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Evaluation of thermosonication in the inactivation of lipoxygenase in hydrosoluble soy extract

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Hydrosoluble soy extract (HSE) is nutritious and highly perishable, requiring it to be submitted to an adequate conservation method. Ultrasound combined with heat (thermosonication) can be an alternative capable of reducing the undesirable effects caused by the conventional thermal treatment in this product. The objective of this work was to evaluate the effect of thermosonication on the inactivation of the enzyme lipoxygenase (LOX) in HSE under 2 ultrasonic amplitude conditions (70 and 90%). Fresh and treated HSE samples were characterized in terms of color parameters, pH, soluble solids and total phenolic content. Temperature was the most important factor in reducing LOX residual activity (RA). Highest LOX inactivation condition (RA = 2.14%) occurred in the range of 90% ultrasonic amplitude at 83ºC for 3 min. In this condition, the specific acoustic energy (SAE) in thermosonicated sample was 596.7 mW/mL. Thermosonication has the potential to minimize the phenolic loses (Total Phenolic Content = 35.25 ± 0.25 mg/100 mL), when compared to heat treatment (Total Phenolic Content = 13.48 ± 0.06 mg/100 mL). Thermosonication has an interesting potential in maintaining the nutritional value of the HSE.

Key words: Hydrosoluble soy extract, enzyme inactivation, lipoxygenase, thermosonication, ultrasound.

INTRODUCTION

Hydrosoluble soy extract (HSE), an aqueous emulsion resulting from the hydration of soybeans, is a food recognized for its nutritional richness and low-fat content, as well as the absence of lactose and cholesterol. The consumption of this drink has increased significantly, especially among lactose-intolerant consumers, vegetarians, vegans and/or those seeking healthier diets (Kubo et al., 2021; Kumar et al., 2021).

Due to the nature of its composition, neutral pH, high water activity and the presence of several metabolic enzymes such as lipoxygenase (LOX), HSE is naturally susceptible to enzymatic and microbiological degradation

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and is therefore a highly perishable product. Thus, to ensure food safety and extend its shelf life, this food must be subjected to adequate conservation processes immediately after it is obtained (Alhendi et al., 2017). Usually, the principle of heat treatment applied to HSE is based on inactivating LOX (biological indicator) due to the undesirable effects of this enzyme on the product, such as rancidity, and its high thermal resistance (Kubo et al., 2021; Kumar et al., 2021).

Lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.11.12) catalyzes the hydroperoxidation reaction, that is, the addition of molecular oxygen to polyunsaturated fatty acid molecules containing cis,cis-1,4-pentadiene.These undesirable reactions on fatty acids promote their degradation and the release of volatile and nonvolatile compounds responsible for undesirable flavors (Alhendi et al., 2017).

Vegetable LOXs are monomeric proteins of approximately 95-100 kDa, with the metal site octahedrally coordinated by five amino acid side chains and a water or hydroxyl ligand. In the case of plant LOXs, these residues are always three histidines, one asparagine and one isoleucine (Ji et al., 2022). LOX isoenzymes are globular proteins that contain a nonheme iron atom, constituting a prosthetic group essential for enzymatic catalysis. When catalyzing the addition of oxygen to linolenic and linoleic acids, which are fatty acids present in HSE, formation of hydroperoxides involved in deteriorative reactions occurs, resulting in the formation of volatile products that modify the original flavor and generate characteristic rancid odor and taste (Ji et al., 2022; Lampi et al., 2020).

Heat treatment is a commonly used method for HSE conservation to ensure food safety and extend shelf life. However, heat can also cause undesirable changes, such as protein denaturation, amino acid deterioration and reactions that ultimately diminish the beverage's sensory quality and nutritional value (Amitabh et al., 2017; Hao et al., 2023).

Thus, given the undesirable effects and consumer eagerness for high-quality food, there is a need for alternative conservation techniques that minimize the damage caused by traditional heat treatment. In this context, several emerging technologies, both thermal and nonthermal, are already being investigated in soybean water-soluble extracts, including ohmic heating (Amitabh et al., 2017), microwave (Kubo et al., 2021; Kumar et al., 2021; Vagadia et al., 2018), high pressure (Andrés et al., 2016), and pulsed light (Alhendi et al., 2017) treatments. Among the nonthermal technologies, ultrasound is highlighted as a promising technique, which, despite presenting satisfactory results in several products, has not yet been investigated for soybean water-soluble extracts.

Ultrasound consists of sound waves that have a frequency above 16 kHz and are not detected by the human ear. It is an emerging technology that ensures food quality and preservation through minimal processing, usually at room or mild temperatures. Enzyme inactivation through ultrasound is related to the phenomenon of cavitation, which involves the formation, growth, and implosion of bubbles when the liquid medium is subjected to sound waves. The collapse of cavitation bubbles leads to localized mechanical and chemical effects (temperatures up to 5,000 K and pressures up to 50,000 kPa), which lead to enzymatic inactivation (Khadhraoui et al., 2021).

When used alone, ultrasound may not be as efficient, so to achieve significant results, it is convenient to combine it with other conservation techniques, such as heat and pressure treatment. Thermosonication involves the simultaneous use of low-frequency ultrasound waves and milder temperatures than those used in conventional thermal processing.

When combined with heat, ultrasound can have a synergistic effect, accelerating the microbiological and enzymatic inactivation rates and, consequently, reducing the rigor of traditional heat treatments. Thus, in addition to decreasing the temperature and/or processing time, this technique has the potential to minimize undesirable changes in the nutritional and sensory quality of the treated food (Dolas et al., 2019). Several studies have revealed the potential of thermosonication in enzymatic inactivation in products such as coconut water (Ribeiro et al., 2017), grapefruit juice (Manzoor et al., 2021; Xu et al., 2023), and hazelnut milk (Atalar et al., 2019).

Therefore, the objective of this work was to evaluate the effect of thermosonication on inactivation of the enzyme lipoxygenase in hydrosoluble soy extract under different ultrasonic amplitudes.

MATERIALS AND METHODS

Preparation of HSE

Hydrosoluble soy extract was obtained as described in (Kwok and Niranjan, 1995) with some modifications. A hundred grams (100 g) of soybeans purchased from the local market in Lavras (Minas Gerais, Brazil) were weighed and soaked in distilled water for 20 h at a ratio of 1:3 (m/v). The remaining water was then discarded, and the grains were washed with distilled water. Then, the swollen beans were ground in an industrial blender for 2 min, with the addition of water at a ratio of 1:3 (m/v). To remove the insoluble material, the extract obtained was manually filtered through nylon fabric and then centrifuged (Thermo Scientific, USA) at 4°C for 20 min at a speed of 10,000 × g. Finally, the supernatant was collected and used as the soybean water-soluble extract in the experiments. This extract was placed in amber glass bottles and kept under refrigeration (~5°C) until thermosonication and conventional thermal treatment.

Based on previous work in literature (Kwok and Niranjan, 1995; Kwok et al., 2002; Vagadia et al., 2018), the ranges of the independent variable temperature (°C) and time (min) of heat treatment on LOX inactivation in HSE were defined. The preliminary experiments were carried out in a 3×3 factorial design, with 3 temperature levels (70, 80 and 90°C) and 3 time levels (3, 5 and 8 min). All experiments were performed in 3 repetitions to calculate the mean and standard deviation (SD). The experimental results

Table 1. Factors and levels of the CCRD 2^2 for the minimization of LOX residual activity.

Factor	Level				
	$-\sqrt{2}$	-1	0	+1	$+\sqrt{2}$
Temperature (ºC) (X_1)	62	65	72.5	80	83
Time (min) (X_2)	1.59	2	3	4	4.41

Source: Authors.

obtained in the 3×3 factorial design were submitted to analysis of variance (ANOVA) at the significance level of 5%.

Statistical analysis

A central composite rotational design (CCRD) 22 + 5 replicates at the central point's + 2 axial points (-1.41 and + 1.41) was used to optimize the independent variables temperature (X_1) and time (X_2) of thermosonication (Table 1) in order to minimize the residual activity of lipoxygenase in HSE. The CCRD 22 experiments were carried out under ultrasonic amplitudes of 70 and 90%.

The results obtained from the CCRD 2^2 were submitted to multiple linear regression analysis, and a quadratic polynomial model (Equation 1) was fitted to the data.

$$\hat{y} = \beta_0 - \beta_i X_i - \beta_{ii} X_i^{\,2} + \beta_{ij} X_i X_j + e \quad (1)$$

Where $\hat{y}$ is the residual enzyme activity (%), β_0 is the intersection of the model, X_i and X_j are the levels of the independent variables, *e* is the error and $\beta_i, \beta_{ii}, \text{and } \beta_{ij}$ is the linear, quadratic and interaction coefficients, respectively.

The suitability of the models was assessed using the multiple determination coefficient (R^2), the significance of the mathematical model ($p<0.05$), the model lack of fit ($p>0.05$) and the significance of the regression coefficients ($p<0.05$). Nonsignificant coefficients ($p>0.05$) were grouped into error *e*. All statistical analyses were performed using SAS® University Edition statistical software. To facilitate visualization and identification of the experimental conditions that minimize the residual activity of LOX in HSE, contour graphs were generated from the values predicted by the obtained mathematical model using SigmaPlot version 14.5.

Conventional heat treatment

To compare the synergistic effect of ultrasound and heat on LOX inactivation with the effect of conventional heat treatment, the hydrosoluble soy extract was subjected to traditional heat treatment (heat effect only) following the temperature and time conditions evaluated in the CCRD 2^2.

Initially, the samples were preheated to the temperature defined by the factorial design for each treatment by immersing a glass beaker containing 50 mL of HSE in a water bath (Model Q215S2 - Quimis, Brazil) at 90°C according to the established experimental conditions (Table 1). The temperature was controlled using a thermometer immersed in the liquid. Once the working temperature was reached, the beaker containing the sample was transferred to a water bath set to the test temperature, where it was kept for the predetermined period of time (3, 5, and 8 min). After each treatment, the samples were immediately cooled in an ice bath to 4°C, and the enzymatic activity of LOX was determined.

The residual activity (RA, %) results obtained for the conventional heat treatment were submitted to regression analysis, and a quadratic polynomial model (Equation 1) was fitted. All tests were performed in triplicate.

Thermosonication

Initially, the samples were preheated to the temperature defined by the experimental design (Table 1) for each treatment by immersing the glass beaker containing 50 mL of hydrosoluble soy extract in a water bath at 90°C. The temperature was monitored by means of a thermometer immersed in the liquid. After reaching the working temperature, the beaker containing the sample was transferred to a QSonica ultrasonicator (Ultronique, Brazil) operating at a frequency of 20 Hz and equipped with a titanium probe measuring 0.3 cm in diameter. Thermosonication was performed by immersing 0.5 cm of the probe in 50 mL of previously heated hydrosoluble soybean extract. The treatment was carried out for the period of time pre-established in the design (Table 1). The sample temperature was maintained throughout the thermosonication process through a jacketed beaker with water circulating at the working temperature. The sample temperature was monitored throughout the process with a thermometer. After each treatment, the samples were immediately cooled in an ice bath to 4 °C, and the enzymatic activity of LOX was determined.

Calculation of ultrasonic power and specific acoustic energy

To calculate the ultrasonic power applied to the samples, a glass beaker containing 50 mL of hydrosoluble soy extract at room temperature (25°C) was subjected to ultrasonic treatment under the two proposed amplitude conditions (70 and 90%) for 2, 3 and 4 min. With the aid of a thermometer and a stopwatch, the increase in temperature as a function of sonication time was recorded at intervals of 30 s. With these data, the power (W) and consequently the specific acoustic energy (mW/mL) for each tested time and amplitude condition were determined (Ribeiro et al., 2017). The potency of each treatment was determined according to Equation (2):

$$P\,(W) = \; m \times c_p \times \left(\frac{dT}{dt}\right) \quad (2)$$

Where *P* is power (W); m is the mass (g) of 50 mL of the hydrosoluble soy extract; c_p is the specific heat of the hydrosoluble soy extract (4649.6 J/kg °C), and $\frac{dT}{dt}$ is the rate of change in temperature during sonication (℃/s). The specific acoustic energy (SAE, mW/mL) under each condition was obtained through the ratio between the power and the sample volume (V, mL) according to Equation (3):

$$SAE = \frac{P}{V} \quad (3)$$

Lipoxygenase enzymatic activity

The lipoxygenase enzyme activity in the control and treated HSE was determined according to the methodology described in literature (Li et al., 2008). The control corresponded to HSE samples without any previous heat treatment (thermosonication or conventional heat treatment). Initially, 0.1 mL of each sample was pipetted into 25 mL test tubes and diluted with 19 mL of distilled water. The diluted solution was stored for later use.

The substrate solution was prepared immediately before carrying out the enzymatic activity analyses. This solution was composed of linoleic acid:ethyl alcohol:0.2 mol/L borate buffer (pH 9.0) (1:1:1,000 v/v), totaling 5 mL, which was mixed in 20 mL of 0.2 mol/L borate

Table 2. Preliminary experimental results of residual activity (RA) of LOX in HSE after thermal treatments.

Temperature (°C)	Time (min)	RA (%)
70	3	87.75 ± 1.62
70	5	75.64 ± 1.11
70	8	22.42 ± 1.68
80	3	3.14 ± 0.44
80	5	3.09 ± 0.50
80	8	2.13 ± 0.64
90	3	2.85 ± 0.55
90	5	2.14 ± 0.33
90	8	0.95 ± 0.28

The RA (%) result is mean ± standard deviation values.
Source: Authors.

buffer (pH 9.0) and 5 mL of distilled water. Then, 2 mL of this substrate solution and 0.95 mL of 0.2 mol/L borate buffer (pH 9.0) were pipetted into a quartz cuvette and mixed by inversion at 25°C.

Afterward, 0.05 mL of the diluted sample was added to the quartz cuvette and immediately mixed by inversion. The decrease in absorbance was observed for 3 min (ΔA_{234}/min) in a VIS 190-1100 nm spectrophotometer (Drawell, DU-8200, China) at a wavelength of 234 nm. A unit of activity (U) was defined as the amount of enzyme required to produce a 0.001 decrease in optical density per minute (Li et al., 2008). The tests were carried out in triplicate.
The enzyme activity was calculated according to Equation (4):

$$EA = \frac{(\Delta A_{234}/min)_{enzyme}}{0.001 \times 0.1} \times f \quad (4)$$

Where EA is the enzymatic activity (U/mL) of lipoxygenase in the HSE; *f* is the HSE sample dilution factor; and $(\Delta A_{234}/min)_{enzyme}$ is the maximum speed of enzyme activity (U). LOX inactivation was evaluated by determining the residual activity (RA, %) of lipoxygenase in the HSE, defined according to Equation (5):

$$RA = \frac{EA}{E_0 A} \times 100 \quad (5)$$

Where EA is the enzymatic activity (U/mL) of lipoxygenase in the HSE sample after treatment and $E_0 A$ is the initial enzymatic activity (U/mL) of lipoxygenase in the HSE sample before treatment, both calculated according to Equation (4):

Physicochemical analysis

The physicochemical characteristics and the total phenolic content of the optimal experimental conditions obtained from the CCRD were determined.

The pH of the HSE samples was determined with a benchtop pH meter (MS Tecnopon, Brazil). The soluble solids content (°Brix) was determined by measuring the refractive index using a digital refractometer (Atago, Brazil). The colorimetric parameters L* (luminosity), a* (red-green axis coordinate) and b* (blue-yellow axis coordinate) were measured in a colorimeter (Konica Minolta, Japan). The total color difference (E*) was calculated according to Equation (6) (Oladunjoye et al., 2021).

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (6)$$

Where º indicates the variation in the parameters, such that $\Delta L^* = (L^* - {L_0}^*)$ is the difference between the luminosity of the thermosonicated samples and the control; $\Delta a^* = a^* - {a^*}_0$ represents the red (positive value) and green (negative value) color intensities of the thermosonicated samples and the control; and $\Delta b^* = b^* - {b^*}_0$ represents the intensities of the yellow (positive value) and blue (negative value) colors of the thermosonicated samples and the control.

Total phenolic content

The total phenolic content (TPC) was determined using the colorimetric method described by Rodríguez-Roque et al. (2013). Briefly, an aliquot of 0.5 mL of HSE (without heat treatment, subjected to thermosonication or conventional heat treatment) was mixed with 0.5 mL of Folin-Ciocalteu reagent and 10 mL of Na_2CO_3 (20% m/v). The volume of the mixture was brought to 25 mL with distilled water using a volumetric flask. The resulting solution was kept in the dark at room temperature (25°C) for 1 h. Then, the absorbance of the samples was measured at 725 nm. The calibration curve for determining the total phenolic content was constructed using gallic acid (GA) as a standard at concentrations from 20 to 75 mg/100 mL. The results were expressed in mg of gallic acid equivalent (GAE) per 100 mL of the extract.

RESULTS AND DISCUSSION

The average results obtained in the preliminary tests using the 3×3 factorial design is presented in Table 2. These results were submitted to ANOVA, followed by regression analysis; the mathematical model obtained for the inactivation of LOX in HSE as a function of thermosonication time and temperature is presented in Equation 7. The adjusted mathematical model was significant ($p<0.05$) and presented significant coefficients ($p<0.05$), a nonsignificant lack of fit ($p>0.05$) and R^2 value higher than 0.94.

$$\hat{y} = 2450.4852 - 54.0057\, x_1 - 59.0509 x_2 + 0.6821 x_1 x_2 + 0.2963 {x_1}^2 \quad (7)$$

Treatments that used temperatures at or above 80°C were found to almost completely inactivate LOX in the HSE, and at 80°C for 8 min, the enzyme showed residual activity similar to that found at 90°C for 5 min. Moreover, at 70°C, with the shortest time period (3 min), LOX presented high resistance to inactivation, and its residual activity remained very high. Although lipoxygenases can be inactivated above 60°C, the effective reduction in their activity is related to processing conditions and their isoforms (Kubo et al., 2021).

For the subsequent step, a temperature range of 62 to 83°C and a time of 1.59 to 4.41 min were selected to optimize the operating conditions of thermosonication, aiming to minimize the LOX residual activity. Importantly, the temperature selection aimed to range from low to high LOX reduction to assess the synergistic potential of ultrasound in inactivating this enzyme.

Table 3. Residual activity of LOX (%) in HSE obtained for treatments submitted to conventional thermal treatment and thermosonicated in the ranges of 70 and 90% amplitude.

Temperature (°C)	Time (min)	Thermal treatment	70%	90%
65	2	72.50	74.19	63.64
65	4	68.71	64.51	60.00
80	2	7.00	2.35	3.87
80	4	3.48	3.13	2.90
62	3	89.93	76.67	70.0
83	3	3.09	3.75	2.14
72.5	1.59	44.12	43.14	24.24
72.5	4.41	11.76	11.76	6.36
72.5	3	33.82	17.31	16.13
72.5	3	35.29	19.23	16.67
72.5	3	23.53	21.15	20.00
72.5	3	28.57	15.38	15.15
72.5	3	35.71	23.53	15.15

Source: Authors.

Optimization of thermosonication operating conditions

In this optimization step, the criterion used to choose the optimal ultrasonic conditions was the greatest inactivation of LOX in the HSE; that is, the selected treatments were those that minimized the residual LOX activity. Additionally, the experimental conditions evaluated with CCRD 2^2 for LOX inactivation in HSE were reproduced to apply a conventional heat treatment (heat effect only) to the HSE. Thus, it was possible to evaluate the synergistic effect of ultrasound and heat in the inactivation of LOX compared to the effect of a traditional heat treatment.

The experimental results of RA obtained from the CCRD 2^2 for ultrasonic amplitudes of 70% and 90% and conventional thermal treatment are presented in Table 3.

The residual activity of lipoxygenase obtained from the CCRD 2^2 for 70 and 90% ultrasonic amplitudes, as well as in traditional heat treatment, was submitted to regression analysis (Table 4). The mathematical models presented in Table 5 describe the relation between the independent variables (time and temperature) considering only the significant terms ($p<0.05$).

According to Table 5, for conventional thermal treatment and thermosonication at amplitudes of 70% and 90%, the parameters of time and temperature significantly affected ($p<0.05$) the residual activity of lipoxygenase; the temperature (X_1) had a quadratic and linear effect, while time (X_2) had only a linear effect. From the models obtained to predict the residual activity of LOX, contour graphs were constructed (Figures 1 and 2) for all the thermal treatments evaluated.

By analyzing the thermosonication and conventional thermal treatment curves (Figures 1 and 2), it can be seen that temperature appears to be the most important factor in reducing enzymatic activity. At higher temperatures, a synergistic effect was observed between heat and sonication for enzyme inactivation because at a temperature of approximately 77°C, for example, the reduction achieved by conventional treatment was 80%, while with thermosonication at an amplitude of 90, 90% inactivation was found.

From analysis of Figures 1 and 2, it was possible to observe that there was no increase in the reduction of enzymatic activity at 70% sonication amplitude compared to conventional thermal treatment. At 90% ultrasonic amplitude, a synergistic effect was observed. This finding demonstrates that although no decrease in enzymatic activity occurred at 70% amplitude compared to that with conventional thermal treatment, at higher amplitudes, such as 90%, the combination of ultrasound and heat had a synergistic effect on LOX enzyme inactivation in HSE.

The difficulty encountered when inactivating LOX at temperatures below 72.5°C may be related to the existence of at least 3 to 4 isoenzymes that differ in their thermal stability. Thus, while the thermolabile fraction can be rapidly inactivated, complete inactivation of the heat-resistant fraction is more difficult, causing the residual activity of the enzyme in the HSE to be high even after applying heat treatment (Kubo et al., 2021).

High temperatures can cause changes in the structure of the enzyme, such as breaking hydrogen bonds and denaturation. On the other hand, ultrasound forms cavitation bubbles capable of altering the structure of proteins by breaking bonds of the peptide chain, generating free radicals. The enzymatic inactivation caused by thermosonication is attributed to the effect between heat and mechanical damage that leads to

Table 4. Regression Analysis from CCRD 2^2.

SV	SS			DF	MS			F calculated			*p* value		
	TT	70%	90%		TT	70%	90%	TT	70%	90%	TT	70%	90%
X_1	8037.66	6991.14	5668.00	1	8037.66	6991.14	5668.00	124.65	142.34	284.56	<0.00	<0.00	<0.00
X_2	262.28	354.82	84.30	1	262.28	354.82	84.30	4.07	7.22	4.23	0.09	0.04	0.09
$X_1 * X_2$	0.02	27.35	1.78	1	0.02	27.35	1.78	0.00	0.56	0.09	0.99	0.48	0.78
$X_1 * X_1$	445.59	770.06	913.14	1	445.59	770.06	913.14	6.91	15.68	45.84	0.04	0.01	0.00
$X_2 * X_2$	12.61	145.12	38.15	1	12.61	145.12	38.15	0.20	2.95	1.92	0.67	0.14	0.22
Lack of Fit	8.29	15.63	84.88	1	8.29	15.63	84.88	0.13	0.32	4.26	0.73	0.59	0.09
Error	386.89	294.70	119.51	6	386.89	49.11	19.92						
Total	9153.35	8598.82	6909.77	12									

SV, Source of variation; X_1, temperature (°C); X_2, time (min);TT, Thermal treatment; SS, Sum of squares; DF, Degree of freedom; MS, Mean square.
Source: Authors.

Table 5. Prediction models for LOX residual activity as a function of temperature (X_1, ºC) and time (X_2, min) for the conventional thermal treatment and thermosonication at different amplitudes.

Attribute	Mathematical models
Conventional thermal treatment	$\hat{y} = 1111.2807 - 25.0259\,X_1 - 6.6117X_2 + 0.1433{X_1}^2$
Thermosonication - 70%	$\hat{y} = 1319.8373 - 31.2767\,X_1 - 6.6597X_2 + 0.1884{X_1}^2$
Thermosonication - 90%	$\hat{y} = 1365.3930 - 33.3117\,X_1 - 3.7483X_2 + 0.2051{X_1}^2$

Source: Authors.

protein denaturation by depolymerization and alteration of its tertiary structure conformation (Wang et al., 2023).

The specific mechanism of enzyme inactivation during sonication may be due to a single or combination of several chemical and physical effects occurring simultaneously. Ultrasonic inactivation mechanisms are specific to the enzyme under investigation and depend on its amino acid composition and conformational structure. For example, lipoxygenase appears to be inactivated by a mechanism mediated by free radicals and by protein denaturation (Ji et al., 2022; Khadhraoui et al., 2021). These radicals, generated during sonication, play an important role in enzymatic inactivation, as they disturb hydrophobic interactions and intramolecular hydrogen bonds, which play important roles in protein stability (Ampofo and Ngadi, 2022; Tian et al., 2004).

Amino acids, such as tryptophan, tyrosine, histidine and cysteine, present in soybeans, are particularly susceptible to degradation by hydroxyl and superoxide free radicals. In additionto proline, the amino acids leucine, isoleucine, lysine, cysteine and glutamic acid easily form peroxides when reacting with OH· radicals (Wang et al., 2023). The free radicals formed react with the enzyme's amino acid residues, making them unable to participate in stabilization of the molecule, bind to the substrate and exert its catalytic function (Ampofo and Ngadi, 2022; Tiwari and Mason, 2011). At the same time, the presence of OH· radicals diminishes the antioxidant properties of foods and can cause off-taste in some foods. Free hydroxyl radicals, OH·,

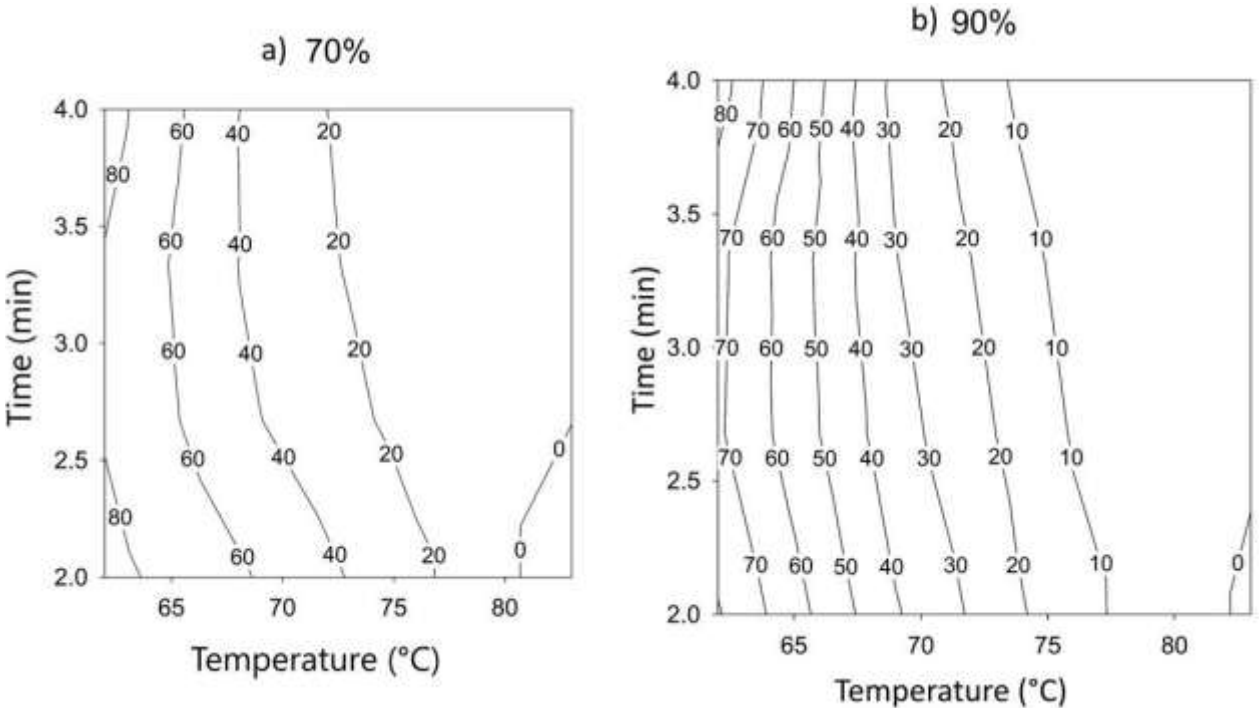

Figure 1. Contour plot of residual activity (%) of LOX as a function of thermosonication time (min) and temperature (°C) in the amplitudes of (a) 70% and (b) 90%.
Source: Authors.

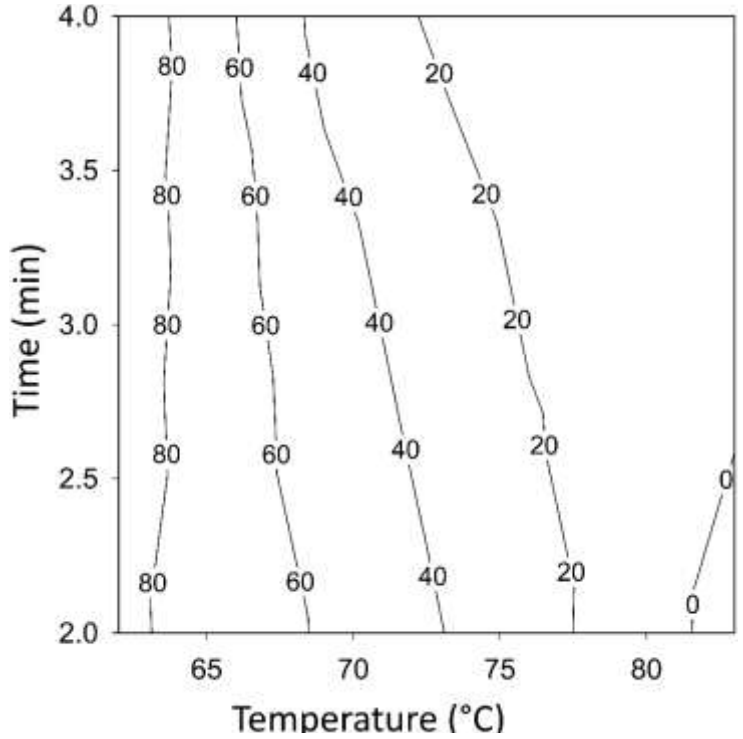

Figure 2. Contour plot of residual activity (%) of LOX, as a function of time (min) and temperature (°C) applied in conventional thermal treatment.
Source: Authors.

Table 6. Specific acoustic energy (SAE) of thermosonicated samples.

Treatment	Amplitude (%)	Time (min)	SAE (mW/mL)
1	70	2	612.8[a]
2	70	3	583.8[ab]
3	70	4	582.7[ab]
4	90	2	610.0[a]
5	90	3	596.7[a]
6	90	4	561.0[b]

Means followed by the same letter do not differ statistically from each other at the level of 5% probability by t-student test.
Source: Authors.

Table 7. Chemical parameters of fresh HSE (control) and HSE submitted to conventional thermal treatment and thermosonication at 70 and 90% of amplitude, both at 83°C for 3 min.

Treatment	L*	a*	b*	ΔE*	pH	°Brix	TPC (mg/100 mL)
Fresh HSE	57.99 ± 2.29[a]	1.58 ± 0.52[a]	10.67 ± 2.19[a]	-	6.49 ± 0.72[b]	6.50 ± 0.36[b]	49.61 ± 0.17[a]
TT	58.40 ± 0.87[a]	1.26 ± 0.60[ab]	11.26 ± 0.43[a]	4.15 ± 0.86[a]	5.99 ± 0.15[b]	7.01 ± 0.10[a]	13.48 ± 0.06[d]
70%	56.79 ± 1.47[a]	0.76 ± 0.30[b]	10.00 ± 0.62[a]	4.18 ± 0.71[a]	6.75 ± 0.50[a]	7.30 ± 0.24[a]	35.25 ± 0.25[b]
90%	57.80 ± 2.27[a]	1.13 ± 0.66[ab]	10.58 ± 0.37[a]	3.83 ± 0.59[a]	6.10 ± 0.11[b]	7.03 ± 0.20[a]	28.35 ± 0.12[c]

Means followed by the same letter do not differ statistically from each other at the level of 5% probability by t-Student test. TT: Thermal treatment. TPC: Total phenolic content. L*: luminosity. a*: red-green axis coordinate. b*: blue-yellow axis coordinate. ΔE*: total color difference.
Source: Authors.

are highly reactive species that have a very high redox potential and represent strong oxidants that can react quickly with most amino acids (Rodríguez-Rico et al., 2022). Different studies have evaluated the LOX activity reduction or inactivation by thermosonication treatments (Manzoor et al., 2021; Xu et al., 2023).

The enzyme activity strongly depends on the ultrasonic intensity, which can be observed at temperatures below 70 °C; the reason is that the samples thermosonicated at the highest amplitude (90%) showed higher enzymatic inactivation than those subjected to the same experimental conditions at the lowest amplitude (70%) (Islam et al., 2014). The increased effect of ultrasound by increasing the amplitude has been related to the increase in the effective size of the cavitation liquid zone and to the range of sizes of bubbles that cavitate at higher amplitudes (Vallath and Shanmugam, 2022).

The specific acoustic energy found for thermosonicated samples at different amplitudes (70 and 90%) and times (2, 3 and 4 min) was in the range of 561-613 mW/mL, with the highest power being obtained at 70% amplitude and a time of 2 min (Table 6).

The lowest residual activity of LOX in HSE (2.1%, Table 3) occurred under in one of the conditions with the highest acoustic power (90%, 3 min at 83°C). This result is attributed to the energy supplied to the liquid medium, which was able to break the hydrogen bonds and disrupt the tertiary structure conformation of the enzyme, that is, expose its hydrophobic groups to the medium, leading to enzyme inactivation and aggregation (Islam et al., 2014).

Effect of thermosonication on the physicochemical characteristics and phenolic content

The physicochemical parameters L*, a*, b*, pH, total soluble solids and the total phenolic content of the samples treated by thermosonication at ultrasonic amplitudes 70 and 90% and by a conventional thermal treatment, both at a temperature of 83°C for 3 min, were evaluated to compare the rigors of heat treatment and thermosonication. These conditions were chosen because they resulted in the highest enzyme inactivation among all experimental treatments.

Fresh HSE corresponded to the hydrosoluble soy extract samples that were not subjected to any thermal treatment. The average results obtained for the physicochemical characteristics and the total phenolic content of the different samples were also compared by Student's t test, and the results obtained are shown in Table 7.

As presented in Table 7, among the parameters evaluated, only the L* and b* color parameters showed no significant difference ($p>0.05$) between treated

(thermal and thermosonication) and fresh HSE (control). For parameter a*, there was a significant difference ($p<0.05$) between samples thermosonicated at 70% amplitude and the control sample (without any treatment). For pH, a significant difference ($p<0.05$) was observed between the control sample and those subjected to conventional thermal treatment and treated at 90% amplitude, while for the soluble solids content (°Brix) and total phenolic content, all treated samples differed significantly ($p<0.05$) from fresh HSE.

Among the color parameters, only for the parameter a*, related to the red-green axis, was a significant difference ($p<0.05$) observed between the samples, and the treatments with ultrasound showed lower mean results than with the traditional heat treatment (Table 7). In this case, the thermosonicated samples showed a coloration closer to green (negative value) than to red (positive value); this result can be explained by the phenomenon of cavitation, which can induce changes in color by accelerating chemical reactions and increasing the rate of diffusion, dispersion, aggregate formation and particle breakage (Alcántara-Zavala et al., 2021; Xu et al., 2023). The average results obtained for the other color parameters were statically similar ($p>0.05$), which suggests that the samples maintained the same luminosity and yellowish color. Based on the color parameters of the control sample, it can be concluded that the color difference between the samples (ΔE) was not significant ($p<0.05$).

The samples differed significantly ($p<0.05$) in relation to pH, mainly for samples thermosonicated at 70% amplitude, where a slight increase in this parameter was observed. Salve and colleagues (Salve et al., 2019) reported in their study with peanut milk that after thermosonication treatment, an increase in pH was associated with higher physical stability of the beverage.

The soluble solids content of the control sample showed a significant difference ($p<0.05$) when compared to that of the other treatments; that is, the high temperature seems to be sufficient to break the cell walls or hydrolyze the polysaccharides (Maghsoudlou et al., 2016). Salve et al. (2019) observed that an increase in °Brix was related to an increase in protein solubility. According to Table 7, compared with samples subjected to the conventional thermal treatment, the thermosonicated samples presented a significantly lower reduction in the total phenolic content. Based on these data, although high temperatures reduced the total phenolic content of the water-soluble soybean extract with both conventional heat treatment and thermosonication, ultrasound still had higher potential to preserve these bioactive compounds. Jabbar et al. (2015) observed a behavior similar to that in the present article because when the ultrasound processing temperature increased, the loss of phenolic compounds in carrot juice also increased, but this loss was lower than that with traditional heat treatment.

Phenolic compounds are incorporated into vacuoles in soluble form or bound to the cell wall (Atalar et al., 2019). The basic principle behind the increased phenolic content involves cavitation in the food components and the pressure exerted during this process, which disrupts cell walls, making it easier to release phenolic compounds bound to the soybean matrix. It has been reported that an increase in the TPC by thermosonication occurs because the concentration of individual phenolic compounds, such as flavonoids, can also increase because of increased hydroxylation of molecules due to the formation of OH· radicals during ultrasound treatment (Ampofo and Ngadi, 2022; Cui et al., 2014).

Atalar et al. (2019) observed that thermal treatment at 85°C for 2 min led to a significant reduction in the TPC in hazelnut milk from 162 µg GAE/g to 150.74 µg GAE/g, while the highest TPC value (178.82 µg GAE/g) was found for hazelnut milk thermosonicated at 75°C at 60% amplitude for 25 min, indicating that temperature had a significant effect on TPC levels.

Conclusion

In this work, the effect of thermosonication on the inactivation of LOX in hydrosoluble soy extract was evaluated, and the synergistic effect of ultrasound and heat was compared with that of conventional thermal treatment in reducing the residual enzyme activity. A central composite rotational design (CCRD) was employed to study the influence of the independent parameters temperature and thermosonication time on the residual activity (RA) of LOX in HSE. Subsequently, the synergistic effect of ultrasound and heat on LOX inactivation was evaluated and compared to that of conventional heat treatments. Finally, HSE samples were characterized by physicochemical analysis and the total phenolic content.

At the highest amplitude (90%), a greater reduction in lipoxygenase enzyme activity in the hydrosoluble soy extract was observed, but compared to that of the traditional heat treatment; the synergistic effect of ultrasound with heating was not as significant. The condition with the highest LOX inactivation (RA=2.34%) was at 70% amplitude (80°C/2 min).

Regarding color, the thermosonicated samples did not show any significant difference relative to the heat-treated sample, but the pH showed a significant increase at 70% amplitude when compared to that of the other treatments. The soluble solids content did not differ significantly between the treatments, whereas a higher total phenolic content was observed in thermosonicated samples, especially at 70% amplitude. Further studies exploring other conditions, especially at higher acoustic energy levels, are strongly recommended. An interesting potential of thermosonication to maintain the nutritional value of the HSE, particularly the content of total

phenolics, was verified in this study.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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