***In vitro* bioaccessibility of isoflavones from a soymilk-based beverage as affected by thermal and non-thermal processing**

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Abstract

High Pressure Processing (HPP) and High Intensity Pulsed Electric Fields (HIPEF) are non-thermal processing technologies used for obtaining safe and high-quality foods and beverages. In the present work, the changes on both the concentration and the bioaccessibility of isoflavones from treated (thermally and non-thermally) and untreated soymilk-based beverages were evaluated. Thermal treatment (TT) was applied at 90 °C for 1 min, HPP: 400 MPa at 40 °C for 5 min and HIPEF: 35 kV cm⁻¹ with 4 µs bipolar pulses at 200 Hz for 1800 µs. Later, beverages were subjected to an *in vitro* gastrointestinal digestion for obtaining the bioaccessibility. Thermal and non-thermal processing increased the isoflavone concentration up to 25-26% in TT and HIPEF treated beverages, and up to 38.52% in HPP treated. After *in vitro* digestion, the concentration of isoflavones in non-thermally processed beverages was higher (70.55% for HIPEF and 98.77% for HPP) than that TT processed (18.52%). HIPEF processing and HPP increased the isoflavone bioaccessibility up to 35.40 and 47.32%, respectively, regarding the untreated product. These results demonstrate that both non-thermal processing technologies HIPEF and HPP are suitable for obtaining high quality and nutritious beverages by improving their isoflavone bioaccessibility.

Industrial relevance

A great variety of functional food and beverages are commercialized in supermarkets around the world. Their high nutritional content, as well as their healthy properties have placed these products on the consumers' preferences. Thermal processing has traditionally been used for inactivating spoilage microorganisms and pathogens. However, several important features of food can be compromised by heat treatment. One of the greatest challenges of the food industry is to find the best processing technology for allowing not only the preservation of bioactive food constituents but also the improvement of nutritional and healthy features. The industrial relevance of the present research is to contribute in elucidating the influence of two non-thermal technologies (high pressure processing and high-intensity pulsed electric fields) on both the concentration and bioaccessibility of isoflavones from a functional beverage, and if they can be used as alternative to thermal processing.

Keywords

Isoflavones, soymilk-based beverage, *in vitro* bioaccessibility, high pressure processing, high intensity pulsed electric fields, thermal treatment.

1. Introduction

Functional beverages are receiving increasing consumer attention due to their easy consumption and their relationship with health and well-being. Nowadays, a great variety of these products are available in the market for satisfying the consumers' preference for tasty, nutritious, healthy and convenient products. The Cumulative Annual Growth Rate of functional beverage market is growing around 7.8 - 9% since 2017, and is projected to continue with this trend in the next years (Nazir et al., 2019). In fact, it has been stated that the functional beverages market will reach up to 40% of the overall consumer demand by 2025 (Bagchi & Nair 2016).

Functional beverages can be formulated by the addition of ingredients that contribute with bioactive compounds, or those that modify the food features (such as texture, stability, flavor, pH). A particular interest has been focused on functional beverages made with soymilk in combination with blends of fruit juices. Both products naturally provide a great variety of health-promoting compounds and the soymilk beany flavor is also masked by mixing with fruit juices (Rodríguez-Roque, 2014). The major bioactive compounds from fruit juices are vitamins, phenolic compounds, carotenoids, tocopherols, as well as other micronutrients (Khaksar, Assatarakul & Sirikantaramas, 2019). On the other hand, soymilk is a healthy colloidal solution containing high amounts of proteins, fats, carbohydrates, phytochemicals (mainly phenolic compounds and tocopherols), saponins, iron and niacin (Ding et al. 2020; Shahidi & de Camargo, 2016). Flavonoids are the most important phenolic constituents from soymilk, specifically isoflavones in their conjugated and aglycone forms (Lee et al., 2018). Isoflavones have been widely studied because they provided health benefits reducing the risk of several diseases such as breast and prostate cancer, osteoporosis, cardiovascular diseases and menopausal symptoms (Baú & Ida, 2015; Pratap et al., 2019). Additionally, soymilk is free from lactose and cholesterol. All these reasons make soymilk an excellent alternative for people with lactose intolerance and milk protein allergy (USDA GAIN Report, 2017).

The concentration and profile of isoflavones mainly depend on the raw material, the type of soy-derived product, as well as the food processing conditions (Morales-de la Peña, Martín-Belloso, & Welti-Chanes, 2018). Processing can modify the composition and concentration of isoflavones in soymilk but also it may induce changes in the food matrix (i.e. pH and viscosity) and in the food microstructure (i.e. cell walls rupture, release of bounded compounds and changes in their solubilisation) (Baú & Ida, 2015). Beverages have traditionally been preserved by thermal

treatment (TT) mainly to avoid microorganism spoilage or contamination with pathogens (Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2013). Nevertheless, heat processing can yield unfavorable soymilk features by reducing the protein solubility, changing the stability, increasing the droplet particle size and decreasing their nutritional and sensorial properties (Shimoyamada, Mogami, Tsuzuki, & Honda, 2014).

Non-thermal food preservation technologies, such as high-intensity pulsed electric fields (HIPEF) or high-pressure processing (HPP), have been developed as alternatives to thermal treatments for obtaining products with improved nutritional, healthy and tasty features. HIPEF processing is based on the application of pulses at high voltage (above 20 kV/cm) and short duration (ms or μ s) to fluid food placed in a treatment chamber confined between two electrodes (Mosqueda-Melgar, Raybaudi-Massilia, & Martín-Belloso, 2012). This processing is effective in inactivating spoilage microorganisms as similar as TT. However, HIPEF processed beverages maintained the nutritional and sensory features of a wide range of juices: strawberry and mango (Odriozola-Serrano et al., 2016), orange, apple, pear and tomato juices (Mosqueda-Melgar, Raybaudi-Massilia, & Martín-Belloso, 2012). On the other hand, HPP uses pressure in the range of 200 to 800 MPa for inactivating microorganisms and enzymes without compromise the physicochemical, nutritional and sensory characteristics of food. In fact, it has been reported that the content of phenolic compounds, flavonoids and anthocyanins slightly increased after HPP processing of a fermented pomegranate beverage (Rios-Corripio, Welti-Chanes, Rodríguez-Martínez, & Guerrero-Beltrán, 2020). An increase in the antioxidant content and the retention of color in non-clarified pomegranate juice after HPP processing was also documented (Chen et al. 2013).

The impact of processing on soymilk isoflavones has been also investigated. In this context, the influence of thermal processing on isoflavone profile from soymilk was evaluated (Baú & Ida, 2015; Zhang, Chang, and Liu, 2015), as well as the effect of ultra-high-pressure homogenization (Toro-Funes, Bosch-Fusté, Veciana-Nogués, & Vidal-Carou, 2014a). The ultrasound was used as pretreatment for increasing the isoflavone content from soymilk (Morales de la Peña, Martín-Belloso, and Welti-Chanes, 2018) and as assisted method for extracting all isoflavone forms contained in soy-based beverages (Rostagno, Palma, & Barroso, 2007). Also, the influence of HIPEF and TT on the isoflavone profile from a fruit juice-soymilk beverage was analyzed (Morales-de la Peña, Salvia-Trujillo, Rojas-Graü, and Martín-Belloso, 2010).

Even though the impact of processing on soymilk isoflavones has been investigated, there is scarce available information dealing with non-thermal processing and even more on the isoflavone bioaccessibility. Once ingested, nutrients must be released from the food matrix into the gastrointestinal tract and thus become available for intestinal absorption (bioaccessibility), as well as for pre-systemic intestinal and hepatic metabolism (Cardoso et al., 2015). Thus, although functional beverages contain a high concentration of nutrients, it does not mean that all this quantity can be absorbed and harnessed by the human organism. For this, it is important to carry out studies assessing the bioaccessibility of bioactive compounds contained in food and beverages. Bioaccessibility is usually evaluated through *in vitro* gastrointestinal digestion by representing a useful and fast approach previous to *in vivo* trials (Tnaker et al., 2020).

It is important to highlight that processing may modulate the release, transformation and absorption of bioactive compounds (Verkempinck, Pallares-Palares, Hendrickx, & Grauwet, 2020), therefore, influencing on their bioaccessibility. However, to the best of our knowledge there is no available information in the literature dealing with the influence of thermal or non-thermal processing on the isoflavone bioaccessibility from functional beverages. For this reason, the aim of this research was evaluating the effect of thermal and non-thermal processing (HIPEF and HPP) on the concentration and bioaccessibility of isoflavones from a soymilk-based beverage (with orange, kiwi, pineapple and mango juices) before and after being subjected to *in vitro* gastrointestinal digestion. The novelty of this research is elucidating if non-thermal technologies are suitable for obtaining a beverage with high concentration of bioaccessible isoflavones, and compare non-thermal technologies (HIPEF and HPP) with thermal processing (TT).

2. Material and Methods

2.1. Materials and reagents

Isoflavone standards (daidzin, genistin, glycitin, daidzein, genistein, glycitein), pepsin from porcine stomach (≥ 250 units per mg solid, P7000), pancreatin from porcine pancreas (P7545), bovine bile (B3883), and the cellulose dialysis membrane (molecular weight cutoff of 12000 Da) were acquired from Sigma-Aldrich (St Louis, MO, USA).

2.2. Soymilk-based beverage

The soymilk-based beverage was prepared by mixing 75% of a blended fruit juice (orange, kiwi, pineapple and mango) with 17.5% of soymilk and 7.5% of sugar. The pH was adjusted to 3.30 ± 0.20 (Crison 2001 pH-meter, Crison Instruments S.A., Alella, Barcelona, Spain) with citric acid. The soluble solid content was analyzed using a refractometer (Atago RX-1000 refractometer, Atago Company Ltd., Japan), obtaining $18.7 \pm 0.3^\circ\text{Brix}$. The electrical conductivity from the soymilk-based beverage was $2.75 \pm 0.02 \text{ S/m}$ (Testo 240 conductivimeter, Testo GmbH & Co, Lenzkirch, Germany). The beverage formulation was selected based on a previous study, where similar concentrations of these fruit juices resulted in a high bioaccessibility of bioactive compounds (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2014; Rodríguez-Roque et al., 2015).

Previously, orange (Valencia variety), kiwi (Hayward variety), pineapple (extra sweet variety) and mango (Palmer variety) fruits were purchased at commercial maturity from a local supermarket in Lleida, Spain. These fruits were washed, peeled and the juice was extracted. The freshly-squeezed juice was filtered with a cheesecloth using a vacuum pump. A blended fruit juice was obtained by mixing 40% of orange, 33% of kiwi, 13.5% of pineapple and 13.5% of mango juices. The soymilk (Yosoy, Girona, Spain) was also purchased from a local supermarket.

2.3. Food processing technologies

The soymilk-based beverage was thermally and non-thermally processed as follows:

2.3.1. High-intensity pulsed electric fields (HIPEF). HIPEF processing was carried out in a continuous-flow bench scale system (OSU-4F, The Ohio State University, Columbus, OH, USA), using square-wave pulses. The treatment system was composed of eight collinear chambers serially connected. Each chamber was made up of two stainless steel electrodes separated by a gap of 0.29 cm. The flow rate was adjusted to 60 mL min^{-1} and controlled by using a variable speed pump (model 752210-25, Cole Palmer Instrument Company, Vernon Hills, IL, USA). HIPEF treatment consisted in application of 35 kV cm^{-1} field strength in the bipolar mode, with $4 \mu\text{s}$ pulse width, 200 Hz pulse frequency and 1800 μs total treatment time. The temperature was always kept below 35°C through a cooling coil connected before and after each pair of chambers and submerged in an ice-water shaking bath. These conditions were selected from previous studies

where the nutritional and microbiological stabilities of soymilk-based beverages were achieved (Morales-de la Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso, 2011; Rodríguez-Roque et al., 2015).

2.3.2. High pressure processing (HPP). HPP was carried out in a hydrostatic pressure unit using a vessel of 2925 mL capacity, a maximum pressure of 900 MPa, and a maximum temperature of 100 °C (High Pressure Iso-Lab System, Model FPG7100:9/2C, Stansted Fluid Power Ltd, Essex, UK). Portions of 300 mL of beverage were vacuum packed in flexible Doypack® bags (Polyskin XL, Flexibles Hispania, S.L.) and introduced into the pressure unit filled with water as pressure medium. The samples were HPP processed at 400 MPa with a holding time of 5 min. The rates of compression and decompression were both 3 MPa s⁻¹. Because of adiabatic compression, the maximum temperature in the vessel was 40 °C at 400 MPa. The pressure, time and temperature were controlled by using a computer program, being constantly monitored and recorded during the process. HPP conditions were selected in accordance with previous studies, where the nutritional and microbiological stabilities of the fruit juices and similar beverages were obtained (Muñoz, De Ancos, Sánchez-Moreno, & Cano, 2007; Sánchez-Moreno et al., 2005).

2.3.3. Thermal treatment (TT). Thermal processing was performed using a tubular stainless-steel heat exchanger coil immersed in a hot water shaking bath at 90 °C during 1 min (University of Lleida, Spain). The flow rate of the beverages was maintained through a gear pump. After thermal treatment, the beverages were immediately cooled down to 5 ± 1 °C in an ice-water bath.

2.4. *In vitro* gastrointestinal digestion

Once the beverages were prepared and processed, they were digested following the procedure described by Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013a). Briefly, each beverage (200 mL) was mixed with 0.2 g of pepsin in a beaker to simulate the gastric digestion. The pH was immediately adjusted to 2 by the addition of 12 M HCl, and the mixture was incubated at 37 °C, 90 rpm for 2 h (incubation chamber with orbital agitation Ovan, Badalona, Spain). Later, the small intestinal digestion consisted in placing 20 mL of gastric digesta, a dialysis membrane (molecular weight cutoff of 12000 Da, containing a water-NaHCO₃ mixture) and 5 mL of pancreatin (4 g L⁻¹) and bile (25 g L⁻¹) solution. This mixture was incubated for 2 h at 37 °C and 90 rpm (incubation chamber with orbital agitation Ovan). The dialysis membrane was removed, rinsed

with distilled water and immediately placed in a cold water bath for 10 min. Finally, non-digested, digested (dialyzed fraction) and processed (HIPEF, HPP and TT) samples were frozen at -45 °C until analysis.

2.5. Isoflavone determination

Extraction, separation, identification and quantification of isoflavones were performed based on the methodology reported by Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013b). Briefly, the extraction and hydrolysis of isoflavones was carried out by adding 4 mL of non-digested or digested samples with 4 mL of 80% ethanol acidified with 1 M HCl. Then, this mixture was incubated at 60 °C during 1 h. After that, samples were cooled, shaken vigorously (for 2 min) and centrifuged (9,000 rpm for 10 min at 4 °C). Supernatants were decanted into a 10 mL volumetric flask and residues were re-extracted with 2 mL of 80% ethanol and centrifuged under the above described conditions. Both supernatants were combined into the volumetric flask and filled up to 10 mL with 80% ethanol. All samples were filtered through a 0.2 µm membrane and kept at -45 °C until HPLC analysis.

The HPLC system consisted in a 600 Controller and a diode array detector (Waters Corporation, Milford, MA, USA), which was set to scan from 200 to 350 nm. Isoflavone separations were performed using a C18 SunFire (3.5 µm) stainless steel column (3 mm x 150 mm) and a C18 SunFire (5 µm) guard column, operating at 37 °C with a flow rate of 0.3 mL/min. A gradient elution was employed with a mixture of two solvents: (A) water/methanol (80:20 v/v) and (B) water/methanol/acetonitrile (40:40:20 v/v/v), as reported by Morales-De La Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso (2010). Isoflavones were identified by comparing their spectrum and retention time with the standards (daidzein, genistein, glycitein, daidzin, genistin and glycitin). The quantification was carried out by integrating the peak areas and using calibration curves (R^2 in the range of 0.9890 to 0.9952, concentration between 0.25 and 30 mg/100 mL). Under the assayed conditions, the limit of detection (LOD) was 0.196 mg/100 mL, while the limit of quantification (LOQ) reached up to 0.327 mg/100 mL. Digested beverages were previously concentrated up to 3 times for accurate measuring their isoflavone concentration. The concentration of total isoflavones was calculated as the sum of each individual compound.

2.6. Bioaccessibility calculations

The bioaccessibility of isoflavones was determined as the quotient between the isoflavone concentration in the digested soymilk-based beverage (IC_{digested}) and that non-digested beverage ($IC_{\text{non-digested}}$) (Eq. 1). The results were expressed in percentage.

$$\text{Bioaccessibility (\%)} = 100 \times \frac{IC_{\text{digested}}}{IC_{\text{non-digested}}} \quad \text{Eq. 1}$$

2.7. Statistical analysis

The food processing technologies and the *in vitro* gastrointestinal digestion were conducted in duplicate, while isoflavones were extracted and analyzed in triplicate. Analysis of variance (ANOVA) of the results followed by the least significant difference test (LSD) were carried out to determine significant differences ($p < 0.05$) in the concentration and bioaccessibility of the bioactive compounds from the beverages in relation to the factors studied in this research (food processing: HPP, HIPEF and TT). All statistical analyses were performed with the program Statgraphics Plus 5.1 (Statistical Graphics Corporation, Inc., Rockville, MD, USA). The results were reported as mean $\pm$ standard deviation.

3. Results and discussion

3.1. Non-digested soymilk-based beverages

The isoflavone concentration of non-digested soymilk-based beverages is presented in Table 1, where three glucosides and three aglycone forms were detected. In untreated beverage, the concentration of total isoflavones, total glucosides and total aglycones was 17.78, 12.30 and 5.47 mg/100 mL, respectively. Similar results were reported in a previous research, where the concentration of total isoflavones reached 20.4 mg/100 mL in a soymilk-based beverage, however, glycitin and glycitein were not detected (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2014). Morales-De La Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso (2010) obtained 14.01 mg/100 mL of total glucosides and 2.2 mg/100 mL of total aglycones.

Once beverages were processed, the concentration of total glucosides and total isoflavones increased independently of the processing type (TT, HIPEF and HPP). However, the concentration

of total aglycones remained unchanged in HIPEF treated beverage. TT increased the concentration of individual glucosides between 10.78 and 25.75% regarding untreated beverage, while individual aglycones augmented up to 14.61%. The concentration of individual glucosides from HIPEF processed beverage showed a similar trend than TT, with increases from 9.02 to 25.32%. On the other hand, daidzein was the aglycone that showed a slight but statistically significant increase in its content after HIPEF processing (2.96%). The highest concentration of isoflavones was obtained in beverages processed by HPP, getting increases up to 17.04 and 38.52% for glucosides and aglycones, respectively.

The increase in the concentration of isoflavones after processing could be related with the higher extractability of isoflavones from the food matrix due to parameters such as pressure, pulsed electric fields and temperature applied during treatments. On this regard, isoflavones are linked to proteins by hydrogen bonding, as well as by hydrophobic interactions (Malaypally & Ismail, 2010; Toro-Funes et al., 2015). Protein denaturalization occur due to both processing types: thermal and non-thermal. TT induces irreversible protein denaturalization and aggregation as a result of heat damage to native protein structure and the breakdown of covalent bonds (Malaypally & Ismail, 2010; Varghese & Pare, 2019). TT (90 °C for 50s) increased around 5 % the total isoflavone content from a soymilk-based beverage (Morales-de la Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso, 2010). A similar trend was found in soymilk thermally processed at 100 °C during 20 min, showing an increase in the content of β -glucosides (Xu & Chang, 2009). The concentration of isoflavones increased 48.7% in soymilk treated by ultra-high temperature (UHT) at 142°C during 6s (Toro-Funes et al., 2015). Combination of temperature with other parameters as amplitude and time in the ultrasound processing also improved the concentration of isoflavones. In a particular case, soymilk contained up to 84% higher isoflavone concentration when ultrasound (amplitude of 100% at 30°C for 20 min) was applied as a pre-treatment in the manufacture (Morales-de la Peña, Martín-Belloso, & Welte-Chanes, 2018).

High pressure processing <400 MPa did not affect hydrogen bonds but the hydrophobic interactions are affected, inducing a reversible dissociation and aggregation (Floury, Desrumaux, & Legrand, 2002). In soymilk treated by ultra-high pressure homogenization (UHPH) at 300 MPa and 75°C of inlet temperature, the concentration of isoflavones increased around 23.5% (Toro-Funes et al., 2015). The degree of extraction of isoflavones in soymilk augmented when UHPH (200 and 300 MPa) was combined with TT (55, 65 and 75 °C), mainly when applying the highest pressure

and temperature conditions (Toro-Funes, Bosch-Fusté, Veciana-Nogués, & Vidal-Carou, 2014b). On the other hand, HIPEF processing at 35 Kv/cm with 4 μ s bipolar pulses at 200 Hz for 800 or 1400 μ s did not show significant differences in the isoflavone content from a soymilk-based beverage (Morales de la Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso, 2010). As previously stated, in the present research total isoflavones increased by 14.5% after HIPEF treatment at 35 kV/ cm with 4 μ s bipolar pulses at 200 Hz for 1800 μ s. These results showed the importance of properly selecting the processing conditions for achieving the highest isoflavone concentration from HIPEF treated products.

3.2. Digested soymilk-based beverages

In this section, the results obtained about the concentration of isoflavones (glucosides, aglycones and total isoflavones) from untreated and treated (TT, HIPEF, HPP) soymilk-based beverages after being subjected to *in vitro* gastrointestinal digestion (digested beverage) are presented (Table 2).

In overall, it was observed a significant increase in the concentration of total isoflavones after digestion ($p < 0.05$), independently of the processing type. Regarding the untreated beverage, the greatest concentration of total isoflavones was obtained from the beverage treated by HPP (increasing up to 69.85%), followed by that HIPEF processed (which augmented up to 43.87%); while the lowest was the TT beverage (increasing only 7.90%).

Particularly, HPP augmented by 14.12, 62.62 and 98.77% the concentration of glycitin, daidzin and genistin, respectively, with respect to untreated product. Increases between 46.75 and 61.11% in the concentration of aglycones after HPP treatment were also observed. The concentration of glucosides increases in the range of 14.71 to 70.55% in the HIPEF processed beverage as follows (ascendant order): glycitin, daidzin and genistin. Similarly, the concentration of daidzein and genistein augmented 36.96 and 42.59%, respectively, in the beverage treated by HIPEF as compared with that untreated. However, the concentration of glycitein remained unchanged ($p > 0.05$) when HIPEF, TT and untreated beverages were compared. On the other hand, the concentration of the glucosides genistin and glycitin from the beverage treated by heat (TT) did not show significant differences in comparison with that untreated; while daidzin increased by 113.08%. A similar trend was followed by aglycones, where no significant changes in the

concentration of daidzein and glycitein from the TT beverage were observed, regarding the untreated product.

The increase in the concentration of isoflavones from digested soymilk-based beverages obtained in the present research, could be explained by the breakdown of linkages between food constituents due to gastrointestinal conditions, thus releasing bioactive compounds such as isoflavones. It has been reported that the isoflavone concentration is affected by changes in the pH conditions, leading to changes in their chemical structure (Baú & Ida, 2015). The acidic pH of gastric digestion had an important influence on the concentration of isoflavones by improving their release up to 22% (Rodríguez-Roque, Rojas-Grau, Elez-Martínez, & Martín-Belloso, 2013b). Isoflavone conjugates were separated into acetyl and malonyl groups after mild acidic conditions (Peñalvo, Nurmi, & Adlercreutz, 2004) and their partition into glucoside and aglycone forms could be modified by acidic pH (Sanz & Luyten, 2006).

On the other hand, soymilk is a rich source of proteins that may bind to isoflavones, which could trigger the formation of insoluble aggregates and make difficult the digestibility of these constituents (Rodríguez-Roque, Rojas-Grau, Elez-Martínez, & Martín-Belloso, 2013b). These compounds are organized into small protein bodies (from 5 to 20 μm) inside the cytoplasmic membrane which once disrupted afford the protein solubilization within an aqueous phase (Varghese & Pare, 2019). It is probable that digestion could disrupt the cytoplasmic membrane favoring the solubilization of proteins linked to isoflavones. This statement could be a reason why the isoflavone concentration augmented in beverages subjected to *in vitro* gastrointestinal digestion.

3.3. Effect of thermal and non-thermal processing on the *in vitro* bioaccessibility of isoflavones

The results obtained from the bioaccessibility of isoflavones (glucosides, aglycones and total isoflavones) from untreated, thermally treated (TT) and non-thermally treated (HIPEF and HPP) soymilk-based beverages is presented in Table 3.

The bioaccessibility of the glucosides genistin, daidzin and glycitin were 23.50, 26.13 and 28.04%, respectively, in untreated beverage. On the other hand, the aglycones genistein, glycitein and daidzein showed a bioaccessibility between 30.39 and 33.70% in the untreated product, being

genistein the compound with the lowest percentage and daidzein that with the highest. In this case, total aglycones were around 30% more bioaccessible than glucosides.

The glucoside bioaccessibility of the TT beverage was in the range of 19.91 and 26.80, while the aglycone bioaccessibility was between 31.37 and 33.65%. These results showed that when the beverage was thermally processed, no significant differences ($p > 0.05$) were observed between the bioaccessibility of daidzin, daidzein, glycitin, glycitein and genistein as compared with that untreated. However, the bioaccessibility of genistin diminished 15.28% in the TT beverage regarding that untreated (Table 3). Similarly, the bioaccessibility of each individual isoflavone was always lower when beverages were thermally processed as compared with the beverages non-thermally processed.

In the case of the HIPEF treated beverage, a significant increase in the bioaccessibility of all analyzed glucosides and aglycones were observed, regarding untreated and TT beverages. The bioaccessibility of the glucosides glycitin, daidzin and genistin was 29.50, 31.30 and 31.82%, respectively, reaching an increment in the bioaccessibility up to 35.40% in comparison with untreated product. When the HIPEF treated beverage was compared with TT beverage, it was observed that genistin showed the highest increment in the bioaccessibility of glucosides with 59.58%. On the other hand, the aglycone bioaccessibility rose between 15.56 and 34.33% when the HIPEF treated beverage was compared with untreated beverage, and up to 38.95% as compared with TT beverage.

Regarding HPP processing, the bioaccessibility of the glucosides glycitin, daidzin and genistin was 28.84, 33.56 and 34.62%, respectively; while the bioaccessibility of aglycones ranged from 42.35 and 44.46%. These results showed that HPP processing followed a similar trend than HIPEF processing by increasing the bioaccessibility of glucosides and aglycones in comparison with both untreated and TT beverages. Genistin and genistein displayed the highest increment in the bioaccessibility of isoflavones with 47.32 and 46.20%, respectively, when HPP beverage was compared with that untreated. The greatest raise in the bioaccessibility of isoflavones was observed in genistin from HPP beverage (73.88% of increment) with respect to TT beverage. On the other hand, no significant differences in the bioaccessibility of isoflavones were observed ($p > 0.05$) when HPP and HIPEF processing were compared, with the exception of total glucosides which showed 9.76% less bioaccessibility in the HIPEF beverage.

These results confirmed that non-thermal processing methods rendered the highest isoflavone bioaccessibility as compared with both untreated and thermally processed beverages. In this sense, the increment in the bioaccessibility of total glucosides from the HIPEF treated beverage reached up to 39.4% as compared with that thermally treated, while that processed by HPP up to 49.1%. Similarly, the bioaccessibility of total aglycones increased by 27.31 and 33.64% in HIPEF and HPP treated beverages, regarding the TT processed beverage.

The *in vitro* gastrointestinal digestion is considered the first step in evaluating the bioavailability of food constituents (Thakur et al., 2020). Several factors influence the bioaccessibility of bioactive compounds, such as the type of food matrix and the interactions between food constituents (Parada & Aguilera, 2007). In this sense, it was found a glucoside and aglycone bioaccessibility of 42% and 17%, respectively, in a commercial soymilk (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2013b). However, when the soymilk was combined with fruit juices, the bioaccessibility of isoflavones diminished up to 51% (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2014). Some phenolic compounds, including isoflavones, may form insoluble aggregates with proteins and iron (Jinapong Supphantharika, & Jamnong, 2008), hindering both the dissolution of the precipitate and the digestibility of these constituents.

Food processing modulates the release, transformation and absorption of bioactive compounds (Verkempinck et al., 2020). Despite the great influence of food processing on the bioaccessibility of several compounds, there is really scarce information on this regard. It has been previously stated that food processing may break the linkage between proteins and isoflavones, thus increasing their concentration and bioaccessibility. Heat could increase or diminish the concentration of isoflavones in soymilk depending on the temperature and time of processing. For instance, the concentration of daidzein and glycitein in soymilk diminished after thermal processing, while genistein was heat stable (Huang Liang, & Kwok, 2006). The latest author also reported that the optimum conditions for processing soymilk were 95°C for 45 min, which was more intense than the treatment applied in the present research (90°C during 1 min). Thermal processing could also lead to chemical changes in soymilk due to Maillard reaction, oxidation and degradation of its constituents (Giri & Mangaraj, 2012). Thus, the decrease in the bioaccessibility of total glucosides in thermally treated beverage reported in the present research could be also related with the decrease in the concentration of isoflavones due to chemical reactions produced by heat.

On the other hand, heat treatments including microwave heating, high temperature short time process, ultra-high temperature processing and ohmic heating had been successfully employed for improving the quality of soymilk in basis of its protein digestibility (Kwok, Liang, & Niranjana., 2002; Vagadia et al., 2018; Varghese & Pare, 2019; Zhang, Li, Tatsumi, & Kotwal, 2003) and for reducing the anti-nutrient content, such as trypsin inhibitors (Chen et al., 2014). The protein digestibility increases by using an appropriate heat treatment due to inactivation of trypsin inhibitors (Yuan & Chang, 2007). Thus, isoflavones linked to those proteins could be released from the food matrix after food processing and thus, becoming available for bioaccessibility.

Regarding non-thermal processing, it is possible that these treatments could disrupt the cytoplasmatic membrane in which proteins are organized into small bodies (Varghese & Pare, 2019), thus favoring the solubilization of proteins linked to isoflavones. This statement could be a reason why the isoflavone bioaccessibility augmented in beverages processed by non-thermal treatments. It is difficult to compare the results obtained in this research with those of literature because to the best of our knowledge, this is the first study on this regard. As processing is one of the main factors influencing the bioaccessibility of bioactive compounds, it is important to continue analyzing the extent to which processing improve the bioaccessibility of isoflavones from functional beverages.

4. Conclusion

Food processing significantly increased the concentration of isoflavones, reaching around 25-26% in both TT and HIPEF treated beverages, while the highest content was achieved in HPP beverages (increment up to 38.52%). After *in vitro* gastrointestinal digestion, the concentration of isoflavones in non-thermally processed beverages was higher than that TT processed; with increases of 18.52%, 70.55% and 98.77% for TT, HIPEF and HPP beverages, respectively.

The bioaccessibility of isoflavones from the TT beverage did not show a significant difference with respect to that untreated, with the exception of genistin which diminished. However, non-thermal processing technologies (HIPEF and HPP) increased the bioaccessibility of both analyzed isoflavones types (glucosides and aglycones). The bioaccessibility of glucosides augmented up to 59.58% and 73.88% in HIPEF and HPP beverages, respectively, regarding the TT beverage. An increment in the bioaccessibility of aglycones reached 36% in the HIPEF beverage and 42% in HPP

beverage as compared with TT. Thus, this research had contributed in elucidating the influence of food processing (thermal and non-thermal) on the *in vitro* bioaccessibility of isoflavones from a complex matrix (a beverage made with soymilk and a blend of fruit juices).

Results demonstrate that both non-thermal processing technologies, HIPEF and HPP, are suitable for obtaining high quality and nutritious beverages by improving their isoflavone bioaccessibility. Future studies are necessary for evaluating: a. The extent to which non-thermal processing may improve the isoflavone bioaccessibility in other food and beverages; b. The optimal processing parameters for achieving the highest isoflavone bioaccessibility; c. To compare the influence of thermal and non-thermal processing on isoflavone bioaccessibility, d. The bioavailability of isoflavones from non-thermally processed products.

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Journal Pre-proof

Table 1. Isoflavone concentration (mg/100 mL) in a non-digested soymilk-based beverage treated with different food processing technologies.^a

	Untreated	TT	HIPEF	HPP
Glucosides				
Daidzin	4.08 ± 0.20c	4.52 ± 0.24b	4.89 ± 0.26ab	5.21 ± 0.29a
Genistin	6.99 ± 0.40b	8.79 ± 0.48a	8.76 ± 0.48a	9.37 ± 0.61a
Glycitin	1.22 ± 0.06d	1.45 ± 0.08b	1.33 ± 0.07c	1.69 ± 0.05a
Total glucosides	12.30 ± 0.59c	14.76 ± 0.57b	14.97 ± 0.62b	16.27 ± 0.55a
Aglycones				
Daidzein	1.35 ± 0.07c	1.47 ± 0.09ab	1.39 ± 0.08b	1.58 ± 0.10a
Genistein	1.78 ± 0.11b	2.04 ± 0.07a	1.81 ± 0.12b	1.97 ± 0.14a
Glycitein	2.34 ± 0.13b	2.30 ± 0.10b	2.22 ± 0.11b	2.68 ± 0.12a
Total aglycones	5.47 ± 0.20c	5.82 ± 0.18b	5.38 ± 0.20c	6.23 ± 0.29a
Total isoflavones	17.78 ± 0.78c	20.58 ± 0.73b	20.36 ± 0.80b	22.50 ± 0.51a

^a Lowercase letter in the same row indicates significant differences ($p < 0.05$) in the isoflavone concentration among treatments.

TT = thermal treatment; HIPEF = High Intensity Pulsed Electric Field; HPP = High Pressure Processing. Total glucosides and aglycones were considered as the sum of individual isoflavones. Total isoflavones were considered as the sum of total glucosides and total aglycones.

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Table 2. Isoflavone concentration (mg/100 mL) in a digested soymilk-based beverage treated with different food processing technologies.^a

	Untreated	TT	HIPEF	HPP
Glucosides				
Daidzin	1.07 ± 0.07d	1.21 ± 0.09c	1.53 ± 0.10b	1.74 ± 0.11a
Genistin	1.63 ± 0.10c	1.74 ± 0.11c	2.78 ± 0.14b	3.24 ± 0.12a
Glycitin	0.34 ± 0.02c	0.37 ± 0.02bc	0.39 ± 0.03b	0.49 ± 0.03a
Total glucosides	3.05 ± 0.10d	3.32 ± 0.04c	4.70 ± 0.11b	5.47 ± 0.17a
Aglycones				
Daidzein	0.46 ± 0.03b	0.48 ± 0.03b	0.63 ± 0.04a	0.70 ± 0.05a
Genistein	0.54 ± 0.04d	0.64 ± 0.04c	0.77 ± 0.06b	0.87 ± 0.06a
Glycitein	0.77 ± 0.04b	0.75 ± 0.06b	0.87 ± 0.05b	1.13 ± 0.09a
Total aglycones	1.76 ± 0.09c	1.87 ± 0.06c	2.27 ± 0.09b	2.71 ± 0.12a
Total isoflavones	4.81 ± 0.16d	5.19 ± 0.10c	6.97 ± 0.18b	8.17 ± 0.26a

^a Lowercase letter in the same row indicates significant differences ($p < 0.05$) in the isoflavone concentration among treatments.

TT = thermal treatment; HIPEF = High Intensity Pulsed Electric Field; HPP = High Pressure Processing. Total glucosides and aglycones were considered as the sum of individual isoflavones. Total isoflavones were considered as the sum of total glucosides and total aglycones.

Table 3. Bioaccessibility of isoflavones (%) in a soymilk-based beverage treated with different food processing technologies.^a

	Untreated	TT	HIPEF	HPP
Glucosides				
Daidzin	26.13 ± 0.68b	26.80 ± 1.85b	31.30 ± 3.59a	33.56 ± 4.05a
Genistin	23.50 ± 2.57b	19.91 ± 1.97c	31.82 ± 2.57a	34.62 ± 1.26a
Glycitin	28.04 ± 2.69ab	25.21 ± 0.77b	29.50 ± 2.52a	28.84 ± 1.52a
Total glucosides	24.82 ± 1.78d	22.53 ± 1.14c	31.40 ± 1.76b	33.60 ± 0.77a
Aglycones				
Daidzein	33.70 ± 3.11b	32.58 ± 3.21b	45.27 ± 5.44a	44.46 ± 5.86a
Genistein	30.39 ± 3.36b	31.37 ± 2.00b	42.71 ± 4.13a	44.43 ± 4.34a
Glycitein	32.97 ± 3.45c	33.65 ± 3.57bc	38.10 ± 2.82ab	42.35 ± 3.18a
Total aglycones	32.25 ± 2.55b	32.55 ± 1.97b	41.44 ± 2.10a	43.50 ± 2.96a
Total isoflavones	27.11 ± 1.98b	25.36 ± 1.34b	34.05 ± 2.08a	36.32 ± 1.02a

^a Lowercase letter in the same row indicates significant differences ($p < 0.05$) in the isoflavone bioaccessibility (%) among treatments.

Bioaccessibility was determined as the ratio of isoflavone concentration in the digested soymilk-based beverage with respect to non-digested beverage (Eq. 1).

TT = thermal treatment; HIPEF = High Intensity Pulsed Electric Field; HPP = High Pressure Processing. Total glucosides and aglycones were considered as the sum of individual isoflavones. Total isoflavones were considered as the sum of total glucosides and total aglycones.

Highlights

Food processing increased the concentration of isoflavones in non-digested beverages

The highest isoflavone bioaccessibility was obtained in High Pressure Processed beverages

High Intensity Pulsed Electric Fields improve up to 35% the isoflavone bioaccessibility

The isoflavone bioaccessibility remained unchanged in thermally treated beverages

Non-thermal processing technologies are suitable for obtaining nutritious beverages