Innovative Food Science and Emerging Technologies 35 (2016) 86–95

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

Combined high hydrostatic pressure and thermal treatments fully inactivate trypsin inhibitors and lipoxygenase and improve protein solubility and physical stability of calcium-added soymilk

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ARTICLE INFO

Article history:
Received 28 July 2015
Received in revised form 12 April 2016
Accepted 15 April 2016
Available online 19 April 2016

Keywords:
High hydrostatic pressure
Soymilk
Calcium
Trypsin inhibitors
Lipoxygenase
Protein solubility

ABSTRACT

The objective of this work was to assess the possibility of obtaining calcium-added soymilk with acceptable characteristics regarding protein solubility and physical stability, and inactivation of trypsin inhibitors and lipoxygenase, through a combined thermal-high hydrostatic pressure treatment. A Doehlert design was applied to study the effect of combining pressure levels (500–700 MPa), initial temperatures (45–65 °C) and $CaCl_2$ concentrations (5–15 mmol L^{-1}). Results showed that protein solubility was a function of $CaCl_2$ concentration ($p < 0.005$), and that inactivation of trypsin inhibitors was a function of pressure level and temperature ($p < 0.005$). Lipoxygenase activity was fully inactivated in most of the conditions tested. Physical stability was improved by the combined treatments: depending on calcium concentration, either no settling was detected in a 5-day period or a less conspicuous phase separation was observed. Our results indicate that some combined thermal-high hydrostatic pressure treatments allow the preparation of calcium-enriched soymilks with improved physical stability without additives such as chelating agents, and acceptable in terms of full inactivation of trypsin inhibitors and lipoxygenase.

Industrial relevance: An analysis of the effect of the combination of high hydrostatic and thermal treatments on calcium-added soymilk is introduced in the present study. Some processing conditions were found to allow the obtention of a product with improved protein solubility and physical stability, and with inactivated trypsin inhibitors and lipoxygenase enzyme. This information could be beneficial considering the reduction of process time and energetic costs, because the assayed combination of thermal and high pressure treatments takes advantage of the instantaneous compression heating. Thus, the results provided in the present work could be useful to prepare an acceptable calcium-added soymilk.

1. Introduction

Soymilk is a non-fermented food prepared from whole soybean. The FDA approved the use of a health claim for soy protein stating that consumption of 25 g of soy protein per day as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease (U.S. FDA, 1999). Soymilk is free of cholesterol and lactose, and its protein content is similar to that of bovine milk. Traditional soymilk manufacturing involves a boiling step, which increases shelf life by reducing microbial load, improves the nutritional value of the milk by inactivating trypsin inhibitors, and enhances flavor by inactivating lipoxygenase (LOX) (Erickson, 1995). Nevertheless, this traditional thermal processing induces detrimental effects on the flavor, nutritional value and color of soymilk. In this way, other processing strategies such as ultra-high pressure homogenization, high hydrostatic pressure (HHP) and pulsed electric field treatments have been applied to soymilk (Lozano, Drake, Benitez, & Cadawallader, 2007; Yuan, Chang, Liu, & Xu, 2008; Smith, Mendonca, & Jung, 2009).

The endogenous enzyme LOX catalyzes a reaction between polyunsaturated fatty acids and oxygen. This reaction leads to the development of undesirable grassy, beany and rancid off-flavors; thus, the inactivation of LOX is required for an acceptable food product (Tressl & Drawert, 1973; Van der Ven, Matser, & Van den Berg, 2005). Ludikhuyze, Indrawati, Van den Broeck, Weemaes, and Hendrickx (1998) analyzed the effect of a heat and pressure combination on a

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http://dx.doi.org/10.1016/j.ifset.2016.04.005
1466-8564/

simple model system of an aqueous solution of commercial LOX. These authors found that pressure application is an alternative to thermal blanching to inactivate LOX and that temperature and pH modify the sensitivity of LOX to HHP.

Trypsin inhibitors (TI) adversely affect enzymes that have a main role in protein digestion. Soybean TI could cause health disorders such as growth suppression and pancreatic hypertrophy (Grant, 1989; Gumbmann, Dugan, Spangler, Baker, & Rackis, 1989). The Kunitz and the Bowman–Birk TI are proteins and represent the main TI present in soybean (Sorgentini & Wagner, 1999). Guerrero-Beltran, Estrada-Girón, Swanson, and Barbosa-Cánovas (2009) evaluated the effect of a heat and pressure combination on inactivation of TI and found that it was 76% at 550 MPa and 80 °C for 15 min.

Calcium is a very important mineral in the diet due to its physiological roles in all tissues. Typical soymilk has much lower calcium content than bovine milk: 20–30 mg Ca^{2+}/100 g versus 120 mg Ca^{2+}/100 g (Chaiwanon, Puwastien, Nitithamyong, & Sirichakwal, 2000). Fortification of soymilk with calcium is hampered by the decrease in colloidal stability of soybean proteins by inducing coagulation and precipitation (at ambient temperature or after heating). Thus, several strategies have been analyzed to improve colloidal stability: addition of lecithin liposomes (Hirotsuka, Taniguchi, Narita, & Kito, 1984) or chelating agents (Pathomrungsiyounggul, Lewis, & Grandison, 2010) and freeze–thaw treatment (Márquez, Salvatore, Otero, Wagner, & Palazolo, 2015). Addition of chelating agents may be associated with disadvantages: hexametaphosphate for example has been related to a decrease in iron absorption and retention (Steinhardt Bour, Souiller, & Zemel, 1984) and sodium citrate at certain concentrations increases darkness and decreases viscosity of soymilks (Prabharaksa, Olek, & Steinkraus, 1989).

Studies have shown that HHP is effective in increasing protein solubility and improving colloidal stability of calcium-added aqueous dispersions of soybean protein isolate when applied at room temperature and that the magnitude of this effect is dependent on pH, calcium concentration and pressure level (Añón, de Lamballerie, & Speroni, 2012; Manassero, Vaudagna, Añón, & Speroni, 2015). Nevertheless, there are no data about the occurrence of this effect at high process temperatures or in a complex matrix such as soymilk. Poliseli-Scopel, Hernández-Herrero, Guamis, and Ferragut (2012) have studied the combination of heat and pressure homogenization in soymilk without calcium addition. These researchers assayed inlet temperatures up to 75 °C and pressure levels up to 300 MPa, and found an effective way to improve physical stability and sterilize soymilk, but stated that 60.8% of the initial TI activity was retained. Thus, no data are available about complete inactivation of TI and LOX in calcium-enriched and HHP-treated soy-based food products.

In this framework, the combination of HHP and thermal treatments (at temperatures lower than those used in traditional thermal treatments) can be studied as an alternative to traditional thermal processing. Moreover, a quantitative analysis of the main process factors seems to be indispensable if calcium addition is proposed. The aim of this work was to optimize the main process parameters ($CaCl_2$ concentration, pressure level and initial temperature) in terms of LOX and TI inactivation, protein solubility and colloidal stability of calcium-added soymilk.

2. Materials and methods

2.1. Experimental design and statistical analysis

Response surface methodology was used to study the simultaneous effects of three process factors, namely initial temperature (T) (45, 50, 55, 60 or 65 °C), pressure level (P) (500, 550, 600, 650 or 700 MPa) and $CaCl_2$ concentration (5, 10 or 15 mmol L^{-1}). The experiment was based on a Doehlert design with three factors (Doehlert, 1970). This experimental design required fifteen trials: twelve points spread over the experimental domain with the central point in triplicate in order to determine the experimental error (Araujo & Brereton, 1996). Table 1 shows the real and coded values of the factor levels. The effect of these factors was evaluated on protein solubility (PS), inactivation of LOX (expressed as percentage, %ILOX) and inactivation of TI (expressed as percentage, %ITI).

Each response variable (PS, %ILOX and %ITI) was analyzed as a function of the coded independent variables (T, P and $CaCl_2$) and a measurement error (ϵ) by using multiple regression analysis. For this, a full quadratic equation containing 10 coefficients was used:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_{ii} x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{i<j=2}^{3} \beta_{ij} x_i x_j + \epsilon$$

where Y is the response variable, β_0 is the constant, β_i, β_{ii} and β_{ij} are the coefficients for linear, quadratic and interaction effects respectively, and x_i are the coded independent variables, which were linearly related to the real values of T, P and $CaCl_2$ concentration. The significance of the equation coefficients for each response variable was assessed using the *F* test with a $p < 0.05$. The independent variables that were found significant at $p < 0.05$ in the full model were retained in the reduced models. These reduced models were used to generate response surface and contour plots.

Optimization was carried out by establishing desirability specifications of acceptable values for each response variable and combining them into their recommended global desirability function (Derringer & Suich, 1980). In the present study, this function was used to select the main process factors and optimize their application to calcium-added soymilk. This method integrates the regression equations obtained for each response variable simultaneously. The desirability function was designed to optimize the inactivation of TI while increasing PS. Consequently, the objective established was the maximization of %ITI and PS. The response variable %ILOX was not included in the analysis because this variable was not significantly affected by the design factors.

All the procedures were carried out using Statistica version 7.0 (Stat Soft. Inc., USA).

2.2. Soymilk preparation

Soybeans (*Glycine max*) belonged to a non-genetically modified variety (INTA ALIM5.09) harvested in 2011 from an Agricultural Experiment Station at the National Institute of Agricultural Technology (INTA) in Marcos Juarez, Córdoba, Argentina (32° 42′ S; 62° 07′ W). Soymilk was prepared from unheated soybeans, which were washed in deionized water and soaked for 14 h at 4 °C (w/v = 1:10). The swollen beans were drained, rinsed with water, drained again and ground with deionized water using a 28:100 bean:water mass ratio that allowed obtaining a total protein concentration of 3% w/w. This ratio was calculated from assays in which different masses of swollen beans were ground with 400 mL of deionized water. Protein concentration was determined by Kjeldahl method (AOAC, 1990) and the conversion factor was 5.8. Soymilks were obtained as follows: the soybeans were ground for 1 min at high speed using a bench blender (model LM852, ATMA, Argentina) and then with three cycles of 1 min at 18,000 rpm using an Ultra-Turrax T-25 homogenizer (IKA®, Werke GmbH & Co., Germany). The soy slurry was filtered through two layers of cheese cloth and the filtrate collected. Calcium was added at different concentrations from stock solutions of 1.0 mol L^{-1} prepared from $CaCl_2$ dihydrate (Sigma, St Louis, USA). After $CaCl_2$ addition, pH was accurately adjusted with 1 mol L^{-1} NaOH to 7.0. The pH value was chosen according to results of Manassero et al. (2015) (the effect of HHP on calcium-added soybean proteins is higher with increasing pH) and from Kwok, Qin, and Tsang (1993) and Ludikhuyze et al. (1998) (TI and LOX are more sensitive to thermal treatment at higher pH values than at lower ones in the range 2.0–9.0).

Table 1
Real and coded values for initial temperature, pressure level and $CaCl_2$ concentration established according to the Doehlert design.

Treatment	Real values			Coded values		
	Initial temperature (°C)	Pressure level (MPa)	$CaCl_2$ concentration (mmol L^{-1})	Initial temperature	Pressure level	$CaCl_2$ concentration
1	55	600	10	0	0	0
2	55	600	10	0	0	0
3	55	600	10	0	0	0
4	55	500	10	0	−1	0
5	55	700	10	0	1	0
6	65	600	10	1	0	0
7	45	600	10	−1	0	0
8	50	550	5	−0.5	−0.5	−0.707
9	50	650	5	−0.5	0.5	−0.707
10	50	550	15	−0.5	−0.5	0.707
11	50	650	15	−0.5	0.5	0.707
12	60	550	5	0.5	−0.5	−0.707
13	60	650	5	0.5	0.5	−0.707
14	60	550	15	0.5	−0.5	0.707
15	60	650	15	0.5	0.5	0.707

2.3. Conditioning and treatment of samples

Samples were placed in plastic bottles (110 mL) and heated in a water bath Thermo Scientific Lindberg/Blue M model RWB3220 (Waltham, MA, USA) until the initial temperature of the treatment was reached (according to the experimental design, Table 1). Temperature–time evolutions during this heating step were measured in samples that were not subjected to HHP treatments, using T type thermocouples and recorded with a Hydra 2625 A data logger (John Fluke Mfg. Co., Inc., Everett, USA). Temperature readings were taken at intervals of 30 s (scanning time) with an accuracy of ±0.1 °C. Preheated bottles were then placed in the canister of the HHP system (Stansted Fluid Power Ltd. model FPG 9400:922, Stansted, UK) and introduced in the high pressure vessel. The compression rate was 300 MPa min^{-1} and the holding time at working pressure was 5 min. The temperature of the HHP system was conditioned according to the corresponding treatment. The initial temperature of the compression fluid (a mixture of propylene glycol and distilled water, 70:30) was conditioned at the same temperature as samples, according to the experimental design (Table 1), by using a heat exchanger attached to the HHP system. In addition, the high pressure vessel was conditioned at the process temperature by a heat exchange jacket that surrounds the vessel. Temperatures of samples and compression fluid were measured during HHP treatments with K type thermocouples (sample thermocouple assay FPG5580.035, Stansted Fluid Power Ltd.). Due to compression heating, an increase in sample temperature was verified during the HHP treatment (Table 2). The temperature during the holding time of 5 min (named process temperature) remained constant due to the thermal conditioning of the vessel and may be considered as a factor in experimental design. However, in the present work, the initial temperature of the samples and compressible fluid were considered in the experimental design and depicted in the tables and figures.

Table 2
Real values for initial temperature, pressure level and $CaCl_2$ concentration established according to the Doehlert design and process temperature.

Treatment	Pressure (MPa)	$CaCl_2$ (mmol L^{-1})	Temperature (°C)	
			Initial	Process
1	600	10	55	85.9
2	600	10	55	84.9
3	600	10	55	87.1
4	500	10	55	79.7
5	700	10	55	86.0
6	600	10	65	94.7
7	600	10	45	74.8
8	550	5	50	73.5
9	650	5	50	78.2
10	550	15	50	73.4
11	650	15	50	77.4
12	550	5	60	84.8
13	650	5	60	89.1
14	550	15	60	83.7
15	650	15	60	87.0

Process temperature corresponds to temperature reached due to compression heating.

2.4. Analysis of samples

Samples from the Doehlert design were characterized by measuring PS, %ILOX and %ITI. Moreover, to validate the optimal process conditions obtained from the desirability function and to compare with other process conditions, we prepared soymilk without further treatments (neither $CaCl_2$ addition nor combination of high pressure and thermal treatments), which was named "control", and soymilks that were subjected to different combination of factors. These samples were characterized in terms of PS, %ILOX, %ITI, viscosity and physical stability.

The treatments for validation and comparison among conditions were carried out in triplicate. These data were subjected to analysis of variance (ANOVA) and Tukey's test.

2.4.1. Protein solubility (PS)

Samples were centrifuged at 10,000 × *g* for 20 min at 4 °C in an Air-cooled Microlitre Centrifuge Z 233 MK-2 Hermle (Gosheim, Germany). Protein concentration was determined in triplicate in the supernatants with the Sigma Bicinchoninic Acid Protein Assay Kit, modified by the incorporation of 7.0 g L^{-1} SDS, using bovine serum albumin as standard (Sigma Chemical Co., St. Louis, MO, USA).

2.4.2. Lipoxygenase (LOX) activity

LOX activity was measured in triplicate in untreated and treated soymilks using a continuous spectrophotometric method based on the enzymatic oxidation of linoleic acid to the corresponding hydroperoxide according to a modified procedure described by Van der Ven et al. (2005) and Wang, Zhou, and Chen (2008). Linoleic acid solution was prepared by adding 0.01 mL of linoleic acid and 0.01 mL of the emulsifier Tween 20 to 4 mL of 0.1 mol L^{-1} borate buffer pH 9.0 at 20 °C. After homogenization, the solution was made up with the same borate buffer to a volume of 60 mL. The oxygen in the substrate was removed by flushing with nitrogen. This solution was daily prepared. The enzyme was extracted from untreated and treated samples as follows: the pH of soymilk was adjusted to 4.5 with 1 mol L^{-1} HCl and centrifuged at 10,000 × *g* for 10 min at 4 °C (in the same centrifuge as that used for PS). Supernatants were designated as the enzyme extract and used for measurement of LOX activity. The reaction was carried out at 25 °C in a quartz cuvette. Then, 2 mL of substrate (linoleic acid solution) and

0.90 mL of 0.1 mol L^{-1} borate buffer pH 9.0 were transferred to quartz cuvette and mixed by inversion. Then, 0.05 mL of diluted enzyme extract (1/20) was added into the quartz cuvette and immediately mixed by inversion. The absorbance was measured immediately at 234 nm for 3 min by a spectrophotometer (Beckman DU 650, USA). A blank was prepared with 0.95 mL borate buffer and 2.0 mL substrate solution. One unit of LOX activity was defined as a change of 0.001 units of absorbance per min and per mL of enzyme extract (Li, Chen, Liu, & Chen, 2008).

The percentage of inactivation of LOX (%ILOX) was defined as

$$\%\text{ILOX} = 100 - 100 \times A/A_0$$

where A is the LOX activity in a treated sample and A_0 is the initial LOX activity in untreated soymilk.

2.4.3. Activity of trypsin inhibitors (TI)

Total TI activity was assessed using the enzymatic method described by González and Carrillo (1987) with some modifications. The enzymatic method is based on the hydrolysis of hemoglobin by trypsin. If trypsin is active, a blue color is formed due to the reaction between the peptides released from hemoglobin and Folin–Ciocalteu reagent (Sigma, St Louis, USA).

The TI were extracted in triplicate from untreated and treated soymilks. The pH of soymilks was adjusted to 4.5 with 1 mol L^{-1} HCl and samples were centrifuged at 10,000 × *g* for 10 min at 4 °C (in an Avanti J20i Beckman, USA). The supernatant (10 mL) was mixed with 0.01 mL of a saturated solution of Na_2CO_3. After homogenization, the solution was made up with phosphate buffer (0.0455 mol L^{-1} Na_2HPO_4, 0.0045 mol L^{-1} NaH_2PO_4) pH 8.0 to a final volume of 25 mL. This TI solution was used for activity assay. An aliquot of 0.5 mL of substrate (2.5 g of hemoglobin (Sigma, St Louis, USA) in 100 mL of urea 8.3 mol L^{-1}, pH 8.0) was incubated at 37 °C for 10 min. Then, 0.05 mL of TI solution was added to the substrate and the reaction was started by adding 0.05 mL of porcine trypsin (Sigma, St Louis, USA) (1350 EU/mL). Control of trypsin activity was evaluated substituting the TI solution with phosphate buffer. The blank of reaction was evaluated by adding 1 mL of trichloroacetic acid (5 g in 100 mL of deionized water) and 0.05 mL of phosphate buffer before addition of porcine trypsin. Samples were incubated a 37 °C for 20 min. The reaction was stopped by adding 1 mL of trichloroacetic acid into control and sample tubes. All tubes were mixed and centrifuged at 16,500 × *g* for 10 min at 20 °C (in the same centrifuge as that used for PS). Then, 0.5 mL of supernatant was mixed with 1 mL of 1.0 mol L^{-1} NaOH and 0.3 mL of Folin–Ciocalteu diluted reagent (1:2 in deionized water). After 1 h, the absorbance at 650 nm was determined using a Synergy HT™ Multi-mode Microplate Reader (BIOTEK Instruments, Winooski, VT, USA).

Percentage of inactivation of trypsin inhibitors (%ITI) was calculated as follows:

$$100\%\text{Trypsin activity} = \text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}}$$

$$\%\text{Trypsin activity} = (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})/(\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}}) \times 100$$

$$\%\text{Trypsin inhibition in sample} = 100 - \%\text{Trypsin activity}.$$

Untreated soymilks were taken as reference (REF) for 100% of TI activity

$$\%\text{TI} = \%\text{trypsin inhibition in sample}/\text{REF} \times 100$$

$$\%\text{ITI} = 100 - \%\text{TI}.$$

2.4.4. Viscosity

Viscosity of soymilks was determined at 20 °C using a rheometer (HAAKE RheoStress 6000, Thermo Electron Corporation, Germany) equipped with a cylindrical sensor system (Z34 DIN, 34 mm in diameter). Samples were thermostated in the sensor for 1 min. Then, the apparent viscosity (η) was recorded by increasing the shear rate from 20 to 500 s^{-1} for 60 s, holding it at 500 s^{-1} for 90 s, and decreasing it from 500 to 20 s^{-1} for 60 s. Viscosity was determined in triplicate for each treatment. The values of η at 400 s^{-1} were reported.

2.4.5. Physical stability

To assess the physical stability of soymilk, samples of 10 mL were poured into transparent glass tubes (internal diameter 13 mm) and stored at 4 °C. Observations were made 0, 1 and 5 days after processing. Physical stability was estimated indirectly as the ratio between the length of the upper separated phase and the total length of the sample. Transparency of the upper phase was naked-eye classified in four levels, and taken as a measure of sedimentation.

3. Results and discussion

3.1. Doehlert design

3.1.1. Protein solubility (PS)

Estimated regression coefficients for PS were obtained from responses by multiple linear regression analysis (Table 3). The model was significant ($p < 0.005$) and explained 93% of the observed responses ($R^2 = 0.93$). PS was significantly modified by $CaCl_2$ concentration and decreased linearly ($p < 0.01$) and quadratically ($p < 0.05$) with the increase in this factor (Table 3). No significant effects of pressure level or initial temperature were observed in the ranges tested of these process factors. Moreover, no significant interactions were observed between factors. For a more complete depiction of the results, a response surface plot was prepared for PS as a function of $CaCl_2$ concentration and pressure level (Fig. 1). The lowest value of PS (50.5% relative to total protein) was observed at the highest $CaCl_2$ concentration (Fig. 1).

Divalent ions, such as calcium, bind soybean proteins through negatively charged amino acid residues and imidazole from histidine, and thus induce their insolubilization (Appu Rao & Narasinga Rao, 1976; Kroll, 1984). Yazici, Alvarez, Mangino, and Hansen (1997) assayed the addition of sodium hexametaphosphate or potassium citrate together with increasing protein concentration and pH (6.8–7.6), and found that these conditions improved the heat stability of soymilks but

Table 3
Regression coefficients and analysis of variance of the regression model for protein solubility (PS), percentage of inactivation of lipoxygenase (%ILOX) and percentage of inactivation of trypsin inhibitors (%ITI) of calcium-added soymilks treated with combination of high hydrostatic pressure and thermal treatments.

Terms		Response variables		
		PS	%ILOX	%ITI
Constant		23.37***	99.05	96.933****
Lineal	$CaCl_2$	−4.950**	−0.081	2.000
	P	0.763	0.191	6.894**
	T	−0.425	0.241	9.787***
Quadratic	$CaCl_2^2$	−5.333*	0.113	−0.729
	P^2	−1.004	0.063	−3.102*
	T^2	−0.904	0.194	−5.827**
Interactions	$CaCl_2 \times T$	−1.600	−0.006	2.163
	$T \times P$	−0.200	−0.244	−6.737*
	$CaCl_2 \times P$	0.375	0.081	−1.475
R^2		0.93	0.07	0.97
p-Value		***	NS	***

Reduced equation for response variables (coded values):
$PS = 21.1857 - 4.95\ CaCl_2 - 5.061\ CaCl_2^2$
$\%ITI = 96.18 + 9.787\ T - 5.799\ T^2 + 6.894\ P - 3.074\ P^2 - 6.737\ T \times P.$
$CaCl_2$: Calcium chloride concentration, P: Pressure level, T: initial temperature. NS: nonsignificant.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.005$.
**** $p < 0.0005$.

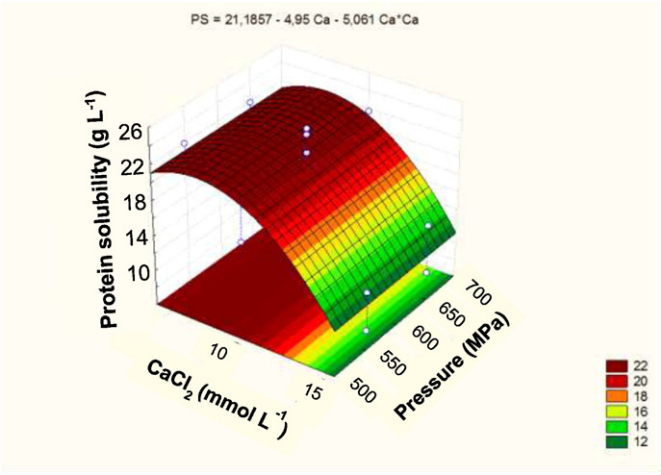

Fig. 1. Response surface plot showing the effect of $CaCl_2$ concentration and pressure level on protein solubility (PS). Experimental values: 0.

exhibited an important decrease in viscosity. Pathomrungsiyounggul, Grandison, and Lewis (2007) also assayed the addition of sodium hexametaphosphate in calcium-fortified soymilk and found that particle diameter was higher when ionic calcium concentration increased.

The stabilizing and solubilizing effect of HHP was not accompanied by an irreversible change in ionic calcium concentration (Manassero et al., 2015); thus, the effects of HHP may represent an advantageous strategy by improving PS and decreasing the incorporation of additives. In the present work, we found no effect of high pressure level because of the range tested, but pressure levels were high enough to reverse calcium-induced insolubilization. Table 4 shows the PS values of control samples and calcium-added samples subjected to different conditions. Añón et al. (2012) reported that differences in the magnitude of the solubilizing effect were found between 400 and 600 MPa, but, in the present study, a plateau was probably reached in the 500–700 MPa range.

Temperature had no significant effect on PS in the range tested. Although soybean proteins are very sensitive to thermal treatment (Sorgentini & Wagner, 1999), the combined effect of 500–700 MPa and 75–95 °C possibly led to indistinguishable high denaturation degrees. The range of pressure assayed was high enough to achieve ca. 75% and 100% of denaturation in β-conglycinin and glycinin, respectively, when applied at room temperature (Speroni, Añón, & de Lamballerie, 2010). On the other hand, the denaturation temperatures of β-conglycinin and glycinin belong to the range of process temperatures achieved in the present work. Cruz et al. (2007) reported 100% denaturation in soymilk treated by ultra-high pressure homogenization at 300 MPa and a reached temperature of 104 °C. Moreover, the pressure-induced thermostabilization that may occur in some proteins is verified with pressure levels up to ca. 100 MPa, above this one, the unfolded states are promoted by HHP-thermal treatment combinations (Sun, Caillot, Mak, Robb, & Clark, 2001). Taken together, these data suggest that proteins were completely denatured in all our samples. This fact may contribute to the lack of effect of temperature or combination of high pressure and thermal treatments on PS.

Table 4
Protein solubility (PS), percentage of inactivation of trypsin inhibitor (%TI), percentage of inactivation of lipoxygenase (%ILOX) and apparent viscosity (η) in calcium-added soymilks subjected to different processing conditions, including the optimal values obtained from desirability function.

Treatments	%PS	%ILOX	%ITI	η (cP)
Control	100.0 ± 0.0^{a}	0.0 ± 0.0^{c}	0.0 ± 0.0^{d}	3.7 ± 0.1^{d}
8.53 mmol L^{-1}	56.3 ± 2.3^{d}	0.0 ± 0.0^{c}	0.0 ± 0.0^{d}	4.8 ± 0.1^{b}
8.53 mmol L^{-1}, 85.8 °C	$59.6 \pm 5.4^{c,d}$	100.0 ± 0.0^{a}	69.1 ± 0.8^{b}	5.8 ± 0.2^{a}
8.53 mmol L^{-1}, initial temp.: 55.8 °C, process temp.: 85.8 °C, 614 MPa	71.9 ± 3.9^{c}	100.0 ± 0.1^{a}	100.0 ± 0.0^{a}	4.5 ± 0.1^{c}
8.53 mmol L^{-1}, initial temp.: 5 °C, process temp.: 33.8 °C, 614 MPa	87.2 ± 1.4^{b}	98.7 ± 0.1^{b}	3.1 ± 0.8^{c}	4.4 ± 0.1^{c}

Values of initial temperature, pressure level and $CaCl_2$ correspond to optimal values obtained from desirability function. Values are expressed as means ± standard deviation. Different letters in the same column indicate significant differences ($p < 0.05$).

3.1.2. LOX inactivation

None of the design factors was significant ($p > 0.005$) for %ILOX in the ranges tested (Table 3). The values of %ILOX ranged between 98.3% (15 mmol L^{-1} $CaCl_2$, 550 MPa, 50 °C) and 100% (5 or 15 mmol L^{-1} $CaCl_2$, 550 o 650 MPa, 60 °C). LOX inactivation was very high and almost constant in the range of pressure and temperature evaluated. Consequently, %ILOX was not affected by any of the design factors evaluated. No residual LOX activity was detected for most of the pressure and temperature combinations studied. Thus, the product obtained would be acceptable and without off-flavors related to enzymatic oxidation.

The high values of LOX inactivation observed in the present study may be explained by the findings of previous works: Heinisch et al. (1995) studied the activity and structure of LOX by Fourier transform infrared spectroscopy and stated that irreversible changes occurred when LOX was treated at 600 MPa and room temperature. Tangwongchai, Ledward, and Ames (2000) reported that a treatment at 600 MPa and 45 °C (measured in the compression fluid) produced irreversible inactivation of LOX. Seyderhelm, Boguslawski, Michaelis, and Knorr (1996) classified LOX as a pressure-sensitive enzyme and reported that LOX activity decreased noticeably after 2 min at 600 MPa and 25 °C in both Tris buffer at pH 7.0 and soymilk. Moreover, Wang et al. (2008) found that irreversible LOX inactivation in soymilk was achieved by combined thermal (5 to 60 °C) and high pressure (200–650 MPa) treatments.

Our results agree with previous findings and show that LOX was fully inactivated with most of the pressure and temperature combinations studied. Moreover, calcium concentration did not interfere with the effects of the combination of high pressure and thermal treatments.

3.1.3. TI inactivation

Estimated regression coefficients for %ITI were obtained from responses by multiple linear regression analysis (Table 3). The model was significant ($p < 0.005$) and explained 97% of the observed responses ($R^2 = 0.97$). Table 3 also shows that pressure level and initial temperature significantly affected %ITI. %ITI increased linearly with the increase in pressure level ($p < 0.01$) and initial temperature ($p < 0.005$) and decreased quadratically with the increase in pressure level ($p < 0.05$) and initial temperature ($p < 0.01$). In addition, significant interaction was observed between pressure level and initial temperature ($p < 0.05$). Calcium concentration had no significant effect on %ITI. For a more complete depiction of the results, a response surface plot of %ITI as a function of initial temperature and pressure level is shown in Fig. 2.

Both TI exhibit an important thermal resistance that may be due to disulfide bonds (DiPietro & Liener, 1989): two in Kunitz TI and seven in Bowman-Birk TI. Birk (1961) reported that purified Bowman-Birk TI was stable when heated in aqueous solution at 100 °C for 10 min. Sorgentini and Wagner (1999) measured a denaturation temperature of Kunitz TI in water of 76 °C, and found two endotherms at 77 and 110 °C for Bowman-Birk TI.

The range of inactivation obtained for TI was between 51.9% (10 mmol L^{-1} $CaCl_2$, 600 MPa, 45 °C) and 100% (15 mmol L^{-1} $CaCl_2$, 550 or 650 MPa, 60 °C). In the center of the design (10 mmol L^{-1} $CaCl_2$, 600 MPa, 55 °C), the average inactivation was 96.7%. Moreover, treatments such as 10 mmol L^{-1} $CaCl_2$, 700 MPa, 55 °C; 10 mmol L^{-1}

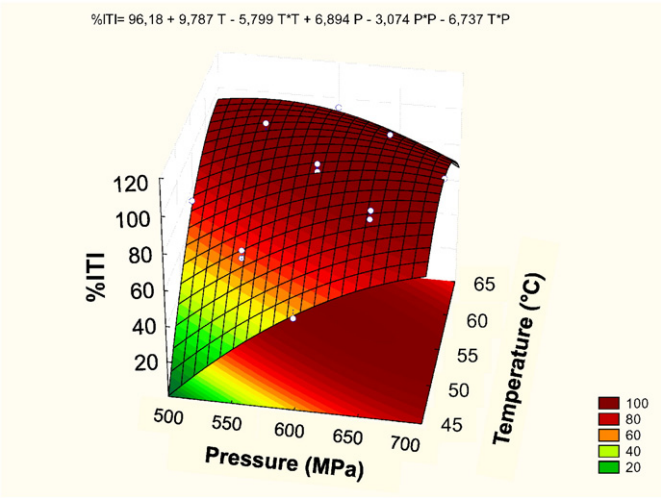

Fig. 2. Response surface plot showing the effect of initial temperature and pressure level on the percentage of inactivation of trypsin inhibitors (%ITI). Experimental values: 0.

$CaCl_2$, 600 MPa, 65 °C; 5 mmol L^{-1} $CaCl_2$, 650 MPa, 50 °C, and 5 mmol L^{-1} $CaCl_2$, 650 MPa, 60 °C caused inactivation higher than 90%.

Salts such as $CaCl_2$ and NaCl may either increase or decrease (depending on the pressure level) the sensitivity of some soybean proteins to high pressure-induced denaturation (Speroni et al., 2010; Añón, de Lamballerie, & Speroni, 2011). Thus, knowledge about the possible effect of calcium concentration on soymilk during combined high pressure and thermal treatments is interesting. The many different methods by which soybeans are processed usually reduce TI levels by up to 90% (Van Buren, Steinkraus, Hackler, El Rawi, & Hand, 1964; Van der Ven et al., 2005). Our data indicate that several of the high pressure and temperature combinations assayed may lead to acceptable soymilks, with respect to TI inactivation. The Bowman-Birk TI has several sites for binding divalent ions (Losso, Munene, Bansode, & Bawade, 2004); calcium association may increase sensitivity to the applied treatment and allow the complete inactivation observed. Thus, the presence of calcium, in addition to the higher holding time (<2 min vs. 5 min), might explain the higher level of TI inactivation observed in the present work in relation to that informed by Van der Ven et al. (2005).

3.1.4. Multi-criteria optimization using the desirability function

In the present work, the desirability function was designed with the criterion that the optimal treatment had to cause maximum increase in PS and inactivation of TI. To define the desirability function, lower and upper limits for each response were specified, based on experimental data. Fig. 3 shows each individual desirability function profile and the global desirability function profile. The predicted optimal process condition leading to the maximal global desirability value (D = 0.999) was the following combination of coded values of the design factors: $CaCl_2$ concentration = −0.2931, initial temperature = 0.15813 and pressure level = 0.28778. The real values of the design factors were: $CaCl_2$ concentration = 8.53 mmol L^{-1}, initial temperature = 55.8 °C and pressure level = 614 MPa. For this condition, the predicted values of the response variables were: 24.46 g L^{-1} for PS and 99.99% for %ITI.

Smith et al. (2009) tested similar conditions (400–600 MPa/75 °C initial temperature), whereas Poliseli-Scopel et al. (2012) assayed less energetic conditions (200 MPa/55 or 75 °C inlet temperature) on soymilk and reported an improvement in microbial shelf-life by decreasing total bacterial count, numbers of psychrotrophs and *Enterobacteriaceae*. Thus, this optimal process condition also probably improves microbiological safety.

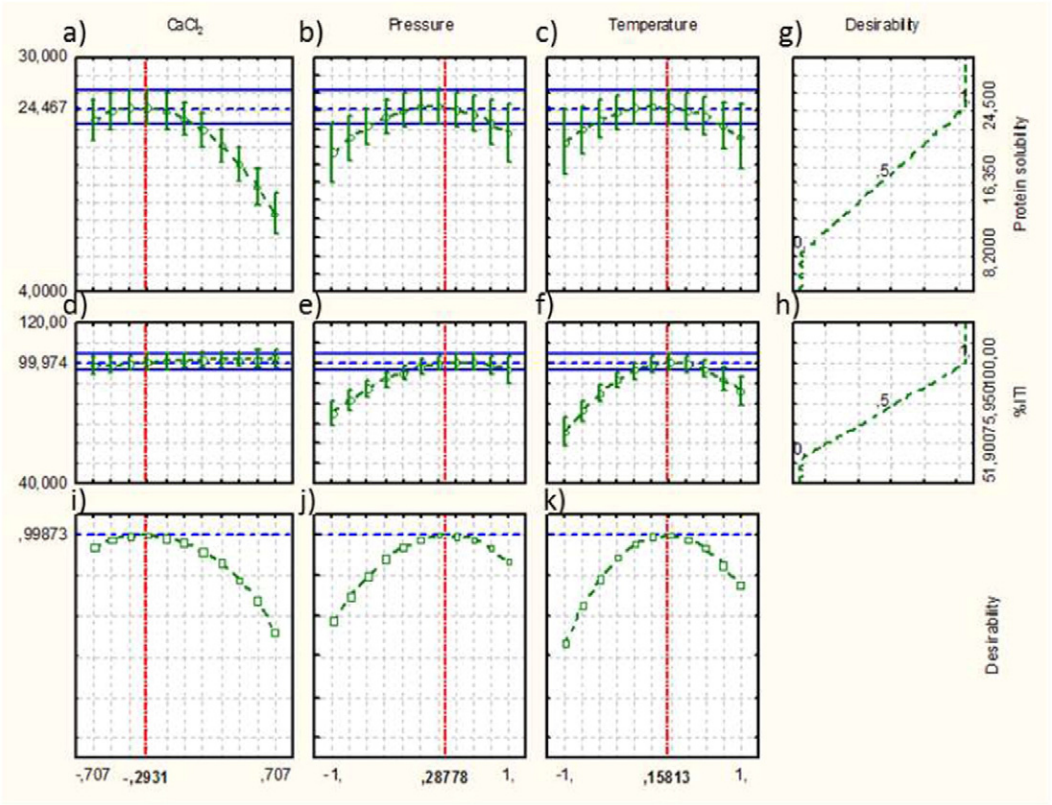

Fig. 3. Simultaneous optimization of process conditions for calcium-added soymilk treated with a combination of high hydrostatic pressure and thermal treatments. Predicted profiles for the response variables (PS and %ITI) at different levels of each design factor ($CaCl_2$ concentration, pressure level and initial temperature), holding the levels of the others factors constant at the estimated optimal value [panels a) to f)]. Predicted profiles for each individual desirability function [panels g) and h)] and for the global desirability function [panels i), j) and k)].

3.2. Comparison between several processing conditions

To validate the optimal process conditions obtained from the desirability function and to distinguish the effects of HHP and thermal treatments alone, we prepared a control soymilk and soymilks added with a $CaCl_2$ concentration of 8.53 mmol L^{-1}. In addition, calcium-added samples were subjected to a thermal treatment at atmospheric pressure (0.1 MPa) and 85.8 °C, or treated at 614 MPa and 85.8 °C, or at 614 MPa and 33.8 °C (Table 4). In the case of the thermal treatment alone, the temperature corresponded to the process temperature achieved due to compression heating in the combination of high pressure and thermal treatments (85.8 °C).

3.2.1. PS, %ITI and %ILOX

The highest values of PS were observed in soymilks without calcium addition and in calcium-added soymilks treated with HHP at room temperature (Table 4). The combination of HHP and thermal treatments increased PS compared with untreated calcium-added soymilks (71.9 ± 3.9% vs. 56.3 ± 2.3%, Table 4). These data suggest that the thermal treatment partially interferes with the solubilizing effect of HHP (71.9 ± 3.9% vs. 87.2 ± 1.4%, Table 4). However, a solubilizing effect of HHP applied at high temperature was evidenced, and interestingly it occurred in a complex matrix such as soymilk, which represents a common foodstuff. On the other hand, Zhang, Li, Tatsumi, and Isobe (2005) reported the coagulation of soymilk to form an HHP-induced tofu when 10 mmol L^{-1} $CaCl_2$ was present during treatment at room temperature. This difference with our results may be due to several factors: the protein concentration used by these authors was higher than that used in our work (44 vs. 30 g L^{-1}), and the pH value decreased after calcium addition (Kroll, 1984), which also favors coagulation, whereas in our work, the pH was adjusted to 7.0 after calcium addition.

Total inactivation of TI (100%ITI) was achieved with the combination of high pressure and thermal treatments, whereas partial inactivation (69.1%ITI) was reached with the thermal treatment alone (Table 4). Calcium-added soymilk treated with HHP at room temperature presented a low value of %ITI (3.1%, Table 4). These results suggest that pressure and temperature interacted synergistically to inactivate TI, and agree with those of Van der Ven et al. (2005), who studied the effect of high pressure and thermal treatments on non-calcium-added soymilks.

LOX was totally inactivated by the thermal treatment alone and by the combination of high pressure and thermal treatments (100%ILOX, Table 4). Also, at room temperature, HHP produced a high level of inactivation (98.7%ILOX, Table 4). These data indicate that LOX is more sensitive to the treatments tested than TI. Nevertheless, both LOX and TI may be successfully inactivated with the application of combined treatments on calcium-added soymilks.

The combination of thermal and high pressure treatments presented in the present study is advantageous because of the almost instantaneous compression heating, which allows saving process time and energy. In these conditions, samples are heated for a time shorter than that corresponding to conventional thermal treatment, and thus some sensory and nutritional properties are probably preserved.

3.2.2. Viscosity

In addition to nutritive value, palatability of soymilk is very important, and it is related to viscosity. Thus, viscosity of some samples was measured to the effect of processing conditions (Table 4). Calcium addition significantly ($p < 0.05$) increased apparent viscosity (η) in untreated samples by formation of soluble and insoluble aggregates. Calcium-added and thermal-treated samples exhibited the highest values of η (5.8 cP). In these samples, thermal-induced unfolding, together with the presence of calcium, promoted the formation of macro-aggregates, with higher molecular weight than before thermal treatment (Yamauchi, Yamagishi, & Iwabuchi, 1991). Bernat, Cháfer, Rodríguez-García, Chiralt, and González-Martínez (2015) found that heated almond milks (subjected or not to homogenization processes) exhibited an increased η, explained as a weak gelation phenomenon promoted by the thermal treatment. These authors also reported that the soluble fiber fraction could also contribute to the increase in η by the extension and hydration of the biopolymer chains induced by the temperature.

Calcium-added and thermal-treated samples (at room temperature or at an initial temperature of 55.8 °C) exhibited a lower η than untreated calcium-added ones (Table 4). This effect may be related to the increase in PS observed in the present work, which was at least in part explained by the breakdown of aggregates into smaller species (Añón et al., 2012). In the case of HHP treatment alone, proteins were unfolded, but aggregates would be smaller than those obtained by thermal treatment (Poliseli-Scopel et al., 2012). It is worth noting that in the combination of high pressure with thermal treatments, η was indistinguishable from that of HHP alone (at room temperature), suggesting that HHP governed the structure and/or size of aggregates. Although changes in η were small (Table 4), they reflect a different mechanism of aggregation. Lakshmanan, de Lamballerie, and Jung (2006), working with soymilk without calcium addition, found no change in η at pH 7.0 after 500 or 600 MPa but observed an increase in η after thermal treatment, and also suggested that different aggregates were formed. However, these authors reported an increase in η at pH 6.0 after the same HHP treatments. Zhang et al. (2005) reported an increase in η of non-calcium-added soymilks after HHP treatment. In that case, the effect might have been due to a lower pH (these authors assayed HHP at the natural pH of soymilk 6.5). Taken together, these data indicate that the pH of soymilk influences the effect of HHP on viscosity.

Soymilk is a complex system with emulsified oil droplets and also dispersed protein particles; both dispersed phases may be modified by processing. Cruz et al. (2007) analyzed the size distribution in ultra-high pressure homogenized and ultra-high temperature treated soymilk, and found a reduction of particle size after homogenization at 200 or 300 MPa, which was not observed after ultra-high temperature treatment. Nevertheless, Malaki Nik, Tosh, Woodrow, Poysa, and Corredig (2009) reported a reduction in particle size by breaking down protein bodies in thermal-treated soymilk (95–100 °C). These differences may be due to different particle size distribution in raw soymilks, protein content, and polypeptide composition. Ono, Choi, Ikida, and Odaoiri (1991) stated that heating of soymilk promotes the degradation of large particles (diameter > 100 nm) to soluble proteins and that soluble proteins are aggregated to form medium-sized particles (40 nm < diameter < 100 nm). Thus, the MW scales of aggregates reported as formed or broken down may have been different in each work. Moreover, the individual contributions of the oil phase and protein aggregates to particle size distribution are not easy to distinguish (Cruz et al., 2007). Therefore, a parameter such as viscosity, which is influenced by both oil droplet and aggregate size distribution, may be a good indicator of the quality or behavior of the whole system.

3.2.3. Physical stability

Physical stability was determined in some samples that belonged to the Doehlert design. Some samples that did not belong to the Doehlert design, but had achieved important effects in terms of TI inactivation and/or PS were also analyzed. These conditions were assayed to distinguish the effects of thermal and of HHP treatments from those of combined HHP and thermal ones. Thus, temperature of thermal treatment at atmospheric pressure was chosen according to process temperature of combined thermal-HHP treatments. In the case of calcium concentrations, 10 mmol L^{-1} was chosen due to the important increase in solubility observed after treatment, whereas 15 mmol L^{-1} was chosen for being the highest concentration assayed in the design.

Destabilization of soymilk, when it occurred, was evidenced as a phase separation that consisted only in sedimentation, no creaming was observed in any sample (Fig. 4). No phase separation was detected at zero time (ratio = 0). After 1 and 5 days, a supernatant appeared in

Fig. 4. Phase separation in soymilks subjected to different treatments after 0, 1 and 5 days. RT: room temperature. Figures correspond to $CaCl_2$ concentration (mmol L^{-1}), temperature (thermal treatment at 0.1 MPa or process temperature of HHP treatments, °C) and pressure level (MPa), respectively.

some samples, limited by a sharp boundary. The supernatants exhibited different turbidities, depending on the composition and treatment. This fact reveals the existence of different aggregates and particles in soymilk samples, each with different colloidal stability. Control soymilk (no calcium addition and no thermal or HHP treatments) was stable during the 5 days. Addition of 10 mmol L^{-1} $CaCl_2$ promoted destabilization; ratios were 0.095 and 0.405, at 1 and 5 days, respectively. The supernatant in 10 mmol L^{-1} $CaCl_2$-added samples was turbid due to the presence of insoluble aggregates and/or oil droplets that did not settle. Addition of 15 mmol L^{-1} $CaCl_2$ also promoted destabilization, with a more noticeable effect than in 10 mmol L^{-1}-added samples. The ratio was higher and the supernatant was more transparent (Table 5). The thermal treatment in both calcium-added samples modified the behavior, accelerating phase separation in the first day, but reaching a smaller ratio after 5 days (Table 5). In the case of 15 mmol L^{-1} $CaCl_2$-added samples, the thermal treatment led to a more transparent supernatant, because of an increased heat-induced sedimentation. These findings are in accordance with several studies about heat-induced changes of colloidal stability of soybean proteins (Kwok & Niranjan, 1995; Kwok, Liang, & Niranjan, 2002; Malaki Nik et al., 2009). The combination of HHP and thermal treatments in 10 mmol L^{-1} $CaCl_2$-added soymilks led to stable samples (ratio = 0 until fifth day). In the case of 15 mmol L^{-1} $CaCl_2$-added soymilks, phase separation was evidenced with turbid supernatants and ratios that depended on temperature and pressure levels.

Table 5
Physical stability of soymilks subjected to different treatments after 0, 1 and 5 days.

	0 day		1 day		5 days	
Sample	Ratio	Transparency of the upper phase	Ratio	Transparency of the upper phase	Ratio	Transparency of the upper phase
Control	0	−	0	−	0	−
10 RT 0.1	0	−	0.095	+	0.405	+
10 85.0 0.1	0	−	0.152	+	0.256	+
10 95.0 0.1	0	−	0.142	+	0.210	+
10 79.7500	0	−	0	−	0	−
10 94.7600	0	−	0	−	0	−
15 RT 0.1	0	−	0.212	++	0.512	++
15 80.0 0.1	0	−	0.369	+++	0.489	+++
15 90.0 0.1	0	−	0.214	+++	0.214	+++
15 77.4 650	0	−	0.582	+	0.633	++
15 83.7 550	0	−	0.320	++	0.433	++

Ratio was defined as the quotient between the length of the upper separated phase and the total length of the sample. Figures of sample names correspond to $CaCl_2$ concentration (mmol L^{-1}), temperature of thermal treatment alone (at 0.1 MPa) or process temperature of high pressure and thermal treatments, and pressure level, respectively. RT: room temperature. Transparency of the upper phase: no separation (−), turbid (+), translucent (++) or transparent (+++).

These results indicate that the combination of HHP and thermal treatments improves the physical stability of $CaCl_2$-added soymilks; the final effects were dependent on the $CaCl_2$ concentration and processing conditions.

Poliseli-Scopel et al. (2012) found that ultra-high pressure homogenized soymilks remained in perfect dispersion state without any visible phase separation, while ultra-high temperature and pasteurized soymilks exhibited an easily observable layer on the bottom of the bottles. Bernat et al. (2015) also reported improved physical stability of almond and hazelnut milks after a sequential high pressure homogenization and thermal treatment. These authors stated that protein solubilization and subsequent formation of a continuous protein matrix occurred when certain processing conditions were applied and favored physical stability.

4. Conclusions

The combination of HHP and thermal treatments was analyzed to obtain acceptable calcium-added soymilks. Our results show that, in the ranges tested, $CaCl_2$ concentration was a significant factor for PS, whereas initial temperature and pressure levels were significant factors to induce TI inactivation. Several conditions may be applied to obtain soymilks with improved PS and with inactivated TI and LOX. The optimal conditions for PS, %ILOX and %ITI were determined using response surface methodology and desirability function, and these were: $CaCl_2$ concentration = 8.53 mmol L^{-1}, initial temperature = 55.8 °C and pressure level = 614 MPa. Compression heating caused an increase in temperature in the range 23–32 °C. This heating contributed to the observed effects because it allows achieving the denaturation temperatures of soybean proteins, but starting from lower temperatures. Compression heating represents an advantage because it is internally generated by compression, needs no temperature difference to induce heat transfer and it is almost instantaneous, allowing the occurrence of short process times. Thus, nutritional and sensory properties may be preserved; this fact should be confirmed by specific assays, but the works of Poliseli-Scopel, Hernández-Herrero, Guamis, and Ferragut (2013, 2014) are promising because ultra-high pressure homogenization (UHPH) did not affect overall soymilk characteristics and in addition, UHPH-soymilks were either not differentiated from pasteurized ones or achieved better sensory acceptance than pasteurized soymilks. On the other hand, Gupta, Kopec, Schwartz, and Balasubramaniam (2011) reported that combined high pressure–temperature treatments of tomato juice improved lycopene extractability. Sensory and nutritional analysis will be performed in a future study, in which a new product will be formulated, based on fruit juice added-soymilk.

In the present study, colloidal stability was also improved with the combination of HHP and thermal treatments, compared with thermal treatment alone: no phase separation in a 5-day period was observed for 10 mmol L^{-1} $CaCl_2$-added soymilks. This effect seems to be a combination of PS, size and composition of protein aggregates, and/or structure of the interfacial film of oil droplets. The solubilizing effect of HHP on calcium-added soybean proteins was demonstrated in a complex food system such as soymilk and under high temperatures.

Our results indicate that the combination of HHP and thermal treatments allows obtaining calcium-added soymilk with several improved properties and without additives such as chelating agents.

Acknowledgments

The authors wish to thank Claudio Sanow from the Instituto Tecnología de Alimentos, Instituto Nacional de Tecnología Agropecuaria (INTA), for his kind assistance during the use of the HHP equipment. This research was supported by the Instituto Nacional de Tecnología Agropecuaria (INTA, grant PNAIyAV 1130033), and the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, grant PIP 11220110101109), Argentina.

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