to homogenization of two stages (18 and 4 MPa) performed in a homogenizer (X68IE+X68P, Niro Soavi, Italy) previous to UHT treatment (142ºC for 6 s) with indirect system equipment (6500/010, GEA Finnah GmbH Ahaus, Germany).

3.3 Production yield

To determine production yield, mass balance was applied to 4 independent elaborations taking into account the weight of dry seeds, soaked seeds, and ground seeds in water, soymilk and pulp. Values were calculated according to law of mass conservation for which, in a determined volume, the sum of ingredients mass is equal to the sum of products mass:

$$\frac{d}{dt}mc = \sum_{i} \dot{m}i - \sum_{e} \dot{m}e$$

Assuming steady state, the equation is reduced at:

$$\frac{dm_{vc}}{dt} = 0$$

$$\sum \dot{m}i = \sum \dot{m}e$$

Where, "*mi*" and "*me*" are, respectively, input mass and output mass.

To calculate global mass balance or component mass balance, input mass should be the same of output mass in the control volume. In this process there is not continuous flow neither time variation. Each production was performed by bath in a close system. Therefore, it is convenient to considerate no consumption neither generation of mass or energy from chemical reaction or mass transference. Productivity of soybeans during soaking, solids recovered, loss of water and overall yield expressed as g/100g was calculated by this method.

Another way to express yield is based in the ratio of soymilk produced by the soybeans seed used:

$$\text{Yield} = \frac{\text{litters of soymilk produced}}{\text{kilograms of seeds}}$$

3.4 Storage of soymilk

Condition A. To perform the fourth step described in the working plan (Figure 1-2), UHPH treated and pasteurized soymilks were stored at 4ºC for 28 days. Samples were transferred aseptically to sterile bottles of 100 mL and divided for each day of analysis: 1, 7, 14, 21 and 28 days of storage. Each bottle was aseptically opened in the correspondent day of analysis.

Condition B. To achieve the fifth step described in the working plan (Figure 1-2), UHPH and UHT soymilks were packaged in a coater paperboard cartons (200 mL Tetra Brick containers) by using a Tetra Pak (TBA9 slim line, Switzerland) aseptic technology. The

tetra brick containers were stored at room temperature during 180 days. Samples were analyzed in triplicate at day 1, 20, 40, 60, 90, 120, 150 and 180 days of storage. For each day of analysis, 3 bricks were randomly selected and then mixed in a glass prepared for this purpose. This procedure was carried out for each treatment.

3.5 Soymilk and soybeans physico-chemical analysis

Soybeans were ground in a crushing machine (Morphy Richards, S., UK) prior to the analysis. Dry matter and ash content were analyzed by reference AOAC method (AOAC, 2000); total nitrogen content was analyzed by Dumas method (FIL - IDF, 2002) in both soybeans and soymilk samples. The pH of soymilk was measured with a pH meter (model GLP 21+ Crison, Spain). Soybeans and soymilk fat content was determined by ASE 200 (Accelerated Solvent Extraction, USA). For soybeans, 2 g of sample were mixed with sea sand (~ 2:1) and then transferring the mix to 11 mL cell extraction. 3 Cycles of 10 min at 105ºC and 1500 psi were applied according to the method proposed by the manufacturer. Petroleum ether was used as extracting solvent which were further evaporated with nitrogen and the residue weighted. For soymilk, 1 g of sample was weighted and mixed with 1.1 g of sea sand and 0.9 g of celite and then transferring the mix to 11 mL cell extraction. 3 Cycles of 1 min at 120ºC and 1500 psi were applied for soymilk samples. Petroleum ether and 2-propanol (60:40) were used as extracting solvents.

3.6 Microbiological analysis

3.6.1 Microbiological quality

Microbiological quality of soymilk samples was assessed by enumerating the following microorganisms: mesophilic aerobic bacteria were counted on PCA medium (Oxoid Ltd, UK) incubated for 48 h at 30ºC. Mesophilic aerobic spore counts were assessed by heat shock at 80ºC for 10 min, quickly cooled in ice and plated on PCA medium (Oxoid) and incubated for 48 h at 30ºC. Enterobacteria counts were determined in violet red bile glucose agar (VRBG, Oxoid) incubated at 37ºC for 24 h. Yeasts and moulds were detected in rose-bengal chlorampenicol medium (Oxoid), incubated at 25ºC for 5 days. *Staphylococcus aureus* was determined in Baird-Parker RPF (bioMérieux) and incubated at 37ºC for 48h. *Bacillus cereus* was determined in brilliance *Bacillus cereus* medium (Oxoid), supplemented with *Bacillus cereus* selective supplement (Oxoid) and incubated at

30ºC for 48 h. *Salmonella sp.* was investigated through sample pre-enrichment at 37ºC for 24 h and then enriched in Muller Kauffman broth (bioMérieux S.A.) and Rappaport Vassiliadis broth (bioMérieux S.A.) at 37ºC and 42ºC for 24 h, respectively. Enrichments were streaked onto XLD (Oxoid) and SM2 agar media (bioMérieux S.A.) and incubated at 37ºC for 24 h.

Sterility test. Soymilk samples were incubated at 30ºC for 20 days. During this period, coagulation and phase separation were checked visually. Mesophilic aerobic bacteria was determined on PCA medium (Oxoid) in all incubated bottles.

3.6.2 Isolate collection and selection

Isolates were obtained from soymilk samples treated by UHPH at 300 MPa and 65ºC of inlet temperature after incubation of 30ºC for 20 days. Spore counts were assessed by heat shock at 80ºC for 10 min, quickly cooled in ice and plated on PCA medium (Oxoid) and incubated for 48-72 h at 30ºC. Typically, colonies representing visually distinct morphology (ranging from 1 to 5 colonies per sample) were selected and streaked for purity on TSA (TSA, Oxoid). Biochemical testing on cultures using the API 50 CHB kit (*bioMérieux*, France) was performed and thereafter, genetic identification was established based on comparative 16S rRNA sequences. Briefly, isolates were cultured on TSA. Bacterial genomic DNA was prepared by using DNeasy tissue kit (Qiaagen, *Valencia*, USA). 16S rRNA genes were amplified by conventional PCR. Amplicons were analyzed on 1% agarose gels containing ethidium bromide. The *sequencing* was performed at *Macrogen* Inc (Korea). DNA sequences of the 16S rRNA were aligned with the Clustal method from MegAlign (DNAStar Inc., USA) assembling with 95% minimum match. The obtained nucleic acid sequences were analyzed with the algorithm Blastn at the National Center for Biotechnology Information (NCBI).

3.6.3 Sporulation conditions

Sporulation of all *Bacillus* strains was performed following the method UNE-EN-13704 standard (Anonymous, 2002). A suspension of *Bacillus* spores was prepared from an exponential phase culture of vegetative bacteria in tryptone glucose broth (0.25% yeast extract; 0.5% tryptone; 0.1% glucose and pH was adjusted to 7.2). Approximately 2–3 mL of this culture were transferred to *Roux flasks containing* meat yeast extract agar (1% meat

extract; 0.2% yeast extract, 0.004% $MnSO_4 \cdot H_2O$; 1.5% agar and pH was adjusted to 7.2) and incubated for 8–10 days at 30°C. The culture was harvested and purified by repeated centrifugation (10000 *g* for 20 min) and washed with sterile distilled water. The suspensions were heat-shocked for 30 min at 75°C in order to kill vegetative cells. All the spore preparations were free (> 97%) of sporulating cells, cell debris and germinated spores, as determined by phase-contrast microscopy. Finally, the spores were stored in double distilled sterile water at 4ºC until use.

3.6.4 Spores recovery

Between 80 and 100 ml of treated samples were used in the analysis. Spore counts were quantified in glucose yeast agar (0.1% casamino acids without vitamins; 0.1% soluble starch; 0.25% glucose; 0.5% yeast extract; 0.01% FeSO4; 0.00001% MnSO4·H2O; 1.5% agar and pH was adjusted to 6.8), incubating at 30°C for 72 h. Lethality was calculated as the difference between the logarithms of colony counts of the untreated and treated samples (log No- log N). Further investigation was carried out to confirm if complete inactivation had been achieved. For this purpose, 50 mL of treated soymilk were incubated at 30ºC for 10 days. During this period, coagulation and phase separation were checked visually. Moreover, a loopful of incubated samples was streaked out on glucose yeast agar, which was incubated at 30°C for 48-72 h.

3.7 Lipid oxidation

3.7.1 Lipoxygenase activity

*LOX extraction.*The extraction was performed following the method described by Van der Ven et al. (2005) with some modifications. Soymilk (30 mL) were placed in polypropylene tubs (32 mm diameter and 115 mm length) and centrifuged for 60 minutes at 12000 *g* at 4ºC. Supernatant was used for enzyme activity assay.

LOX assay. The assay was performed following the method described by Axelrod et al. (1981). The reaction was carried out at 25ºC in quartz cuvette of 1.0 cm light path in spectrophotometer (Cecil 9000, UK) at 234 nm. The assay mixture contained 2.975 mL of borate buffer, pH 9.0, 0.025 mL of sodium linoleate substrate (10 mM), and 0.030 mL of LOX extract (enzyme). After each addition the mixture was stirred. The blank cuvette contained no enzyme.

The activity was expressed as absorbance per minute read (abs/min) in the spectrophotometer and transformed into units of lipoxygenase activity (ULA). One ULA was defined as a change of 0.1 units of absorbance per minute of enzyme extract.

3.7.2 Hydroperoxide index

Lipid hydroperoxides in soymilk were determined by using the method described by Ostdal et al. (2000) with some modifications. The reaction consists of the oxidation of ferrous to ferric ion by hydroperoxides in the presence of ammonium thiocyanate to produce ferric thiocyanate whose absorbance can be measured at 500 nm. Soymilk (0.4 mL) was mixed with water (1.6 mL). Then 2 mL of methanol were added and stirred. Chloroform (4 mL) was added and vortexed for approximately 30 s. After centrifugation for 10 min at 12000 *g*, 1 mL of the chloroform phase was transferred to a test tube and mixed with 1 mL of Fe (II)/thiocyanate in methanol/chloroform (1:1). The mixture was allowed to react for 5 min at room temperature before the absorbance at 500 nm was read. Soymilk samples were analyzed in triplicate, and data were expressed as meq peroxide/L of sample as described by (Hornero-Méndez et al., 2001). The calibration curve was prepared according to the methodology described by FIL - IDF (1991). Measure is based on spectrophotometric reading at 500 nm of a series of dilutions that contain Fe^{3+} in chloroform/methanol (70:30).

$$meq / L = \frac{(As - Ab)}{55.84 \text{ x } 2 \text{ x } m \text{ x } Vs}$$

Where *As* is the absorbance of the sample, *Ab* is the absorbance of the blank; 55.84 is the atomic weight of Fe; *2* is the factor to convert miliequivalents (meq) of Fe to meq of peroxide; *m* is the slope of Fe^{3+} calibration plot; *Vs* is the sample volume in litters.

3.8 Trypsin inhibitor activity

Method A. Trypsin inhibitor (TI) extraction was prepared following the method described by Van der Ven et al. (2005). Soymilk samples were diluted (1:5) with 0.015M of NaOH and 0.5M of NaCl and stirred for 2 h at room temperature. After mixing, the mixture was

ultra-centrifuged (Beckman, model L8 60M, USA) at 30000 *g* for 20 min at 20ºC. Supernatant was used for TI assay.

TI determination was based in the method described by Hamerstrand et al. (1981). Dissolutions of Tris Buffer: hydroxymethyl aminomethane (1.21 g) and 0.59 g of $CaCl_2$ dihydrated were dissolved in 180 mL of distilled water with adjusted pH 8.2 and then made up to 200 mL with distilled water. BAPA solution: N_α-Benzoyl-L-arginine 4-nitroanilide hydrochloride (0.080 g) was dissolved in 2 mL of dimethyl sulfoxide and diluted to 200 mL with Tris Buffer. Trypsin solution: trypsin (0.0040 g) was diluted to 200 mL with 0.001N HCl.

Soymilk TI extract (2 mL) was added in four tubes. 2 mL of trypsin solution were added in the first 3 tubes and then placed in a constant temperature bath of 37ºC for 10 min. Five milliliters of BAPA solution (prewarmed to 37°C) were rapidly added into each tube. The mixture was stirred immediately and the tubes were replaced in the bath. The reaction was stopped exactly 10 min later by adding of 1 mL of 30% acetic acid. In the fourth tube was then added 2 mL of trypsin solution. Two extras tubes (trypsin standard and blank) were prepared applying the same procedure without soymilk. The absorbance was determined at 410 nm (Cecil 9000, UK) and inhibition percentage was calculated according to the relation described by Kumar et al. (2003) with some modifications:

$$\text{Percentage of inhibition} = \frac{100 \times \text{Absorbance of the sample}}{\text{Absorbance of the standard trypsin}}$$

Method B. Due to infrastructural conditions of the laboratory and for accurate results, some modifications were applied in the analytical method described above, respecting the sequence proposed by the authors. Soymilk samples were diluted twice with 0.02 N NaOH (the pH was adjusted to 8.4-10.0) and stirred for 2 hours. The extract was then centrifuged at 12000 *g* for 35 min and filtered through Whatman paper No. 42. The TI extract was diluted so that 2 mL of the extract dilution inhibited 40% - 60% of the trypsin used as standard in the analysis. Values of inhibition superior to 60% cause loss of linearity of the trypsin inhibitor reaction, causing turbidity of the sample extract. Percentage of inhibition was previously determined in untreated soymilk extract in order to find out the optimal volume of treated soymilk extract needed to be used in the assay.

TIA assay was assessed following the modifications of method A: 3 tubes of TI soymilk extract were prepared and blank sample consisted of distilled water without sample extract.

To define TI activity, one trypsin unit (TU) was arbitrarily described as the increase of 0.01 absorbance units per 10 mL reaction mixture at 410 nm (Kakade et al., 1969). Absorbance values obtained from soymilk TI extracts were subtracted from the values of trypsin standard and the TI activity was expressed as mg of TI/mL of sample extract by assuming that 1 µg of pure trypsin was equivalent to 0.019 absorbance units.

3.9 Particle size determination

Particle size distribution was performed by laser light-scattering, supported by the patented multi-wavelength system (PIDS) which provides accurate results in the 0.04 and 2000 µm regions. An LS^{TM} 13 320 Series Particle Size analyzer (Beckman Coulter, USA) using soymilk optical model with a refractive index of 1.46 (Malaki-Nik et al., 2008) and dispersant phase (water) of 1.332 was used for this purpose. Soymilk samples were diluted until obscuration of 2% or 7%, depending on the sample. Particle size distribution was characterized by the Sauter mean diameter, $d_{3,2}$ (particle diameter that has the same specific surface as that of the full distribution) and by the $d_{4,3}$ (diameter of the sphere of equivalent volume to measured particles).

3.10 Particle sedimentation

Centrifugation method. Approximately 30 g of soymilk were poured into flexible plastic tubes (32 mm diameter, 115 mm length) and centrifuged at 1046 *g* for 45 min at 20ºC. Values were expressed as percentage (w/w) of solid deposition obtained after centrifugation.

Particle migration method. Soymilk samples were transferred into borosilicate glass tubes of 27.5 x 70.0 mm up to 40 mm of height and sodium azide (0.04% NaN_3) was added. Three tubes of each treatment and untreated soymilk were prepared and stored at 4ºC. On each day of analysis samples were carefully placed in the Turbiscan equipment (LAB expert Formulaction, France) and a near infrared light from top to bottom measured the percentage of light backscattered through the sample at 25ºC. Results were expressed in base to changes of backscattering (ΔB) in the bottom of the tube (chapter 8) and by the determination of the height of solids layer settled during the period of storage (chapter 9). The latter was calculated using the tool “sediment phase thickness” of the manufacturer software.

3.11 Transmission electron microscopy

Transmission electron microscopy (TEM) of soymilk was determined following the method described by Cruz et al. (2007). Soymilk was mixed with 3% glutaraldehyde in a bijou bottle and then mixed with warm 2% low-temperature gelling agar at 1:1 ratio. The mixture was allowed to gel and was chopped into 1 mm^3 cubes. Then cubes were washed with 0.1 M sodium cacodylate buffer, pH 7.2 for 30 min and again for 1 h, and then left for a further 1 h prior to replacement with 1 ml of a solution containing equal amounts of 2% osmium tetroxide and 50% of 0.1 M cacodylate/HCL buffer. This was left to stand for 2 h before being replaced with 1 ml of 1% uranium acetate for 30 min. Product cubes were washed with water before dehydration. Dehydration included washing with 50, 70 and 90% of ethanol for 5, 30 and 180 min, respectively. 100% ethanol was changed after 30 and 60 min. Ethanol was poured off and the bottle was filled with incomplete resin [20 ml epoxy resin, 20 ml dodecylsuccinic anhydride (DDSA) and 1 drop of dibutyl phthalate] and placed on a rotator overnight before replacement with complete resin [incomplete formulation with addition of 0.6 ml of the plasticiser benzyldimethylamine (BDMA)] and then placed on the rotator for a further 4 h. One cube of sample was added to each of three moulds containing fresh complete resin which were then baked overnight at 60ºC. Samples were cut (0.03–0.05 µm) using a Reichert Ultracut microtome and mounted in 3 mm copper grids and stained using uranyl acetate and lead citrate before examination in a Philips 201 transmission electron microscope at an accelerating voltage of 60 kV (NL-5600 MD Philips, The Netherlands).

3.12 Surface hydrophobicity

Surface hydrophobicity of soymilk protein was measured using 8-Anilino-1-naphtalene sulfonic acid (ANS) as reactive (Shimoyamada et al., 2008). Soymilk samples (50 µL) were diluted with 50 µL of 0.01 M phosphate buffer (pH 7.0) and mixed with 20 µL of 8 mM ANS solution and 4 mL of the same buffer. The resulting mixtures were subjected to fluorescence spectrometry (Eclipse Spectrophotometer, Varian Inc., USA) and fluorescence was measured (excitation, 390 nm; emission, 470 nm). This analysis was only carried out at day 1 (chapter 8 and 9).

3.13 Color measurements

Soymilk color was measured using a Hunter Lab colorimeter (MiniScan XE Hunter Associates Laboratory Inc., USA). D65 was used as illuminant with an observation angle of 10º using the ring and disk set for translucent liquids (HunterLab, 2008). Data was acquired in the CIELab color space, L^* (luminosity), a^* (red-green) and b^* (blue-yellow) were then used to calculate the total color difference ΔE by means $\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2)]^{1/2}$ equation. ΔL, Δa and Δb are the differences in the tristimulus coordinates between reference sample and treated soymilks.

3.14 Headspace analysis of volatile compounds

Changes in volatile compounds produced by thermal and high pressure treatments could strictly modify odor and flavor of soymilk. Solid-phase microextraction (SPME) is a simple and sensitive technique which allows a direct extraction of the volatile compounds present in soymilk. In addition, this method is economic and ecologic because it does not consume large quantities of solvent.

3.14.1 SPME – gas chromatography mass spectrometry

Volatile compounds analysis was performed following the method described by Achouri et al. (2006) with some modifications. The SPME fiber used was 85 µm CAR-PDMS (Supelco, USA). 1.5 mL of soymilk sample (previously homogenized using ultrasound equipment with temperature control for 10 min) were placed in tubes (4 mL) and incubated for 30 min at 40ºC. The adsorbed volatiles were desorbed in the injector port in splitless at 300ºC for 1 min. Headspace of the volatile compounds was analyzed using an automated gas chromatography (model: HP 6890 Series II, Agilent, USA). The column model used was 0.25 µm in a 60 m x 0.25 mm (TRB-Was, Agilent technologies). The mass spectrometry (MS) detector was used in the electron impact ionization (model: HP 5972 Agilent, USA) with a mass range of 30 – 250 *m/z*. Before each analysis, the fiber was preconditioned for 1 h at 300ºC. The temperature was programmed in 2 stages. The initial temperature was kept at 35ºC for 9 min, and then increased at 5 ºC/min to 110ºC for 10 min. In the second stage, the temperature increased at 10 ºC/min to 250ºC for 10 min.

Retention indices, relative to C_8-C_{26} *n*-alkanes were determined injecting 1 µl of each standard solution (Alkane standard solution C_8-C_{20} from Sigma-Aldrich, and Connecticut

ETPH calibration mixture C_9-C_{36} were used as standards) in triplicate with a split ratio of 1:200. Signals were processed using Agilent MSD Productivity ChemStation Enhanced Data Analysis software (Agilent technology, USA). The identification of some volatile compounds (hexanal, pentanal, 1-octen-3-one, 2,3-pentanodione, 1-hexanol, 1-pentanol, 1-octen-3-ol and 2-penthyl furan) were confirmed by comparing their retention times and mass spectra with those of authentic reference compounds injected under the same operating conditions Otherwise, tentative identifications were made based on comparisons with the mass spectra data of NIST08 and Wiley 7n1 libraries and retention index of the literature. Main, molecular, and qualifier ions were selected for each compound indentified.

3.15 Sensory analysis

The attributes required for the training as well as for the sensory analysis were based on the study described by Torres-Penaranda et al. (1998) and Torres-Penaranda and Reitmeier (2001).

Selection and training of panelists. Twelve judges from students and staff at *Universitat Autònoma de Barcelona* with a previous experience evaluating different products were pre-selected based on availability, interest or habitual consumption of soymilk. Panelists were trained in 3 sessions for approximately 30 minutes on different days. In each session, judges were exposed to different tastes: sweet (10 g/L of saccharose), salt (5 g/L of sodium chloride), bitter (0.3 g/L caffeine) and acid (0.1 g/L citric acid) in order to check if judges were able to identify each taste. Moreover, attributes of flavor and aroma such as astringency (alum in water 0.3 g/L), green or beany flavor (50 g/L of fresh soybeans in water), grassy aroma (0.02 g/L cis-3-hexen-1-ol) and 2 solutions of oxidized aroma (1 mg/L 2-nonenal and 10 mg/L 2-heptenal), were prepared and exposed to the panelist. At the end of each meeting, judges evaluated a commercial soymilk sample according to the attributes used in the session.

Sensory tests applied. Sensory tests were divided into 3 parts. The first one included a triangular test, where panelists were asked to identify possible differences between UHPH-treated soymilk and heat-treated soymilk, both presented in random order. The second part included a descriptive test, where the following seven attributes were evaluated: beany flavor, grassy aroma, oxidized aroma, astringent mouthfeel, thickness and darkness. In addition, judges were instructed to describe uncommon flavor perceived. Responses were

recorded on an intensity scale from 0 to 5 points, where 0 = not intense and 5 = extremely intense. In the last part a preference test was applied using a hedonic scale with 9 categories ranging from “dislike extremely” to “like extremely”. Soymilk samples (~ 30 mL) at room temperature was presented in white plastic cups with a 3-digit random code

3.16 Statistical analysis

To carry out the preliminary study of soymilk elaboration, two individual soymilk extractions were performed. For the initial screening and shelf-life evaluation of refrigerated UHPH-treated soymilk, three individual treatments were carried out and analyses of the treated samples were performed in triplicate. Results were analyzed by ANOVA using GLM procedure of SAS (SAS Institute, 2004) to determine differences. SNK (Student Newnan Keuls) test was used for comparing sample data in chapter 4, 5 and 6 and Tukey test was used in chapters 7, 8 and 9. Principal component analysis (PCA) was performed to reduce the data in two dimensions and identify patterns of variation in the results of volatile profile. R software (R software, New Zealand) was used for this purpose. Results of triangular test were analyzed using Chi-square test of SAS to accept or reject the null hypothesis. Hedonic test data were analyzed by two-sample *T-test* of SAS. Data analyses were based on a significant level of $P < 0.05$.

3.17 References

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Chapter 4

Optimization of soymilk elaboration at pilot plant scale

4

Optimization of soymilk elaboration at pilot plant scale

4.1 Introduction

Soybean variety, geographic area of cultive and climate are the main factors influencing soymilk elaboration (Liu, 1999). Some soybean varieties have become well known for their superior processing properties, but storage and environmental conditions during the growing season affects soybean seed size and composition and consequently soymilk yield and quality (Mullin et al., 2001; Alpaslan & Hayta, 2002).

Main steps of soymilk elaboration include: soaking, grinding, filtration, heat treatment and packaging. Soaking reduces the power requirement for grinding, breaks out some oligosaccharides resulting in a better dispersion of solids during extraction, improving production yield.

The best known methods and extensively applied in industries 40 years ago are Cornell and Illinois methods. Cornell method apply heat grinding between 80ºC and 100ºC and Illinois method apply blanching for 10-20 min at 100ºC before grinding with cold water (Liu, 1999). Due to technology improvement occurred in the last decade, nowadays industries commonly apply modern and automatic methods in the soymilk production. Heat treatments using steam injection at high temperatures is a continuous flow method extensively used nowadays.

Production yield is an important factor for all industrial process. There are several ways to express this parameter. Some of them include expressing by weight or volume of soymilk from original soybeans quantity and by total solids calculated as percentage of protein or solids recovery in the final product (Liu, 1999). Additionally to production yield, trypsin inhibitor activity and lipoxygenase activity play an important role in the soymilk quality. The presence of trypsin inhibitors affects trypsin hydrolytic action causing reduction of nutrients absorption (Liu, 1999). In animal feed, for example it has been associated with growth suppression and pancreatic hypertrophy. On the other hand, the effects of trypsin inhibitors in humans are not fully clear, however its reduction is highly recommended to soy products and generally is achieved by heat treatments (Friedman & Brandon, 2001).

Lipoxygenase is an enzyme which catalyzes the hydrolysis of polyunsaturated fatty acids (Vijayvaragiya & Pai, 1991), favoring lipid oxidation which result in the formation of off-flavors (Yuan & Chang, 2007).

Soymilk used in previous studies in our research group was provided by a commercial company. However, in order to control the influence of the elaboration process of the soymilk, the first goal of this study was to establish and standardize the conditions of soymilk base product production. For this purpose, two temperatures of soymilk extraction, 60 and 80ºC (see 3.1 method A) were used and analytical techniques such as chemical composition of soybeans and soymilk (see 3.5), production yield (see 3.3), lipoxygenase activity (see 3.7.1) as well as trypsin inhibitor activity (see 3.8 method A), were evaluated as quality parameters to select the best condition of soymilk base product elaboration.

4.2 Results and discussion

4.2.1 Chemical composition

Composition of Majesta variety soybeans was the following: 9.94 ± 0.04 g/100g dry matter; 39.67 ± 0.32 g/100g protein; 21.20 ± 0.29 g/100g fat; 23.29 ± 0.24 g/100g carbohydrate and 5.72 ± 0.11 g/100g ash. Similar results were reported by (Cai et al., 1997) and (Mullin et al., 2001) for different soybean varieties. Composition of soymilk extracted at 60ºC and 80ºC are shown in Table 4-1. Results indicated that the temperature used in soymilk extraction did not influence dry mater and fat composition. However, soymilk extracted at 60ºC presented values of protein slightly higher than those observed at 80ºC ($P < 0.05$). This difference could be attributed to the protein solubility. At 80ºC soymilk protein may have partially reduced its solubility, as a consequence of denaturation process that takes place at high temperatures. The filtration step could therefore be essential for the protein recovery, because part of protein precipitated could form complexes with particles and macromolecules in the pulp, reducing then its availability. Kwok & Niranjan (1995) observed that combination of temperature and time applied during soymilk extraction may increase solid and protein recovery from the seeds or, on the contrary, insolubilized proteins and decrease yield. Results of soymilk composition were similar to those reported in commercial soymilks (Liu, 1999; Cruz et al., 2007; Liu & Chan, 2012).

Table 4-1. Dry matter, fat and protein content[1] of soymilk extracted at 60ºC and 80ºC.

	Extraction at 60ºC	Extraction at 80ºC
Dry matter	6.28 ± 0.11[a]	5.78 ± 0.24[a]
Fat content	1.53 ± 0.08[a]	1.36 ± 0.17[a]
Protein content	3.21 ± 0.04[a]	3.10 ± 0.04[b]

[a-b] Different superscripts in the same raw are significantly different ($P < 0.05$).

[1] Mean values (g/100g) of chemical composition of soymilk base product.

4.2.2 Production yield

Productivity of soybeans during soaking step was 228.06 ± 3.49 (% w/w). This result indicates an increase of 2.28 times the original weight of the seeds. Soaking step would be completed when seeds reach 2.4 times the initial weight (Wang, 1986). However, a partial hydration of twice initial weight of the seeds is very common in soymilk extraction (Liu, 1999).

Yield production of soymilk elaborated at 60ºC and 80ºC is shown in Table 4-2. No significant differences were observed for all parameters studied. Total solids recovery from soybeans is decisive for the content of lipids and proteins of soymilk as well as its nutritional value. Amount of 56.68 and 52.09% obtained for soymilk extracted at 60ºC and 80ºC, respectively, are in agreement with the typical soymilk production, commonly known as Cornell method as described by Prawiradjaja (2003). The same author reported values of 55-65% (w/w) of total solids recovery using Cornell method, whereas Illinois method values were about 86-89% (w/w). In the Illinois method, all parts of the soybeans including the pulp are incorporated into the soymilk, favoring high percentage of solid extraction (Golbitz, 1995; Kwok & Niranjan, 1995).

The relation soybean:soymilk produced is another way to express production yield. A relation of 8.17 means that total volume of soymilk produced was 8.17 times the total seeds used. Traditional method of soymilk extraction reaches values between 8-10 times the original values of the seeds weighted before the soaking step (Liu, 1999). Total loss of water was about 13% for the whole process with an overall yield about 81%. According to conventional industrial process, 19% of losses are higher than desired. Grinding and filtration are the crucial steps to avoid or to reduce losses during soymilk elaboration (Liu, 1999). In this study, grinding step produced the major losses compared to the rest of elaboration steps, reaching values around of 68%. Infrastructure conditions of production

used in this study did not allow elaborating soymilk with better overall productivity. On the other hand, results of soybean:soymilk obtained in this study as well as results of total solids recovery are in agreement with different methods of soymilk production, traditional or industrial. However, some studies have reported high overall productivity by adding sugar in the soymilk formulation using conventional method of extraction (Prawiradjaja, 2003).

Table 4-2. Production yield[1] of soymilk extracted at 60ºC and 80ºC.

	Extraction at 60ºC	**Extraction at 80ºC**
Solids recovered	56.68 ± 6.16[a]	52.09 ± 3.30[a]
Soybean:soymilk[2]	8.17 ± 0.74[a]	8.19 ± 0.01[a]
Loss of water	13.03 ± 9.67[a]	13.46 ± 0.04[a]
Overall yield	81.68 ± 7.39[a]	81.81 ± 0.14[a]

[a] Different superscripts in the same raw are significantly different ($P < 0.05$).

[1] Mean values (g/100g) of production yield parameters of soymilk base product.

[2] The relation was based in volume of soymilk (L) per kilograms of soybeans.

4.2.3 Lipoxygenase activity

Soybeans contain about 20% of lipids, being 80% of them composed by unsaturated fatty acids. Linoleic acid (51%) is the most predominant unsaturated fatty acid, followed by oleic acid (23%) and linolenic acid (7%). Most of theses fatty acids are present in soymilk and makes a perfect substrate of lipoxygenase enzyme (Liu, 1999; Prawiradjaja, 2003).

Lipoxygenase (LOX), an iron-containing dioxygenase, catalyse the oxidation of polyunsaturated fatty acids containing *cis,cis*-1,4-pentadiene units to the corresponding conjugated hydroperoxydiene derivatives by the addition of molecular oxygen (Wang et al., 2008). In advanced stages of oxidation reaction, secondary products are formed such as aldehydes and ketones. These volatile compounds are part of the off-flavors which limit the acceptance of soymilk. According to Min et al. (2005), off-flavor compounds are often formed during soaking and grinding steps in soymilk elaboration. For this reason, controlling parameters such as time and temperature in the soaking and grinding steps may play an important role in the sensory perception and overall quality of soymilk.

Results showed that lipoxygenase activity was influenced by the temperature. Soymilk extracted at 60ºC presented a residual activity of 6.31 ULA (units of lipoxygenase activity), while no activity in soymilk extracted at 80ºC was detected. According to Yuan and Chang (2007), lipoxygenase is denatured approximately at 80ºC, which causes inactivation of its catalytic function. In addition, soybeans cultivars, year of production and geographic location have been reported to affect the content and activity of soybean lipoxygenase. Inactivation of lipoxygenase can be achieved by blanching soaked soybeans in boiling water for 10 min or by dropping dry seeds directly into boiling water for 20 min (Kwok & Niranjan, 1995).

4.2.4 Trypsin inhibitor activity

Trypsin inhibitors (TI) are substance with antinutritional properties. They affect protein digestibility, may cause pancreas hyperactivity and their presence in animal feed has been associated with growth suppression and pancreatic hypertrophy (Van der Ven et al., 2005). These substances have a proteolytic activity which reduces the availability of trypsin, an important protease in the animal digestive function (Friedman & Brandon, 2001). Two types of trypsin inhibitors are present in soybeans: Kunitz trypsin inhibitor (KT) and Bowman-Birk (BB) inhibitor. BB is considered more heat stable than KT due to higher proportion of disulfide bonds which stabilize the molecular composition required for biological activity (Kwok & Niranjan, 1995). Therefore the extent of destruction of TI (KT and BB fractions) in soymilk should be achieved for maximum nutritive value.

Values obtained of TI inactivation were calculated according to the absorbance values of trypsin used as standard. As a result, soymilk extracted at 80ºC showed higher values of TI inactivation (76.3%) compared to soymilk extracted at 60ºC (65.0%). Using different conditions of temperature in soymilk processing, Kwok et al. (1993) obtained 70% of TI inactivation from initial activity of untreated sample applying 93ºC for 20 min. Lei et al. (1981) found 30% and 34% of inactivation for soybeans water-extract heated at 70ºC and 80ºC, respectively, during 30 min. To reach an 80% of inactivation requires heating of 100ºC for 14 min and 30 min for a 90% of inactivation at same temperature (Liu, 1999). Other methods using high temperatures and/or high holding time reported achieve 90% of TI inactivation. For instance, Kwok et al. (2002) obtained 90% of inactivation from initial activity of untreated sample applying 93ºC during 60-70 min or 121ºC for 5-10 min. Ultra high temperature (UHT) treatments at 154ºC also reach 90% of TI inactivation, but it is

necessary to apply heating during 60 seconds (Kwok et al., 1993). However, overheating reduce nutritive value and cause destruction of important amino acids, such as cysteine, arginine and lysine (Skrede & Krogdahl, 1985). The results of TI inactivation obtained in this study are in agreement with the authors cited, indicating that time and temperature plays a fundamental role in the TI inactivation.

4.3 Conclusions

Soymilk samples extracted at 60ºC and 80ºC did not present significant differences in the values of dry matter and lipids in chemical composition results. However values obtained of protein content showed that proportion water:soybeans in the soymilk extraction as well as parameters such as temperature and time, affected total protein content. On the other hand, overall yield were not affected by these conditions ($P < 0.05$).

Soymilk base product elaborated at 80ºC was efficient in the lipoxygenase inactivation and caused a partial inactivation of the trypsin inhibitor activity. The method of soymilk elaboration applied was able to produce a product with good quality and feasible to available infrastructure of UAB pilot plant.

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Chapter 5

Comparison of ultra-high pressure homogenization and conventional thermal treatments on the microbiological, physical and chemical quality of soymilk

5

Comparison of ultra-high pressure homogenization and conventional thermal treatments on the microbiological, physical and chemical quality of soymilk.

5.1 Introduction

Commercial soymilk is produced mainly by conventional technologies, such as UHT (ultra high temperature). High temperatures, however, cause undesirable chemical changes which include destruction of amino acids and vitamins, browning reactions, and development of cooked flavors (Kwok & Niranjan 1995). In addition, working at high temperatures can accelerate the oxidation process, which is related with the formation of off-flavors and overall quality of soymilk.

Consumers demand high quality food products, which mean that they have to guaranty good nutritional quality, long shelf-life and high physical stability. To define the overall quality of a product a number of parameters have to be studied. In addition to good hygienic quality, soymilk should have low oxidation values, low antitrypsin activity, high emulsion stability and high nutritional values.

A previous study dealing with UHPH treated soymilk (Cruz et al., 2007) resulted in microbial reduction similar to pasteurization and an excellent physical stability. The objective of the present work was to study the influence of UHPH conditions (combination of inlet temperature and pressure) to obtain high quality soymilks. Special attention was to paied in finding out conditions for commercial sterility of the product. For this purpose six UHPH treatments (see 3.2 UHPH A) were performed by combining, 200 MPa and 300 MPa with different inlet temperatures (55, 65 and 75ºC). UHPH-treated soymilks were compared with those pasteurized and UHT-treated (see 3.2). Microbiological quality (see 3.6.1), particle size determination (see 3.9), particle sedimentation (see 3.10 centrifugation method), lipid oxidation (see 3.7) and trypsin inhibitor activity (see 3.8 method B) were applied as quality parameters.

5.2 Results and discussion

5.2.1 Soymilk chemical composition

Soymilk basic composition was not affected by the treatment applied. Data are given as mean values regardless of the treatment applied to soymilk (heat treatments or UHPH): 2.68 ± 0.17 g/100g protein; 1.92 ± 0.17 g/100g fat; 5.53 ± 0.39 g/100g dry matter; 1.35 ± 0.05 g/100g carbohydrate; 0.18 ± 0.05 g/100g ash. Mean pH value was 6.65 ± 0.05. Authors such as Liu (1999), Wang et al. (2001) and Cruz et al. (2007) presented similar composition of soymilk in their studies.

5.2.2 Temperature changes in UHPH processing

Temperature of soymilk reached in the high pressure valve in combination with very short time (approximately 0.7 s in this UHPH machine) at this temperature determined the quality characteristics of UHPH-treated samples. Temperature increase during UHPH treatment (Table 5-1) was about 20ºC between 200 and 300 MPa at the different inlet temperatures used in this study. This temperature increase was close to those observed by other authors. Pereda et al. (2007), who pressurized milk in the same UHPH equipment at inlet temperatures of 30 and 40ºC and pressures from 100 to 300 MPa, found the same temperature change. Thiebaud et al. (2003) reported a temperature increase of 18.5ºC per 100 MPa, in the pressure range 100 to 300 MPa for milk samples. The increase of temperature during UHPH treatment is the consequence of the adiabatic heating generated in the machine in addition to the turbulence, shear, and cavitation forces that the food experiments when passing through the ultra high pressure valve (Thiebaud et al., 2003; Hayes & Kelly, 2003).

Table 5-1. Temperature changes[1] of UHPH-treated soymilks during processing.

Ti (ºC)	Treatment (MPa)	T1 (ºC)	Tf (ºC)
55	200	105.7 ± 0.58	27.1 ± 1.0
	300	128.3 ± 1.53	27.3 ± 1.1
65	200	111.7 ± 1.15	27.0 ± 1.0
	300	130.7 ± 1.15	26.2 ± 0.8
75	200	117.0 ± 2.00	25.6 ± 2.7
	300	135.7 ± 1.53	26.2 ± 2.2

[1] Ti = inlet temperature; T1 = temperature after the homogenization valve; Tf = temperature after final heat exchanger.

5.2.3 Microbiological quality

Table 5-2 shows the microbiological counts (log cfu/mL) of BP, heat treated (pasteurized and UHT) and UHPH samples. In general, UHPH treatments at 200 and 300 MPa were more effective than pasteurization against almost all the microorganisms. However, significant differences ($P < 0.05$) were detected between UHPH samples at 200 MPa (55 and 65ºC inlet temperature) and pressurized samples at 300 MPa. According to some authors, the reduction in microbiological counts was pressure dependent, showing better inactivation as homogenization pressure increased (Thiebaud et al., 2003; Hayes et al., 2005). In contrast, Pereda et al. (2007) reported that no significant differences were detected between UHPH milk samples at 200 and 300 MPa at 30 and 40ºC inlet temperature, immediately after treatment. However, they concluded that milk treated at 300 MPa, 30ºC of inlet temperature had similar or better microbial shelf-life than pasteurized milk.

Additionally to the pressure applied in the UHPH treatment, inlet temperature could also be considered as a factor affecting microbial lethality, especially when samples were treated at 200 MPa. In this study, the highest temperature reached was 135°C at 300 MPa, 75ºC inlet temperature. All treatments at 200 MPa were more effective than pasteurization in reducing aerobic spore counts. UHPH samples treated at 200 MPa, 55ºC inlet temperature showed no significant differences ($P \geq 0.05$) in the total counts of mesophilic aerobic bacteria and *B. cereus* counts with pasteurized samples, while UHPH treatment at 200 MPa, 65ºC significantly reduced *B. cereus* and total mesophilic bacteria and spore counts compared with BP, pasteurized and 200 MPa, 55ºC samples. Cruz et al. (2007) who worked with soymilk at 200 MPa, 40ºC inlet temperature, obtained a reduction in the total

mesophilic bacteria counts of 2.42 log units, which was slightly higher than the reduction obtained in this study at inlet temperature of 55ºC and similar to those obtained at 65ºC inlet temperature, where reduction reached was of 1.6 and 2.58 log units, respectively.

Counts below the detection level for *B. cereus* (< 5 cfu/mL) and mesophilic aerobic bacteria and mesophilic aerobic spores (< 0.5 cfu/mL) were obtained for 200 MPa, 75ºC and 300 MPa at 55, 65 and 75ºC inlet temperatures. Although Cruz et al. (2007) obtained in soymilk samples a significant reduction of total mesophilic bacteria and spore counts (4 and 2 log units, respectively) after UHPH treatment at 300 MPa, 40ºC, it was not achieved a complete inactivation. Picart et al. (2006) reported a reduction on the total bacteria in milk samples of 2.9 log units at 300 MPa, 24ºC inlet temperature, while Pereda et al. (2007) reported in milk samples reductions about 3.3 log units at 300 MPa, 30 or 40ºC inlet temperatures. However, Smiddy et al. (2007) found a reduction of about 5 log units for UHPH-treated milk at 250 MPa, 55 and 70°C. As it was above mentioned, differences observed in microbial counts between this study and others could be explained by the effect caused of the maximum temperature reached in the UHPH treatment.

Table 5-2. Microbial populations (log cfu/mL ± SD) of BP and treated soymilks.

Treatment	Total bacteria	Total spores	*B. cereus*
BP	5.02 ± 2.24^{a}	3.46 ± 1.21^{a}	3.55 ± 2.37^{a}
Pasteurized	3.46 ± 1.23^{b}	2.85 ± 0.95^{a}	2.56 ± 1.91^{b}
UHT	ND[1]	ND	ND
200MPa 55ºC	3.39 ± 1.55^{b}	1.75 ± 0.69^{b}	2.31 ± 1.38^{b}
200MPa 65ºC	2.44 ± 1.59^{c}	0.85 ± 0.57^{c}	ND[2]
200MPa 75ºC	ND	ND	ND
300MPa 55ºC	ND	ND	ND
300MPa 65ºC	ND	ND	ND
300MPa 75ºC	ND	ND	ND

[a-c] Values in the same column with different superscripts are significantly different ($P < 0.05$).

[1] ND = no detected.

[2] *Bacillus cereus* growth was detected only in one production (3.40 ± 0.12 log cfu/mL).

Despite the fact that in some UHPH treatments, apparently were achieved a complete microbial inactivation, after incubation at 30ºC for 20 days to test the sterility of treated samples, it was observed that only the UHT samples and those treated at 300 MPa, 75ºC

did not show bacterial growth. Samples treated at 200 MPa, 65 and 75ºC inlet temperature coagulated after 2 days of incubation and those treated at 300 MPa, 65ºC showed microbial growth of 3.97 ± 0.08 cfu/mL after one week of incubation and coagulated on day 20. Spores showed a high resistance. According with Suárez-Jacobo et al. (2010), who worked with fruit juice, all vegetative cells were already destroyed at 200 MPa, and the differences between 200 and 300 MPa were only accounting for the destruction of spores. Cruz et al. (2007) and Pereda et al. (2007), also detected that bacterial spores were not completely eliminated by UHPH treatments with inlet temperatures of 30 or 40ºC at 300 MPa.

Coliform counts (data not shown) were only detected in one production of BP (2.57 ± 0.02 log cfu/mL). However, coliforms counts were below the detection level after pasteurization, UHT and UHPH treatments (< 0.5 cfu/mL). Cruz et al. (2007) found enterobacteria counts below detection level (initial count of 2.3 log cfu/mL) in soymilk samples when homogenized at 200 and 300 MPa, and Hayes et al. (2005) and Pereda et al. (2007), did not detect coliforms in milk samples, after treatment at 200 MPa and above.

Yeasts and moulds, *Salmonella* spp. and *Staphylococcus aureus* (data not shown) were not detected in both BP and treated soymilks. Tahiri et al. (2006) obtained inactivation of *Saccharomyces cerevisiae* and *Penicillum* spp. in orange juice by 2.5 and 4.0 log units, respectively, working at 200 MPa, 25ºC. Suárez-Jacobo et al. (2010) obtained reductions of yeast and mould counts around 5 log units in apple juice treated at 200 MPa and 300 MPa and Donsì et al. (2009) achieved a reduction of 5 log units for *S. cerevisae* inoculated in sterile water at 250 MPa, showing high susceptibility of some yeasts to UHPH treatment.

5.2.4 Colloidal stability: particle size and sedimentation

In soymilk, particles dispersed in the aqueous phase are of different characteristics such as oil droplets, native protein aggregates, and other aggregates formed from oil droplets and proteins and/or polysaccharides (Cruz et al., 2007; Malaki-Nik et al., 2008). The most common stability problems of vegetable beverages are the creaming of oil droplets and the sedimentation of solid particles; both phenomena depending to a great extent on particle size distribution. Colloidal stability was assessed by measuring solids sedimentation after centrifugation of soymilk and by means of particle size distribution (Table 5-3). It was observed that UHPH treatments reduced solids sedimentation induced by centrifugation compared with conventional thermal treatments applied in this study. Samples treated at

200 and 300 MPa at any inlet temperature did not show significant differences ($P \geq 0.05$) in solids sedimentation values. In the same way, samples did not exhibit additional sedimentation increase after 15 days of storage (data not shown). Another test to qualitatively evaluate the physical stability of samples consisted of detecting any destabilizing phenomena in glass bottles of treated soymilks for one week while maintained at rest. UHPH-treated soymilks remained in perfect dispersion state without any visible phase separation, while UHT and pasteurized soymilk exhibited a layer on the bottom of the bottles easily observable. All these results indicated that applying pressure in such a magnitude compared with those conventional applied at the food industry (in this case 18 MPa in a single or double step) is very effective for dispersing solid particles, even during storage periods. However this latter aspect should further be study.

Table 5-3. Solids sedimentation and particle size parameters of BP and treated soymilks.

Treatment	Stability index[1]	$d_{3.2}$ (µm)[2]	$d_{4.3}$ (µm)[3]
BP	7.03 ± 0.55[a]	0.51 ± 0.05[a]	12.98 ± 3.82[a]
Pasteurized	3.32 ± 0.30[b]	0.68 ± 0.04[b]	48.00 ± 10.2[b]
UHT	3.08 ± 0.23[b]	0.39 ± 0.03[c]	15.65 ± 1.83[a]
200MPa 55ºC	1.32 ± 0.11[c]	0.13 ± 0.02[d]	4.82 ± 2.27[c]
200MPa 65ºC	1.34 ± 0.10[c]	0.14 ± 0.02[d]	0.14 ± 0.01[d]
200MPa 75ºC	1.38 ± 0.12[c]	0.14 ± 0.01[d]	3.09 ± 0.37[e]
300MPa 55ºC	1.55 ± 0.40[c]	0.12 ± 0.01[d]	4.39 ± 1.16[ce]
300MPa 65ºC	1.61 ± 0.36[c]	0.11 ± 0.01[d]	1.32 ± 0.55[d]
300MPa 75ºC	1.38 ± 0.20[c]	0.11 ± 0.01[d]	3.39 ± 1.83[ce]

[a-e] Different superscripts in the same column are significantly different ($P < 0.05$).

[1] Mean values ± SD (g/100g w/w) of solids sedimentation after centrifugation.

[2] Mean values ± SD (µm) of average diameter (surface weighted mean diameter).

[3] Mean values ± SD (µm) of average diameter (volume weighted mean diameter).

Analyzing particle size distribution in soymilk is useful not only to determine the individual particle size but also to detect aggregates, which behave as big particles. Analysis of particle size was performed by examining the distribution curves and through the parameters $d_{3.2}$ and $d_{4.3}$ which are the average diameter of particles based on surface area and that based on volume of particles, respectively. The latter is especially sensitive to

the presence of aggregates. All samples exhibited a bimodal curve of particle size distribution, which was characterized by the presence of two peaks (Figure 5-1). UHPH-treated soymilks were characterized by a big peak in the range of 0 - 0.6 µm which represented 80 - 95% of the total volume of particles, and a second small peak (> 3 µm) which represented 5 - 20% of the total volume of particles, depending on pressure applied. UHT and pasteurization treatments were characterized by a peak in the range of 0.1 - 1.6 µm which represented approximately 65 and 20% respectively of the total volume of particles, and a second large peak (> 3 µm) representing about 35% of total volume of particles for UHT and 80% for pasteurized soymilk.

Particle size parameters exhibited a significant ($P < 0.05$) decrease in UHPH-treated soymilks compared to BP and to those soymilks processed by heat treatment (Table 5-3). However, pasteurized soymilk showed unexpected high values of particle size parameters compared to BP. This might be due to the aggregation of particles caused by using a single effect homogenizer as part of the pasteurization line in the present study. This phenomenon, well known in dairy industry (Walstra et al., 1999), makes using double effect homogenizers to be preferred. The second valve in double effect homogenizers, such as this used in UHT-treated soymilk in this study, have the objective of dispersing aggregates formed in the first valve, into smaller particles. Another data that supports this explanation is the fact that particle sedimentation was similar in both heat treated soymilks, pasteurized and UHT.

UHPH technology produced a considerably reduction of the mean particle size and also caused the reduction of aggregates volume fraction. On the other hand, in UHPH treatments the combination of pressure and inlet temperature did not produce significant variations in the mean diameter of particles, while it affected the production of aggregates. However, no specific relation was observed between UHPH treatment applied and aggregates formation. Despite the presence of small quantity of aggregates in the UHPH-treated soymilks, physical stability of these samples was not affected as demonstrated by the observed values of particles sedimentation. Thus UHPH dramatically increased colloidal stability of soymilk. The presence of aggregates in UHPH-treated soy-based food products has also been described by Floury et al. (2002) in emulsions stabilized with soy proteins, and Cruz et al. (2007) in soymilk. The formation of large particles was observed at 300 MPa for milk samples (Thiebaud et al., 2003) and for soymilk (Cruz et al., 2007). They attributed the aggregates presence to unfolded whey proteins, which in this condition may interact with other proteins and/or fat globules. In the UHPH treatment, phenomena of

cavitation, shear and turbulence are involved simultaneously, affecting the macromolecular conformation of proteins. In the case of soybean, globulins are affected by the decrease in solubility, due to the denaturation produced by UHPH treatment above 200 MPa, with the consequent aggregation (Floury et al., 2002).

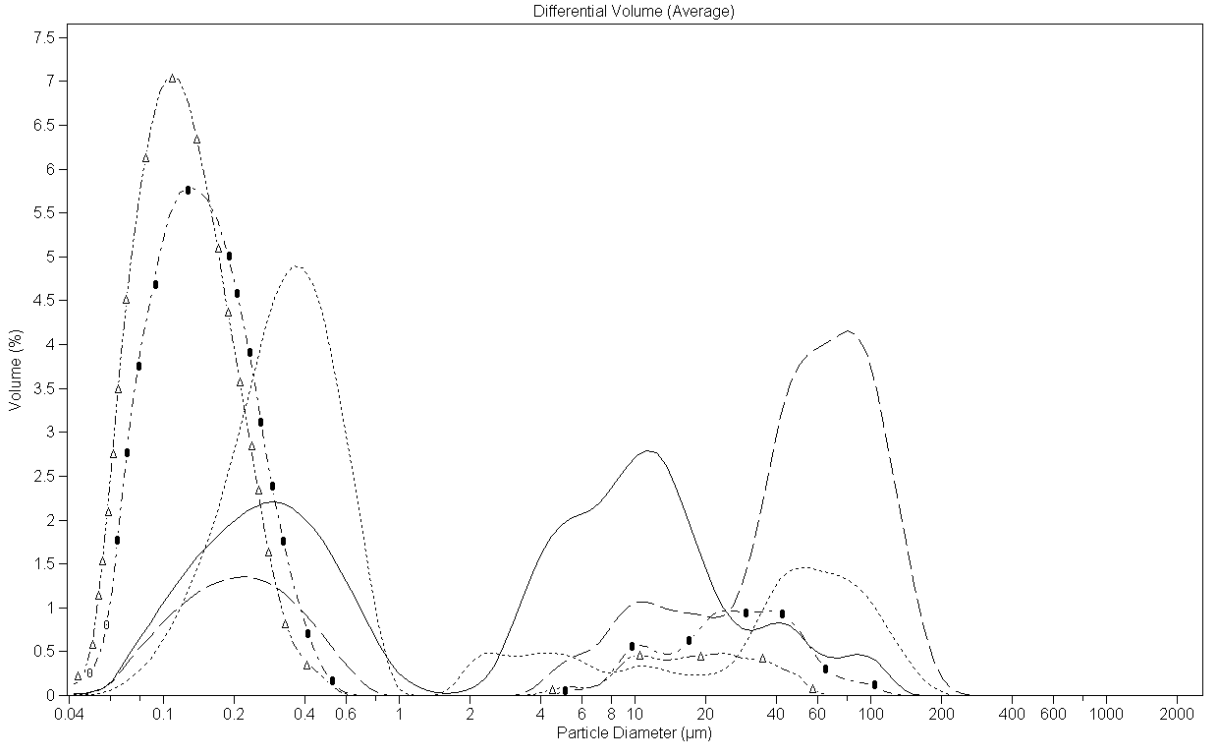

Figure 5-1. Particle size distribution determined by light-scattering of BP (—), pasteurized (--), UHT (…), 200 MPa, 75ºC(-●-) and 300 MPa, 75ºC (-Δ-).

Malaki-Nik et al. (2008) have study the colloidal stability of soymilk in terms of particle size distribution as influenced by heat treatment and homogenization. They concluded that heating of soymilk disrupts large aggregates of soy proteins and causes a decrease in the particle size. Their conclusions may be partially in agreement to those observed in this study. However, results have to be interpreted in their specific context. They applied a mild thermal treatment (60ºC) to obtain the BP sample which was further heat treated (95–100ºC for 7 min) or heated and subsequently homogenized (69 MPa). However, in the present study, soymilk BP was obtained by a method which included heating at 80ºC, causing therefore a partial denaturation of soy proteins. Subsequent heat treatments and

homogenization of soymilks were made in different conditions and sequence as those performed by Malaki-Nik et al. (2008). The same authors, Malaki-Nik et al. (2009) have further investigated the influence soy protein subunits by studying different soy varieties and confirmed the influence of protein subunit composition in the colloidal stability of soymilks. However, it is important to note that changing the elaboration process of soymilk might produce different results.

5.2.5 Chemical stability: oxidation and trypsin inhibitor activity

Lipid oxidation involves the formation of hydroperoxides as primary products in reactions which are developed through free radicals. There are many factors which favor lipid oxidation: presence of oxygen, light and enzymes such as lipoxygenase, and high temperatures. In this study, the oxidation process was evaluated by measuring the lipoxygenase activity and the hydroperoxide concentration at days 1 and 15 after processing of soymilks. As primary reaction, lipoxygenase catalyzes the hydroperoxidation by molecular oxygen of linoleic acid and other polyunsaturated lipids (Axelrod et al., 1981). Lipoxygenase activity was determined because it is an initiator of hydroperoxides formation and it may give some information about raw material manipulation previously or during processing. In this study, lipoxygenase was completely inactivated during the elaboration of soymilk BP and consequently, all subsequent treatments applied to soymilk did not present any lipoxygenase activity (dates not shown). During the elaboration of the BP sample, soybeans were ground at 80ºC for 20 minutes which explains total inactivation of lipoxygenase. Kwok and Niranjan (1995) reported different ways of lipoxygenase inactivation: grinding with water between 80ºC and 100ºC for 10 minutes, blanching soaked soybeans in boiling water for 10 minutes, and boiling dry whole beans in water for 20 minutes.

Hydroperoxide determination was carried out on the first day and 15 days after soymilk storage at 4ºC (Figure 5-2). On the first day, soymilk samples presented significantly lower hydroperoxide values than at day 15 at cold storage as expected ($P < 0.05$). The initial hydroperoxide values of samples did not exhibit a tendency when comparing UHPH and heat treatments, although the index ranged in a narrow interval between 0.2 and 0.4 meq/L for all samples. After 15 days of cold storage all treated soymilks presented higher hydroperoxide index and the tendency was defined: heat treated soymilks had higher values of oxidation index than UHPH treatments, 300 MPa at any inlet temperature

showed the lowest hydroperoxide index. Pereda et al. (2008) who applied UHPH to milk reported that 300 MPa produced samples with less hydroperoxides index compared to 200 MPa. However, the secondary oxidation, studied through malondialdehyde formation, was higher at 300 MPa, which indicated an evolution in the oxidation process to final products as also confirmed by the higher hexanal content of these samples.

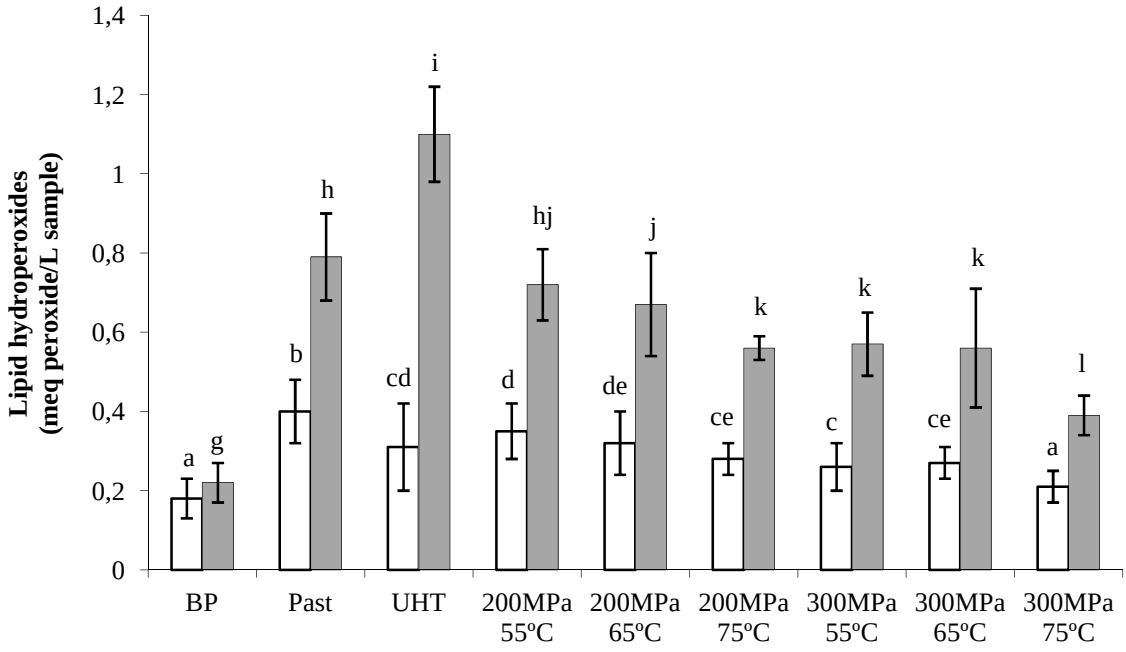

Figure 5-2. Hydroperoxides values (mean ± SD) of BP, heat treated (pasteurized and UHT) and UHPH soymilks at day 1 (□) and day 15 (■).

[a-e] Different letters above bars of day 1 indicate that samples are significantly different ($P < 0.05$).

[g-l] Different letters above bars of day 15 indicate that samples are significantly different ($P < 0.05$).

Trypsin inhibitors are substances with anti nutritional properties which are present in animal and vegetal tissues, such as soybeans. They reduce the availability of trypsin, an important protease in the animal digestive function for splitting proteins to render dipeptides and tripeptides (Guerrero-Beltrán et al., 2009). Consumption of raw or inadequately cooked soy products may cause a decrease in protein digestibility and nutritive value (Yuan et al., 2008). For this reason, reaching the maximum inactivation of trypsin inhibitors is an objective in the production of soy products. However, trypsin inhibitors (TI) are very stable due to the presence of disulfide bonds in their molecular

structure (Wolf, 1977). In the present study, original soybeans used to prepare soymilk presented a total content of 9.25 g of TI/L of sample extract.

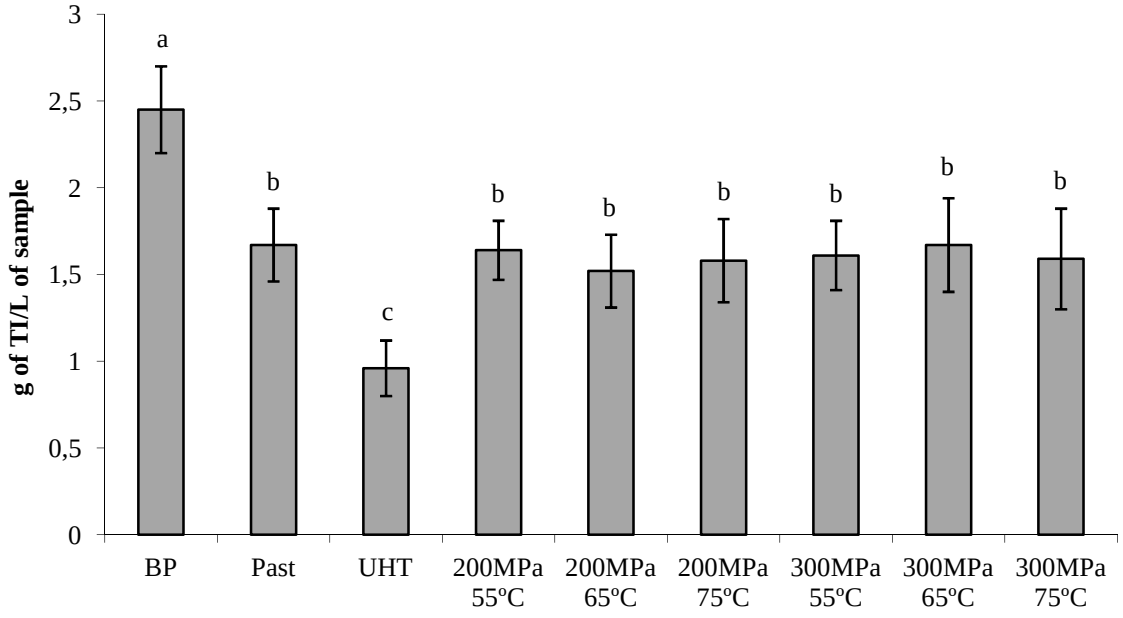

Figure 5-3. Trypsin inhibitor values (mean ± SD) of BP, heat-treated and UHPH soymilks.

[a-c] Different letters above bars of each treatment indicate that samples are significantly different ($P < 0.05$).

Figure 5-3 shows results of residual TI activity after treatment of soymilks. According to these results, UHPH treatments caused similar inactivation compared to the pasteurized sample (about 37% of initial activity in BP sample). However, to reach the same inactivation effect on soymilks, it was necessary that pasteurization acted during 30 s at 95ºC, whereas in UHPH treatment the holding time at high temperature (from 105 to 135ºC depending on inlet temperature and pressure applied) was about 0.7 s. So, to reach a significantly higher reduction of TI, it was necessary to combine higher temperatures and longer holding time such as in UHT treatment applied in this work (142ºC for 6 s). Under these conditions, TI inactivation achieved 60.8% of the initial TI activity in the BP sample. This result is in accordance with values observed in commercial UHT soymilks (data not shown). Taking into account the TI values of soybeans, UHPH and pasteurized soymilks achieved total inactivation about 80%, whereas UHT-treated soymilk achieved 90%.

Kwok et al. (1993) reached about 90% of inactivation (considering TI values of untreated sample) in soymilk at 93ºC with holding time of 60 min or 154ºC using UHT treatment for

about 60 s. Miyagi et al. (1997) boiled soymilk by traditional cooking method at 100ºC for 30 min and reduced TI to about 10% of the initial activity (whole soybeans). All the above-mentioned studies applied higher temperature during longer holding times compared to UHPH treatment applied in this work.

5.3 Conclusions

The present study demonstrates that UHPH was effective in the reduction of microbial populations. Inlet temperature was decisive to achieve complete reduction in combination with pressure. UHPH at 300 MPa and 75ºC inlet temperature was able to produce sterile soymilks. In terms of physical stability, UHPH conditions applied produced high stability products, with a considerable reduction of particle size and no deposition layer of particles observed during the first week compared to that produced in heat-treated samples. The hydroperoxides index also showed lower values in UHPH-treated samples compared to heat-treated samples, although in order to assess the complete oxidation caused by treatments, further analysis must be completed by means of determination of the secondary oxidation products, as well as profile of volatile compounds and finally sensorial analysis. UHPH treatments did not achieve the same level of TI inactivation as UHT treatment. However, any study has established up to now healthy problems derived of the consumption of soymilk in humans.

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Chapter 6

Study of the potential inactivation of UHPH treatment on selected Bacillus spores isolated from soymilk

6

Study of the potential inactivation of UHPH treatment on selected Bacillus spores isolated from soymilk

6.1 Introduction

The ability of UHPH to inactivate microorganisms has been already recognized by some authors, Wuytack et al. (2002), Briñez et al. (2006) and Donsì et al. (2009). Several microbial strains were exposed to UHPH in order to evaluate the sensitivity of the microorganisms to the treatment and the efficiency in sanitization process. Investigations reported that microbial inactivation was influenced by microbial strains, medium of suspension, pressure and temperature in the homogenization valve.

Sporogenesis is a characteristic of some bacteria that permit organisms to withstand environmentally harsh conditions, allowing long-term survival and favoring contamination of many foods. *Bacillus* genus is a class of bacteria able to form spores and usually detected in soy foods, such as soymilk. The characteristic of endospores to be able to survive traditional heat treatments (pasteurization and UHT) widely used in dairy industry, allows, under good conditions, growth of vegetative bacteria at refrigeration temperatures (Feijoo et al., 1997; Van Opstal et al., 2004).

The few data available of the high-pressure homogenization effects on spore inactivation indicate a resistance of spores to this treatment. Therefore, the purpose of this study was to evaluate the kinetics of inactivation of *Bacillus cereus* and *Paenibacillus taichungensis* previously isolated and purified from UHPH-treated soymilk at 300 MPa, 65ºC inlet temperature. The spores of these microorganisms were then inoculated in sterile soymilk followed by UHPH treatments at 300 MPa and 55, 65, 75 and 85ºC of inlet temperature. For this purpose, a benchtop ultra high pressure homogenizer (see 3.2 UHPH B) was used for soymilk treatment. The methodology applied was as follow: isolation and selection of the strains (see 3.6.2), sporulation conditions (see 3.6.3) and spores recovery (see 3.6.4).

6.2 Results and discussion

6.2.1 Spore-former occurrence

Spore-forming bacteria prevalence in soymilk treated at 300 MPa, 65ºC of inlet temperature was investigated. Several genus and species of *Bacillus* were identified: *Lysinibacillus* and *Paenibacillus (P. taichungensis* and *P. glucanolyticus*). Of these *Bacillus* species, *B. cereus* and *Paenibacillus taichungensis* were the most frequently encountered on UHPH soymilk. Precisely, these indigenous spore-forming bacteria were selected to evaluate their lethality at 300 MPa and 55, 65, 75 and 85ºC inlet temperatures.

The contamination of foods with bacterial spores is well documented in several foodstuffs. The multiplication of the vegetative cells formed after spore germination and outgrowth can occur at a wide range of temperature (spore-forming bacterial species which include psychrotrophic, mesophilic or thermophilic). Water activity and pH can be the cause of foodborne poisoning or food spoilage (Carlin, 2011). In the present study, the more resistant aerobic mesophilic spore-forming bacteria found in soymilk treated by UHPH at 300 MPa and 65ºC of inlet temperature coincide partially with those found by Postollec et al. (2012). They investigated the occurrence of spore-forming bacteria in 90 foodstuffs (raw materials, dehydrated ingredients and processed foods) and reported that the most frequently encountered *Bacillus* species were *B. cereus, B. subtilis* and *B. licheniformis*, isolated from dehydrated vegetables. In food industry, hygiene management and processing largely contribute to lower spore-forming bacterial contamination ensuring quality and safety of final products (Postollec et al., 2012). *Bacillus cereus,* for instance, is widely distributed in natural and commercial products due to the strong resistance of its spores to physical and chemical disinfectant agents. The organism is associated with two types of gastrointestinal syndromes, denominated as emetic type and diarrhoeal type (Ju et al., 2008; De Jonghe et al., 2010). Food spoilage can be produced by two different ways: by survival of viable spores to the treatment (pasteurization/UHT) with subsequent growth, causing therefore the characteristic spoilage; or by extracellular enzymes (proteases, lipases and lecithinases) that are synthesized prior to heat treatment. According to Rodríguez-Lozan et al. (2010), conventional treatments are able to eliminate viable organisms, but do not inactivate the preformed heat-stable enzymes.

Paenibacillus has recently been recognized as a separate genus from *Bacillus* and many new species of *Paenibacillus* continue to be identified (Ivy et al., 2012). This specie have

been isolated from various different pasteurized foodstuffs (Guinebretiere et al., 2001) and even by ultra high temperature (UHT) treatment applied in milk (Scheldeman et al., 2004). Recently, *Paenibacillus* spp. has described as spoilage organisms. Some species appear to be predominantly psychrotolerant with an ability to grow in milk and possibly other foods at temperatures as low as 6ºC (Ivy et al., 2012).

6.2.2 UHPH effect on spores survive

Spores of *Bacillus* genus are greatly resistant to conventional treatments, including homogenization (Popper & Knorr, 1990). In this study, the effect of UHPH on spores of *B. cereus* and *P. taichungensis* suspended in sterilized soymilk has been evaluated by measuring the capability of spores to survive and proliferate in solid medium after treatment. Among of 7-8 log cfu/mL of each bacterial strain were inoculated in sterilized soymilk before treatment. In order to evaluate UHPH efficiency in the total elimination of inoculated strains and the possible recovery of sub-lethally injured cells, samples of drastic UHPH conditions (75ºC and 85ºC of inlet temperature) were incubated for 10 days at 30ºC. Table 6-1 shows UHPH effect in the reduction of *B. cereus* and *P. taichungensis* in sterile soymilk. All treatments applied caused significant reduction in the colony counts of the two strains inoculated ($P < 0.05$).

In general, *B. cereus* and *P. taichungensis* achieved great log reductions for all UHPH treatments. *P. taichungensis* reached complete inactivation at 85ºC of inlet temperature, despite samples presented higher initial load ($P \geq 0.05$), the resistance to the treatment was observed being higher for *B. cereus*. Considering the strains lethality calculated according to *log No- log N* equation described in chapter 3 (see 3.6.4), highest reduction for *P. taichungensis* was detected at 75 and 85ºC of inlet temperature (around 6 log units), whereas 65 and 55ºC achieved values between 3.17 and 2.15 log units, respectively. For *B. cereus* the maximum lethality was achieved at 85ºC of inlet temperature with values about 4.60 log units, whereas 75, 65 and 55ºC reached values of 3.37, 2.55 and 1.12 log units, respectively. Taking into account inlet temperature between bacterial strains, only significant differences were observed at 75ºC and 85ºC ($P < 0.05$). These results indicated that inlet temperature played an important role in the strains resistance against treatment. In addition, as inlet temperature increased, high microbial inactivation was reached. During the period of incubation, no coagulation and no microbial growth were observed for *P. taichungensis* treated at 300 MPa, 85ºC.

Table 6-1. Counts (log cfu/mL ± SD) of two bacterial strains inoculated in sterilized soymilk before and after treatment.

Treatments	*B. cereus*	*P. taichungensis*
Before treatment	5.28 ± 0.55^{ax}	6.01 ± 0.24^{ax}
300 MPa at 55ºC	4.08 ± 0.22^{bx}	3.19 ± 0.57^{bx}
300 MPa at 65ºC	2.90 ± 0.99^{bcx}	1.98 ± 0.93^{cx}
300 MPa at 75ºC	1.78 ± 1.45^{cx}	0.20 ± 0.30^{dy}
300 MPa at 85ºC	0.54 ± 0.47^{d}	ND[1]

[a-d] Different superscripts in the same column are significantly different ($P < 0.05$).

[x-y] Different superscripts in the same row are significantly different ($P < 0.05$).

[1] No coagulation was detected after sample incubation at 30ºC for 10 days.

Feijoo et al. (1997) investigated spores inactivation of *Bacillus licheniformis* in ice cream high-pressure homogenized. They reached reductions of 0.5 log units applying pressures from 50 to 200 MPa at 33ºC of inlet temperature. Chaves-López et al. (2009) evaluated the influence of high-pressure homogenization treatment at 150 MPa (one, two or three passes) in the inactivation of *Bacillus cereus* spore and *Bacillus subtilis* spore suspended in sterilized distilled water. When single stage was applied, a negligible reduction of colony counts at 150 MPa was observed. In fact, they obtained inactivation values of 0.5 log cfu/mL for *B. subtilis* and 0.3 log ufc/mL for *B. cereus*. However, applying three consecutive stages of treatment, high reduction of colony counts (about 5 log units) were obtained. In the present study, UHPH treatment at single stage was enough to reach about 6 log units, indicating that pressure of 300 MPa was more effective than number of passe at 150 MPa applied in that study.

Treatment parameters such as inlet temperature, temperature after homogenization vale, outlet temperature and pressure were monitored during UHPH treatment. Table 6-2 shows changes of temperature and pressure during soymilk processing. Treatments at 55ºC, 65ºC, 75ºC and 85ºC of inlet temperature achieved maximum temperature after high-pressure valve of about 109ºC, 118ºC, 128ºC and 137ºC respectively. These values may explain why spores lethality reached high values in samples treated at 85ºC, as expected. In the UHPH processing, the food fluid is brought to high pressure in few seconds and then forced through a very small orifice, the valve gap of few micrometres in width. The

resulting pressure drop generates intense mechanical forces and elongational stress in laminar flow at the valve entrance and in the valve gap. Turbulence, cavitation and impacts with solid surfaces occur at the gap outlet. Due to these phenomena, the food fluid experiences a short-heating phenomenon that increases as pressure increase (Dumay et al., 2012). According to Sharma et al. (2009), spore inactivation requires a combination of shear and temperature. Phenomena of turbulence, high shear, and cavitation forces increase as pressure increase, resulting in thermal dissipation on the product. The resultant temperature elevation has a positive effect in the destruction of bacterial spores. Some studies have reported germination stimulation of spore accompanied by sub-lethal heating, rendering germinated spore more sensitive to destructive action of temperature and mechanical forces as were vegetative cells (Hyung-Yong et al., 1999; Ananta et al., 2001). Therefore, the flow of inoculated soymilk through the homogenizing valve may have stimulated spores germination, rendering them sensitive to heat and mechanical forces (Diels & Michiels, 2006; Chaves-López et al., 2009). Nevertheless, it is worth to point that the holding time at high temperature was lower than 0.3 s, resulting in a minimal thermal damage respect to usual heat treatments applied in food industries. Although the residence time was very low, it was essential to reach high levels of spore inactivation.

Table 6-2. Temperature and pressure changes[1] of UHPH-treated soymilk during processing.

Pressure	Ti (ºC)	T1 (ºC)	Tf (ºC)
305.0 ± 1.73	55.6 ± 0.55	108.8 ± 0.84	4.80 ± 1.10
302.3 ± 2.52	65.2 ± 0.45	117.8 ± 1.10	7.60 ± 2.88
307.7 ± 3.21	74.6 ± 1.14	127.6 ± 1.52	7.60 ± 1.95
302.0 ± 2.65	84.0 ± 1.41	137.2 ± 1.30	8.20 ± 2.86

[1] Ti = inlet temperature; T1 = temperature after the homogenization valve; Tf = temperature after final heat exchanger. Mean values ± SD from 3 individual productions.

Additionally to processing parameters, the type of foodstuff may affect the efficacy of the treatment. Available results indicated that UHPH treatments were more effective against microorganisms in saline buffered solutions than complex matrices (Vachon et al., 2002;

Diels et al., 2005). Foodstuff is a complex chemical system in which most of components, such as protein, lipids and carbohydrates may affect microbial tolerance to heat or pressure, making difficult to study the possible protective mechanism of the interactions among those components (Roig-Sagués et al., 2009). Therefore, the possible reason of higher resistance of *B. cereus* to the UHPH treatment than *P. taichungensis* could be due to soymilk composition and to the components interaction in addition to cellular structure of the spore strain. Structurally, an endospore consists of a core, surrounded by a cortex of peptidoglycan, a spore coat of protein and in some species a delicate thin layer called exosporium. Mature core of endospore differs greatly of cytoplasm of vegetative cell from which it is derived. In addition, it is rich in calcium dipicolinate, contains small water content (10-30%) from the vegetative cell, becoming gel consistency the cytoplasm core (Diels & Michiels, 2006). These characteristics of spore confer high resistance to the treatment in addition to the matrix characteristics. On the other hand, when cell water content is about 10% the resistance to pressure and temperature remained unaffected. However, beyond 10% moisture, the resistance of spores is gradually reduced, enabling a particular pressure-temperature combination to achieve an adequate level of spore reduction (Ananta et al., 2001). In addition, water content from the matrix, in this case soymilk could be transferred to the spore cells, increasing its sensibility to pressure-temperature effect (Chaves-López et al., 2009). This approach was first elucidated from heat inactivation studies of spores in fat systems (Molin & Snygg 1967; Harnulv et al., 1977).

6.3 Conclusions

Bacterial endospores inactivation is the main objective in food sterilization, due to high resistance to several chemical and physical agents as well as heat treatments. The main potential spores identified and isolated from soymilk belonged to *Bacillus* genus, in particular *Bacillus cereus* and *Paenibacillus taichungensis*. UHPH treatment was able to reduce noticeably these bacterial strain inoculated in sterilized soymilk. It was observed that as the inlet temperature increased, the levels of inactivation increased, especially at 85ºC. Inactivation of *Paenibacillus taichungensis* was more effective instead of *Bacillus cereus* at any inlet temperature, reaching complete inactivation at 85ºC. The maximum temperature achieved in the high-pressure valve was 137ºC when inlet temperature of 85ºC was applied. The combined effect of high-pressure and temperature was determinant in the

bacillus strains inactivation, taking into account that the holding time at high temperature was very low. Therefore, the use of UHPH system could be an alternative to increase shelf-life and microbial safety in soymilk processing, avoiding the typical thermal damage caused by conventional technologies.

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Chapter 7

Characterization of volatile profile in soymilk treated by UHPH

7

Characterization of volatile profile in soymilk treated by UHPH

7.1 Introduction

The main off-flavors that could be associated to the limited soymilk acceptation by consumers have been described as green, grassy, paint, rancid, astringent, and bitter (Torres-Penaranda, & Reitmeier, 2001; N'Kouka, et al., 2004; Lozano, et al., 2007). Soy odors in soymilk are primarily derived from enzymatic oxidation. Moreover, auto-oxidation of linoleic and linolenic acids also plays an important role in the off-flavor generated by degradation products in the last steps of oxidation reaction (Min, et al., 2005). Thus, the profile of volatile compounds in soymilk is strongly related to its quality and acceptability.

Identification of volatile compounds in soymilk treated by UHPH and comparing them with those identified by conventional thermal treatments could provide information related to sensory quality and better acceptation of soymilk. The aim of this study was to characterize the volatile profile of soymilk treated at 200 MPa at two inlet temperatures (55 and 75ºC) and 300 MPa at 80ºC of inlet temperature (see 3.2 UHPH A). UHPH soymilks were then compared to UHT and pasteurized treated samples. SPME analysis (see 3.14) was applied in order to characterize soymilk volatile profile.

7.2 Results and discussion

7.2.1 Volatile compounds

In addition to those natural compounds from soy seeds, responsible for the characteristic volatile profile, other compounds may be generated and the amount of the original compounds may be changed during processing of soymilk. An important part of those compounds is consequence of lipid auto-oxidation. This important lipid degradation in soy and derived products depends on soymilk processing conditions such as light incidence,

partial pressure of oxygen and processing temperature. Oxidation may also take place by the enzymatic pathway through the lipoxygenase action. In both, enzymatic and no enzymatic auto-oxidation, hydroperoxidation of polyunsaturated fatty acids such as linoleic and linolenic acids by molecular oxygen is the primary reaction (Boatright & Lei, 1999; Kakumyan, et al., 2009). The hydroperoxides formed are very unstable and they can easily be transformed to final products such as aldehydes, ketones, furans, alcohols, polymers, etc. Maillard reaction and Strecker degradation might also contribute to the formation of volatiles compounds through saccharides and amino acids reaction in thermally processed soymilk (Lozano et al., 2007; Plutowska & Wardencki, 2007).

Fifty-seven compounds were identified in the headspace of soymilks studied in the present work, which consisted of untreated, pasteurized, UHT and UHPH treated at 3 different combinations of temperature and pressure. Table 7-1 shows total abundance of volatile compounds grouped by chemical families in BP, pasteurized, UHT and UHPH soymilks. Chemical families included aldehydes, ketones, alcohols, furans, esters and acids were listed in Tables 7-2 to 7-6.

Adehydes and alcohols were the most representative compounds in all chromatographic profiles studied for all treatments (Table 7-1) in agreement with the study reported by Achouri et al. (2006). In general and considering the total of volatiles for each family of compounds, soymilk treated at 200 MPa, 55 and 75ºC inlet temperature respectively, caused similar effects to the pasteurized and unprocessed soymilks. Only some differences were found between specific treatments and families (Table 7-1) which are later discussed without altering general results. At 300 MPa, the abundance of compounds of all chemical families increased with the increase of pressure and inlet temperature, reaching similar results to UHT soymilk for all groups except for furans for which UHT obtained the highest abundance values ($P < 0.05$). These results indicated that the adiabatic temperature increase caused by high pressure could be the main reason for the formation of volatile compounds. Temperature after homogenization valve in UHPH treatments at 200 MPa reached values between 106ºC and 117ºC when inlet temperatures of 55ºC and 75ºC, respectively, were applied and 144ºC were reached for soymilk treated at 300 MPa, 80ºC inlet temperature. The residence time at these temperatures in the UHPH treatment was only 0.7 seconds as described in chapter 5, while pasteurization reached 95ºC for 30 seconds and UHT 142ºC for 6 seconds. The differences of temperatures after homogenization valve between 200 and 300 MPa were the main cause of the changes observed in the volatile profile among UHPH treatments. On the other hand, differences

observed between UHT and 300 MPa, 80ºC indicated that high pressure additionally to the temperature could have an important effect on formation of the volatile profile, especially in the oxidative process during treatment application, as discussed below.

Table 7-1. Total of volatile compounds[1] by chemical family in BP and treated soymilks.

	BP	Pasteurized	UHT	200 MPa 55ºC	200 MPa 75ºC	300 MPa 80ºC
Aldehydes	150.64 ± 19.45^{a}	188.14 ± 11.28^{a}	252.42 ± 44.02^{b}	158.19 ± 16.05^{a}	184.64 ± 9.03^{a}	244.47 ± 7.40^{b}
Alcohols	248.92 ± 60.31^{ab}	244.61 ± 30.46^{ab}	207.47 ± 8.02^{ab}	171.26 ± 20.92^{a}	174.46 ± 17.40^{a}	258.62 ± 38.48^{b}
Ketones	28.20 ± 3.97^{a}	36.19 ± 2.71^{a}	47.12 ± 1.82^{b}	35.28 ± 2.55^{a}	34.06 ± 5.41^{a}	46.45 ± 5.12^{b}
Furans	19.84 ± 3.57^{a}	28.87 ± 1.51^{ab}	104.83 ± 9.43^{c}	20.36 ± 0.76^{a}	21.77 ± 0.70^{ab}	32.56 ± 2.05^{b}
Esters	9.95 ± 0.67^{ab}	8.96 ± 0.77^{a}	12.22 ± 1.37^{b}	6.48 ± 1.38^{c}	6.01 ± 0.77^{c}	9.79 ± 0.67^{ab}
Acids	26.50 ± 3.96^{a}	39.39 ± 2.27^{b}	44.30 ± 8.95^{bc}	33.83 ± 3.58^{ab}	40.73 ± 6.05^{bc}	49.18 ± 4.64^{c}

$^{a-c}$ Different superscript in the same row are significantly different ($P < 0.05$).

[1] Integrated area counts. Mean values x 10^5 ± SD.

7.2.2 Aldehydes

UHT treatment and 300 MPa, 80ºC UHPH condition caused the most significant effect in the total aldehydes compounds. The abundance of this chemical family influenced by treatment is shown in Table 7-2. Hexanal was the most abundant compound identified followed by pentanal. Other compounds were also detected in low levels such as acetaldehyde, propanal, heptanal, benzaldehyde and 2-hexanal. Except acetaldehyde, all other aldehyde levels changed after treatment ($P < 0.05$).

Of the aldehydes identified in soymilk, hexanal is the most studied because it is indicative of the oxidation degree of product and, as reported, it plays the most important role in the sensory off-flavors. Commonly it is related to beany, grassy and green flavors in soymilk (Yuan & Chang, 2007a). According to Hashim and Chaveron (1995), hexanal has a very low sensory threshold, so content above 25 µg/kg allows its detection. Therefore, hexanal content could be decisive in the sensory quality and as a result soymilk with a low hexanal content would receive a better acceptance by the consumers. Table 7-2 shows that UHPH treatments at 200 MPa achieved similar levels of hexanal compared to pasteurized and BP soymilks. However, soymilk treated at 200 MPa, 75ºC inlet temperature reached values slightly higher than those observed at 200 MPa, 55ºC, although this difference was not

significant ($P \geq 0.05$). These results are in accordance to the temperature increase during UHPH application. High temperatures combined with oxygen have an important role in the oxidative processes that take place during and after treatment. Alternatively, 300 MPa, 80ºC and UHT treatments reached the highest levels of hexanal. Similar results were observed by Pereda et al. (2008), who analyzed the effect of UHPH in the volatile profile in milk. They found that 300 MPa, 30 and 40ºC inlet temperatures showed higher content of hexanal compared to 200 MPa at the same inlet temperatures. Several authors (Min et al., 2005; Yuan & Chang, 2007ab; Achouri et al., 2007; Achouri et al., 2008) found also hexanal as the main compound in heat-treated soymilk.

Table 7-2. Abundance[1] of aldehydes detected in the volatile fraction of soymilks

			Treatments[4]					
Name	ID[2]	KI[3]	BP	Past	UHT	P1	P2	P3
Hexanal	MS, RI, P	1092	122.20 a	143.14 a	200.03 b	123.35 a	141.37 a	192.48 b
Pentanal	MS, RI, P	990	9.88 a	14.77 a	22.22 b	12.34 a	14.54 a	20.23 b
Acetaldeyde	MS		4.29 a	4.63 a	4.77 a	2.74 a	4.23 a	4.66 a
Benzaldehyde	MS, RI	1563	1.86 a	6.36 c	3.10 ab	3.66 b	4.28 b	4.66 bc
Propanal	MS, RI	804	2.78 a	5.53 b	4.22 ab	4.05 ab	4.00 ab	4.41 ab
2-Butenal	MS, RI	1056	1.16 ab	2.01 bc	0.62 a	3.15 c	5.65 d	2.22 bc
3-Methylbutanal	MS, RI	929	0.91 a	1.52 ab	1.36 ab	0.93 a	1.23 ab	2.15 b
Heptanal	MS, RI	1192	2.44 ab	2.64 a	4.37 d	1.43 c	1.59 ab	3.80 d
2-Hexenal	MS, RI	1219	1.48 a	2.03 ab	3.87 c	2.14 ab	2.49 ab	2.73 bc
2-Heptanal	MS, RI	1345	1.00 a	1.55 ab	2.67 bc	1.34 ab	1.63 abc	2.77 c
Nonanal	MS, RI	1407	0.91 a	1.21 ab	1.69 b	0.96 ab	1.17 ab	1.54 ab
2,4-Hexadienal	MS, RI	1430	0.48 a	0.65 ab	1.02 c	0.63 a	0.73 abc	0.95 bc
Octenal	MS		0.22 a	0.37 abc	0.72 b	0.31 ac	0.37 abc	0.61 bc
2,4-Heptadienal	MS, RI	1520	0.13 a	0.32 bc	0.96 d	0.31 b	0.37 b	0.68 c
2-Pentenal	MS, RI	1145	0.90 ab	1.41 c	0.80 ab	0.84 ab	0.97 a	0.59 b
Total			150.64 a	188.14 a	252.42 b	158.19 a	184.64 a	244.47 b

[a-d] Different letters in the same row are significantly different ($P < 0.05$).

[1] Integrated area counts. Mean values x 10^5.

[2] Identification: MS = Mass spectra, RI = Retention index compared to Pherobase database, P = Positively identified by comparison with authentic standard.

[3] Kovats retention index calculated.

[4] BP = Base product; Past = Pasteurization; P1 = 200 MPa, 55ºC; P2 = 200 MPa, 75ºC; P3 = 300 MPa, 80ºC.

Percentage of hexanal detected related to total volatile compounds in all treated soymilks varied between 25 and 30%. UHT, 200 MPa, 75ºC and 300 MPa, 80ºC were those treatments which reached the highest hexanal percentage with values around 30% compared to 25% of BP. Wilkens and Lin (1970) reported that hexanal comprises about 25% of the total volatile profile in soymilk. On the other hand, Suratman et al. (2004) found 34%-49% of hexanal related to total volatiles in soymilk added of cyclodextrins and heated at 95ºC for 15 min. Reported hexanal percentage in soymilk by Achouri et al. (2008) was between 50 and 66% taking into account a total of 14 volatile compounds identified in soymilk made from soybeans stored for 10 months. They concluded that making soymilk from stored soybeans for 3 months, resulted in a product with lower volatile compounds formed. Thus, controlling process conditions, method of treatment as well as soybean quality, could help to minimize the hexanal formation and improve soymilk sensory quality.

Pentanal was the second most detected compound in the aldehydes group (Table 7-2). It was formed from oxidation of linoleic acid by the catalytic action of lipoxygenase, mainly in the grinding step of soybeans, when lipoxygenase was still active (Min et al., 2005; Mizutani & Hashimoto, 2004). The two treatments performed at 200 MPa did not produce pentanal level differences from untreated soymilk. On the other hand, UHT and 300 MPa soymilks showed higher values ($P < 0.05$) than those observed at 200 MPa (Table 7-2). Minor detected compounds such as heptanal, nonanal and 2-hexenal were also identified by Wilkens and Lin (1970), Suratman et al. (2004) and Min et al. (2005). These compounds in soymilk may be related to grassy flavor and oxidized aroma. In general, results indicated that temperature reached during UHPH treatment, in addition to the pressure, was decisive in aldehydes formation.

7.2.3 Ketones

Ketone compounds are derived mainly from linoleic acid (Wilkens & Lin, 1970). Table 7-3 shows the chromatographic areas of ketones in soymilk studied samples. UHT and 300 MPa, 80ºC treatments significantly ($P < 0.05$) increased total ketones composition while pasteurization and UHPH treatments at 200 MPa reached equivalent values to BP sample. The main compound detected was ketone, followed by 2,3-octanedione, 2,3-pentanedione, 2-butanone, 2-heptanone and 1-octen-3-one. Some of these compounds were also identified by Wilkens and Lin (1970), Boatright and Lei (1999) and Lozano et al. (2007)

on soymilk. As observed in Table 7-3, 2-pentanone, 1-octe-3-one, 2-octanone and 3-octanone, were not affected by treatment applied compared to BP ($P \geq 0.05$). UHT treatment reached higher values of ketone and 2-heptanone and 300 MPa, 80ºC had higher levels of 2-butanone and 2,3-octanedione compared to the rest of the treatments. UHPH treatments at 200 MPa did not show significant differences between them and BP sample, except for acetophenone, 2,3-octanedione and 2,3-pentanedione. The most interesting compound of the ketones group is 1-octen-3-one. It has been described by many authors to cause an undesirable flavor in soymilk. It was related to green-beany odor (Wilkens & Lin, 1970) and mushroom aroma (Boatright & Lei, 1999; Lozano et al., 2007). In addition, its sensory threshold was described to be about 7 µg/mL in heat-treated soymilk (Yuan & Chang, 2007a).

Table 7-3. Abundance[1] of ketones detected in the volatile fraction of soymilks

			Treatments[4]					
Name	ID[2]	KI[3]	BP	Past	UHT	P1	P2	P3
Ketone	MS, RI	826	12.05 abc	12.99 bc	25.10 d	9.87 ab	6.95 a	15.91 c
2,3-Octanedione	MS, RI	1333	3.72 a	5.46 ab	4.36 a	7.34 bc	8.00 c	8.47 c
2,3-Pentanedione	MS, RI, P	1072	3.77 a	5.17 b	3.30 a	6.39 bc	7.24 c	6.14 bc
2-Butanone	MS, RI	910	2.30 a	2.83 a	2.68 a	3.57 a	3.05 a	6.14 b
2-Heptanone	MS, RI	1190	2.07 a	3.85 b	5.63 c	2.59 ab	2.76 ab	3.36 ab
1-Octen-3-one	MS, RI, P	1314	1.49 a	1.79 a	1.64 a	1.94 a	1.90 a	1.94 a
2-Octanone	MS, RI	1295	1.02 a	1.51 a	1.30 a	1.25 a	1.27 a	0.99 a
2-Pentanone	MS, RI	1070	0.62 a	0.87 a	0.95 a	0.78 a	1.22 a	1.00 a
3-Octen-2-one	MS, RI	1427[A]	0.54 a	0.64 a	1.20 b	0.64 a	0.67 a	1.30 b
3-Octanone	MS		0.46 a	0.62 a	0.64 a	0.52 a	0.50 a	0.55 a
Acetophenone	MS, RI	1693	0.16 a	0.47 bc	0.32 ab	0.41 bc	0.49 bc	0.63 c
Total			28.20 a	36.19 a	47.12 b	35.28 a	34.06 a	46.45 b

[a-d] Different letters in the same row are significantly different ($P < 0.05$).

[1] Integrated area counts. Mean values x 10^5.

[2] Identification: MS = Mass spectra, RI = Retention index compared to Pherobase database and (A) Kobayashi et al. (1995), P = Positively identified by comparison with authentic standard.

[3] Kovats retention index calculated.

[4] BP = Base product; Past = Pasteurization; P1 = 200 MPa, 55ºC; P2 = 200 MPa, 75ºC; P3 = 300 MPa, 80ºC.

Due to low sensory threshold, hexanal and 1-octen-3-one may compromise sensory quality of soymilk as described by Yuan and Chang (2007a). Authors reported that a 1-octen-3-one content was affected by soybean material, heating method, and heating time. They achieved a drastic reduction of 1-octen-3-one compared to unprocessed soymilk according to the combination of temperature and time parameters during treatment.

In addition to 1-octen-3-one, acetophenone and 2,3-pentanedione respectively possess penetrating green and buttery unpleasant odors which may compromise the acceptance of soymilk (Boatright & Lei, 1999; Lozano et al., 2007). However, the conditions applied in the present work, the levels of this compound were not affected by any treatment applied.

7.2.4 Alcohols

Volatile alcohols were the second most detected group in all treated soymilks, even in untreated soymilk. Table 7-4 shows the relative abundance area of alcohols for all soymilks studied. Most of them were associated with green and beany aromas which are characteristic off-flavors of soymilk. No changes were observed in the total alcohols due to heat and UHPH treatment taking into account BP sample as reference level, although a slight decrease was observed in treatments at 200 MPa ($P \geq 0.05$). Some compounds were not affected significantly by the type of treatment: 1-octen-3-ol, (Z)-2-penten-1-ol, 2-heptanol, 1-octanol, 2-octanol, (Z) 3-hexen-1ol, 3-octanol and 2-ethylhexanol. In general, treatments, except pasteurization, presented only one significantly different compound ($P < 0.05$) to the untreated sample. Ethanol was one of them and moreover, it was the most abundant among this family of compounds. Its origin was reported to be from oxidation of linolenic acid (Wilkens & Lin, 1970). As shown in Table 7-4, ethanol levels decreased in all treatments compared to BP and showed specially a stronger reduction in 200 MPa UHPH treatments. Min et al. (2005) identified ethanol in soymilk samples as the second most abundant compound detected. On the other hand, Wilkens and Lin (1970) found ethanol as the least abundant alcohol compound in soymilk. 1-Hexanol and 1-pentanol were also identified as relevant compounds in the present study in terms of high volatile levels. Both compounds may be originated from linoleic acid as reported by Wilkens and Lin (1970) and Kobayashi et al. (1995), although a possible alternative of 1-hexanol formation could be through hexanal reduction by alcohol dehydrogenase before treatment (Kakumyan et al., 2009). 1-Hexanol and 1-pentanol were associated to beany and green odors (Achouri et al., 2008) and 1-hexanol may also be related to harsh grassy and painty

odors (Wang et al., 2001). Therefore, these compounds may play an important role in the overall quality aroma of soymilk. UHPH treatment did not affect 1-hexanol levels as shown in Table 7.4, but pasteurization treatment caused a significant effect with higher values detected. For 1-pentanol, no significant effect of treatment was found, except for 300 MPa, 80ºC (high levels). Relevant levels of 1-octen-3-ol and 1-penten-3-ol were also identified in the alcohols group. These compounds were associated respectively to mushroom and pungent aroma (Lozano et al., 2007) and their formations were linked to the oxidation of linoleic acid and linolenic acid, respectively (Wilkens & Lin, 1970). All treatments applied did not affect levels of 1-octen-3-ol, but slight differences, although not significant, were found between 200 MPa, 55ºC (low values) and 300 MPa, 80ºC (high levels).

Table 7-4. Abundance[1] of alcohols detected in the volatile fraction of soymilks

			Treatments[4]					
Name	ID[2]	KI[3]	BP	Past	UHT	P1	P2	P3
Ethanol	MS, RI	944	143.76 a	102.99 ab	103.54 ab	65.93 b	63.84 b	133.41 a
1-Pentanol	MS, RI, P	1260	32.58 a	33.36 a	27.53 a	31.20 a	32.90 a	43.14 b
1-Hexanol	MS, RI, P	1359	31.76 a	62.91 b	34.15 a	39.28 a	41.70 a	37.47 a
1-Octen-3-ol	MS, RI, P	1454	15.88 a	16.94 a	17.54 a	11.82 a	12.36 a	15.63 a
1-Penten-3-ol	MS, RI	1175[A]	12.72 ab	12.07 ab	13.10 ab	10.64 a	10.86 ab	14.14 b
3-Methyl-1-butanol	MS, RI	1260	2.00 ab	4.45 c	1.40 b	2.59 a	2.63 a	3.09 a
(Z)-2-penten-1-ol	MS, RI	1328	2.52 a	2.77 a	1.72 a	1.88 a	1.93 a	2.44 a
2-Methyl-1-butanol	MS, RI	1217	1.88 ab	2.34 b	1.32 a	2.01 ab	2.07 b	2.11 b
1-Butanol	MS	-	1.05 ab	1.20 ab	1.56 b	0.93 a	1.10 ab	1.42 ab
1-Heptanol	MS, RI	1460	0.94 a	1.13 ab	1.34 b	0.93 a	1.01 ab	1.24 ab
2-Heptanol	MS, RI	1323	0.73 a	0.99 a	0.83 a	0.77 a	0.80 a	0.78 a
1-Octanol	MS, RI	1562	0.73 a	0.71 a	0.69 a	0.74 a	0.63 a	0.71 a
2-Octanol	MS, RI	1421	0.66 a	0.86 a	0.73 a	0.71 a	0.73 a	0.63 a
(Z)-3-hexen-1-ol	MS, RI	1466	0.59 a	0.78 a	0.54 a	0.57 a	0.62 a	0.67 a
3-Octanol	MS, RI	1395	0.40 a	0.48 a	0.40 a	0.38 a	0.38 a	0.41 a
2-Ethylhexanol	MS, RI	1492	0.40 a	0.27 a	0.66 a	0.59 a	0.61 a	0.88 a
(E)-2-penten-1-ol	MS	-	0.33 ab	0.37 ab	0.42 ab	0.29 a	0.28 a	0.47 b
Total			248.92 ab	244.61 ab	207.47 ab	171.26 a	174.46 a	258.62 b

[a-c] Different letters in the same row are significantly different ($P < 0.05$).

[1] Integrated area counts. Mean values x 10^5.

[2] Identification: MS = Mass spectra, RI = Retention index compared to Pherobase database and (A) Vichi, et al. (2003), P = Positively identified by comparison with authentic standard.

[3] Kovats retention index calculated.

[4] BP = Base product; Past = Pasteurization; P1 = 200 MPa, 55ºC; P2 = 200 MPa, 75ºC; P3 = 300 MPa, 80ºC.

1-Hexanol, 1-pentanol and 1-octen-3-ol were also identified by Suratman et al. (2004) and Achouri et al. (2008) and 1-penten-3-ol by Wilkens and Lin (1970) and Lozano et al. (2007). Volatile compounds identification reported by these authors was carried out in heat-treated soymilk.

7.2.5 Furans

Generally, furans in soymilk are associated with unpleasant flavor besides being an indication of color changes due to treatment applied. Furans may be formed by the oxidation of unsaturated fatty acids or by Maillard reaction products (Achouri et al., 2007; Yuan & Chang, 2007a). UHT treatment (Table 7-5) caused the most significant effects in furans composition ($P < 0.05$). Levels of all compounds increased substantially comparing those of UHPH treatments, pasteurization and untreated soymilk. The Maillard reaction which favors furans formation take place when processing at high temperatures, for instance 121ºC, 143ºC and 154ºC (Kwok & Niranjan, 1995). Processing conditions (time and temperature) of UHT treatment used in the present study were probably the reason for the occurrence of browning reactions, contributing to the increase of furan compounds.

Table 7-5. Abundance[1] of furans detected in the volatile fraction of soymilks

			Treatments[4]					
Name	ID[2]	KI[3]	BP	Past	UHT	P1	P2	P3
2-Penthyl furan	MS, RI, P	1235[A]	10.61 a	13.96 a	40.89 b	10.97 a	11.96 a	16.36 a
2-Ethyl furan	MS, RI	965[A]	6.56 a	9.70 a	35.00 b	6.42 a	6.65 a	11.03 a
2-Propyl furan	MS, RI	1044	1.22 a	1.58 a	22.11 b	1.42 a	1.54 a	3.17 a
2-Vinyl furan	MS, RI	1085	0.74 a	0.75 a	2.98 b	0.71 a	0.68 a	0.98 a
2-n-Butyl furan	MS, RI	1135	0.46 a	0.53 a	2.61 b	0.59 a	0.65 a	0.57 a
2-Methyl furan	MS, RI	881	0.24 a	0.35 ab	1.25 c	0.26 a	0.28 a	0.46 b
Total			19.84 a	26.87 ab	104.83 c	20.36 a	21.77 ab	35.56 b

[a-c] Different letters in the same row are significantly different ($P < 0.05$).

[1] Integrated area counts. Mean values x 10^5.

[2] Identification: MS = Mass spectra, RI = Retention index compared to Pherobase database and (A) Vichi et al. (2003), P = Positively identified by comparison with authentic standard.

[3] Kovats retention index calculated.

[4] BP = Base product; Past = Pasteurization; P1 = 200 MPa, 55ºC; P2 = 200 MPa, 75ºC; P3 = 300 MPa, 80ºC.

Treatments at 200 MPa (Table 7-5) and that processed by pasteurization did not affect furan composition compared to BP sample. UHT and 300 MPa, 80ºC produced a significant increase in the levels of 2-methyl furan compared to BP, noticeably reaching UHT soymilk levels. The most abundant compound detected was 2- penthyl furan which is the main compound related with grassy and beany off-flavors (Boatright & Lei, 1999; Min et al., 2005; Achouri et al., 2006). 2-Penthyl furan is formed from linoleic acid by singlet oxygen that could be obtained by soy riboflavin or by atmospheric air under light (Min et al., 2005). Moreover, it has a low perception threshold (Yuan & Chang, 2007a). Results showed that UHPH treatment did not affect levels of 2-penthyl furan which, on the contrary, occurred in UHT soymilk ($P < 0.05$).

7.2.6 Esters and Acids

Ester and acid were the minority compounds in the whole volatile profile of soymilks in this study. In Table 7-6, a total of 3 acids were identified in soymilk: butanoic acid, pentanoic acid and hexanoic acid. Prevalence of all acids detected increased significantly due to treatment applied, either heat or UHPH. However, no changes were observed in 200 MPa, 55ºC compared to BP sample. The predominant compound detected was hexanoic acid. In general, its levels increased in all treatments compared to soymilk BP, with a more important increase in 300 MPa, 80ºC ($P < 0.05$). Lozano et al. (2007) found butanoic acid and hexanoic acid in heat-treated soymilk. These compounds were related to cheese aroma and sweaty odor, respectively. According to Wilkens and Lin (1970), hexanoic acid is formed by hexanal oxidation in presence of oxygen which in turn causes a fetid odor.

In the esters group, treatments at 200 MPa caused a significant decrease in the total esters composition, while esters remained stable in heat treatments and 300 MP, 80ºC compared to untreated soymilk. Methyl acetate and ethyl acetate were the most abundant compounds detected (Table 7-6). For methyl acetate, only UHT treatment caused a significant increase in the levels detected ($P < 0.05$) and, on the other hand, ethyl acetate decreased significantly in all treatments applied. Achouri et al. (2006) found ethyl heptanoate, ethyl octanoate and isoamyl acetate in 6 different commercial soymilks, and Kato, et al. (1981) found ethyl methanoate and ethyl acetate in roasted soybeans, but in this study only ethyl acetate was identified. There is no information in the literature about esters formation or about sensory effect on soymilk, however, they have been related with floral and fruity flavors in other products.

Table 7-6. Abundance[1] of esters, acids and others compounds detected in the volatile fraction of soymilks

Name	ID[2]	KI[3]	Treatments[4] BP	Past	UHT	P1	P2	P3
Esters								
Ethyl acetate	MS, RI	897	6.02 a	4.45 b	3.70 bc	2.84 bc	2.89 bc	4.84 ab
Methyl acetate	MS, RI	839	3.02 abc	3.69 ab	7.33 d	2.33 bc	2.18 c	3.85 a
n-Hexyl acetate	MS, RI	1278	0.41 a	0.38 a	0.48 a	0.46 a	0.46 a	0.45 a
n-Amyl acetate	MS, RI	1178	0.32 a	0.44 ab	0.55 ab	0.70 b	0.33 a	0.42 ab
Total			9.95 ab	8.96 a	12.22 b	6.48 c	6.01 c	9.79 ab
Acids								
Hexanoic acid	MS, RI	1946	24.52 a	35.44 bc	39.38 bc	30.65 ab	36.72 bc	44.46 c
Pentanoic acid	MS, RI	1747	1.84 a	3.63 bc	4.64 c	2.92 ab	3.66 bc	4.39 bc
Butanoic acid	MS, RI	1638	0.14 a	0.32 ab	0.28 ab	0.27 ab	0.35 b	0.33 b
Total			26.50 a	39.39 b	44.30 bc	33.83 ab	40.73 bc	49.18 c
Others								
Carbon dissulphide	MS		1.48 a	1.42 a	0.43 b	1.22 a	1.32 a	1.38 a

[a-d] Different letters in the same row are significantly different ($P < 0.05$).

[1] Integrated area counts. Mean values x 10^5.

[2] Identification: MS = Mass spectra, RI = Retention index compared to Pherobase database, P = Positively identified by comparison with authentic standard.

[3] Kovats retention index calculated.

[4] BP = Base product; Past = Pasteurization; P1 = 200 MPa, 55ºC; P2 = 200 MPa, 75ºC; P3 = 300 MPa, 80ºC.

7.2.7 Principal component analysis (PCA)

PCA is a statistical analysis for resolving sets of data into orthogonal components, whose linear combinations (principal components, PC) approximate the original data to any desired degree of accuracy. In most cases, two components are sufficient to explain a great proportion of the variation in the original parameters. Figure 7-1 shows the distribution of the samples in the PC1 and PC2 according to volatile composition. 38% and 19% of the variability was explained by PC1 and PC2, respectively. As observed in PC1 there is a clear separation between drastic treatment conditions and mild treatments. UHT and 300 MPa, 80ºC are located on the negative side of PC1 and treatments at 200 MPa, pasteurized and untreated soymilks are located on the positive side.

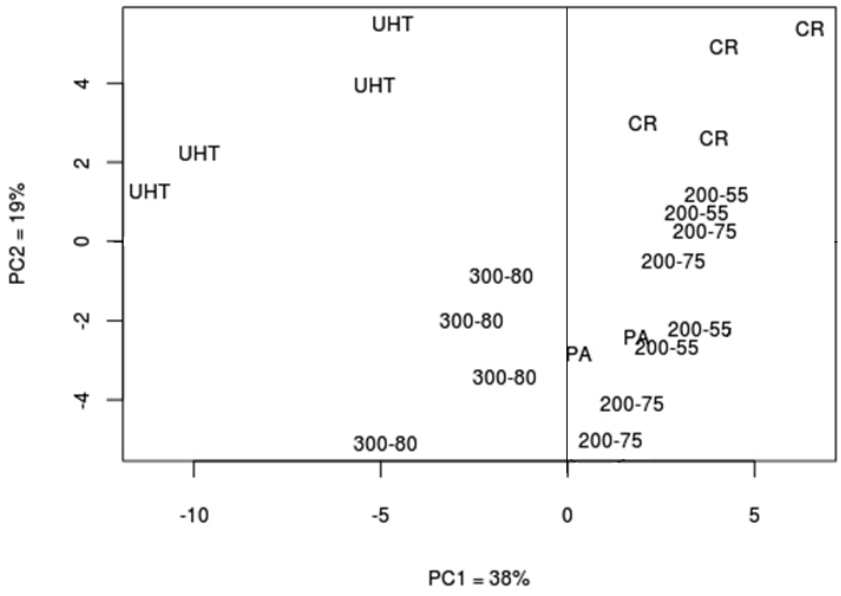

Figure 7-1. Loadings plot after principal component analysis of the individuals in the plane defined by two first principal components (PC1 and PC2).

Regarding PC1 model, several aldehydes, 3 ketones (ketone, 3-octen-2-one, 2-heptanone), two alcohols (1-butanol and 1-heptanol), 2 acids (pentanoic and hexanoic acids) and all furans achieved very high loadings (Table 7-7). UHT shows higher values of these compounds compared to 300 MPa, 80ºC. Due to processing parameters of UHT treatment, thermal damage was higher in UHT soymilk than 300 MPa, 80ºC soymilk. Therefore, samples are distributed along the PC1 component according to treatment intensity. In addition, 300 MPa, 80ºC soymilk shows a subtle approach between pasteurized and 200 MPa treatments. Concerning PC2, propanal and primarily benzaldehyde achieved high values in the aldehydes group. Some ketones (2,3-pentanedione, acetophenone, 1-octen-3-one and 2,3-octanedione), some alcohols and all furan compounds obtained very high values in the PC2 (Table 7-7). Moreover, the higher values of all furan compounds are related to UHT soymilk. As mentioned above, this class of compounds as well as aldehydes, some ketones and alcohols, are related to off-flavors of soymilk. On the contrary, benzaldehyde, which was reported as having a desirable almond flavor (Boatright

& Lei, 1999), shows negative loading in the PC2. This compound is more representative in 300 MPa, 80ºC treatment.

Differences in the holding time at high temperature and pressure applied during the process played an important role on the type of compound affected. To support this affirmation, treatments at low UHPH pressures and temperatures had similar results to pasteurized and untreated soymilk. Therefore, the real impact of pressure on volatile formation was associated to the temperature generated during UHPH process as a consequence of inlet temperature. High holding times at high temperature was more beneficial to volatile formation than combination of pressure and middle and high temperature at low holding time.

Table 7-7. Loading and percentage variance accounted by the first two principal components of soymilk volatile profile.

	Principal Component	
	PC1	PC2
Aldehydes		
2-Butenal	0.093	-0.149
2-Pentenal	0.062	-0.056
Benzaldehyde	-0.038	-0.246
Acetaldeyde	-0.072	0.086
Propanal	-0.078	-0.195
3-Methylbutanal	-0.093	-0.148
Nonanal	-0.180	-0.038
2-Heptanal	-0.187	-0.078
Heptanal	-0.188	0.061
2,4-Hexadienal	-0.190	-0.087
Octenal	-0.193	-0.045
2-Hexenal	-0.194	-0.044
Hexanal	-0.197	-0.021
Pentanal	-0.198	-0.056
2,4-Heptadienal	-0.204	-0.010
Ketones		
2,3-Pentanedione	0.065	-0.249
2,3-Octanedione	0.004	-0.255
1-Octen-3-one	-0.033	-0.243
2-Octanone	-0.039	-0.115
2-Butanone	-0.052	-0.200
Acetophenone	-0.053	-0.273
2-Pentanone	-0.069	-0.178
3-Octanone	-0.122	-0.029

	Principal Component	
	PC1	PC2
Ketones		
Ketone	-0.176	0.106
3-Octen-2-one	-0.180	-0.045
2-Heptanone	-0.198	0.009
Alcohols		
2-Methyl-1-butanol	0.113	-0.164
3-Methyl-1-butanol	0.069	-0.175
(Z)-3-hexen-1-ol	0.032	-0.078
1-Hexanol	0.017	-0.196
(Z)-2-penten-1-ol	-0.002	-0.076
1-Pentanol	-0.025	-0.177
Ethanol	-0.029	0.128
2-Octanol	-0.033	-0.093
3-Octanol	-0.042	-0.103
1-Octanol	-0.044	-0.017
2-Heptanol	-0.061	-0.122
2-Ethylhexanol	-0.080	-0.121
1-Octen-3-ol	-0.110	0.045
1-Penten-3-ol	-0.127	-0.006
(E)-2-penten-1-ol	-0.150	-0.053
1-Butanol	-0.175	0.019
1-Heptanol	-0.189	-0.037
Furans		
2-n-Butyl furan	-0.178	0.102
2-Ethyl furan	-0.181	0.119
2-Propyl furan	-0.182	0.122
2-Vinyl furan	-0.185	0.116
2-Methyl furan	-0.190	0.102
2-Penthyl furan	-0.195	0.094
Esters		
Ethyl acetat	0.027	0.129
n-Hexyl acetat	-0.050	-0.044
n-Amyl acetat	-0.053	0.009
Methyl acetat	-0.169	0.145
Acids		
Butanoic acid	-0.078	-0.245
Hexanoic acid	-0.156	-0.186
Pentanoic acid	-0.172	-0.145
Ohters		
Carbon dissulphide	0.139	-0.054
Porcentage of variance explained	38	19

7.3 Conclusions

The soymilk aroma profile was characterized primarily by aldehydes and alcohols. Compounds of these chemical families were the most detected in all treatments applied as well as untreated soymilk. Furan and ketone compounds were identified in low levels, but they are not less relevant. In general, the main compounds detected in soymilk by other authors were also identified in the present study, primarily compounds responsible for generating off-flavors. On the other hand, not all compounds identified possess a disagreeable aroma. For example, it is well recognized that benzaldehyde has an almond aroma, octanol has an odor slightly reminiscent of roses and 2-heptanone has a flowery odor. Hence, the characteristic aroma of soymilk is not formed by an individual compound, but by a mixture of them which can be formed in higher or lower levels according to the treatment applied. Pasteurization and UHPH treatments at 200 MPa produced slight changes in the volatile profile compared to untreated soymilk. Although UHPH treatment at 300 MPa, 80ºC inlet temperature achieved similar results to UHT treatment, PCA analysis indicated higher levels of furans in UHT soymilk. High temperature and high holding time were the main processing parameters responsible for affecting changes in volatile profile. Therefore, UHT could be the treatment that produces soymilk with lower sensory acceptance due to results of furan compounds combined with high levels of aldehydes, ketones and alcohols chemicals group. On the other hand, UHPH treatment at 300 MPa, 80ºC with low levels of furan compounds could have good sensorial acceptation.

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Chapter 8

Characteristics of soymilks pasteurized by UHPH

8

Characteristics of soymilks pasteurized by UHPH

8.1 Introduction

The consumption increase experimented by soymilk in the last years is accompanied by a specific segment of consumers due to the healthy characteristics. In fact, in North American markets, in addition to UHT treatment, soymilk is also commercializing under different conditions of pasteurization as demanded by consumers (The Soyfoods Association of America, 1996) and this product require to be stored under refrigeration to maintain a shelf-life of about one week (Kwok & Niranjan, 1995). The alternative treatment of soymilk in the market is that UHT-treated. However, despite the benefits of the heating process, such as microbial safety and extended shelf-life, UHT processing induces important modifications in sensory characteristics, resulting in changes in color, and loss of nutritive value (Achouri et al., 2007; Lozano et al., 2007). Pasteurization by thermal treatments applied at food industry is considered less aggressive to the overall quality parameters of the product. The low temperature applied in pasteurization, compared to UHT and sterilization treatments, is probably the main factor responsible for this advantage. However, the holding time used in the pasteurization process can be decisive in the detrimental effects of nutritional and quality attributes of soymilk. Therefore, the present study aims to evaluate the UHPH effect on changes in quality parameters of soymilk in order to produce a fresh product to be stored at refrigeration. For this purpose, pressure of 200 MPa at 55 and 75ºC inlet temperature respectively were applied on soymilk and compared with thermally pasteurized soymilk (95ºC, 30 s). Samples were kept in bottles at 4ºC to determine shelf-life (see 3.4 condition A). UHPH conditions (200 MPa, 55 and 75ºC) were selected based on microbial and chemical stability of soymilk from a previous study (chapter 5). Microbiological analysis applied included mesophilic aerobic bacteria and spore and enterobacteria counts (see 3.6.1). Quality parameters evaluated during storage of samples were: particle size (see 3.9), particle sedimentation (see 3.10 centrifugation and particle migration methods), hydroperoxide index (see 3.7.2), TEM (see 3.11), color (see 3.13), surface hydrophobicity (see 3.12), volatile profile evolution (see 3.14) and sensory analysis (see 3.15).

8.2 Results and discussion

8.2.1 Microbiological quality and pH measurements

Figure 8-1 shows microbiological results and pH measurements of untreated, UHPH and pasteurized soymilk during 28 days of storage at 4ºC. Initial counts of mesophilic bacteria and spores of untreated soymilk were respectively 2.13 log cfu/mL and 1.54 log cfu/mL. Enterobacteria counts were not detected in any sample (detection limit < 0.5 cfu/mL) (data not shown). UHPH treatments at 200 MPa were more effective in the microbial inactivation than pasteurization treatment, being 75ºC of inlet temperature the most efficient UHPH condition. Pasteurized soymilk showed an increasing in mesophilic bacteria counts of 4.2 log units from day 1 to day 28, whereas soymilk treated at 200 MPa of pressure and 55 and 75ºC of inlet temperature of just 3.2 and 0.3 log units respectively. During storage, mesophilic spore counts increased of 2.0 and 0.8 log units respectively for pasteurized and 200 MPa, 55ºC soymilks. In general terms, pasteurized soymilk presented higher bacterial counts compared to UHPH soymilks. From day 1 to day 14, mesophilic bacteria increased progressively in pasteurized and 200 MPa, 55ºC soymilks, but beyond this point an accelerated increase occurred mainly in pasteurized soymilk. In parallel with bacterial growth, pH values of pasteurized sample decreased considerably after 14 days of storage. On the last day of measurements, pH reached values about 6.49, indicating a slight acidification produced by this microbial growth. UHPH-treated soymilks revealed similar pH evolution during the period of storage. A decreasing in the pH values was observed after 21 days of storage, with values on the last day of analysis of 6.73 and 6.77 for 200 MPa, 55 and 75ºC of inlet temperature, respectively.

The marked microbiological growth observed in pasteurized soymilk, was related to the recovery of health and sub-lethally injured cells during storage or by spore germination that remained after treatment, while this recovery was not observed in soymilk treated at 200 MPa and 75ºC of inlet temperature. Phenomena, such as cavitation, shear forces and turbulence take place at high pressure causing destructive stresses of the bacterial cell (Middelberg, 1995; Donsì et al., 2009).

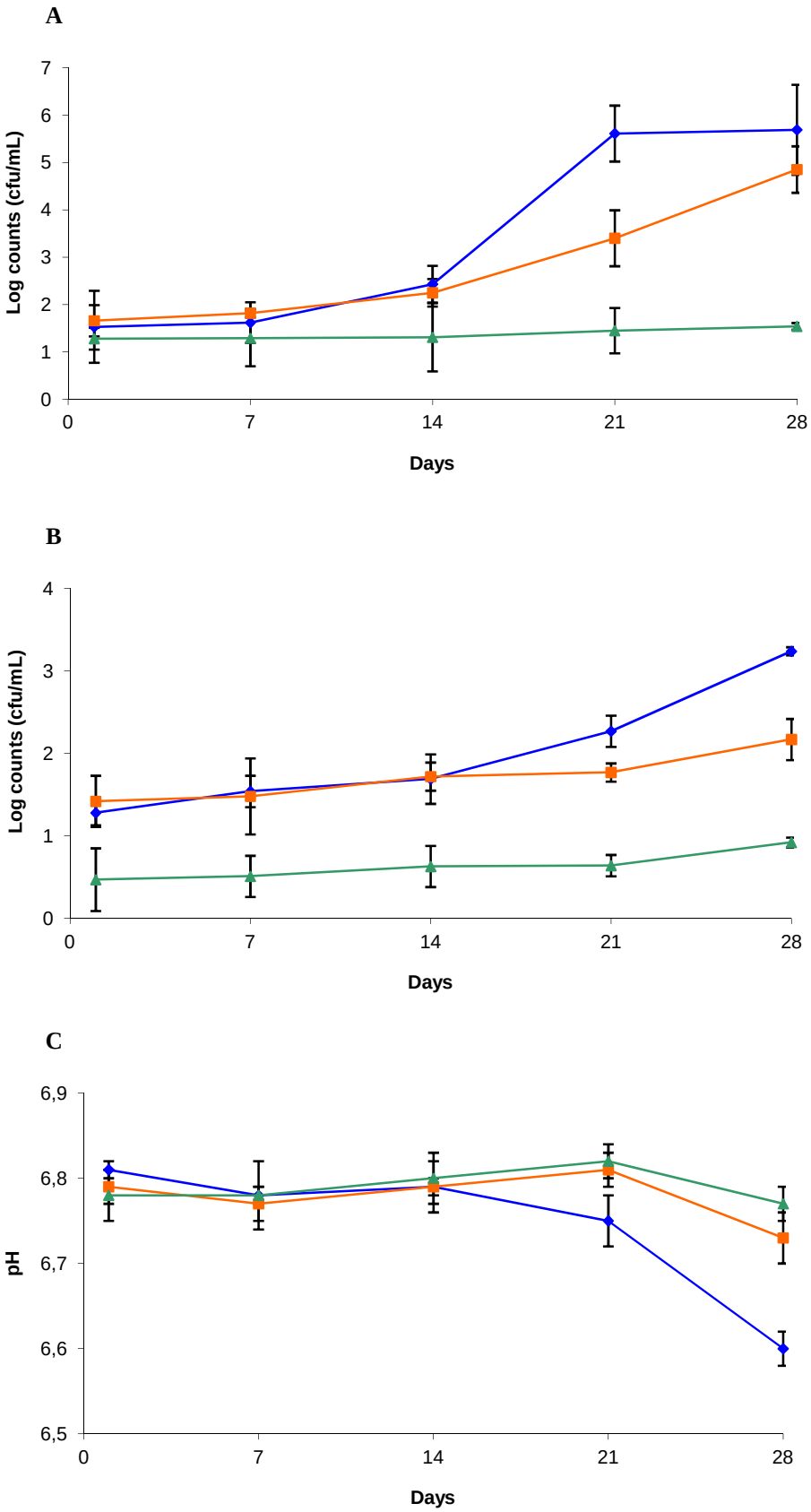

Figure 8-1. Development of (A) mesophilic aerobic bacteria counts, (B) mesophilic aerobic spores counts and (C) pH measurements of pasteurized soymilk (-♦-), 200 MPa, 55ºC (-■-) and 200 MPa, 75ºC soymilks (-▲-).

Similar increase of spores counts were observed in milk samples treated in the same UHPH equipment at 200 MPa, 30ºC and 40ºC (Pereda et al. 2007). However, mesophilic bacteria growth of that study presented higher counts than the present study with values of 7.4 and 3.9 log cfu/mL, respectively, after 21 days of storage at 4ºC. These differences may be attributed especially to the inlet temperature applied in each study and to the characteristics of indigenous microbiota of milk.

8.2.2 Microstructure description

Proteins in the untreated soymilk are distributed between the continuous phase (in form of soluble proteins, aggregates and protein bodies which are high density particles from seed grinding) and the surface of oil droplet. According to the intensity of the homogenization process, the protein dispersion may improve considerably and its capacity of adsorbing at the oil-water interface provide a better coverage and dispersion of the oil droplets finely distributed in the continuous phase. As a result, the soymilk stability can be improved dramatically.

Surface hydrophobicity measurements are related to the exposure of buried hydrophobic zones of the native proteins which are measured through fluorescence emission derived from the interaction between ANS (8-Anilino-1-naphtalene sulfonic acid) and those hydrophobic groups of proteins (Bouaouina et al., 2006; Miriani et al., 2011). This analysis gives information about protein structural modifications from native to unfolding state during processing. The major soy globulin proteins are glycinin and β-conglycinin and probably are the main fractions affected by the treatment conditions. During processing, soy proteins can undergo structural changes which may affect properties such as solubility and stability. Unmasking of inner regions makes the protein more active to hydrophobic interactions and to disulfide binding (S-S) between proteins, fat globules and small particles finely distributed in the aqueous phase, creating a new interface o/w which may modify the emulsion stability (Floury et al., 2002; Bouaouina et al., 2006; Shimoyamada et al., 2008). In Figure 8-2 can be seen the increase in hydrophobicity of UHPH-treated soymilks compared with pasteurized and BP samples. The homogenization (18 MPa) and subsequent heat treatment (95ºC, 30 s) of the pasteurized soymilk, favored the exposition of the inner hydrophobic regions of the soy globulins to bind to ANS. The same effect but more intense was observed in UHPH-treated samples with no relevant differences between UHPH conditions applied.

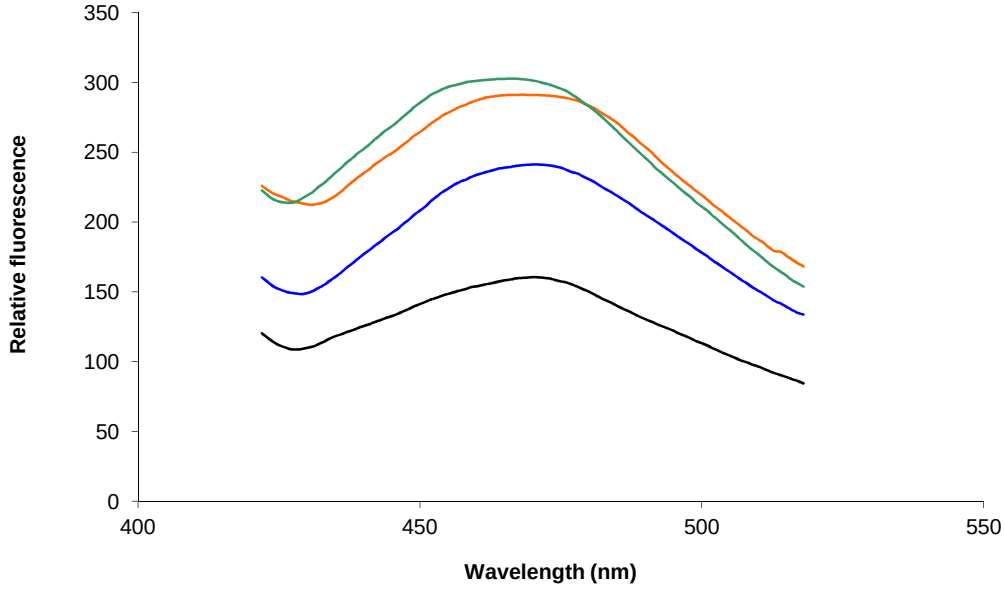

Figure 8-2. Protein surface hydrophobicity of BP (—), soymilk pasteurized (—), UHPH-treated soymilk at 200 MPa, 55ºC (—) and 200 MPa, 75ºC (—).

The large aggregates and/or native large particles detected by particle size determination in pasteurized and BP samples could partially have masked some of these hydrophobic regions, making them unavailable for hydrophobicity determination. Since for UHPH samples, the severe homogenization linked to the temperature in the high pressure valve experienced by soymilk, produced strong disruption of large particles, fat globules and protein aggregates. As a consequence, an efficient exposition of hydrophobic regions of the protein was obtained, allowing the complex protein-ANS.

Bouaouina et al. (2006) observed that surface hydrophobicity of UHPH-treated whey protein increased gradually as pressure increased, showing better stabilizing properties by the strong increase of hydrophobicity. Shimoyamada et al. (2008) observed an increasing in the hydrophobicity of soymilk during heating process. Their results were related to the improvement of soymilk stability by the soluble aggregates formed due to the soy globulins denatured.

Protein solubility is controlled by a delicate balance between repulsive and attractive intermolecular forces. Although each protein possesses a unique, well-defined structure in the native state, after drastic treatment such as UHPH or heat treatment, protein change its conformation, creating several non-specific structures according to the type and extent of treatment. For instance, β-conglycinin fraction undergoes a molecular rearrangement

involving a re-organization of its quaternary without affecting its tertiary structure (Floury et al., 2002; Miriani et al., 2011). Disulfide bonds were reported as being responsible for the preservation of the soy globulin tertiary structure due to high resistance to UHPH treatment of aqueous soy protein solution as reported by Floury et al. (2002). Therefore, the results of surface hydrophobicity of UHPH soymilks may indicate that protein denaturation occurred during UHPH treatment could not be related to loss of solubility, but on the contrary with better soymilk stability. However, extreme values could indicate complete protein denaturation which would result in the formation of sediments.

Micrographs of BP, pasteurized and UHPH-treated soymilk at 200 MPa, 75ºC are shown in Figure 8-3. In BP samples (Figure 8-3A), several groups of protein-fat globule aggregates and fat globules are observed distributed in the continuous phase. Most of fat globules are located far away to each other and they did not have spherical format as expected. In addition, a large aggregate of protein-fat globule is observed, masking hydrophobic zones of the soy protein. The distorted shapes of the oil droplets suggest that interactions between the aggregated proteins are stronger than surface tension forces (Malaki et al., 2008). For pasteurized sample (Figure 8-3B) a better distribution of oil droplets and small protein-fat aggregates could be seen. In this case, the homogenization process and heat treatment dispersed partially protein in the surface of fat globule favoring the formation of spherical droplets. On the other hand, large protein aggregates, possibly glycinin and β-conglycinin fractions, could be seen. The rearrangement of the protein structure caused by the heating process, has unmasked some hydrophobic zones allowing interactions between proteins. These results are in accordance to that obtained in particle size determination (Table 5-3), which indicated particles of large volume of pasteurized soymilk. Great spherical fat globules dispersion, some protein macromolecules and small protein-fat globule aggregates are observed in UHPH-treated soymilk (Figure 8-3C). Partially unfolded proteins and their molecular interactions in UHPH-treated soymilk could have resulted in the protrusion of very small fat globules into a protein aggregate. Therefore, a great dispersion of the soy protein in the surface of oil droplets and micro particles finely distributed in the aqueous phase was obtained in UHPH treatment. In addition to the reduced particle size (Table 5-3), a high number of micro particles solubilized in the continuous phase were produced. Because of this, several remained protein molecules are available for binding with ANS in UHPH soymilks. Cruz et al. (2007) and Malaki-Nik et al. (2008) also observed small aggregates of protein and fat globules in homogenized soymilk.

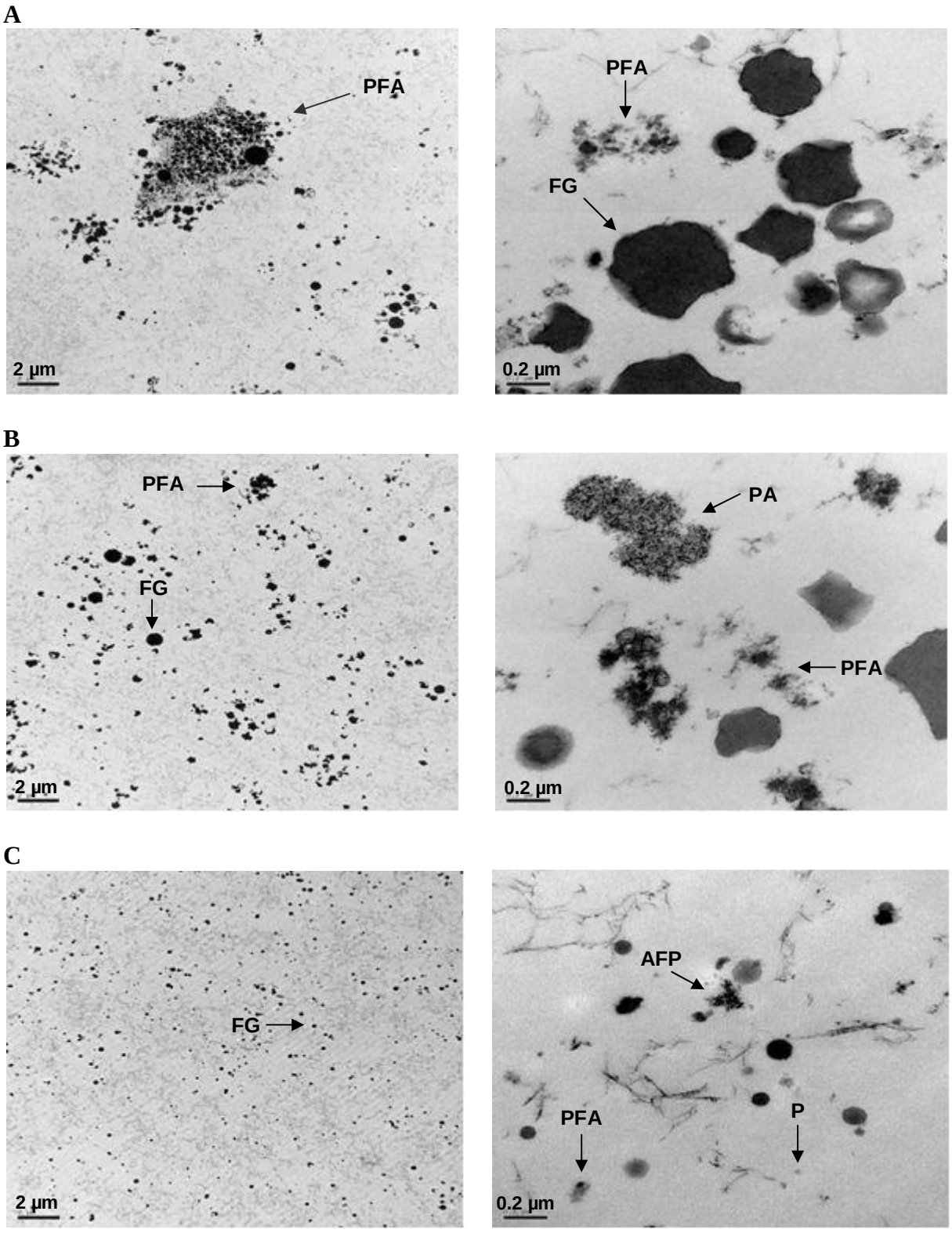

Figure 8-3. Transmission electron micrographs of (A) BP, (B) pasteurized soymilk and (C) UHPH-treated soymilk at 200 MPa, 75ºC. Different colloidal structures are indicated: (FG) fat globule, (PFA) protein-fat globule aggregates, (PA) protein aggregates, (P) protein and (AFP) aggregate of fat droplets protruded into protein.

8.2.3 Physical stability

Soymilk is a water extract of soybeans in form of oil-in-water emulsion. The most relevant functional property of proteins in this system is to cover oil droplets of the lipid fraction for maintaining a good dispersibility of those in the continuous phase during storage. Thus, soy proteins make soymilk to be a very stable emulsion. In spite of this, creaming of oil droplets and sedimentation of solid particles are the primary mode of destabilization of vegetable beverages. Both phenomena are dependent to a great extent on particle size distribution (Durand et al., 2003; Poliseli-Scopel et al., 2012).

Particles in soymilk include not only fat globules, but also small particles, such as protein bodies, protein aggregates and even protein-fat globule and globule aggregates (Cruz et al., 2007). Untreated and pasteurized soymilk presented highest values of particle size parameters (Table 5-3, chapter 5), indicating that conventional homogenization at low pressure (18 MPa) applied before heat treatment did not produce an additional decrease compared to the colloidal mill in the grinding step of soymilk elaboration. On the other hand, pasteurization with single effect homogenization used in this study was not enough to disperse aggregates formed into small particles. As expected, UHPH-treated soymilk showed higher reduction in the particle size parameters compared to pasteurized soymilk. According to the parameters evaluated, no significant differences were observed in particle size between UHPH soymilks (Table 5-3) in spite of inlet temperature and pressure. Previous results of particle size were supported by particle sedimentation through low-speed centrifugation during 28 days of storage (Table 8-1).

Particle sedimentation measured by centrifugation is indirectly related to the stability of the system. Under the same conditions, centrifugation was applied to all samples, forcing big particles and aggregates to separate from the bulk, either to the top, in the case of fat globules, or to the bottom, in the case of solid particles. This analysis can be considered as indicative of the sedimentation potential of soymilk during long storage periods and especially as a comparative measurement among treatments applied. Pasteurized soymilk presented higher amount of solids settled by centrifugation than UHPH soymilks throughout 28 days of storage (Table 8-1). As storage time increased, a slight increase in the percentage of sediments was observed, and differences were especially marked from 14 days of storage. Solids sedimentation values in UHPH samples were very reduced and only slight increase was observed at day 28 ($P < 0.05$).

Solid sedimentation results indicated that the state of particle dispersion of the UHPH samples provided enough stability during 28 days of storage, whereas homogenized soymilk by pasteurization did not resist the low centrifugal force revealed by solids accumulation in the bottom of the tube. Similar results were previously described in chapter 5. Applying similar centrifugation method of particle sedimentation in soymilk heated (70 to 115ºC), Shimoyamada et al. (2008) observed that the interaction between soy globulin fractions played an important role in the colloidal stability, mainly at high temperatures. They reported that the combination of denatured β-conglycinin and native glycinin caused lower stability of soymilk dispersion, whereas the combination of denatured β-conglycinin and denatured glycinin increased the stability.

Table 8-1. Solids sedimentation[1] of treated soymilks during refrigerated storage.

Treatment	Storage day				
	1	7	14	21	28
BP	5.97 ± 0.47^{a}				
Pasteurized	3.68 ± 0.17^{bx}	3.83 ± 0.12^{axy}	4.01 ± 0.09^{ay}	3.71 ± 0.08^{ax}	3.96 ± 0.08^{ay}
200 MPa 55ºC	1.34 ± 0.10^{cx}	1.36 ± 0.13^{bx}	1.53 ± 0.16^{bx}	1.48 ± 0.19^{bx}	1.86 ± 0.41^{by}
200 MPa 75ºC	1.19 ± 0.05^{dx}	1.18 ± 0.11^{cx}	1.34 ± 0.06^{cx}	1.28 ± 0.04^{cx}	1.95 ± 0.41^{by}

[a-d] Different superscript in the same column are significantly different ($P < 0.05$).

[x-y] Different superscript in the same row are significantly different ($P < 0.05$).

[1] Mean values ± SD (g/100g w/w) of solids sedimentation after low-speed centrifugation.

To evaluate the real impact of the treatment and its influence on the colloidal system stability, particles migration along the bottle containing the soymilk sample was performed in a Turbiscan^{R} equipment. This system measure the percentage of backscattered light which depend on the particle size and particle concentration. In such a way, it can be detected destabilization phenomena such as aggregation, sedimentation and creaming; depending on what part of the bottle the backscattered light is produced. Results of percentage of light backscattered in the bottom of the analysis tube (from 1.1 to 2.0 mm) are shown in Figure 8-4. An increase in backscattering intensity indicates that particle size and/or particle concentration has increased, while a decrease in backscattering intensity indicates a decrease in particle size and/or particle concentration.

All samples, including untreated soymilk (BP), increased the percentage of backscattering over time. BP and pasteurized soymilks showed the most pronounced increase, mainly in the first 7 days of storage when an accelerated rate was observed. Beyond this point, % of backscattering of these samples increased at a slow rate.

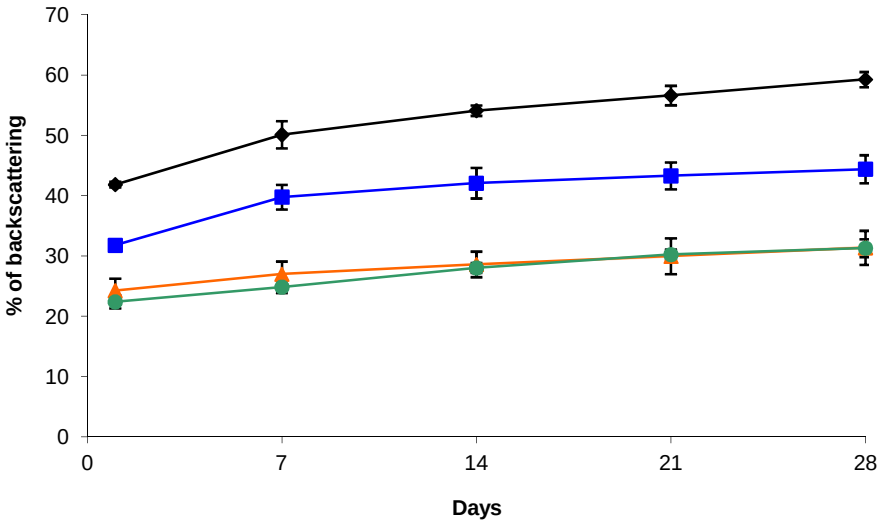

Figure 8-4. Percentage of backscattering in the bottom of the tube of soymilk BP (-♦-), pasteurized soymilk (-■-), soymilk UHPH treated at 200 MPa, 55ºC (-▲-) and 200 MPa, 75ºC (-♦-) during storage at 4ºC.

The difference in backscattering from day 1 to day 28 for the BP and pasteurized soymilks was 18 and 13% respectively, indicating, as expected high concentration of particles in the bottom of BP samples. In the same way, backscattering values of pasteurized soymilk was, respectively 10 and 15% higher than UHPH soymilk in the first and last day of analysis. On the other hand, UHPH soymilks showed a difference between first and last day of analysis of just 7.6%, indicating low particle concentration at the bottom of the analysis tube which resulted in higher stability of the soymilk during storage than pasteurized and BP samples. UHPH conditions presented a similar increase of backscattering at slow rate on all days analyzed. These results were supported by centrifugation and particle size parameters.

Durand et al. (2003) investigated stability of milk and of different vegetable beverages, including soymilk. All samples were treated by commercial UHT process and changes in backscattering intensity were analyzed during 12 h of measurements. They observed that milk and soymilk samples did not exhibit sedimentation phenomenon, but slight amount of

creaming in the surface of the sample. Creaming phenomenon was attributed to the tendency of oil droplets to coalesce by the large particles in the floating portion at the top of the tube. On the contrary, the present study revealed different levels of sedimentation of untreated and treated soymilks analyzed for 28 days as mentioned above.

Figure 8-5 shows ΔB (backscattering difference calculated of each measurement taking into account the first one as reference) versus sample length of the tube for BP, pasteurized and UHPH-treated soymilks during the different storage periods. The increase in ΔB at the bottom of the tube (between 0 mm and 5 mm) indicates an increasing in particle concentration and thus, indicative of the sedimentation phenomenon. The strong decrease of particle size as a consequence of UHPH treatment (chapter 5), have allowed particle migration along the tube with discrete particle concentration at the bottom, which was not visually perceptible. Creaming phenomenon of emulsion destabilization is identified by an increasing in ΔB at the top of the tube (near 40 mm), indicating the migration of the oil droplets due to the density difference between continuous and disperse phases. As observed in the figure no creaming was detected in any of the treated soymilks. Straight line at zero ΔB across the sample length indicates stable sample in which no changes occurred. UHPH-treated soymilk exhibited lower variation in the straight line compared to pasteurized treatment, indicating higher stability of the sample.

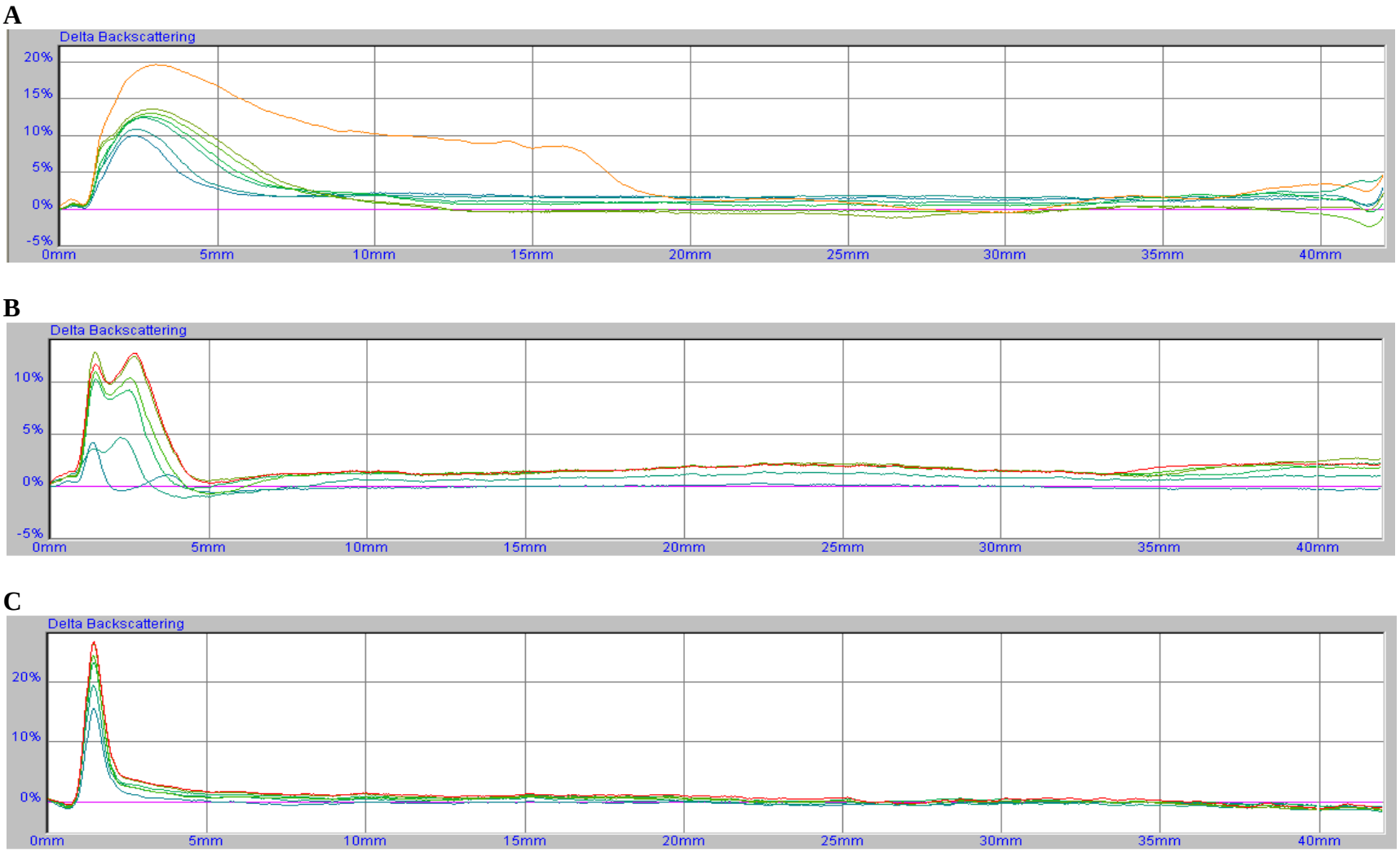

Figure 8-5. Delta backscattering for (A) BP, (B) pasteurized soymilk and (C) UHPH-treated soymilk at 200 MPa, 75ºC for 28 days of storage.

8.2.4 Chemical stability

Hidroperoxide formation in processed foods causes several deterioration reactions which negatively affect quality and storage life of food products. Stability reduction, changes in color parameters and formation of off-flavors are the main undesirable modifications which affect soymilk and its consumer acceptance (Gray & Monahan, 1992; Hornero-Méndez et al., 2001). Determination of hydroperoxide index in soymilk allows the evaluation of the initial stages of oxidation and its evolution through time. As a consequence, high number of non-volatile and volatile secondary compounds are originated. Results of hydroperoxides formation are shown in Table 8-2.

Table 8-2. Hydroperoxide index values[1] of untreated and treated soymilks

Treatment	Storage day				
	1	7	14	21	28
BP	0.38 ± 0.02^{a}				
Pasteurized	0.48 ± 0.02^{xb}	0.77 ± 0.03^{ya}	0.82 ± 0.03^{yza}	0.79 ± 0.07^{ya}	0.92 ± 0.10^{za}
200 MPa 55ºC	0.43 ± 0.01^{xc}	0.51 ± 0.02^{yb}	0.54 ± 0.03^{yb}	0.56 ± 0.03^{yzb}	0.63 ± 0.09^{zb}
200 MPa 75ºC	0.44 ± 0.01^{xc}	0.44 ± 0.01^{xc}	0.47 ± 0.02^{xc}	0.66 ± 0.03^{yc}	0.66 ± 0.04^{yb}

[a-c] Different superscript in the same column are significantly different ($P < 0.05$).

[x-z] Different superscript in the same row are significantly different ($P < 0.05$).

[1] Mean values ± SD (meq peroxide/L sample) of hydroperoxides.

Results showed slight increases of the hydroperoxide index for all treated soymilks during storage ($P < 0.05$), being pasteurized soymilk with highest values. This sample showed an accelerated increase in the first 7 days and remained fairly constant during the rest of the period of analysis, whereas UHPH treatments presented a slow rate of hydroperoxides formation. Although significant differences were obtained between UHPH treatments, its evolution was quite similar during storage time, reaching equivalent values on the last day of analysis ($P \geq 0.05$). Similar results were obtained by Pereda et al. (2008) who did not observe relevant differences between day 1 and day 18 of cold storage in milk pasteurized and treated at 200 MPa, 40ºC.

Secondary compounds formed by the oxidative process are commonly involved in unpleasant quality changes due to the generation of aldehydes, ketones, alcohols and furans compounds. Some of these chemical families are primarily derived from lipoxygenase

action on unsaturated fatty acids as described in chapter 7 and most of them are part of the volatile profile of foods.

Pasteurization and UHPH effects on total volatiles profile of soymilk during 28 days of storage at 4ºC is shown in Figure 8-6A. Significant changes were observed after pasteurization treatment compared to BP, and no changes were observed during in the last day of storage. No significant differences were found in the volatile profile between UHPH soymilk samples just after treatments. However, 200 MPa, 55ºC UHPH-treated soymilk showed a significant increase on the last day of storage while 200 MPa, 75ºC treated sample remained invariable.

Achouri et al. (2007) treated 3 different soymilk formulations at 142ºC for 4 s and found results of volatile profile fairly stable in two of them between 1 and 4 weeks of storage at 4ºC. Although they applied drastic thermal treatment, similar results were found in the present study in the same period of evaluation.

In base to found results, 28 days of storage at 4ºC did not produce important changes in hydroperoxide index and total volatiles compounds which could cause perceptible sensory modifications, primarily for 200 MPa, 75ºC by the total volatile results (Figure 8.6A). The high levels of hydropexides and secondary products of pasteurized soymilk is an indication of high oxidation rate compared to UHPH soymilks. Probably these oxidation levels could be linked to the holding time and temperature applied in pasteurization process.

As described in chapter 7, hexanal was the main compound formed as a consequence of lipid oxidation among all chemical families identified. Due to the large impact on the flavor of soymilk by its low sensory threshold and by its high detection, hexanal is commonly used as an indicator of secondary products formed during the oxidation process (Plutowska & Wardencki, 2007; Yuan & Chang, 2007). Figure 8-6B shows the ratio of hexanal to total volatiles of untreated and treated soymilks. UHPH treatment caused significant increase of hexanal compared to pasteurization treatment in the first day of analysis ($P < 0.05$). However, on the last day an important decrease was observed in UHPH soymilks, whereas pasteurized soymilk increased considerably ($P < 0.05$). Hexanal compound can be degraded in presence of oxygen to render carboxylic acid (Wilkens & Lin, 1970) and it can be also reduced to n-hexanol (Kakumyan et al., 2009).

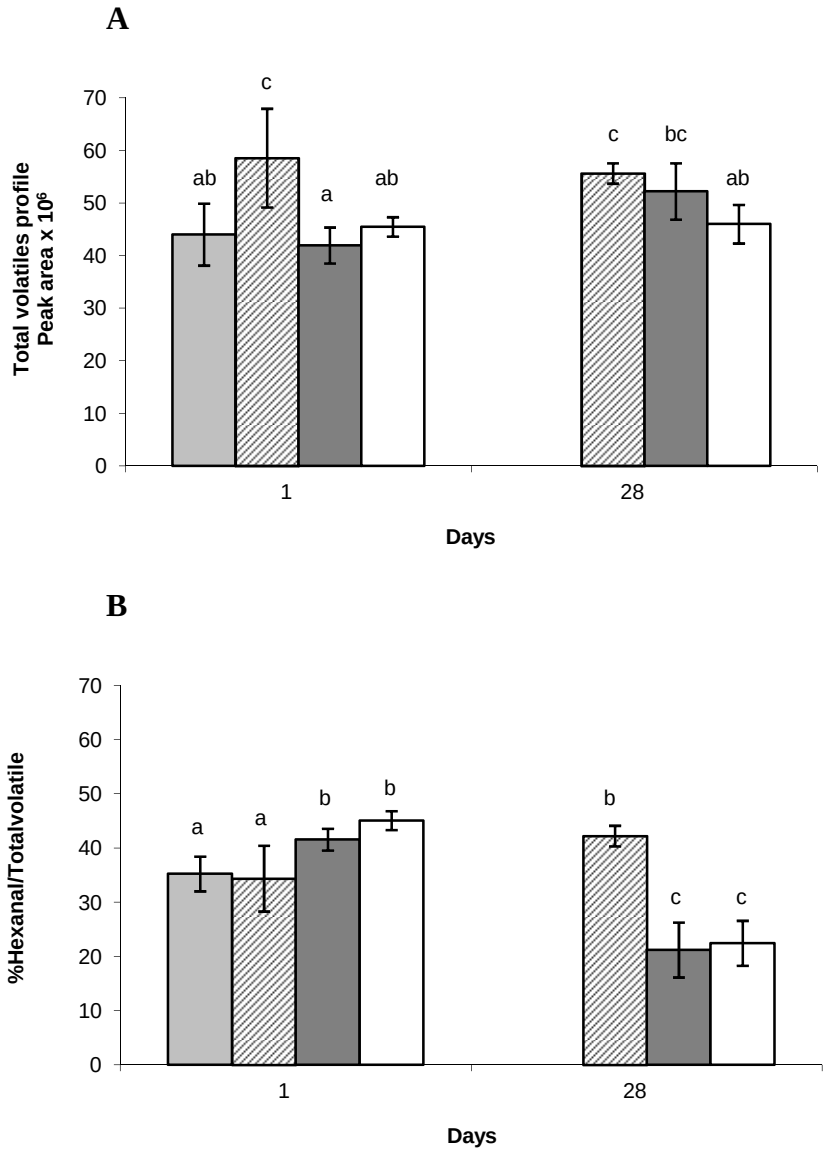

Figure 8-6. Evolution of (A) Total volatiles compounds and (B) percent ratio of hexanal to total volatiles of soymilk ( ) BP, ( ) pasteurized, ( ) 200 MPa at 55ºC and ( ) 200 MPa at 75ºC over a period of 28 days at 4ºC.

The dramatic reduction in fat globules size and the increase in surface hydrophobicity in UHPH-treated soymilks is indicative, as mentined above of a well covered surface of oil droplets by proteins through hydrophobic zones exposed (Dunkley et al., 1962; Huppertz & Kelly, 2006). Due to protein rearrangement, phospholipids were partially transferred to the aqueous phase, but some of them were retained in the particles for binding among protein and lipids molecules (Ono et al., 1996). The new interface formed could have caused a protective effect of the proteins against lipid oxidation. Therefore, a low oxidation

rate of UHPH soymilks took place during storage compared to pasteurized soymilk. For UHPH-treated milk samples, Hayes et al. (2005) suggested that at higher pressures there would be more exposed fat interface allowing a great proportion of casein to be adsorbed on the fat globule. The protective effect of caseins was the reason for the low hydroperoxides and hexanal values obtained in the study reported by Pereda et al. (2008) in milk treated at 200 MPa.

8.2.5 Sensory analysis

Sensory analysis applied in this study included the main attributes relevant to a global perception of the product characteristics. It is a subjective analysis that can be related to instrumental color evaluation and volatile compounds detection. L^* (luminosity) of color evaluation is the main parameter that indicate darkness/whiteness which is visually perceptible for a judges panel. On the other hand, volatile compounds can have an impact on sensory quality of soymilk due to the off-flavors generated by the compounds. Some of them are: hexanal (beany and grassy), 1-hexanol (beany, grassy), 1-pentanol (grassy), hexanoic acid (sweaty), 1-octen-3-ol (mushroom), 2-penthyl furan (beany and grassy), pentanal (buttery), 2,3-pentanedione (buttery), benzaldehyde (almond) and 1-octen-3 one (beany and grassy) (Wang et al., 1997; Boatright & Lei, 1999; Lozano et al., 2007).

Sensory analysis of treated soymilk was carried out after 15 days of storage at 4ºC using two different methods of evaluation: triangular and descriptive testing. The first one was used to identify possible differences between treated soymilks. According to results, 82% of the judges were not able to detect any difference between UHPH soymilks and pasteurized soymilk. The second part, descriptive test was performed to assess the most relevant attributes in soymilk by panelist, such as beany flavor, grassy aroma, oxidized aroma, astringent mouthfeel, thickness and darkness. Results of panelist evaluation are shown in Figure 8-7. Pasteurized soymilk were identified by judges as containing higher notes of grassy and oxidized aroma ($P \geq 0.05$) while soymilk treated at 200 MPa, 55ºC showed higher notes of beany and similar notes of grassy ($P \geq 0.05$). On the other hand, 200 MPa, 75ºC soymilk obtained low notes of these attributes ($P \geq 0.05$).

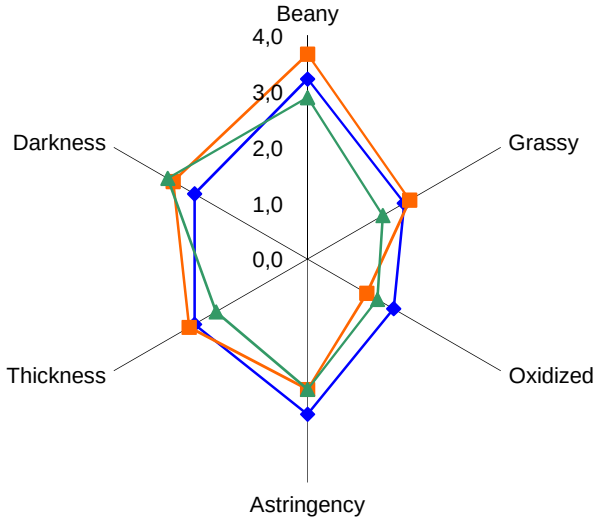

Figure 8-7. Effect on sensory attributes of soymilk pasteurized (-♦-), UHPH-treated soymilk at 200 MPa, 55ºC (-■-) and 200 MPa, 75ºC (-▲-).

Beany flavor, grassy and oxidized aroma can be considered the main off-flavors in soymilk (N'Kouka et al., 2004). These off-flavors perceived by the judges were the consequence of chemical changes that occurred due to the oxidative process, generating secondary compounds during elaboration, treatment and storage of soymilk. Table 8-3 lists the main volatile compounds detected at day 1 and day 28 of BP and treated soymilks. All compounds listed are considered as the main volatile compounds identified in soymilk by the sensory impact and on the other hand by the total levels detected. Among of compounds detected, hexanal and ethanol were the most abundant, followed by 1-hexanol and 1-pentanol. UHPH-treated soymilk showed a significant decrease in hexanal detection during storage while pasteurized soymilk remained stable. 1-Pentanol, benzaldehyde 1- and octen-3-one did not experience modification due to period of storage and between treatments applied. 2-Penthyl furan increased significantly after pasteurization treatment, however decreased during storage ($P < 0.05$), reaching similar levels to UHPH soymilks at day 28. 1-Hexanol and ethanol increased in UHPH soymilks during storage, whereas a slight decrease was observed in pasteurized soymilk. On the other hand, hexanoic acid increased in pasteurized soymilk while it decreased in UHPH soymilks. In general, inlet temperature of UHPH soymilks did not exhibit significant differences with similar levels for all volatile compounds and similar tendency during storage.

Table 8-3. Main volatile compounds[1] detected in untreated and treated soymilks

Compound	ID[2]	Base product	Pasteurization 1	Pasteurization 28	200 MPa 55ºC 1	200 MPa 55ºC 28	200 MPa 75ºC 1	200 MPa 75ºC 28
Hexanal	ABC	113.93 x	146.53 abx	165.30 a	123.35 bx	89.96 c	141.37 abx	83.31 c
Ethanol	AB	131.20 x	108.40 ax	90.09 a	65.93 ay	121.25 a	63.84 by	114.14 a
1-Hexanol	ABC	39.52 x	71.00 ax	47.35 a	39.28 ax	78.44 b	41.70 ax	63.45 a
1-Pentanol	ABC	35.25 x	47.73 ax	41.61 a	31.20 ax	41.84 a	32.90 ax	35.18 a
Hexanoic acid	AB	27.29 x	37.22 aby	44.09 a	30.65 bcx	21.59 c	36.72 aby	23.39 c
1-Octen-3-ol	ABC	15.40 x	18.45 ax	16.38 a	11.82 by	16.80 a	12.36 bx	11.92 b
2-Penthyl furan	ABC	10.06 x	16.96 ay	13.79 a	10.97 bx	15.65 a	11.96 bx	13.38 a
Pentanal	ABC	10.19 x	16.05 ay	18.81 b	12.34 cx	9.20 d	14.54 bcy	9.80 d
Propanal	A	3.34 x	7.06 ay	5.46 a	4.05 bx	4.49 b	4.00 bx	5.39 a
2,3-Pentanedione	ABC	4.13 x	5.64 abx	5.44 ab	6.40 cbx	4.78 cd	7.24 cy	4.10 d
Benzaldehyde	AB	2.27 x	5.47 ay	5.30 a	3.66 ax	7.05 a	4.28 ax	4.34 a
2,3-Octanedione	AB	4.23 x	4.70 abx	6.03 ac	7.34 dcy	4.06 b	8.00 dy	3.47 b
2-Butanone	AB	2.30 x	3.08 ax	2.24 a	3.57 bx	2.69 a	3.05 ax	2.14 b
1-Octen-3-one	ABC	1.97 x	2.32 ax	2.15 ax	1.94 ax	1.97 ax	1.90 ax	1.89 ax

[a-d] Different letters in the same row of treated samples are significantly different ($P < 0.05$).

[x-y] Different letters in the same row of treated sample at day 1 are significantly different from BP ($P < 0.05$).

[1] Integrated area counts. Mean values x 10^5.

[2] Identification: A = Mass spectra, B = Retention index compared to literature database, C = Positively identified by comparison with authentic standard.

In the compounds mentioned above, beany and grassy are, in general, the main sensory attributes associated to unpleasant flavors, although benzaldehyde confers a desirable almond flavor. On the other hand, sensory threshold plays an important role in the judges' perception. Hexanal, 1-octen-3-one and 2-penthyl furan were reported to possess low sensory threshold, with hexanal being the most perceptible. Content of around 25 µg/kg allows its detection (Hashim & Chaveron, 1995). Considering results of volatile compounds and specifically compounds of interest, pasteurized soymilk should have had the highest notes of beany, grassy and oxidized attributes, mainly by the thermal effect of the treatment on the oxidation (hydroperoxides and volatile compounds). However, 200 MPa, 55ºC soymilk reached the highest note of beany and similar values of grassy for pasteurized soymilk. Results of astringent mouthfeel may be associated to non-volatile compounds such as phenolic acids and isoflavones (Min et al., 2005) which are minor

components well known as being present in soybeans (Murkies et al., 1998; Rochfort & Panozzo, 2007). Thickness may be associated to the homogenization intensity in the processing. Because of that, it was expected that soymilks treated at 200 MPa (55 and 75ºC) had similar notes of thickness, instead of pasteurized soymilk ($P \geq 0.05$). However, sensory results showed that UHPH treatment at 55ºC of inlet temperature obtained similar notes of pasteurized soymilk. Standardization of measurements and subjectivity of panelists were possible problems affecting results of sensory analysis, although 3 training sections were applied to the judges. On the other hand, soymilk is a complex of proteins, lipids and carbohydrates combined with several micro components, which make soymilk a difficult matrix for an objective evaluation of the relation between sensorial analysis and volatile profile. Similar observations were reported by Torres-Penaranda and Reitmeier (2001) who carried out a sensory descriptive analysis of soymilk-heat treated samples and commercial soymilk samples.

A study carried out by Lozano et al. (2007) with heat-treated soymilk by different conditions of time and temperature (143ºC 14 s, 143ºC 59 s, 154ºC 24 s), obtained high notes of astringent mouthfeel in treated samples and high notes of beany flavor as temperature and holding time increased ($P \geq 0.05$). In the present study, the trend of beany formation due to an increase of processing temperature was not detected. UHPH treatment at 200 MPa and 75ºC of inlet temperature achieved approximately 116ºC in the homogenization valve while 55ºC of inlet temperature achieved 105ºC, both temperatures for 0.7 s. Thus, inlet temperature used in the UHPH treatment did not affect sensory results, but chemical changes occurring during storage, sample manipulation and panelist subjectivity were probably the reasons for the slight differences observed in beany, grassy and oxidized results among UHPH treatments.

Table 8-4 shows results of color parameters, L^*, a^*, b^* and ΔE of BP and treated soymilks. The first attribute of color, lightness value (L^*), is associated with luminous intensity which described light-reflecting or transmitting capacity of an object (Kwok et al., 1999). UHPH treatments caused a significant decrease in the L^* parameter after treatment indicating a slight darkening compared to pasteurized and BP soymilks. Similar results were observed by sensory analysis, with UHPH soymilks being darker than pasteurized samples in the panel opinion ($P \geq 0.05$). During storage, L^* values of UHPH treatments remained stable, except 200 MPa, 55ºC which showed an increasing beyond 21 days. On the contrary, pasteurized soymilk showed similar L^* values to BP and remained fairly stable during the period of storage.

Table 8-4. Color parameters[1] of untreated and treated soymilks

Treatment	Day	*L**	*a**	*b**	ΔE^{BP}
BP	1	85.03 ± 0.26^{ax}	-0.39 ± 0.43^{x}	14.43 ± 0.54^{x}	-
Pasteurized	1	85.23 ± 0.07^{ax}	0.01 ± 0.43^{ax}	14.34 ± 0.27^{ax}	0.64 ± 0.26^{a}
	7	85.24 ± 0.08^{a}	0.01 ± 0.42^{a}	14.22 ± 0.14^{a}	0.60 ± 0.25^{a}
	14	85.39 ± 0.25^{a}	-0.87 ± 0.27^{b}	13.39 ± 0.24^{b}	1.25 ± 0.23^{b}
	21	85.42 ± 0.14^{a}	-0.94 ± 0.62^{b}	13.39 ± 0.24^{b}	1.45 ± 0.15^{b}
	28	85.38 ± 0.30^{a}	-0.77 ± 0.28^{b}	13.18 ± 0.63^{b}	1.44 ± 0.48^{b}
200MPa 55ºC	1	81.00 ± 0.27^{ay}	-2.88 ± 0.05^{ay}	9.78 ± 0.29^{ay}	6.66 ± 0.37^{ab}
	7	80.50 ± 0.38^{a}	-2.87 ± 0.06^{a}	9.26 ± 0.18^{b}	7.31 ± 0.34^{a}
	14	80.77 ± 0.24^{a}	-2.70 ± 0.32^{a}	9.21 ± 0.18^{b}	7.14 ± 0.20^{a}
	21	82.41 ± 0.72^{b}	-2.57 ± 0.12^{a}	9.74 ± 0.44^{ab}	5.81 ± 0.72^{bc}
	28	82.45 ± 0.55^{b}	-1.85 ± 0.57^{b}	10.15 ± 0.74^{a}	5.24 ± 0.87^{c}
200MPa 75ºC	1	80.36 ± 0.38^{ay}	-2.68 ± 0.19^{ay}	9.65 ± 0.26^{ay}	7.27 ± 0.17^{a}
	7	80.64 ± 0.56^{a}	-3.11 ± 0.50^{a}	9.65 ± 0.26^{a}	7.15 ± 0.24^{a}
	14	80.16 ± 0.10^{a}	-2.80 ± 0.25^{a}	9.31 ± 0.29^{a}	7.47 ± 0.20^{a}
	21	80.77 ± 0.24^{a}	-2.88 ± 0.21^{a}	9.17 ± 0.11^{a}	7.22 ± 0.13^{a}
	28	81.33 ± 0.43^{a}	-2.80 ± 0.47^{a}	9.28 ± 0.23^{a}	6.94 ± 0.27^{a}

[1] Mean values ± SD of color parameters. ΔE was calculated taking into account BP as reference sample.

[a-c] Different superscript in the same column of treated samples are significantly different ($P < 0.05$).

[x-y] Different superscript in the same column between treatments at day 1 are significantly different ($P < 0.05$).

Particle distribution and concentration (droplet characteristics) of UHPH and pasteurized soymilks were different due to the new interface of the soymilk dispersion caused by the treatment conditions. Thereby, pasteurized soymilk showed similar lightness to BP instead of UHPH samples. For *a** (red-green) parameter, a significant decrease was obtained after both UHPH treatments compared to BP. However, *a** values remained stable during the period of storage. In this way, no relevant modifications were experimented in pasteurized soymilk during 28 days compared to BP. UHPH treatment caused a significant decrease in the *b** (yellow-blue) values on the first day of analysis. As observed for *L** and *a** parameters, *b** values did not exhibit important changes due to storage conditions.

The ΔE parameter is a single value which takes into account differences between *L**, *a**, and *b** of the sample and standard. The ΔE values were calculated taking into account BP as reference sample. As shown in Table 8-4, color difference (ΔE values) was more important for UHPH treatments than for pasteurized soymilk. These differences may be attributed primarily to the *L** parameter contribution due to treatment applied. Similar results of *L** and ΔE were obtained by Cruz et al. (2007) for soymilk treated by UHPH at 200 MPa and 40ºC of inlet temperature. Achouri et al. (2007) reported a significant

increase in the ΔE values after 21 days of storage at 4ºC of different blends of soymilk treated at 142ºC for 4 s (UHT).

In the last part of sensory analysis, preference test was applied using hedonic scale of 9 categories. Unexpectedly results indicated that soymilk treated at 200 MPa, 55ºC was the most accepted sample for about 67% of the judges ($P < 0.05$), even though it achieved high notes of beany off-flavor. These results demonstrate the subjectivity of the judges in the sensory analysis, indicating that new training sections and sensory analyses should be performed to achieve reliable results. Nevertheless, they give a global idea of sensory evaluation and show a tendency towards UHPH processing being able to produce soymilk with improved sensory qualities.

8.3. Conclusions

The study carried out in this chapter has shown that UHPH-treated soymilks achieved high microbial inactivation compared to pasteurization treatment during 28 days of storage at 4ºC, being 200 MPa, 75ºC the most stable microbiologically. UHPH soymilk was characterized by great exposure of hydrophobic zones of the protein, which helped to improve the protein dispersion in the interface o/w observed by the oil droplets distribution in the micrographs. As a result, high colloidal stability was detected for UHPH samples showing low solids sedimentation during storage. Physical and chemical aspects evaluated of UHPH-treated samples during storage showed, on the other hand, high color stability and high stability against initial stages of oxidation. Considering hexanal compound as indicator of secondary product of oxidation, an important decrease was detected in the same period of storage compared to pasteurized sample. In base to panel opinion, UHPH treatment did not affect overall soymilk characteristics and in addition, soymilk achieved better sensory acceptance than pasteurized soymilk, especially at 200 MPa, 55ºC. Therefore, UHPH technology was able to produce soymilk as fresh product stored under refrigeration conditions microbiologically and physically stable and with good sensorial acceptance.

8.4 References

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Chapter 9

Aseptically packaged UHPH-treated soymilk

9

Aseptically packaged UHPH-treated soymilk

9.1 Introduction

In order to extend the shelf-life and facilitate distribution, soymilks are usually subjected to intense heat treatment for sterilization (Kwok & Niranjan, 1995). Although UHT treatment can produce a soymilk microbiologically stable for at least a year, it may change quality aspects that could compromise shelf-life (Wang et al., 2001).

Several aspects define the quality characteristics of soymilk such as physical stability of the product, including color, chemical changes and microbial growth, and all of them are crucial factors to take into account in the shelf-life determination of products packaged under aseptic conditions. Additionally to shelf-life determination, volatile profile could be considered as an important factor related to the consumer acceptation, due to the typical beany flavor generated by the compounds. Sensory analysis allows evaluating the impact of volatile compounds by analyzing general sensory attributes during storage of the treated soymilk. In general, acceptance of the product, appropriate terminologies as well as appropriate standard to evaluate objectionable flavors are essentials to achieve reliable results (Torres-Penaranda and Reitmeier, 2001). Moreover, it is important for emerging technologies, such as UHPH, to gain the acceptance and understanding of consumers apart from its opinion about the final product.

Thus, the goal of this study was to evaluate quality aspects of soymilk treated at 300 MPa, 80ºC of inlet temperature in comparison with UHT-treated soymilk. The UHPH condition was selected based in the microbiological and chemical stability results given in chapter 5 and 6. Samples were stored at room temperature for 180 days (see 3.4 condition B). Microbial analysis applied included mesophilic aerobic bacteria and mesophilic aerobic spores, enterobacteria and *Bacillus cereus* counts (see 3.6.1). Analysis performed consisted of: particle size (see 3.9), particle sedimentation (see 3.10 centrifugation and particle migration methods), hydroperoxide index (see 3.7.2), color (see 3.13), surface hydrophobicity (see 3.12), volatile profile evolution (see 3.14) and sensory analysis (see 3.15). Particle size, volatile profile and sensory analysis were performed on 1, 90 and 180 days of storage.

9.2 Results and discussion

9.2.1 Microbiological quality and pH measurements

Microbiological quality of soymilk is an important factor that contributes to the chemical changes during storage and determines the shelf-life, especially for product aseptically packaged.

Results of unprocessed soymilk were: mesophilic aerobic bacteria of 3.18 ± 0.73, mesophilic aerobic spores of 2.18 ± 0.53, enterobacteria of 0.49 ± 0.19 and *Bacillus cereus* of 2.29 ± 0.78 log cfu/mL and pH 6.80 ± 0.02. After both treatments, microbial growth was below the detection limit for all samples (< 0.5 cfu/mL). Because injured cells may not grow on media immediately after treatment (Smith et al., 2009), around of 10 bricks of each treatment at day 1 was incubated at 30ºC for 20 days and 55ºC for 10 days to evaluate mesophilic and thermophilic growth respectively. As a result of incubation time, there was no microbial growth. These results are in accordance with the study described in chapter 5, where soymilk UHT treated and UHPH treated at 300 MPa, 75ºC of inlet temperature did not show microbial growth neither after treatment nor after 20 days of incubation at 30ºC. Likewise to the day 1, UHPH and UHT soymilk remained sterile during for all storage days of analysis (20, 40, 60, 90, 120, 150 and 180 days). Supporting these microbiological results, pH measurements did not change significantly and remained constant around the neutral value during storage at room temperature (Figure 9-1). The good microbiological quality of the soymilk BP have played important role in the UHPH efficience of microbial inactivation. In this way Donsì, et al (2006) and Tahiri et al. (2006) reported that UHPH-treatment effectiveness increased at low initial bacterial concentration. Similarly, high inlet temperature combined with high pressure causes better microbial inactivation (Thiebaud et al., 2003).

In the literature there is not enough information about soymilk treated by UHPH and aseptically packaged to compare with this study. However, different studies have been published about UHT treatments in soymilk. For instance, Achouri et al. (2007) subjected soymilk samples to UHT treatment (142ºC, 4 s) followed by aseptic packaged in coated paperboard and stored for 3 months at 3 controlled temperatures of 4, 22 and 38ºC. They reported counts below the limit of detection (< 10 cfu/mL). Nevertheless, a significant decrease in the pH measurements after 1 month of storage was observed. Beyond this point, the pH increased (values around 7) and remained stable during the rest of storage

period. The pH decreasing was attributed to chemical interactions caused by lipolysis and proteolysis reactions.

Examples of different studies based on the same UHPH technology, but with different foodstuffs such as, apple juice, milk and orange juice are shown below. Pereda et al. (2007) studied milk treated by different UHPH conditions and stored at 4ºC for 21 days. On day 21, milk treated at 300 MPa and 40ºC of inlet temperature showed mesophilic aerobic bacteria of 7.1 log cfu/mL and mesophilic aerobic spores of 2.6 log cfu/mL. Suárez-Jacobo (2011) applied UHPH treatment at 300 MPa and 4ºC of inlet temperature in apple juice followed by aseptic packaging and storage for 60 days at 4, 10, 20 and 30ºC. In the end of storage period, mesophilic aerobic bacteria were below of 2 log cfu/mL. Velazquez-Estrada (2011) studied UHPH-treated orange juice at 300 MPa and 20ºC of inlet temperature and aseptically packaged. Samples stored at 20ºC achieved more than 75 days of shelf-life compared to pasteurized orange juice. Both apple and orange juice samples have low pH compared to milk and soymilk. This factor did not favor microbiological growth compared to those latter products with neutral pH. Therefore, the different levels of microbial inactivation achieved by UHPH treatment, mainly at 300 MPa is strongly dependent of inlet temperature and microbial initial load of unprocessed sample.

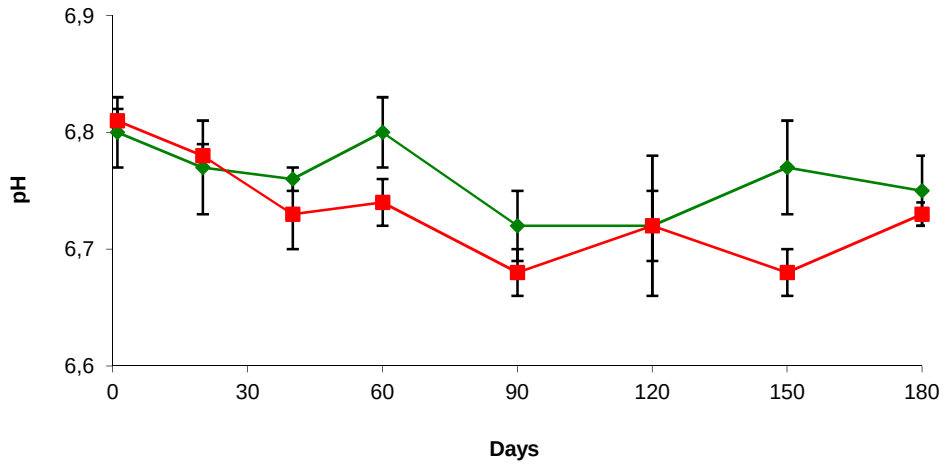


Figure 9-1. pH measurements of UHPH soymilk (-♦-) and UHT soymilk (-■-).

9.2.2 Colloidal stability

Soymilk is a diluted emulsion which, in consequence, may experiment destabilization phenomena during storage. The main problems associated to vegetable beverages, commonly as a consequence of the heat treatment and homogenization process, are creaming and solids sedimentation which adversely affects the quality of final product.

To understand possible changes in physical stability due to treatment applied and during storage, surface hydrophobicity, particle size and sedimentation by centrifugation and particle migration were evaluated.

Surface hydrophobicity determination of BP, UHT and UHPH soymilks is shown in Figure 9-2. Hydrophobic interactions and disulfide binding (S-S) are the main path of complexes between soy protein, oil droplets and micro particles due to the exposition of protein hydrophobic groups determined by fluorescence emission through the complex between ANS and protein (Fukushima, 2001; Floury et al., 2002; Bouaouina et al., 2005). In BP soymilk, with large fat globules, and protein bodies and large aggregates of protein-fat globules (Figure 8-3A), most of the protein hydrophobic zones are masked for binding to ANS reactive, leading then to low fluorescence values. This result was confirmed by particle size parameters of BP samples (D_{50} = 0.64 ± 0.09 µm, $d_{3,2}$ = 0.44 ± 0.03 µm and $d_{4,3}$ = 17.11 ± 2.61 µm) where particles of large volume were detected. UHT soymilk, on the other hand, produced higher fluorescence values than BP. The double effect homogenization (18 and 4 MPa) and the high temperature (142ºC) of treatment caused higher particle disruption allowing better dispersion of particles than BP soymilk. The high temperature applied in the treatment also favored protein denaturation, changing from globular structure to unfolded state. This new protein conformation increased the exposition of protein hydrophobic zones, leading to the formation of large aggregates (fat-protein and protein-protein) and leaving part of them free for binding to the ANS. As a result UHT soymilk was characterized by a dispersion of large particles which experienced slight modifications during storage (Table 9-1).

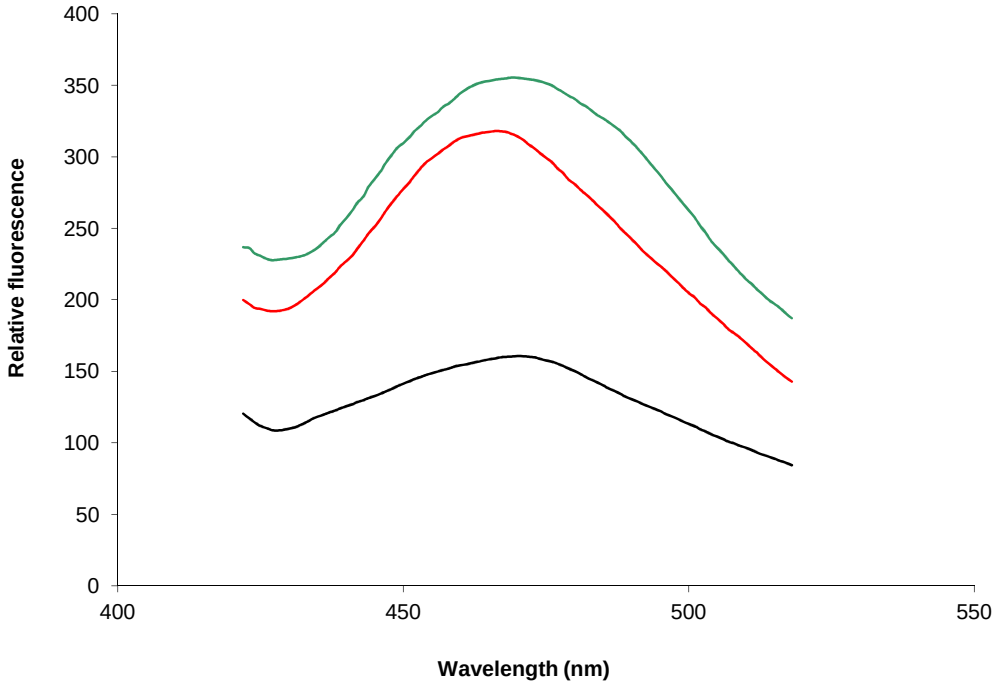


Figure 9-2. Protein surface hydrophobicity of BP (—), UHT-treated soymilk (—) and UHPH-treated soymilk at 300 MPa 80ºC (—).

Soymilk treated at 300 MPa, 80ºC achieved the highest fluorescence values compared to UHT treatment. UHPH treatment caused such protein rearrangement which led to a high exposition of hydrophobic zones of the soy protein to the aqueous phase and hence to the ANS, forming micro-soluble aggregates well dispersed in the continuous phase. Although the high surface hydrophobicity is related to protein denaturation with consequent loss of solubility, the presence of soy lipids, particularly phospholipids that have strong emulsifying capacity, improved the stability of soymilk emulsion due to increased interaction of proteins with lipids (Liu, 1999). In this way, particle size parameters (Table 9-1) evidenced that aggregates formed by UHPH treatment ($d_{4,3}$ parameter) were significantly lower than those by UHT treatment, remaining stable during the period of storage. The high intensity of homogenization in UHPH treatment produced strong particle size reduction which favored a better stabilization of the interactions between micro particles and protein and fat globule during storage. On the other hand, the double-stage homogenization applied in UHT soymilk was not enough to produce an additional dispersion of aggregates formed into continuous phase in the first step such as single-stage of UHPH treatment.

Table 9-1. Changes in particle size distribution of UHT and UHPH soymilks

Parameters	Day	UHT	UHPH
D_{50}[A]	1	0.45 ± 0.03^{xa}	0.17 ± 0.01^{ya}
	90	0.47 ± 0.06^{xa}	0.18 ± 0.01^{yb}
	180	0.32 ± 0.06^{xb}	0.17 ± 0.01^{ya}
$d_{3,2}$[B]	1	0.36 ± 0.02^{xa}	0.23 ± 0.19^{xa}
	90	0.35 ± 0.02^{xa}	0.17 ± 0.01^{ya}
	180	0.26 ± 0.05^{xb}	0.16 ± 0.01^{ya}
$d_{4,3}$[C]	1	14.03 ± 0.72^{xa}	6.14 ± 1.64^{ya}
	90	15.46 ± 2.03^{xab}	5.07 ± 1.15^{ya}
	180	16.57 ± 1.94^{xb}	6.38 ± 0.83^{ya}

[a-b] Different superscripts in the same column are significantly different ($P < 0.05$).

[x-y] Different superscripts in the same row are significantly different ($P < 0.05$).

[A] Mean values ± SD (µm) of diameter below which 50% of the volume of particles are found.

[B] Mean values ± SD (µm) of average diameter (surface weighted mean diameter).

[C] Mean values ± SD (µm) of average diameter (volume weighted mean diameter).

During storage no changes in $d_{4,3}$ parameter was observed for UHPH soymilk, whereas an increase tendency in this parameter was observed for UHT treatment. It means that large aggregates in UHT-treated soymilks were formed and their evolution during storage slightly increased. In traditional UHT treatment, homogenization step commonly occur prior to heat treatment. Thus, aggregates formed in the heating of soymilk were not re-dispersed in the continuous phase. Large aggregates of soy globulin (glycinin and β-conglycinin) were observed during heating of soymilk at high temperature (Tay et al., 2005). Similar results of particle size parameters ($d_{3,2}$ and $d_{4,3}$) for UHT and 300 MPa soymilks were described in chapter 5 and by Cruz et al. (2007).

As commented in chapter 8, solids sedimentation measured by low-speed centrifugation is a good tool to quantify, under specific conditions, sedimentation potential during long-term storage. Table 9-2 show results of solids settled during 180 days.

Solids sedimentation in UHT soymilks was higher than UHPH soymilk, as expected. Sedimentation values of both treatments did not show relevant changes during storage,

although a slight increase was observed in UHT soymilk. According to the previous results of particle size and surface hydrophobicity, UHT-treated soymilk was characterized by large aggregates, whose behaviour was like a large particle. Large particles can become insoluble, allowing easily their deposition when samples were submitted to centrifugation or storage during a long time. Using equivalent method, Cruz et al. (2007) observed similar values of solids sedimentation for UHT and 300 MPa soymilks. In their study, samples were analyzed at days 1, 30 and 60 of cold storage (4ºC). Results indicated a significant increase of sediments for 300 MPa samples after 30 days of storage, remaining unchanged in the last day of analysis.

Table 9-2. Solids sedimentation[1] of treated soymilks during storage.

Day	UHT	UHPH
1	4.67 ± 0.38^{ax}	2.34 ± 0.48^{ay}
20	5.06 ± 0.79^{abx}	2.42 ± 0.23^{ay}
40	5.23 ± 0.98^{abx}	2.11 ± 0.14^{aby}
60	4.99 ± 0.61^{abx}	2.37 ± 0.17^{ay}
90	5.97 ± 0.61^{bx}	2.09 ± 0.09^{aby}
120	5.96 ± 0.47^{bx}	2.19 ± 0.08^{aby}
150	6.17 ± 0.75^{bx}	2.13 ± 0.08^{aby}
180	6.03 ± 0.84^{bx}	2.00 ± 0.17^{by}

[a-b] Different superscript in the same column are significantly different ($P < 0.05$).

[x-y] Different superscript in the same row are significantly different ($P < 0.05$).

[1] Mean values ± SD (g/100g w/w) of solids sedimentation after low-speed centrifugation. BP value was 5.77 ± 0.50 g/100g.

An appropriate instrumental (Turbiscan^R) method to evaluate colloidal stability is that based on migration and/or interaction of particles in samples at resting conditions during storage. Light backscattered along the sample bottle, from the bottom to the top is recorded in a single measurement. Changes of backscattering (ΔB) are associated to changes in the homogeneity, particle size and concentration, and thus the stability of the sample (Durand et al., 2003). ΔB indicates variation of backscattering from single measurements at different times during storage. The increase in ΔB at the bottom or at the top of the tube

indicates an increase in particles or oil droplets concentration in this part of the tube, and hence sedimentation or creaming phenomena respectively can be detected. In all samples analyzed, no creaming phenomenon was observed. Therefore, only the occurrence of sedimentation was detected as relevant destabilization mechanism in this type of product. Using a tool for calculation provided by the Turbiscan manufacturer, solids sedimentation was expressed as the height of solid layer in the bottom of the tube during the storage period of 180 days at 4ºC (Figure 9-3).

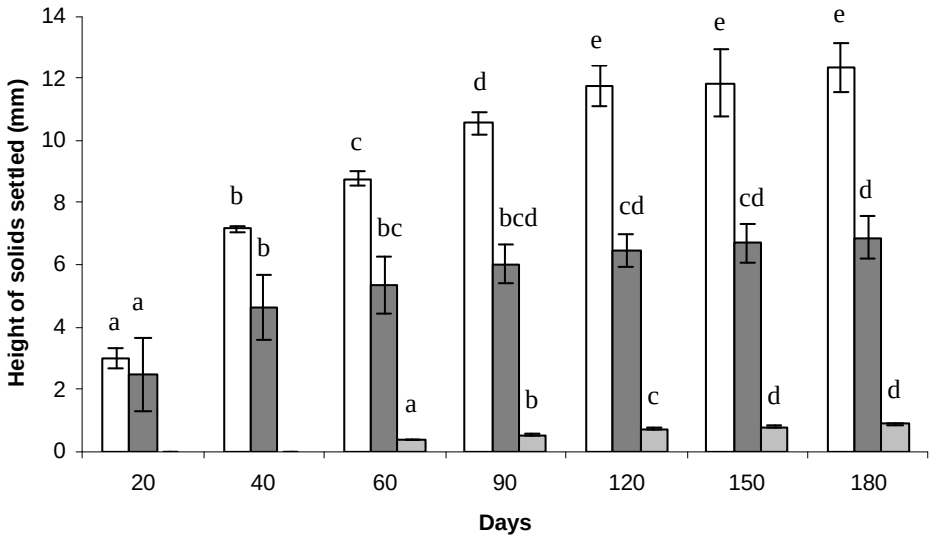


Figure 9-3. Height of solids settled of (□) BP, (■) UHT and (□) UHPH soymilks.
[a-e] Different letters above each bars for each treatment indicate significant differences ($P < 0.05$).

The intense homogenization applied in UHPH treatment was enough to achieve high colloidal stability of soymilk. UHPH-treated soymilk showed spontaneous solids layer deposition from 60th day, reaching a maximum height of just 0.87 mm in the last day of storage. However, UHT soymilk which was subjected to much lower homogenization intensity compared to UHPH soymilk, reached 6.87 mm of solids settled after 180 days of storage. These results are in accordance to those obtained by centrifugation and by particle size parameters, where UHT reached high values indicating low colloidal stability. In relation to the kinetics of sedimentation, UHT soymilk showed values of 0.094 (mm/day) in the lineal stage (the first 60 days of storage), reaching 5.35 mm of solids layer height. UHPH soymilks exhibited an order of magnitude lower with maximum rate of 0.0056 (mm/day) between day 60 and day 90. On the other hand, BP samples subjected to

colloidal mill in the soymilk elaboration, the sedimentation rate was 0.16 (mm/day) in the lineal stage (first 45 days), with 7.2 mm of solids settled. The large aggregates formed during storage of UHT soymilks, was evidenced by the spontaneous particle migration to the bottom of the tube. In the case of UHPH soymilks, the thin layer of sediments detected in the bottom of the analysis tube was dramatically smaller than UHT, what prove a much better stability of the product than those treated by conventional processes.

Therefore, homogenizing conditions had a fundamental role in the kinetic of particle migration. Durand et al. (2003) observed creaming phenomenon at slow rate in commercial soymilk treated by UHT evaluated during 12 h in Turbiscan equipment. This phenomenon was attributed to the tendency of oil droplets and fat globules to coalesce producing a clarification zone in the bottom of the analysis tube. In the present study, no coalescence phenomenon was observed.

9.2.4 Chemical stability

The most important problem that limits the extensive use of soy products is related to strong off-flavors caused by oxidative reactions. Lipid oxidation is one of the main causes of deterioration that negatively affect quality, shelf-life as well as consumer acceptance of soymilk (Hornero-Méndez et al., 2001). Moreover, the secondary products of lipid oxidation cause undesirable implications in human health and contribute to decrease the nutritional value of the product (Angulo et al., 1998). Determination of hydroperoxide concentration gives information about initial state of oxidation reactions, being the first stage of the further formation of volatile compounds as secondary products.

The evolution of hydroperoxide index during storage of soymilks exhibited an inverse tendency in UHT and UHPH samples (Table 9-3). In UHT soymilks, the hydroperoxide index remained quite stable during the first 40 days and then experienced an important increase between 40 and 90 days of storage. In UHPH samples, a decrease of the hydroperoxide index values was found during storage. Fat globules breakdown, as well as molecules conformation, especially protein and lipids, may have an important role in the oxidation reactions. Due to the great disruption of fat globules in UHPH soymilk, the exposition of oil droplets surface increased considerably compared to UHT soymilk. This new state of fat globules could have initially contributed to peroxidation reactions, resulting for UHPH soymilk higher hydroperoxides index values than UHT soymilk at day 1. However, the protein rearrangement caused by UHPH treatment and by the natural

presence of phospholipids, allowed an efficient covering of the oil droplets surface by proteins. These interactions between proteins and oil droplets that occurred during the first days of storage resulted in a protective effect against the formation of new hydroperoxide radicals. In this way, Pereda et al. (2008) reported that homogenized milk was less susceptible to oxidation due to the covering action of casein on the oil droplets surface. In UHT soymilk, the large aggregates formed during treatment and possibly during the period of storage, probably reduced this protective action of the proteins on the oil droplets surface, allowing a gradual increase of hydroperoxide index values during storage.

Table 9-3. Hydroperoxide index values (meq/L) of UHT and UHPH soymilks during storage at room temperature (Mean values ± SD).

Day	UHT	UHPH
1	0.18 ± 0.05^{ax}	0.32 ± 0.01^{ay}
20	0.16 ± 0.02^{ax}	0.21 ± 0.05^{by}
40	0.20 ± 0.04^{ax}	0.14 ± 0.01^{cy}
60	0.30 ± 0.05^{ax}	0.10 ± 0.01^{dy}
90	0.47 ± 0.02^{bx}	0.08 ± 0.02^{dy}
120	0.55 ± 0.21^{bcx}	0.07 ± 0.02^{dy}
150	0.49 ± 0.13^{bcx}	0.05 ± 0.03^{dy}
180	0.67 ± 0.24^{cx}	0.08 ± 0.06^{dy}

[a-d] Different superscript in the same column are significantly different ($P < 0.05$).

[x-z] Different superscript in the same row are significantly different ($P < 0.05$).

To evaluate the impact of oxidation products on soymilk, volatile profile determination is an indicator of secondary products with potential off-flavor characteristics (Plutowska & Wardencki, 2007). Volatile compounds can be formed by different ways. Oxidation of the polyunsaturated fatty acids is the main pathway of formation, commonly initiated in the soaking and grinding steps of soymilk elaboration. In this case, lipoxygenase is the main enzyme responsible for catalyzing oxidation reactions by means of molecular oxygen (see 7.2.1 in chapter 7). Once hydroperoxides lipids are formed, hydroperoxylyase catalyzes the formation of secondary products, such as aldehydes, alcohols, ketones, furans, esters and acids (Mizutani & Hashimoto, 2004; Min et al., 2005ab). Table 9-4 lists the main volatile compounds detected at day 1, 90 and 180 days of BP and treated soymilks selected according to the level of detection, as well as the impact on the sensory characteristics.

Among of compounds detected in soymilk, the literature often points to hexanal as indicator of the degree of oxidation (Plutowska & Wardencki, 2007; Pereda et al., 2008). Additionally to hexanal, compounds such as pentanal (Brunton et al., 2000, Min et al., 2005b), 1-hexanol (Kobayashi et al., 1995), 1-octen-3-ol (Wilkens & lin, 1970), 2-heptanone and 2-penthyl furan (Mtebe & Gordon, 1987) are formed from fatty acids oxidation, therefore could also indicate the oxidation degree. According to the *P* values of each compound presented in Table 9-4, hexanal was affected only by the time storage factor, while pentanal was not affected by any parameter. In the alcohols group, 1-octen-3-ol did not change by any parameter evaluated and 1-hexanol changed significantly by all parameters studied, showing the latter an increasing during storage in UHPH soymilk. Levels of 2-heptanone and 2-penthyl furan were affected by the treatment, time of storage and by the interaction among parameters. 2-Heptanone presented an increase in UHPH soymilk while a decrease was observed in UHT soymilk during the days analyzed. On the other hand, 2-penthyl furan increased in both treatments, with high levels in UHT soymilk.

Aldehyde compounds are commonly associated to off-flavor in soymilk. Beany and grassy flavors as well as oxidized aroma generated by the compounds have unpleasant sensory perception which noticeably affects the soymilk acceptance (Vara-Ubol et al., 2004; Yuan & Chang, 2007). Results showed that hexanal, pentanal and acetaldehyde, were the most abundant compounds in the aldehydes group. Benzaldehyde, 2-heptanal, heptanal and 2-hexenal experienced significant changes in the levels detected due to treatment and period of storage ($P < 0.05$). For most of them, their levels decreased during storage (Table 9-4).

Among the aldehydes identified, hexanal is the most studied and is commonly considered as the main contributor to off-flavor in soymilk (Wilkens & Lin, 1970; Achouri et al., 2006; Yuan & Chang, 2007). Considering the total volatiles at day 1 listed in Table 9-4, hexanal was the dominant compound with 32% for UHT and UHPH treatments and 26% for BP (Figure 9-4). However at day 90, the total of hexanal in relation to the total of volatiles reduced significantly to 23% and 20% for UHT and UHPH soymilks respectively. The same tendency was observed at day 180 of storage, reaching UHPH soymilks the lowest value (12%). Similarly, Achouri et al. (2007) detected hexanal values from 14% to 52% in 3 types of heat-treated soymilk and aseptically packaged, stored at 4, 22 and 38ºC for 12 weeks. They concluded that storage conditions was the main factor affecting hexanal profile with a significant decrease observed after 6 weeks, mainly at 4 and 22ºC. In the present study, the stable values of hydroperoxide index and the decrease of hexanal levels in UHPH soymilk could lead to better sensory response.

Table 9-4. Main volatile compounds[1] detected in soymilk treated by UHT and UHPH.

Name	ID[2]	KI[3]	BP	UHT			UHPH			P value		
				1	90	180	1	90	180	Treatment	Time	Interaction
Aldehydes												
Hexanal	MS, RI, P	1092	122.2 x	200.03 ay	120.68 b	109.16 bc	192.48 ay	132.36 b	82.07 c	0.239	0.001	0.066
Pentanal	MS, RI, P	990	9.88 x	22.22 ay	16.89 a	16.49 a	20.23 ay	14.84 a	16.28 a	0.474	0.070	0.908
Acetaldeyde	MS		4.29 x	4.77 ax	4.25 a	4.59 a	4.66 ax	7.59 b	6.15 b	0.002	0.115	0.019
Benzaldehyde	MS, RI	1563	1.86 x	3.10 ay	5.33 b	6.12 b	4.66 bcz	3.53 ac	3.62 ac	0.004	0.031	0.001
2-Heptenal	MS, RI	1345	1.66 x	2.67 ax	0.46 bc	0.33 c	2.77 ax	1.50 b	3.66 a	0.001	0.001	0.001
Heptanal	MS, RI	1192	2.01 x	4.37 ay	2.23 b	0.24 c	3.80 ay	2.86 b	0.26 c	0.873	0.001	0.026
2-Hexenal	MS, RI	1219	1.48 x	3.87 ay	1.30 b	1.40 b	2.73 cz	2.97 ac	1.52 b	0.240	0.001	0.001
Ketones												
Ketone	MS, RI	826	9.61 x	25.10 ay	28.65 ab	33.25 b	15.91 cx	13.30 c	12.91 c	0.001	0.116	0.001
2-Heptanone	MS, RI	1190	2.07 x	5.63 ay	6.93 a	1.17 b	3.36 cx	7.41 b	10.19 b	0.015	0.001	0.038
2-Butanone	MS, RI	910	3.58 xy	2.68 ay	3.84 ac	5.81 b	6.14 bx	4.22 c	5.11 bc	0.011	0.015	0.001
2,3-Pentanedione	MS, RI, P	1072	3.89 x	3.30 x	ND	ND	6.14 y	ND	ND	0.001	-	-
2,3-Octanedione	MS, RI	1333	3.72 x	4.36 ax	0.28 b	0.24 b	8.47 cy	0.41 b	0.45 b	0.001	0.001	0.001
1-Octen-3-one	MS, RI, P	1314	1.49 x	1.64 ax	1.79 a	1.94 a	1.94 ax	1.86 a	2.18 a	0.124	0.211	0.749
Acetophenone	MS, RI	1693	0.16 x	0.32 ax	0.49 ab	0.68 b	0.63 by	0.51 ab	0.34 a	0.938	0.788	0.001
Alcohols												
Ethanol	MS, RI	944	143.76 x	103.54 ay	87.60 a	91.41 a	133.41 bx	151.25 b	152.29 b	0.001	0.977	0.522
1-Hexanol	MS, RI, P	1359	32.60 x	34.15 ax	32.65 a	33.10 a	37.47 ax	90.55 b	136.57 c	0.001	0.005	0.004
1-Pentanol	MS, RI, P	1260	34.02 xy	27.53 ay	29.13 a	36.05 ac	43.14 bcx	50.92 b	50.56 b	0.001	0.085	0.525
1-Penten-3-ol	MS, RI	1175 A	13.63 x	13.10 ax	11.59 a	13.55 a	14.14 ax	14.01 a	13.15 a	0.187	0.666	0.325
1-Octen-3-ol	MS, RI, P	1454	15.88 x	17.54 ax	15.59 a	13.47 a	15.63 ax	16.72 a	15.57 a	0.780	0.532	0.559
Furans												
2-Penthyl furan	MS, RI, P	1235 A	11.71 x	40.89 aey	57.40 b	81.20 c	16.36 dx	49.06 be	59.66 b	0.001	0.001	0.010
2-Ethyl furan	MS, RI	965 A	7.55 x	35.00 ay	39.72 a	61.16 b	11.03 cx	24.10 d	39.55 a	0.001	0.001	0.102

Name	ID[2]	KI[3]	BP	UHT			UHPH			P value		
				1	90	180	1	90	180	Treatment	Time	Interaction
Furans												
2-Propyl furan	MS, RI	1044	1.66 x	15.11 ay	15.65 a	26.35 b	3.17 cx	5.94 c	13.13 a	0.001	0.001	0.030
Esters												
Ethyl acetate	MS, RI	897	4.80 x	3.70 ax	4.11 a	8.81 b	4.84 ax	9.33 b	16.22 c	0.054	0.001	0.001
Methyl acetate	MS, RI	839	3.22 x	7.33 aby	6.43 b	8.43 a	3.85 cx	7.58 ab	8.67 a	0.001	0.001	0.044
Acids												
Hexanoic acid	MS, RI	1946	25.67 x	39.38 ay	27.21 b	21.93 b	44.46 ay	26.58 b	18.19 b	0.924	0.001	0.349

[a-c] Different letters in the same row are significantly different ($P < 0.05$).

[x-y] Different letters in the same row of treated sample at day 1 are significantly different from BP ($P < 0.05$).

[1] Integrated area counts. Mean values x 10^5.

[2] Identification: MS = Mass spectra, RI = Retention index compared to Pherobase database and (A) (Vichi et al., 2003), P = Positively identified by comparison with authentic standard.

[3] Kovats retention index calculated.

Ketone was the most abundant compound detected in ketones group followed by 2-heptanone and 2-butanone. Latter two compounds were affected by the time of storage and all three were affected by the type of treatment ($P < 0.05$), while acetophenone and 1-octen-3-one were not affected significantly by treatment and time parameters. 2-Heptanone, acetophenone, 1-octen-3-one were described to attribute, respectively, flowery odor, penetrating green and mushroom odor properties (Boatright & Lei 1999). 1-Octen-3-one had small peak in the chromatogram compared to ketone compounds and hexanal in the aldehydes group. However, 1-octen-3-one as hexanal, has a very low sensory threshold (0.005 mg/L), making its contribution very important to the overall soymilk sensory quality (Yuan & Chang, 2007). Additionally to 1-octen-3-one, 2,3-pentanedione was detected only in the first day of analysis and possess buttery unpleasant odor (Boatright & Lei, 1999).

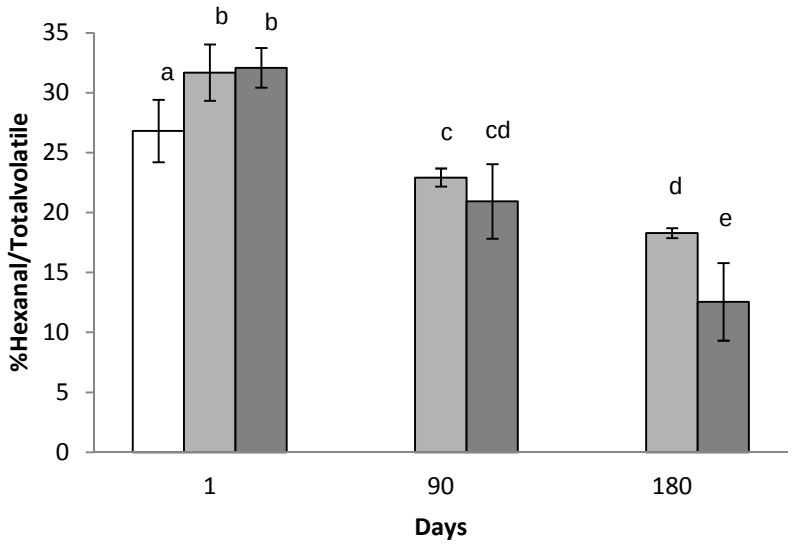


Figure 9-4. Percentage ratio of hexanal to total volatiles of (□) BP, (▒) UHT and (■) UHPH soymilks.
[a-c] Different letters above bars indicate that samples are significantly different ($P < 0.05$).

In the alcohols group, ethanol, 1-hexanol, 1-pentanol, 1-octen-3-ol and 1-penten-3-ol were the main compounds detected, with ethanol being the most abundant. They are commonly associated to grassy and beany flavor (1-hexanol) and mushroom flavor (1-octen-3-ol). 1-Hexanol is the main compound of alcohol chemical family related to off-flavor in soymilk. It can be formed through oxidative evolution process of hexanal (Yuan & Chang, 2007;

Kakumyan et al., 2009) that could partially justify the decrease of hexanal during storage, especially in samples treated by UHPH. In general, alcohols are produced by the reduction of their corresponding aldehydes by chemical reactions (Molimard & Spinnler, 1996). Ethanol and 1-pentanol values showed a significant difference between treatments, but did not present significant difference over time for a same treatment (Table 9-4). On the contrary, 1-hexanol values showed significant increase during storage for UHPH soymilks, whereas remained stable for UHT soymilk. Observing all alcohol compounds, UHPH soymilks obtained higher values than UHT soymilks, mainly for ethanol and 1-hexanol which the highest values were reached after 180 days of storage. Furan compounds and specially 2-pentyl furan are associated to beany odor and taste in soymilk. As reported by Achouri et al. (2007), 2-pentyl furan was the main compound detected followed by 2-ethyl furan in heat-treated soymilk. UHT treatment produced a significant increase of all furan compounds detected at day 1 as reported in chapter 7. However, furan compounds tended to increase significantly during storage time ($P < 0.05$). On the other hand, the increase in the levels of furan compounds for UHPH soymilks was observed after 90 and 180 days of storage, showing significant lower values than UHT soymilks. Taking into account the total furan composition after 6 months of storage (Table 9-4), 64% of the levels detected was represented for UHT soymilks and 36% for UHPH soymilk. The formation of this chemical family is related to thermal damage derived of high temperature used during processing. In UHT treatment the process conditions (142ºC for 6 s) were the main factor in the formation of furans. At these conditions, take place Maillard reactions that favor furan compounds formation (Achouri et al., 2007).

Ester and acid compounds were detected at low levels for both treatments. Ethyl acetate obtained a significant increase ($P < 0.05$) during storage primary for UHPH soymilk, reaching the highest value in the last day of analysis. Methyl acetate increased significantly after UHT treatment and remained stable during storage, whereas it was not affected by UHPH treatment at day 1, but increased significantly at day 90 maintaining this level at day 180. As reported in chapter 7, there is no information about sensory effect of ester compounds on soymilk, but they could be associated to floral and fruit flavors. Hexanoic acid was the most abundant acid compound detected. It could be originated by hexanal oxidation in presence of oxygen, and produce a harsh and fetid sensory odor (Wilkens & Lin, 1970). Significant increases were observed for UHPH and UHT treatment at day 1. However an important decrease was detected ($P < 0.05$) for both samples in the period of storage, reaching values close to untreated soymilk.

Not all compounds detected in this study possess a disagreeable odor. For example, benzaldehyde has a cherry or almond aroma and 2-heptanone has flowery odor (Aparicio et al., 1997; Wilkens & Lin, 1970; Boatright & Lei, 1999). However, these compounds have small peak in the chromatogram compared to undesirable compounds detected in the total volatiles of soymilk.

9.2.5 Sensory analysis and color quality

Thermal treatment applied to soymilk as well as long periods of storage may produce color changes affecting food quality and sensory response of consumers. In addition to the heat effects, homogenization process of soymilk may also produce color changes. When a light interact on the surface of an emulsion, part of it is reflected, while the rest is transmitted into the emulsion. As the transmitted light propagates through the emulsion it may be absorbed by any chromophores or scattered by any droplets and/or particles. The light returning from the emulsion is therefore the result of reflected, transmitted, scattered, and absorbed light (Chantrapornchai et al., 1998). Table 9-5 shows evolution of color parameters (L^*, a^* and b^*) during storage of UHT and UHPH soymilks over 180 days.

Luminosity (L^*) values, is associated with darkness or lightness of the product. A significant difference was found in L^* value between UHT and UHPH treatments. UHT soymilk showed higher L^* values than UHPH and BP soymilks, remaining this tendency during storage ($P < 0.05$). In emulsions, L^* parameter is mainly related with particle concentration and size distribution, and probably with protein denaturation degree (Rhim et al. 1988ab; Chantrapornchai et al., 1998). In general, L^* value of the emulsions increased with decreasing droplet size diameter (Chantrapornchai et al., 1998), so it was expected in UHPH soymilk higher L^* values than UHT soymilk. However, state of particle aggregation could have influenced the light reflection and scattering through the UHT and UHPH samples, affecting L^* parameter result. On the other hand, it is important to highlight that the L^* value was fairly stable during storage for both treatments. Regarding a^* and b^* parameters, slight differences, although significant, were found between UHT and UHPH soymilks during storage. Cruz (2008) observed similar results of color parameters. In that study, UHT-treated soymilk showed higher L^* values than UHPH-treated soymilk immediately after treatment.

The ΔE (color difference) parameter is a single value which takes into account differences between L^*, a^*, and b^* of the sample and standard. Considering BP as standard, the ΔE

gives an idea of the influence of treatment contributing to the overall color. UHT soymilk at day 1 and during storage showed higher color difference than UHPH samples. On the other hand, considering UHT soymilk as standard, the resultant ΔE can be the value of UHPH processing for comparing to the most conventional commercial product. Making a general observation of the results, the color variation for both treatments remained stable during storage. On the contrary, Achouri et al. (2007) reported a drastic change in the color difference (ΔE) of heat-treated soymilk during 12 weeks of storage. At the first 6 weeks, a significant increase in the ΔE values was observed. Beyond this point, the values decreased and then slight modifications were detected during the rest of the storage period.

Table 9-5. Changes in color[1] parameters during storage in Tetra Brik containers

Parameters	Day	UHT	UHPH
*L**	1	85.55 ± 0.41^{ax}	82.20 ± 0.53^{ay}
	90	86.71 ± 0.28^{bx}	82.69 ± 0.14^{by}
	180	86.23 ± 0.35^{cx}	82.16 ± 0.09^{ay}
*a**	1	0.17 ± 0.24^{ax}	-0.83 ± 0.13^{ay}
	90	0.32 ± 0.22^{ax}	-1.01 ± 0.24^{ay}
	180	0.34 ± 0.12^{ax}	-0.18 ± 0.90^{bx}
*b**	1	12.63 ± 0.37^{ax}	12.16 ± 0.75^{ax}
	90	12.13 ± 0.14^{ax}	12.34 ± 0.06^{ay}
	180	12.58 ± 0.35^{ax}	12.50 ± 0.39^{ax}
ΔE^{BP}	1	3.64 ± 0.27^{ax}	2.12 ± 0.52^{ay}
	90	4.86 ± 0.23^{bx}	2.09 ± 0.24^{ay}
	180	4.27 ± 0.43^{cx}	1.79 ± 0.41^{ay}
ΔE^{UHT}	1	-	2.90 ± 0.90^{a}
	90	-	4.25 ± 0.06^{b}
	180	-	4.26 ± 0.10^{b}

[x-y] Different superscripts in the same row indicate significant differences ($P < 0.05$).

[a-c] Different superscripts in the same column indicate significant differences ($P < 0.05$).

[1] Mean values ± SD of color parameters. BP results were: $L^* = 82.13 \pm 0.15$; $a^* = 0.20 \pm 0.40$; $b^* = 13.36 \pm 0.76$ and $\Delta E^{UHT} = 2.65 \pm 0.16$.

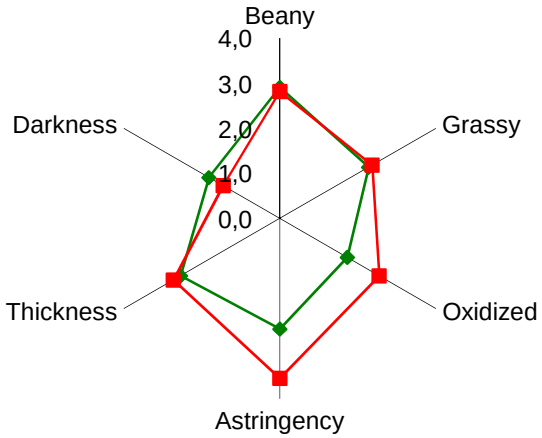
Sensory analysis gives information about consumer acceptance of soymilks and the detection of possible changes occurred during long periods of storage. Sensory analysis was carried out at day 1, 90 and 180. In the first part of the analysis, judges were instructed to identify possible differences between UHT and UHPH soymilks by means of triangular test. To evaluate results, chi-square test was applied considering as null hypothesis that no

differences between treatments were perceived by the judges. Triangular test showed the following *P* values: day 1 ($P = 0.2733$), day 90 ($P = 0.4652$) and day 180 ($P = 0.1441$). This result indicated that judges did not identify differences between UHT and UHPH soymilks.

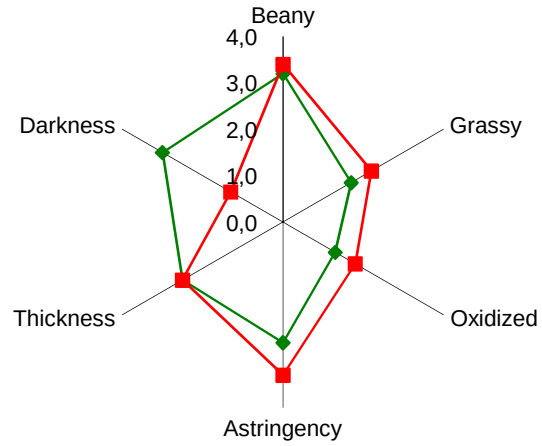
The second test performed to assess sensory analysis was descriptive test. In this test some characteristic attributes of soy products such as beany flavor, grassy aroma, oxidized aroma, astringent mouthfeel, thickness and color (darkness) were evaluated. Results for each day of analysis are shown in Figure 9-5. For astringency mouthfeel, panel evaluation at day 1 classified UHT soymilks with higher mouthfeel astringency than UHPH soymilks ($P < 0.05$). Similar tendency was obtained at day 90 of storage, whereas no significant differences were observed at day 180. Astringent mouthfeel may be originated by phenolic compounds such as isoflavones and saponins which were reported being the main non-volatile off-flavor in soy products (Matsuura et al., 1989). Regarding dark color at day 1, judges classified UHPH soymilk slightly darker than UHT soymilk in all days of analyzis, showing only significant differences at day 90. This result is in accordance to that observed in L^* values of instrumental color evaluation. Considering thickness attribute, no significant differences were observed between days analyzed ($P \geq 0.05$). Likewise, beany flavor and grassy and oxidized aromas did not show significant differences between treatments during the period of storage (Figure 9-5ABC). However, these attributes in UHT soymilk obtained notes slightly higher than UHPH soymilk at 90 and 180 days of storage, with oxidized aroma being the most relevant difference. These attributes are related to hexanal detection. As previously mentioned, hexanal was the most abundant compound detected in the whole volatile profile (Figure 9-4) and moreover, it has very low sensory threshold so its detection plays an important role in the sensory perception.

Adittionaly to hexanal, total furan compounds in UHT soymilks showed higher values than UHPH soymilks in the same days of analysis (Table 9-4), emphasizing the possible relation of the sensory perception with volatile profile. Oxidized aroma could be originated by a mix of aldehydes such as, nonanal, heptanal, pentanal and 2,4-heptadienal which were related to aged or rancid flavor (Boatright & Lei, 1999; Vara-Ubol et al., 2004). Grassy aroma and green odors in general are primarily associated to furan compounds, mainly to 2-pentyl furan, although hexanal and 2-hexenal were also reported to have these characteristics (Wilkens & Lin, 1970; Boatright & Lei, 1999; Suratman et al., 2004; Lozano et al., 2007). Therefore, hexanal and furan values could explain the panel sensation of beany, grassy and oxidized flavor for UHT-treated soymilk.

A



B



C

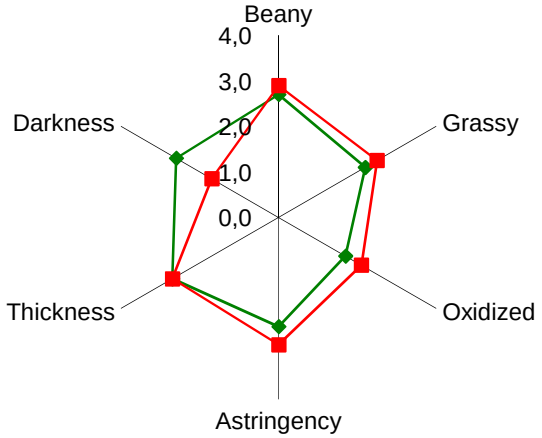


Figure 9-5. Sensory attributes evaluation of (-■-) UHT and (-♦-) UHPH soymilks at (A) day 1, (B) day 90 and (C) day 180.

Few papers have studied the impact of soymilk treatment on volatile compounds and their sensory characteristics. Lozano et al. (2007) studied soymilk treated by three UHT conditions by combining time and temperature. They considered a total of 10 attributes evaluated after treatment, resulting astringency and beany flavor the most perceived attributes by panelist. That result is in accordance with the present study for UHT samples at day 1 of analysis as well as at day 90.

Storage time of treated samples did not affect levels of each attribute evaluated by the panel ($P \geq 0.05$). This result indicates that chemical changes occurred during storage did not impact the organoleptic characteristics of soymilk, as also observed in the results of triangular test, where judges did not identify differences between treatments. Preference test was the last part of sensory analysis. This test gives information about the acceptance of UHT and UHPH treatments. To perform the interpretation of the results, it was considered that the null hypothesis indicated that samples were equal in the panelist preference. According to the results, no significant differences were detected ($P \geq 0.05$), so the type of treatment was not relevant in the panel preference. However panel evaluation at each day of analysis (bold values) demonstrated a trend of UHPH soymilk to be the preferred sample (Table 9-6). Hence overall sensory results suggested that high temperature is more detrimental to soymilk flavor than high pressure treatment. These results are very positive for UHPH technology, because it would be possible to produce soymilk with improved sensory characteristics and good acceptation.

Table 9-6. Preference results of panel evaluation[1]

	Day	UHT	UHPH
Like	1	3	**7**
	90	3	**7**
	180	5	**4**
Dislike	1	**7**	2
	90	**5**	2
	180	**4**	3

[1] Number of judges that selected its preference according to the category gave.

9.3 Conclusions

The present study has shown that UHPH-treated and aseptically packaged soymilk was able to achieve 6 months of storage at room temperature, without any microbial growth as

commonly reached for conventional UHT treatments. Moreover, it showed higher colloidal stability with smaller spontaneous solids sedimentation than UHT treatment. Although hydrophobicity results indicated that UHPH treatment produced high degree of protein denaturation, the new state of particle distribution contributed to improve the physical stability of soymilk during storage. In this sense, UHPH treatment achieved high color stability with small color difference compared to UHT soymilk. On the other hand, UHPH-treated soymilk obtained stable levels of primary oxidation degree and an important decrease of hexanal values used as indicator of advanced levels of oxidation over storage. Almost all compounds associated to soymilk off-flavors were detected in the volatile profile of UHT and UHPH soymilks. Nevertheless, UHPH treatment did not produce changes in the soymilk which could affect the panel perception for differing UHT and UHPH soymilks and for selecting the preference. Moreover, overall attributes as well as judges preference analyzed for each treatment in each day, allow concluding that UHPH-treated soymilk has a slight tendency to be the sample with better characteristics in the panelist opinion. This result is very promising for UHPH technology, turning it into a clear alternative to heat treatments producing soymilk with long shelf-life and better global qualities than conventional technologies. On the other hand, it is important to note that soybeans variety and its chemical composition of lipids and protein, so as the method of soymilk elaboration and treatment, are the main factors which indicate what phenomena (physical and chemical) can undergo soymilk during its shelf-life.

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Chapter 10

Conclusions

10

Conclusions

1. The soymilk elaboration procedure developed at the UAB pilot plant produced a good quality soymilk without lipoxygenase activity and with reduced trypsin inhibitor activity. The extraction performed at 80ºC was the selected temperature as it produced a better yield than 60ºC and a good standard of composition similar to commercial soymilk.

2. The combination of pressure and inlet temperature in UHPH treatment reduced significantly microbial populations and particle size compared to conventional thermal treatments. UHPH soymilks achieved high colloidal stability and low oxidation index compared to heat treatments. Nevertheless, UHPH treatments did not achieve the same level of trypsin inhibitor inactivation as obtained in UHT treatment.

3. The UHPH condition of 300 MPa and 75ºC inlet temperature was able to produce commercial sterile soymilks. On the other hand, the main potential spores identified and purified from soymilk treated at 300 MPa, 65ºC of inlet temperature after incubation time belonged to *Bacillus* genus, in particular *Paenibacillus taichungensis*, *P. glucanolyticus* and *Bacillus cereus*.

4. UHPH treatments at 300 MPa and 55, 65, 75 and 85ºC of inlet temperature were able to reduce noticeably of *P. taichungensis* and *B. cereus* spores inoculated in sterilized soymilk. Inactivation of *P. taichungensis* was more effective than *B. cereus* at any inlet temperature, reaching complete inactivation at 85ºC.

5. Fifty seven volatile compounds were identified in soymilk. The aroma profile was characterized primarily by aldehyde and alcohol compounds. Ketone, furan, ester and acid compounds were detected in low levels, although not less relevant. Pasteurization and 200 MPa treatments were less favorable to the formation of volatile compounds, reaching results close to untreated soymilk. UHT and 300 MPa

were the treatments which favored volatile formation. UHT-treated soymilk produced high levels of furan compounds in contrast to 300 MPa.

6. UHPH treatments at 200 MPa (55 and 75ºC) showed high colloidal stability during 28 days of storage at 4ºC compared to pasteurized soymilk. In terms of lipid oxidation, UHPH-treated soymilk showed lower values of hydroperoxide index than pasteurized soymilk and a significant reduction of hexanal levels during storage under refrigeration conditions. On the other hand, the UHPH condition of 75ºC remained microbiologically stable during storage, although 200 MP, 55ºC soymilk reached acceptable results in the period of storage studied.

7. UHPH-treated soymilk at 300 MPa, 80ºC aseptically packaged was able to achieve 6 month of storage at room temperature, without microbial growth. Although UHPH soymilk showed high hydrophobicity values after treatment, the colloidal stability was highly stable compared to UHT soymilk. Moreover, it showed high color stability and stable levels of primary and secondary compounds of lipid oxidation over the period of storage.

8. In UHPH treatments, where fresh soymilk and soymilk with extended shelf-life was obtained, panelists were not able to identify differences between UHPH-treated and heat-treated soymilks. Almost all compounds which are associated to soymilk off-flavors (beany, grassy and oxidized attributes) were detected in the volatile profile of the UHPH and heat-treated soymilks. However, overall sensory results allow the conclusion that UHPH-treated soymilk had a tendency to be the sample with better sensory characteristics.

9. Finally, UHPH treatment (200 MPa, 75ºC) was able to produce soymilk as a fresh product stored under refrigeration conditions for 28 days with better qualities than pasteurization treatment (95ºC, 30 s). On the other hand, soymilk treated at 300 MPa, 80ºC aseptically packaged and stored at room temperature proved to be a clear alternative to conventional thermal treatments, such as UHT.

Chapter 11

Appendix

Appendix 1

List of articles published

Articles:

Poliseli-Scopel, F. H., Hernández-Herrero, M., Guamis, B. & Ferragur, V. (2012). Comparison of ultra high pressure homogenization and conventional thermal treatments on the microbiological, physical and chemical quality of soymilk. *LWT – Food Science and Technology. 46*, 42-48. DOI: 10.1016/j.lwt.2011.11.004

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