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Optimisation of germination time and temperature on the concentration of bioactive compounds in Brazilian soybean cultivar BRS 133 using response surface methodology

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ABSTRACT

The objective was to optimise the effect of germination time and temperature on the concentration of soluble protein, lunasin, BBI, lectin, saponins and isoflavones in soybean seeds from cultivar BRS 133. Isoflavone and saponin concentrations were analysed by HPLC. Lunasin, Bowman-Birk inhibitor and lectin were analysed by ELISA and western blot. The effects of the variations in germination time and temperature on bioactive compounds were analysed using the response surface methodology (RSM), with a 2^2 central composite rotational design. Germination of soybean for 42 h at 25 °C resulted in an increase of 61.7% of lunasin, decrease of 58.7% in lectin and 70.0% in lipoxygenase activity. Optimal increases in the concentrations of isoflavone aglycones were observed in combination of 63 h of germination and 30 °C. A significant increase of 32.2% in the concentration of soy saponins was observed in combination of 42 h of germination at 25 °C.

1. Introduction

The majority of processed soybean products have been derived from dry mature soybeans. The development of products from germinated soybeans could further increase the versatility and utilisation of soybeans. Germination processes have been developed to overcome the disadvantages of soybean seed used in food products (Zhu, Hettiarachchy, Horax, & Chen, 2005). Soybean is a complex matrix of several bioactive compounds, including peptides and proteins, isoflavones, saponins, and other compounds with cancer-preventive properties. For example, lunasin is a novel and promising chemopreventive peptide from soybean (Jeong, Park, Lam, & de Lumen, 2003). Lunasin and BBI are bioactive soy peptides that have been shown to be effective suppressors of carcinogenesis *in vitro* and *in vivo* model systems (Park, Jeong, & de Lumen, 2007). Lectin is an antinutritional factor that lowers soybean protein quality (George, Bhide, Thengane, Hosseini, & Manjaya, 2008), but is currently being employed as a therapeutic agent in cancer treatment studies (Gonzalez de Mejia & Prisecaru, 2005). Soybean seeds are a relatively rich source of lipoxygenase enzymatic activity. It has been shown that short periods of germination (72 h) can substantially reduce the odour and flavour scores of full-fat soybean flour due to reduced lipoxygenase activity (Suberbie, Mendizabal, & Mendizabal, 1981).

Soybeans are a rich source of biologically active phytochemicals such as isoflavones and saponins. There are three types of isoflavones in soybean, and each type exists in four different chemical forms: the aglycones – (daidzein, genistein, and glycitein); and the β-glucoside conjugate forms – (daidzin, genistin, and glycitin); as well as their malonyl-glucoside forms and their acetylglucoside forms (Berhow, 2002). However, limited information is available on the effect of soybean germination on isoflavone content and

Abbreviations: BBI, Bowman-Birk inhibitor; ELISA, enzyme-linked immunosorbent assay; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; RSM, response surface methodology.

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composition (Zhu et al., 2005). The soy saponins are plant triterpenoids which are accumulated in a multiple glycoside forms in the seeds. Germination induces a substantial increase in the concentration of estrogenical compounds and almost all phytosterols, particularly β-sitosterol. A diet rich in germinated soybean seeds may possibly have beneficial effects in preventive obesity (Bau, Villaume, & Méjean, 2000).

The objective of this study was to optimise the effect of germination time and temperature on the concentration of soluble protein, lunasin, BBI, lectin, saponins and isoflavones in soybean seeds from cultivar BRS 133 using response surface methodology.

2. Materials and methods

2.1. Materials

The soybean cultivar BRS 133, with a weight of 129 g per 1000 seeds, was developed as part of the breeding programme of Embrapa Soybean, Brazil. This cultivar was selected because of its high levels of isoflavones (Mandarino, Carrão-Panizzi, & Crancianinov, 2006). Soybeans seeds of BRS 133 (8.4% moisture) were cleaned with sodium hypochlorite (100 mg/kg) for 10 min, and then rinsed three times with distilled water and kept at room temperature for 8 h. Germination was carried out in germination chambers using paper trays containing 500 g of seeds. Germinated seeds were then frozen at −30 °C for 4 h, freeze-dried, and milled to produce germinated soybean flour.

Immunoaffinity purified lunasin (98%) from soy and mouse monoclonal antibody against the lunasin epitope – EKHIMEKIQGRGDDDDD were provided by Dr. Ben O. de Lumen, University of California at Berkeley. Purified A and B group soy saponins were prepared in the Peoria USDA laboratory (Berhow, Kong, & Duval, 2006).

The primary polyclonal antibody that is specific for lectin from soybean was provided by Dr. Theodore Hymowitz from the Department of Crop Sciences, University of Illinois at Urbana-Champaign). The lectin anti-serum was obtained by immunizing young male New Zealand white rabbits with a subcutaneous injection of 5 mL emulsion containing 5 mg of pure lectin, 1 mL of distillated water and 1 mL of Freund's complete adjuvant. Six weeks after the first immunisation, rabbits showing response to the antibodies (measured 20 days after the first injection) were injected again with a similar dose and bled two weeks later (Orf, 1979).

2.2. Protein extraction

Fifty mg of soybean flour and 1 mL of extracting buffer (0.05 M Tris–HCl buffer, pH 8.2) were placed in an Eppendorf tube. After mixing, the samples were sonicated in an ultrasonic bath (Branson Ultrasonic Corporation, Danbury, CT) for 70 min, mixing every 10 min to avoid settling, at 40 °C using a recirculation bath (Endocal model RTE-9, Neslab Instruments, Portsmouth, NH). The samples were centrifuged at 20,000*g* for 40 min at 8 °C in an Eppendorf Centrifuge (model 5417R, Brinkmann Instruments, Westbury, NY), and the supernatant was decanted to a new Eppendorf tube.

2.3. Soluble protein concentration determination by DC assay

The protein concentration was determined using the Bio-Rad *DC* Microplate Assay Protocol (Bio-Rad Laboratories, Hercules, CA). Briefly, 5 μL of samples (1:20 dilution) were placed in a 96-well plate and treated with 25 μL of Bio-Rad A (alkaline copper tartrate solution) and 200 μL of Bio-Rad reagent B (dilute Folin reagent) (Bio-Rad Laboratories, Hercules, CA). The plate was gently agitated and incubated for 15 min at room temperature. After incubation, the absorbance was measured at 630 nm. The protein concentration was calculated using pure bovine serum albumin standard curve ($y = 0.0002x - 0.0021$, $R^2 = 0.997$).

2.4. Enzyme-linked immunosorbent assay (ELISA) for lunasin and BBI

Lunasin concentration in soy flour from germinated seeds was analysed by ELISA (Gonzalez de Mejia, Vasconez, de Lumen, & Nelson, 2004) with the following modifications. Samples of 100 μL of protein extracts (1:5000 dilution) were placed in a 96-well plate and stored for 14 h at 4 °C. Lunasin mouse monoclonal antibody (1:4000 dilution) was used as the primary antibody and anti-mouse IgG alkaline phosphatase conjugate (1:7000) (Sigma Chem, St. Louis, MO) as the secondary antibody. The reaction was stopped adding 25 μL of 3 N NaOH at 30 min and the absorbance (405 nm) read at 35 min. A similar procedure was used for BBI analysis. Samples of 100 μL of protein extracts (1:10,000 dilution) were placed in a 96-well plate, except that BBI mouse monoclonal antibody (1:1000 dilution) (Agdia, Inc., Elkhart, IN) was used as the primary antibody and anti-mouse alkaline phosphatase (AP) conjugated IgG (1:2000) as the secondary antibody. Standard curves were determined using purified lunasin ($y = 0.0054x + 0.001$, $R^2 = 0.993$) and purified BBI ($y = 0.0108x + 0.0465$, $R^2 = 0.998$).

2.5. Enzyme-linked immunosorbent assay (ELISA) for lectin

Lectin concentration in soy flour from germinated seeds was analysed by ELISA (Vasconez–Costa, 2004) with the following modifications. One hundred microlitres (100 μL) of protein extracts (1:10,000 dilution) was placed in a 96-well plate and stored for 14 h at 4 °C. Lectin mouse polyclonal antibody (1:500 dilution) was used as the primary antibody, and anti-mouse IgG alkaline phosphatase conjugate (1:1000; Sigma) as the secondary antibody. The reaction was stopped adding 25 μL of 3 N NaOH at 30 min and the absorbance (405 nm) read at 35 min. Standard curves were determined using purified lectin ($y = 0.0101x + 0.0025$, $R^2 = 0.998$).

2.6. Gel electrophoresis of soy proteins extracts

To the supernatant of each protein extract (20 μL) was added 20 μl of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) with 5% 2-mercaptoethanol in Eppendorf tubes which were then heated at 100 °C for 3 min. The samples (20 μL) and the standard (5 μl) were loaded in the wells of the gel. The gel was run in Mini Protean-3 cell (Bio-Rad, Laboratories) using 10–20% gradient Tris–glycine SDS buffer as the running buffer. The power was set at 400 mA (200 V) constant for 30 min. Gels were fixed with peptide fixing solution for 30 min in methanol/acetic acid/water (10:40:50) and were stained with Bio Safe Coomassie $G = 250$ (Bio-Rad, Laboratories) overnight and the destained with a 10% solution of acetic acid. Gels were read in a Kodak Image Station 440 CF, where the respective molecular masses and band intensities were recorded. Amino acid sequences of major soy proteins were retrieved from UniProtKB/Swiss-Prot Release 54.1 of 21st August 2007. The theoretical molecular weight of each protein was calculated from amino acid sequence with ProtParam programme (http://ca.expasy.org/tools/protparam.html). Identification of lipoxygenase band (92.9 kDa) was confirmed by comparing theoretical molecular weight with experimental data.

2.7. Western blot procedures

Identity of lunasin was established by western blot analysis in the protein extract of germinated soybean flours. Samples were

centrifuged (20,000g) at 8 °C to eliminate any precipitate. Unstained gels were soaked in 20 mL of blotting buffer (20% methanol, 80% 1x Tris–glycine SDS) for 15 min. A Western blot sandwich was assembled by the following order: a sponge, filter, gel, polyvinylidene difluoride (PVDF) membrane Inmobilon™-FL (Millipore Corporation), and another filter and sponge being careful to avoid formation of bubbles and then developed for 1 h at 110 V and 4 °C. After the complete transfer, membrane was then saturated by incubation in 5% nonfat dry milk (NFDM) in 0.01% TBST (0.1% Tween 20 in Tris-Buffered saline) buffer for 1 h at 4 °C, and washed three times for 5 min with fresh changes of 0.01% TBST. The washed gel was incubated with lunasin mouse monoclonal antibody (1:1000 dilution) prepared in 1% NFDM in TBST buffer for 16 h at 4 °C. After washing the incubated membrane, the membrane InmobilonTM-FL (Millipore Corporation) was incubated with anti-mouse IgG alkaline phosphatase conjugate (1:10,000 dilution) prepared in 1% NFDM in TBST buffer for 3 h at room temperature. The membrane was prepared for detection using chemiluminescence reagent (Lumigen TM, GE Healthcare, Buckinghamshire, UK).

2.8. Isoflavone concentration determination by HPLC

Approximately 250 mg defatted soybean flour was extracted in test tubes with 3.0 mL of dimethyl sulfoxide: methanol (1:4 v/v) placed in sealed containers and heated at 50 °C for 18 h. The extracts were centrifuged and the supernatants were filtered using 0.45 micron filters. For isoflavones quantification 20 μL aliquots of the extracts were injected into a Shimadzu (Columbia, MD) HPLC system (LC-10AT VP pumps) equipped with SPDM10A VP photodiode array detector an (CTO-10AS VP) oven column to maintain temperature at 40 °C, all operating under Class VP software. Isoflavone separation was carried out in a C-18 reverse-phase column YMC – Pack ODS-AM, 250 mm × 4.6 mm, 5 μm particle size (YMC Co, Ltd.). The initial gradient conditions consisted of 100% H_2O containing 0.025% trifluoroacetic acid (TFA), and 0% acetonitrile, to 45% H_2O and 55% acetonitrile, over 25 min. with a flow rate of 1 mL/min. Isoflavones were detected at 260 nm and quantified by comparison with standard curves of genistin, daidzin and glycitin. The concentrations of the malonyl-glucosides and the aglycones were calculated from standard curves of their corresponding β-glucosides, using the similarity of the molar extinction coefficients of malonyl-isoflavones and β-glucosides. Isoflavone concentrations were expressed in mg/100 g of defatted samples.

2.9. Saponin concentration determination by HPLC

Saponins from the soybean flour were extracted with dimethylsulphoxide/methanol (1/1) solution at room temperature for 4 h, followed by a 15 min sonication at 50 °C and another 2 h extraction at room temperature. The extracts were then filtered through a 0.45 μm nylon filter. HPLC analysis were carried out on a Hewlett–Packard Series 1100 HPLC system equipped with an Inertsil ODS-3 reverse-phase C-18 column (250 mm × 4.6 mm ID) and particles size of 5 μm, with a metaguard column (Varian) and a G1316A column oven. The system was controlled by HPChem Station version A.06.01. For saponin analysis, a linear water-acetonitrile gradient from 30% to 50% in 45 min was used, with 0.025% TFA added to both solvents. The flow rate was 1 mL/min and the effluent was monitored at 210 nm. Saponin concentrations were calculated by using standard curves prepared from a standardised mix of B group saponins prepared in the Peoria laboratory. The nanomolar extinction coefficient for soy saponin I, was used to quantify the remaining B group saponins, the A group saponins, and the DMPP conjugated B group saponins. Identification of saponins peaks was confirmed by comparison of retention times to standards and/or LC–MS analysis (Berhow et al., 2006).

2.10. Experimental design

Variation effects in germination time and temperature were analysed using the response surface methodology (RSM), with a 2^2 central composite rotational design. The independent variables studied were germination time (12, 21, 42, 63 and 72 h) and germination temperature (18, 20, 25, 30 and 32 °C). Symbols and coded factor levels for these variables are given below (response surface were obtained using ±|α| = 1.41):

Independent variables		Levels				
Coded	Real	−α	−1	0	+1	+α
$\mathbf{X}_1$	Time of germination (h)	12	21	42	63	72
$\mathbf{X}_2$	Temperature of germination (°C)	18	20	25	30	32

2.11. Statistical analysis

Statistics 5.0 (Statsoft, USA) was used to determine the effects of the independent variables, to calculate regression coefficients (R^2), carry out analysis of variance (ANOVA) and build the response surface, at a 5% significance level.

The following second order polynomial model was fitted to the data:

$$\mathbf{Y} = \beta_0 + \beta_1 X_1 + \beta_2 \mathbf{X}_2 + \beta_{11} \mathbf{X}_1^2 + \beta_{22} \mathbf{X}_2^2 + \beta_{12} \mathbf{X}_1 \mathbf{X}_2 \quad (1)$$

where $\mathbf{Y}$ is the response variable, $\mathbf{X}_1$ and $\mathbf{X}_2$, are the coded process variables and $\boldsymbol{\beta}_0$, $\boldsymbol{\beta}_1$, $\boldsymbol{\beta}_2$, $\boldsymbol{\beta}_{11}$, $\boldsymbol{\beta}_{22}$, $\boldsymbol{\beta}_{12}$ are the regression coefficients. A stepwise methodology was followed to determine the significant terms in Eq. (1).

3. Results and discussion

Lunasin, BBI and lectin concentrations in non-germinated freeze-dried soybean flour were 3.1 ± 0.1, 5.9 ± 0.3, 4.2 ± 0.2 mg/g flour, respectively, with a total soluble protein of 248.1 ± 2.2 mg/g flour.

Table 1
Observed response values for soluble protein (mg/g flour), lunasin (mg/g SP), BBI (mg/g SP), lectin (mg/g SP), and lipoxygenase (%) with different combinations of germination time and temperature for BRS 133.

Exp	Coded level		Response values				
	$\mathbf{X}_1$ (h)	$\mathbf{X}_2$ (°C)	Soluble protein (SP) (mg/g flour)	Lunasin (mg/g SP)	BBI (mg/g SP)	Lectin (mg/g SP)	Lipoxygenase (%)
1	−1 (21)	−1 (20)	281.6	15.4	28.3	12.7	9.4
2	+1(63)	−1 (20)	192.1	19.6	21.5	10.6	6.7
3	−1 (21)	+1(30)	201.9	18.5	27.1	12.7	8.9
4	+1 (63)	+1(30)	216.7	10.8	34.9	6.5	4.0
5	−α (12)	0 (25)	282.3	17.5	27.3	12.9	7.4
6	+α (72)	0 (25)	211.7	13.2	26.4	11.0	4.2
7	0 (42)	−α (18)	305.2	12.5	28.4	11.9	8.1
8	0 (42)	+α (32)	184.4	10.5	31.3	12.5	6.8
9	0 (42)	0 (25)	208.4	21.2	28.7	7.6	4.0
10	0 (42)	0 (25)	208.5	21.0	28.4	7.3	4.0
11	0 (42)	0 (25)	208.4	21.0	28.8	7.3	4.0

$\mathbf{X}_1$ = Germination time.
$\mathbf{X}_2$ = Germination temperature.
SP = Soluble protein.
BBI = Bowman-Birk inhibitor.

Table 2
Observed values of soluble protein (mg/g flour), lunasin (mg/g flour), BBI (mg/g flour) and lectin (mg/g flour) in soy flour with different combinations of germination time and temperature for BRS 133.[A]

Exp.	Soluble protein (mg/g flour)	Lunasin (mg/g flour)	BBI (mg/g flour)	Lectin (mg/g flour)
1	281.6 a	4.3 a	8.0 b	3.6 a
2	192.1 bc	3.8 c	4.1 d	2.0 c
3	201.9 bc	3.7 c	5.5 c	2.6 b
4	216.7 b	2.3 e	7.6 b	1.4 d
5	282.3 a	5.0 a	7.7 b	3.6 a
6	211.7 b	2.8 d	5.6 c	2.3 bc
7	305.2 a	3.8 c	8.7 a	3.6 a
8	184.4 c	1.9 e	5.8 c	2.3 bc
9	208.4 bc	4.4 b	6.0 c	1.5 d
10	208.5 bc	4.4 b	5.9 c	1.5 d
11	208.4 bc	4.4 b	6.0 c	1.5 d

BBI = Bowman-Birk inhibitor.
[A] Means with different letters in the same column are significantly different ($p < 0.05$).

The experimental responses in terms of soluble protein (SP) (mg/g flour), lunasin (mg/g SP), BBI (mg/g SP), lectin (mg/g SP) and lipoxygenase (%) are presented in Table 1. The observed values of soluble protein, lunasin, BBI and lectin in soy flour with different combinations of germination time and temperature are summarised in Table 2.

3.1. Soluble protein concentration in germinated soy flour

The soluble protein concentration (SP) in the protein extracts from the flour obtained from germinated soybean seeds varied from 184.4 to 305.2 mg/g. The regression model for this parameter was statistically significant ($p < 0.05$) with $R^2 = 0.91$ which indicates a good adjustment of the model to the experimental data, in this case, the non-significant interaction term can be removed to make the regression equation simple with an $R^2 = 0.82$. The 2nd order adjusted model for soluble protein concentration is presented in Eq. (2) and the response surface in Fig. 1a.

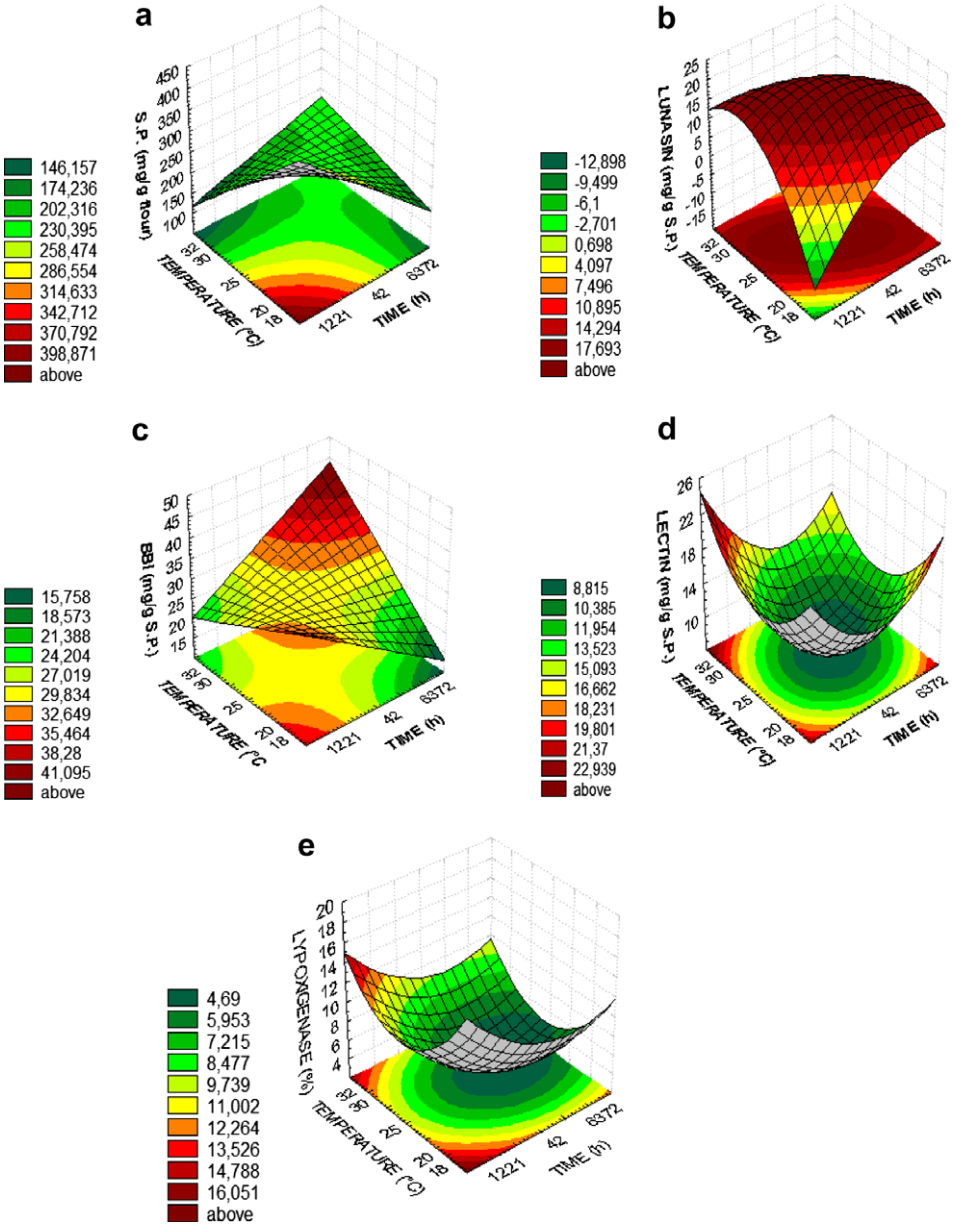

Fig. 1. Response surface model of soybean seed BRS 133 germinated flour showing time versus temperature. (a) Soluble protein. (b) Lunasin. (c) BBI. (d) Lectin. (e) Lipoxygenase.

$$\text{Soluble protein (mg/g flour)} = \mathbf{229.01} - \mathbf{24.17x_1} - \mathbf{30.53x_2} + \mathbf{30.71x_1x_2} \quad (2)$$

High values of SP were observed with low germination times from 12 h ($-\alpha$) to 21 h (-1) and 18 °C ($-\alpha$) to 20 °C (-1) of germination temperature. Maintaining the germination time constant at 21 h (-1) (Comparing Experiments 1 and 3), an increase in germination temperature from 20 °C (-1) to 30 °C (+1) promoted a decrease of 31.6% in soluble protein.

3.2. Lunasin identity and lunasin concentration in extracted protein

Identification of the lunasin band (5.45 kDa) was confirmed by western blot analysis. The results for lunasin were similar to those reported for different soybean genotypes by Gonzalez de Mejia et al. (2004). The lunasin concentration in the protein extracts from germinated soybean flour varied from 10.5 to 21.2 mg/g SP. The regression coefficient for the complete model was 0.95. In this case, the non-significant interaction term can be removed to make the regression equation a 2nd order adjusted model for lunasin concentration with an R^2 = 0.91. This is presented in Eq. (3) and the response surface in Fig. 1b.

$$\text{Lunasin (mg/g SP)} = \mathbf{21.08} - \mathbf{2.45x_1^2} - \mathbf{4.38x_2^2} - \mathbf{4.03x_1x_2} \quad (3)$$

Higher values of lunasin were observed at 21 h (-1) than 63 h (+1) germination time, and also at 20 °C (-1) than 30 °C (+1) germination temperature. The optimal condition was exactly the central point (0,0) with 42 h germination time at 25 °C. In this case, germination process contributed to an increase in lunasin levels from 12.3 mg/g SP in the non-germinated soybean flour to 21 mg/g SP in germinated soybean flour, resulting in an increase up to 61.7% in this bioactive compound.

3.3. Bowman-Birk inhibitor concentration in extracted protein

The BBI concentration in the protein extracts obtained from the germinated soybean flour varied from 21.5 to 34.9 mg/g SP. The regression coefficient for the complete model was 0.90. In this case, the non-significant interaction term can be removed to make the regression equation of 2nd order adjusted model for BBI concentration with an R^2 = 0.85. This is presented in Eq. (4) and the response surface in Fig. 1c.

$$\text{BBI (mg/g SP)} = \mathbf{28.43} - \mathbf{2.03x_1} + \mathbf{3.65x_1x_2} \quad (4)$$

Lower values of BBI concentration in SP were observed at higher germination times [63 h (+1) to 72 h (+α)] and lower germination temperatures [18 °C ($-\alpha$) to 20 °C (-1)] or lower germination times [12 h ($-\alpha$) to 21 h (-1)] and higher germination temperatures [30 °C (+1) to 32 °C (+α)]. In this case, BBI concentration decreased only in Experiment 2 (63 h of germination time at 20 °C) in about 8.8% in relation to the non-germinated soybean flour. Germination degrades trypsin inhibitor slowly (Bau, Villaume, Nicolas, & Méjean, 1997). Collins and Sanders (1976) found that 24 h soaking process had only a slight effect altering BBI in soybean; after 24 h soaking and 3-day germination, BBI decreased only about 13% for Kanrich variety, 4% for Soylima variety and 8% for Dare variety.

3.4. Lectin concentration in extracted protein

The lectin concentration in the non-germinated freeze-dried soybean flour was 17.0 mg/g SP. Germination resulted in decreased lectin concentration in the protein extracts of germinated flour, which varied, from 6.5 to 12.9 mg/g SP. The regression coefficient for the complete model was 0.92; but in this case, the non-significant term can be removed to make the regression equation simple with an R^2 = 0.89. The regression equation obtained for the second-degree adjusted model in terms of coded factors is presented in Eq. (5) and the response surface in Fig. 1d.

$$\text{Lectin (mg/g SP)} = \mathbf{7.40} - \mathbf{1.12x_1} + \mathbf{2.05x_1^2} + \mathbf{2.15x_2^2} \quad (5)$$

The lower values of lectin concentration in SP were observed in germination temperatures ranging from 20 °C (0) to 30 °C (+1) and with 42 h (0) to 63 h (+1) of germination time. This is an important effect of germination improving the biological and nutritional value of germinated soybeans and its utilisation in human foods and animal feed (Bau et al., 1997). The optimal condition was the central point (0,0) with 42 h germination time at 25 °C. In this case, the germination process contributed to a decrease in lectin levels in comparison to the non-germinated soybean flour, resulting in a decrease to 58.7% in this protein.

Table 3
Isoflavone concentrations of soybean BRS 133 at different germination times and temperatures.[a]

Isoflavone (mg/100 g)	Raw	1	2	3	4	5	6	7	8	9	10	11
Aglycones												
Daidzein	11.0	5.7	3.5	8.3	45.3	3.1	13.8	2.9	15.2	12.7	12.6	13.0
Genistein	14.4	7.5	5.3	9.9	45.0	3.6	16.7	5.8	17.8	14.5	15.0	14.5
Glycitein	1.3	0	0	0	0	0	0	0	0	0	0	0
β-Glucosides												
Daidzin	42.5	65.4	29.5	37.4	57.6	30.9	33.1	36.5	24.1	38.8	40.3	38.6
Genistin	36.1	31.9	32.8	31.2	29.3	27.7	36.9	33.3	23.8	33.9	34.5	35.1
Glycitin	10.4	0	0	0	0	0	0	0	0	0	0	0
Acetylglucosides												
Acetyldaizin	0	0	0	0	0	0	0	0	0	0	0	0
Acetylgenistin	0	0	0	0	0	0	0	0	0	0	0	0
Acetylglycitin	0	0	0	0	0	0	0	0	0	0	0	0
Malonylglucosides												
Malonyldaidzin	131.6	171.9	139.7	124.3	123.6	165.3	119.4	136.8	115.8	122.9	122.5	122.1
Malonylgenistin	100.8	57.6	36.4	38.2	8.0	57.4	38.1	38.7	35.8	29.6	31.5	29.5
Malonylglycitin	42.0	113.2	148.0	111.2	28.6	116.6	104.2	126.9	46.2	110.7	108.6	110.8
Total aglycone	26.7	13.1	8.8	18.2	**90.3**	6.7	30.5	8.7	33.0	27.3	27.5	27.5
Total isoflavone	390.0	453.1	395.2	360.4	337.5	404.6	362.0	380.9	278.8	363.2	364.9	363.6

[a] Time and temperature of experiments: 1 (21 h, 20 °C), 2 (63 h, 20 °C), 3 (21 h, 30 °C), 4 (63 h, 30 °C), 5 (12 h, 25 °C), 6 (72 h, 25 °C), 7 (42 h, 18 °C), 8 (42 h, 32 °C), 9 (42 h, 25 °C), 10 (42 h, 25 °C), 11 (42 h, 25 °C).

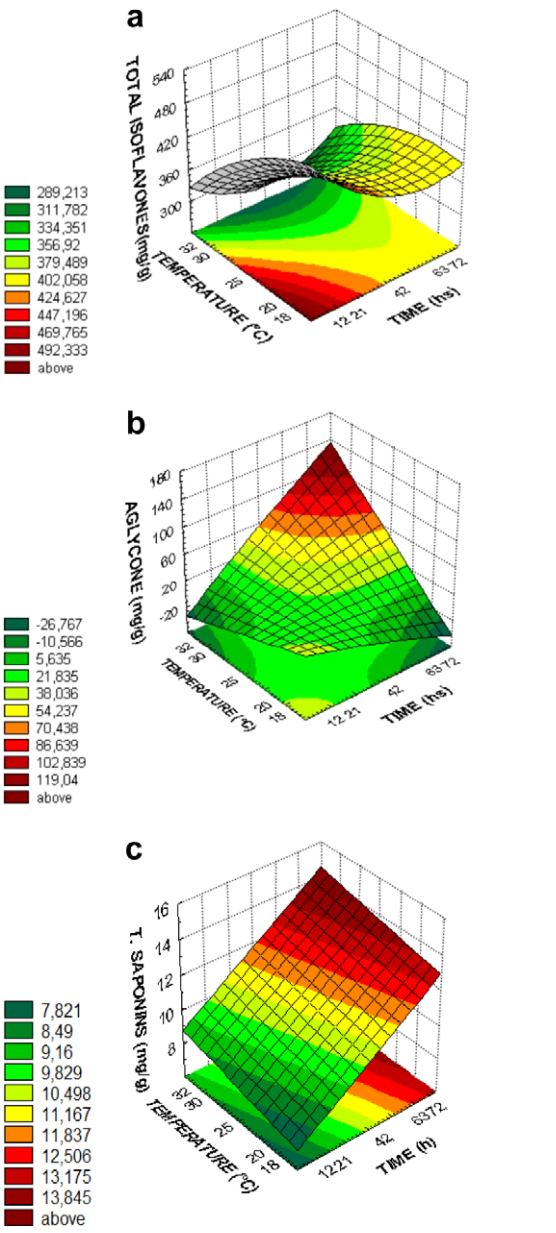

Fig. 2. Response surfaces of germination time versus germination temperature for soybean seeds BRS 133. (a) Total isoflavones. (b) Total aglycones. (c) Total saponins.

3.5. Lipoxygenase concentration

The lipoxygenase concentration of the germinated soybean flour varied from 4.0% to 9.4%, whilst the lipoxygenase concentration of the non-germinated freeze-dried soybean flour was 13.3%. The regression model for this parameter was statistically significant ($p < 0.05$) and had an $R^2 = 0.95$. The 2nd order adjusted model ($R^2 = 0.93$) for lipoxygenase concentration is presented in Eq. (6) and the response surface in Fig. 1e.

$$\text{Lipoxygenase}\ (\%) = \mathbf{4.01} - \mathbf{1.52x_1} + \mathbf{1.05x_1^2} - 0.62x_2^2 + \mathbf{1.86x_1x_2} \quad (6)$$

Lower values of lipoxygenase in SP were observed from 25 °C (0) to 30 °C (+1) germination temperature and higher germination times [42 h (0) to 72 h (+α)]. The values in bold in Eq. (6) are statistically significant ($p < 0.05$) to each other. The optimal condition was the central point (0,0) with 42 h germination time at 25 °C. In this case, germination process contributed to a decrease in lipoxygenase from 13.3% in the non-germinated soybean flour to 4% in the germinated soybean flour, resulting in a decrease of 70.0%. It has been reported that commercial full-fat soy flour has no lipoxygenase activity and the stability of its lipid composition is constant (Suberbie et al., 1981). Germination caused reduction in the level of specific activity of lipoxygenase-1 (Bordingnon, Olivera, & Mandarino, 1995).

3.6. Isoflavone concentration

The total isoflavone concentration of the non-germinated freeze-dried soybean flour was 390 mg/100 g of which 26.7 mg/100 g was composed of the aglycones daizein, glycitein and genistein. The total isoflavone concentration of germinated soybean varied from 278.8 to 453.1 mg/100 g of defatted samples for the different treatments (Table 3). The regression coefficient for the equation obtained for the complete model was 0.90. In this case, the non-significant terms were removed, to make the regression equation simple with an $R^2 = 0.73$. The regression second-degree complete model in terms of coded factors is presented in Eq. (7) and the response surface in Fig. 2a.

$$\text{Total isoflavone (mg/100 g of defatted sample)} = \mathbf{363.89} - \mathbf{17.60x_1} + \mathbf{17.18x_1^2} - \mathbf{36.84x_2} - \mathbf{9.54X_2^2} + \mathbf{8.72x_1x_2} \quad (7)$$

The highest isoflavone concentration was obtained with lower germination times [12 h (−α) to 21 h (−1)] and between 18 °C (−α) to 25 °C (0) temperature.

The total aglycone content in germinated soybean flour varied from 6.7 to 90.3 mg/100 g of defatted sample for the different treatments. The regression coefficient for the complete model was 0.86, in this case, the non-significant term were removed to make the regression equation simpler with $R^2 = 0.85$. The regression equation obtained for the second-degree adjusted model in terms of coded factors is presented in Eq. (8) and the response surface in Fig. 2b.

$$\text{Total isoflavone aglycones (mg/100 g of defatted sample)} = \mathbf{26.51} + \mathbf{12.69x_1} + \mathbf{15.12x_2} + \mathbf{19.10x_1x_2} \quad (8)$$

Higher concentration of total isoflavone aglycone forms were found in germinated soy flours ranging from higher germination times [63 h (+1) to 72 h (+α)] and higher germination temperatures [30 °C (+1) to 32 °C (+α)]. The optimal conditions were 63 h of germination time at 30 °C resulting in an increase of up to 90.3 mg/100 g (26.8%) in these bioactive compounds. In this case, the hydrolysis of the glucoside during soaking and germination process contributed to increase genistein levels from 14.4 mg/100 g in non-germinated soybean flour to 45.0 mg/100 g in germinated soybean flour. When germination time increased to 72 h at 25 °C, a decrease in genistein levels was observed (16.7 mg/100 g) possibly due to the conversion of genistein to other isoflavone forms (Zhu et al., 2005). The acetylglucoside forms, glycitin and glycitein were not detected within the ranges studied.

3.7. Saponin concentrations

The total saponin glycoside concentration in the non-germinated freeze-dried soybean flour was 9.8 mg/g. The total saponin concentration in the flours from germinated soybean seeds varied from 8.2 to 12.9 mg/g in the different treatments (Table 4). Higher saponin concentrations in germinated soybean seeds have been reported (Bau et al., 2000; Zhu et al., 2005).

The regression coefficient for the complete model was $R^2 = 0.95$, in this case, the non-significant term were removed to make the

Table 4
Saponin concentrations of soybean BRS 133 at different germination times and temperatures.[A]

SAPONINS (mg/g)	Raw	1	2	3	4	5	6	7	8	9	10	11
Group B and DMPP group B												
Soy saponins I	1.67	1.55	1.68	1.66	2.08	1.57	1.69	1.68	2.05	2.26	2.31	2.24
Soy saponins II	0.23	0.13	0.23	0.13	0.14	0.16	0.35	0.24	0.18	0.24	0.24	0.24
Soy saponins III	0.74	0.67	0.78	0.73	0.96	0.68	0.79	0.77	0.87	0.97	0.96	0.96
Soy saponins IV	0.13	0.04	0.06	0.04	0.12	0.07	0.17	0.18	0.18	0.18	0.18	0.18
Soy saponins V	0.29	0.28	0.31	0.29	0.34	0.28	0.29	0.27	0.34	0.35	0.33	0.34
Soy saponins βg	2.25	1.04	1.65	1.60	6.44	2.05	2.44	2.15	5.38	3.58	3.60	3.64
Soy saponins βa	0.24	0.21	0.26	0.23	0.23	0.20	0.21	0.12	0.12	0.26	0.27	0.29
Soy saponins γg	0.94	0.05	0.10	0.11	0.06	0.05	0.15	0.10	0.12	0.08	0.07	0.09
Soy saponins γa	0.14	2.47	2.26	2.43	0.93	2.03	2.28	2.61	1.04	1.32	1.38	1.30
Soy saponins αg	0.55	0.17	2.58	0.17	0.32	0.20	2.14	0.50	0.53	0.24	0.24	0.24
Total group B	7.18	6.61	9.91	7.39	11.62	7.29	10.51	8.62	10.81	9.48	9.58	9.52
Group A acetyl-saponins												
Soy saponins aA1	2.30	1.05	0.96	1.04	0.30	1.01	1.11	1.09	0.34	0.87	0.79	0.84
Soy saponins aA2	0.23	0.17	0.15	0.29	0.55	0.20	0.41	0.29	0.31	0.17	0.18	0.18
Soy saponins aA7	0.04	0.35	0.65	0.38	0.39	0.41	0.72	0.66	0.31	0.54	0.55	0.56
Total group A	2.57	1.57	1.76	1.71	1.24	1.62	2.24	2.04	0.96	1.58	1.52	1.58
Total saponins (A + B)	9.75	8.18	11.67	9.10	**12.86**	8.91	12.75	10.66	11.77	11.06	11.10	11.10

[A] Experiments: 1 (21 h, 20 °C), 2 (63 h, 20 °C), 3 (21 h, 30 °C), 4 (63 h, 30 °C), 5 (12 h, 25 °C), 6 (72 h, 25 °C), 7 (42 h, 18 °C), 8 (42 h, 32 °C), 9 (42 h, 25 °C), 10 (42 h, 25 °C), 11 (42 h, 25 °C).

regression equation simple with an R^2 = 0.93. The regression equation obtained for the second-degree adjusted model in terms of coded factors is presented in Eq. (9) and the response surface in Fig. 2c.

$$\text{Total saponins (mg/g)} = \mathbf{10.83} - \mathbf{1.58x}_1 + \mathbf{0.46x}_2 \quad (9)$$

Higher levels of total saponins were observed at higher germination times [63 h (+1) to 72 h (+α)]. The optimal condition was 63 h germination time at 30 °C. In this case, the germination process contributed to an increase in saponins from 9.8 mg/g in the non-germinated soybean flour to 12.9 mg/g in germinated soybean flour, resulting in an increase of 32.2%.

3.8. Radicles and cotyledons of soybean germinated

The best treatments for the germinated Brazilian soybean cultivar BRS 133 was 42 h at 25 °C (highest concentration of lunasin and lowest concentration of lectin and lipoxygenase), and 63 h at 30 °C (highest concentration of isoflavone aglycones and total saponins).

4. Conclusions

Germination time and temperature had a strong influence on the composition and concentration of bioactive compounds in the germinated soybean flour from the Brazilian soybean cultivar BRS 133. Germination of soybean cultivar BRS 133 for 42 h at 25 °C resulted in an increase of 61.7% of lunasin, decrease of 58.7% in lectin and a decrease of 70.0% in lipoxygenase activity. A significant increase in the concentration of isoflavone aglycone (daidzein and genistein) and the total saponins were observed in 63 h of germination time at 30 °C. In relation to genistein, germination conditions (63 h at 30 °C) contributed to an increase of 212.3%.

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