

**Green extraction of isoflavones from okara using subcritical water:
kinetics, optimization, and comparison with other water-based
sustainable methods**

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Abstract

Water has been explored as a green extraction agent for isoflavone extraction from okara. First, subcritical water (subW) was explored using two different okaras: (1) washed and dried (OKW) and (2) non-pretreated (OKC). Genistein family was the most abundant, with very low quantities in the glycitein family. Kinetic data revealed interconversion of malonyl-glycosides to β -glycosides in subW, with increasing degradation rate constants for all isoflavones with temperature. A maximum of 1229 μg isoflavone/g of dried okara was obtained at 120 °C after 30 min for OKC. Microwave (MAE) and ultrasound (UAE) technologies achieved 72.8 % and 75.4 %, of the yield of subW. Productivity at the maximum was of 41.0, 82.5 and 92.8 μg isoflavone /g dry okara min for subW, MAE, UAE. However, higher fraction of the more bioavailable forms β -glycoside + aglycone was obtained by subW.

Keywords

okara, isoflavones, subcritical water, kinetics, microwave-assisted extraction, ultrasound-assisted extraction.

1. Introduction

Okara is the by-product generated in the soybean (*Glycine max*) industry. Soybean is the most widely cultivated and consumed legume globally. Okara is generated during the filtration stage of soybean-derived products, such as tofu, soy milk, and soy protein isolate. More than 14 million tons of okara are produced every year, with approximately 1.1 kg produced per kilogram of soybeans processed (Mok et al., 2019). Despite its significant potential as a valuable by-product, okara is often discarded due to challenges related to its preservation and tendency to spoil rapidly. Okara presents high nutritional value, it is rich in fiber, proteins with good amino acid profile, lipids with beneficial fatty acids, B-group vitamins, antioxidants and essential minerals. It also contains a noteworthy concentration of isoflavones, as soybeans are the most important source of these flavonoids (Mok et al., 2019; Nkurunziza, Pendleton, Sivagnanam, et al., 2019). Therefore, it is worth exploring sustainable methods to valorize this by-product, with a focus on extracting the valuable fraction of bioactive compounds as an initial step in a cascade approach.

Isoflavones are flavonoids with an estrogen-like structure, commonly referred to as phytoestrogens, that confer functional properties and health benefits. Some of the most notable health benefits associated with isoflavones in blood plasma include anti-inflammatory effects, protection against colon cancer, anti-obesity properties, diabetes

management, prevention of osteoporosis, enhancement of bone health, and support for the gut microbiota (Hsiao et al., 2020). These benefits are particularly relevant during periods of senescence and perimenopause, where supplementation with isoflavones, due to their structural similarity to estrogens, may be especially advantageous (Hsiao et al., 2020).

Twelve isoflavones have been isolated from soybeans, classified into four main groups: aglycones (genistein [GE], daidzein [DE], and glycitein [GYE]), β -glycosides (genistin [G], daidzin [D], and glycitin [GY]), malonyl-glycosides (malonyl-genistin [MG], malonyl-daidzin [MD], and malonyl-glycitin [MGY]), and acetyl-glycosides (acetyl-genistin [AG], acetyl-daidzin [AD], and acetyl-glycitin [AGY]) (Jankowiak et al., 2014).

Malonyl-glycosides contain a malonyl group, which makes them the most thermolabile form, and they are the most common in nature. β -Glycosides contain only a glycoside group and are also common in nature. Aglycones, which are the native forms, have a non-polar structure and are less common in nature; however, they are the most bioavailable forms, followed by β -glycosides. The higher the bioavailability, the greater their capacity to provide health benefits (Hsiao et al., 2020; Nkurunziza, Pendleton, Sivagnanam, et al., 2019).

Isoflavone extraction typically involves at least two steps: extraction using a large amount of organic solvent, followed by purification through multiple chromatography columns. In this study, a green method for extracting isoflavones without using organic solvents was proposed using water under subcritical conditions. Subcritical water (subW) is an innovative green technology that uses only water as a solvent at temperatures from 100

to 374 °C and pressures high enough to maintain water in its liquid state. Water exhibits unique properties under subcritical conditions. Its dielectric constant decreases due to hydrogen bond dissociation, while the ionic product of water (K_w) increases, resulting in a higher concentration of hydronium and hydroxide ions. This makes water a great solvent for both polar and non-polar substances, and it also exhibits acidic or basic catalytic properties, eliminating the need for additional chemical catalysts in the process (Barea et al., 2024; Nkurunziza, Pendleton, Sivagnanam, et al., 2019).

Many prior studies on isoflavone extraction have focused on using hydroalcoholic mixtures to achieve high extraction efficiency with 50-70% aqueous ethanol solutions (Jankowiak et al., 2014). When a significant amount of ethanol is needed in the solvent to work at low liquid-to-solid, either a large volume of ethanol or a drying pretreatment step is required (Jankowiak et al., 2014). Consequently, subW extraction offers an eco-friendly and sustainable alternative to conventional organic solvent-based methods for isoflavone extraction. The unique properties of water under subcritical conditions enhance its ability to dissolve bioactive compounds. By adjusting the temperature and pressure, both yield and selectivity can be optimized. Additionally, owing to its increased ionic product content, subW enables the simultaneous extraction and hydrolysis of isoflavone glycosides into aglycones, which are more bioavailable, without the need for extra acid hydrolysis steps.

The objective of this study was to investigate the potential of water under subcritical conditions as an extraction agent for the sustainable valorization of okara, proposing a first step under mild conditions for the recovery of isoflavones from okara, focusing mainly on the effect of extraction temperature. It is important to note that okara has a high

moisture content exceeding 80 %, which contributes to its rapid spoilage. A key strength of this study is the examination of isoflavone extraction from crude okara without any pretreatment, as water was chosen as the extraction agent. The results obtained by subW extraction were compared with conventional solvent extraction using different hydroalcoholic mixtures, as well as other water-based emerging extraction technologies, such as microwave and ultrasound-assisted extraction processes. A comparison will be established considering the extraction yield, isoflavone distribution, productivity, and energy consumption per gram of isoflavone in the extract. Additionally, a comprehensive kinetic study was conducted to explore the interconversion pathways between different groups of isoflavones under different extraction conditions by subW extraction.

2. Experimental

2.1. Materials

Okara was supplied by *Frias Nutrición S.A.U.* (Burgos, Spain), with a moisture content of 82.5 ± 0.1 % (w/w), making it highly susceptible to spoilage. Upon receipt, a portion of okara was conveniently washed and dried. When okara was tentatively dried without washing, either in a convection oven at 45 °C or vacuum oven, it spoiled rapidly. Therefore, experiments were carried out with okara previously washed with distilled water at a ratio of 1:1 (w/v) (equivalent to 9.6 % of dry matter in water). The mixture was homogenized for 1 min and filtered. The washed okara (hereafter referred to as OKW) was subsequently dried in a convection oven at 45°C for 24 h to a final moisture content of 5 % (w/w). OKW was stored at 5 °C without spoiling during storage. In contrast, another portion of the received okara was immediately frozen in individual small plastic bags without any prior treatment (no washing or drying) and is referred to as crude okara

(OKC). To allow for a better comparison between both types of okara, all results in this study were expressed using the corresponding dry basis.

Isoflavone standards (purity ≥ 98 %) used for calibration in HPLC were purchased from various suppliers: daidzin (**D**) and genistin (**G**) from *TargetMol*; glycitin (**GY**) and malonylgenistin (**MG**) from *ChemCruz*; daidzein (**DE**) from *Thermo Scientific*; glycitein (**GYE**) from *Chengdu Biopurify Phytochemicals*; genistein (**GE**) from *Fluorochem*; and malonyl-daidzin (**MD**) from *ChemFaces*.

2.2. Conventional extraction

Conventional solvent extraction of isoflavones was performed in an orbital shaker (Grant Instruments OLS 200, Shepreth Cambridgeshire, England). Extraction conditions were selected based on a previous study by Jankowiak et al. (2014). The authors identified 50 °C for 1h as the optimal extraction conditions. In this work, extractions were conducted at 50 °C for 30 min and 60 min using various ethanol-water mixtures, including 100 % ethanol, 70 % ethanol, 50 % ethanol, 30 % ethanol, and 100 % water. OKW was dissolved at a concentration of 5 % (w/v), equivalent to 4.75 % (w/v) on a dry basis. Times shorter than that reported by Jankowiak et al. (2014) were also considered to optimize yield and productivity.

2.3. Subcritical water (subW) extraction process

Subcritical water extraction was performed in a 0.5 L batch reactor covered with a ceramic heating jacket (230 V, 4000 W, $\varnothing$ 95 mm, 160 mm height). A Pt100 sensor and PID system enabled the control and registration of the temperature, while a pressure gauge with a purge valve maintained the pressure inside the reactor during the process.

For each extraction, 200 mL of 4.75 % in a dry basis (w/v) OKW suspension was charged into the reactor. Higher okara loadings were not feasible owing to swelling of the raw material and stirring challenges. The treatment was conducted at various temperatures ranging from 110 °C to 160 °C at a pressure of 5 MPa using N₂ as the pressurization agent. The operating temperature and pressure were selected considering previous work when dealing with subW extraction of flavonoid compounds from food wastes such as onion skin wastes, concluding that extraction of phenolic compounds such as flavonoids was temperature sensitive with subW under mild conditions, 145 °C, resulting in the optimum extraction conditions, when covering the temperature range from 105 to 180 °C, observing degradation at temperatures higher than 160 °C (Benito-Román, 2020). Regarding the operating pressure, its effect on the performance of subW was observed to be non-significant, compared with temperature and time, as long as water remained in the liquid state. After determining the optimal extraction temperature, isoflavone extraction was performed from crude okara (OKC) using a 200 mL suspension at the same dry basis concentration of 4.75 % (w/v).

Isoflavone extraction kinetics were followed by withdrawing the samples at regular time intervals through the sampling port. After a certain treatment time, the reactor was cooled to 90 °C and depressurized to collect the final hydrolysate.

2.4. Microwave assisted extraction

The microwave assisted extraction (MAE) was conducted using a flexiWAVE microwave equipment ($T_{\max} = 300$ °C, $p_{\max} = 10$ MPa, Milestone SRL, Sorisole, Italy). Hydrolysis was performed in Teflon 100 mL batch vessels with constant stirring and temperature control. A heating ramp was set for 5 min with microwave power limited to 1000 W, with

the actual average power used ranging from 150 to 200 W during the 5 min heating phase and 75–100 W to maintain the temperature during the rest of the process. Extractions were performed with OKC, using vessels containing 25 mL of OKC suspensions at 4.75 % (w/v) on a dry basis, at different temperatures (110 – 140 °C) and treatment times (5, 10, and 15 min). The temperature extraction conditions were chosen based on previous results of subW extractions and are of the order of extraction conditions reported for MAE tested by Tsubaki et al. (2009) in the range of 110 to 140 °C, with extraction times ranging from 5 to 45 min. The results were compared with those from subW extractions.

2.5. Ultrasound assisted extraction

A 750 W ultrasonic processor with a 13 mm probe (Vibra-Cell™ 75042, Sonics Material™ Inc., U.S.A.) was used to extract the isoflavones from okara. The samples were processed at a constant ultrasound frequency of 20 kHz and 100 % of amplitude, 79 µm according to the manufacturer's instructions. In each run 200 mL of an OKC aqueous suspension at 4.75 % (w/v) on a dry basis were placed in a thermostated vessel ($\Phi = 4.8$ cm, $V = 199$ cm³) to regulate temperature by circulating water through the jacketed vessel from a thermostat bath, performing extractions at 50 °C. When dealing with UAE, the working temperature should be below the saturation vapor pressure of water at ambient pressure; therefore, 50 °C was selected for comparison with the hydroalcoholic conventional solvent extraction. The US probe was submerged at a constant depth of 2 cm from the bottom of the vessel (Illera et al., 2018). The treatment was administered in pulses, 5s on and 5s off, with a maximum treatment time of 30 min (1 h total process).

Samples were collected every 5 min to follow the kinetics, with additional aliquots collected at the start and at 2.5 min.

2.6. Analysis of the extracts

2.6.1. Extraction yield

An aliquot (1.5 - 2 mL of the final extract was filtered through a 0.22 μ m syringe filter and dried at 105 °C to remove the solvent until a constant weight was achieved. The extraction yield was calculated as the ratio between the weight of the dry extract obtained after removing the solvent by considering the initial volume of the collected extract and the weight of the dried okara at the beginning of the extraction process.

2.6.2. Isoflavones (determination and quantification)

Isoflavones in the different extracts were analyzed using an HPLC/DAD system (Agilent 1260 Infinity II/HP Series 1100) equipped with a column system consisting of a precolumn (Phenomenex® AJ0-9000:2.1-4.6 mm) and an LC Column (Kinetex® 5 μ m Bypheyl: 4.6 mm x 250 mm) with a porosity of 100 Å. Prior to analysis, the extracts were filtered through 0.22 μ m syringe filters. The column temperature was maintained at 25°C. A gradient of two mobile phases was used for separation: (A) 1 % acetic acid in 0.005 M ammonium acetate (prepared in deionized water) and (B) 1 % acetic acid in 0.005 M ammonium acetate (prepared in ACN). The flow rate was set at 0.8 mL/min, with the gradient flow transitioning over 90 min from an initial concentration of 98 % (A) and 2 % (B) to final concentrations of 20 % (A) and 80 % (B).

A Diode Array Detector (HP Agilent 1100 HPLC G1315A DAD) was used to measure the signals and spectra of each sample from 190 nm to 950 nm. The wavelength used to

measure and compare all chromatograms was 254 nm, complemented by measurements at 280 nm, 330 nm, and 370 nm. Additionally, analyzing the full spectrum helps match each peak with the corresponding isoflavone standard. Standard solutions of eight isoflavones were used for calibration and identification. **G**, **D**, **GE**, **DE**, **GYE**, and **MD** were dissolved in methanol, whereas **MG** and **GY** were dissolved in ethanol and dimethyl sulfoxide (DMSO), respectively, owing to their low solubility in methanol. For data acquisition and treatment OpenLab CDS 2.5 was used.

The results are expressed as the amount of isoflavones per gram of dry okara. The purity of the isoflavones in the extract was expressed as the ratio of the mass of isoflavones to the mass of the total dry extract.

2.6.3. Total nitrogen

A Total Organic Carbon Analyzer Shimadzu (TOC-V CSN) was used to quantify the total nitrogen content. Through catalytic thermal decomposition, nitrogen-containing compounds are oxidized during combustion. The resulting nitrogen oxides (NO_x) were detected using a chemiluminescence detector and correlated with the original nitrogen concentration of the samples. Potassium nitrate (KNO₃) was used as a calibration standard.

2.6.4. Total phenolic content

Total phenolic content (TPC) was determined using the Folin-Ciocalteu reagent (VWR). Samples (100 µL) were diluted with 2.8 mL), and subsequently, Folin-Ciocalteu reagent (100 µL) was added, followed by the addition of 2.0 mL of sodium carbonate 7.5 % (w/v). After incubation for 1 h, absorbance was measured at 750 nm. Gallic acid was used as the

standard for calibration, and the results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry sample.

2.6.5. Reducing capacity

The reducing capacity of okara extracts was assessed using the Ferric Reduction Antioxidant Power assay (FRAP), as proposed by (Benzie & Strain, 1996). The standard used for the calibration was an $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ solution (0.1 M), and the results were expressed as μmol of Fe^{2+} per gram of dry okara.

2.7. Statistical analysis

All samples were analyzed at least twice and expressed as the mean $\pm$ standard deviation of the replicates. Data were analyzed using software Statgraphics19 X64 and, the significance of the differences between sample results was determined based on an analysis of variance with Fisher's Least Significant Difference (LSD test) at $p \leq 0.05$.

3. Results and discussion

3.1. Isoflavones extraction on pre-treated Okara (OKW)

3.1.1. Isoflavones extracted by conventional extraction

The discussion focuses on the eight isoflavones detailed in the Material section (section 2.1) among the 12 existing isoflavones. Acetyl forms and malonyl-glycitin were excluded because of the absence of the corresponding standards in this study. Notably, Jankowiak et al. (2014) studied the extraction of isoflavones from okara using different solvents, including a 70 % ethanol aqueous solution and concluded that malonyl glycitin accounted for only 1.5 % of the total isoflavone content. Furthermore, among the three acetyl-glycosides, only acetyl-genistin was detected in the extracts, but it accounted for only

0.6 % of the total extracted isoflavones. When water was used as a solvent, Jankowiak et al. (2014) reported extraction percentages of 2.9 % for malonyl-glycitin and 1 % for acetyl-genistin. Tsubaki et al. (2009) also identified malonyl-glycitin as the least concentrated isoflavone when extraction was performed with water as solvent, noting its high susceptibility to degradation (ignoring also the presence of acetyl-glycosides). Therefore, it can be concluded that the sum of the eight predominant isoflavones (G, D, GY, GE, DE, GYE, MG, and MD) is a good approach for total isoflavone extraction.

Table 1 presents the total isoflavone extraction yield, as well as the yield of each individual isoflavone, for the aqueous, ethanol, and different ethanol-water mixtures used as solvents in this study. Extraction from OKW using only water at 50 °C for 30 min resulted in a total isoflavone yield of 281 ± 3 µg/g of dry okara (Table 1). As the ethanol concentration in the extraction solvent increased, the total isoflavone yield continuously increased, reaching a maximum of 439 ± 10 µg/g of dry okara in 50 % ethanol, representing a 56 % increase in yield. Further increase in ethanol concentration did not result in higher yields; for example, at 70 % ethanol, the yield decreased slightly to 411 ± 11 µg/g dry okara. The lowest yield was obtained using pure ethanol, with only 14 ± 1 µg of total isoflavones per gram of dry okara, indicating that isoflavones have a limited solubility in ethanol. Table 1 also lists the dielectric constant (ξ) values for the corresponding ethanol-water mixtures at 50 °C, as reported by Akerlof (1932). The dielectric constant is a physical property that influences, among other properties, the solvent capacity. According to Fakhree et al. (2010), knowledge of the dielectric constant of mixed solvents can help predict the solubilities of bioactive compounds. Considering the dielectric constant values for the ethanol an aqueous mixture at 50 °C as reported by

268 Akerlof (1932) it can be established that the highest extraction yield occurred at dielectric
269 constant value between ethanol and water, with a value of $\xi = 42.92$ corresponding to 50
270 % ethanol-water mixture, whereas both higher dielectric constant values (e.g., pure water,
271 $\xi = 69.85$) and lower values (e.g., pure ethanol, $\xi = 20.87$) were associated with lower
272 extraction yields.

273 The most abundant forms in the extract were the malonyl-glycosides, followed by β -
274 glycosides with only a small low proportion of aglycones, except in the case of pure
275 ethanol. For the optimal solvent, 50 % ethanol-water mixture, malonyl-glycosides
276 accounted for 66.5 % of the total isoflavones (slightly lower than the 71.3 % obtained
277 with water alone), β -glycosides- accounted for 22.9 % (lower than the 27.0 % extracted
278 with water), while the aglycones made up 10.6 % (almost seven times higher than the
279 1.6 % extracted with water). This pattern is consistent with other studies that have shown
280 that okara produced on an industrial scale predominantly contains malonyl glycosides and
281 β -glycosides, which are considered the more natural forms of isoflavones, similar to those
282 found in soybeans. These forms are typically present when lower temperatures and shorter
283 extraction times are used. Aglycones tend to accumulate only under more severe
284 extraction conditions (Moras et al., 2017; Tsubaki et al., 2009). These findings align with
285 those of (Jankowiak et al., 2014), who reported higher concentrations of aglycones (386
286 $\mu\text{g/g}$ dry okara, 37.9 % of the total isoflavones) compared to β -glycosides (35.1 %) and
287 malonyl-glycosides (26.4 %) when using a 70 % ethanol-water solution, attributing this
288 result to the production of okara at laboratory scale starting from crude soybean and
289 submitting it to harsh conditions. In any case, focusing on valorization studies on

industrial-scale raw materials, which could be more suitable for potential scaling-up processes, may be a more interesting approach.

Extractions for 1 h did not result in a significant increase in the concentrations of total or individual isoflavones. The total isoflavone yield after 1 h was approximately 4% higher with the 50 % ethanol solvent and almost 2 % lower with pure water. Therefore, only the 30-minute extraction data are presented here (additional data for the 1-hour extraction can be found in the Supplementary Material).

Table 1. Isoflavone extraction yield of OKW using different hydroalcoholic mixtures as solvents at 50 °C for 30 min. Values with letters (a-e) show significant differences in each row when applying the LSD method at $p \leq 0.05$.

$\mu\text{g ISF /g dry okara}$	100% EtOH	70% EtOH	50% EtOH	30% EtOH	0% EtOH
Malonyl-daidzin (MD)	2.1 ± 0.3^a	130 ± 3^c	145 ± 1^d	145 ± 0.3^d	112 ± 0.1^b
Malonyl-genistin (MG)	2.03 ± 0.05^a	130 ± 4^c	147 ± 1^d	145 ± 2^d	87.9 ± 0.2^b
Daidzin (D)	1.1 ± 0.1^a	34.9 ± 0.9^d	34.9 ± 0.3^d	32.6 ± 0.7^c	30.8 ± 0.2^b
Glycitin (GY)	0.24 ± 0.02^a	11.5 ± 0.5^b	12 ± 2^b	11.5 ± 0.1^b	13 ± 2^b
Genistin (G)	1.76 ± 0.01^a	59 ± 2^c	54 ± 1^d	46 ± 3^c	32.5 ± 0.1^b
Daidzein (DE)	4.0 ± 0.5^a	27.1 ± 0.4^c	27 ± 2^c	17.8 ± 0.02^b	3.46 ± 0.02^a
Glycitein (GYE)	0.39 ± 0.09^a	3.3 ± 0.3^b	3.6 ± 0.9^b	2.87 ± 0.01^b	1.0 ± 0.1^a
Genistein (GE)	2.07 ± 0.01^a	15.0 ± 0.1^c	15 ± 2^c	7.2 ± 0.1^b	0.11 ± 0.01^a
Total Malonyl-glycosides	4.1 ± 0.4^a	260 ± 7^c	292 ± 2^d	290 ± 2^d	199.9 ± 0.3^b
Total β -glycosides	3.1 ± 0.1^a	105 ± 3^c	101 ± 3^d	90 ± 4^d	76 ± 2^b
Total aglycones	6.5 ± 0.6^a	45.4 ± 0.8^c	46 ± 5^c	27.9 ± 0.1^b	4.6 ± 0.1^a
Total ISOFLAVONES	14 ± 1^a	411 ± 11^c	439 ± 10^d	408 ± 6^c	281 ± 3^b
Dielectric constant *	20.87	32.86	42.92	53.79	69.85

(*) Akerlof (1932)

3.1.2. Isoflavones extraction by using water under subcritical conditions

The extraction efficiency of organic solvents and subW can be compared based on their dielectric constants, which is not the only factor affecting the isoflavone extraction

process but is an important parameter to consider when determining the optimal conditions for the extraction of some biocompounds. The results presented in Table 1 indicate that the 50 % ethanol-water mixture yielded the highest isoflavone extraction, corresponding to a dielectric constant value of 42.92. The physical properties of water under subcritical conditions undergo significant changes with temperature, showing a decrease in its dielectric constant and higher hydrolytic power owing to the increase in its ionic product. Therefore, subW has been explored as a potential greener alternative to hydroalcoholic mixtures for isoflavone extraction. An increase in the temperature in the subcritical region led to a decrease in the dielectric constant of water, becoming similar to that of organic solvents. The temperature range covered in this study was from 110 °C to 160 °C, which corresponds to dielectric constant values of approximately (graphical lecture at 100 bar) 53 and 41, respectively, of the order of ethanol-water mixtures with 30-50 % content of the alcohol (see Table 1) (Galamba et al., 2019). Therefore, in the temperature range covered in this work, subW exhibits dielectric constant values similar to those found in the most effective traditional isoflavone extractions using hydroalcoholic mixtures but avoids the use of any organic solvent. This reduction enhances the solvent effectiveness of water, allowing it to dissolve substances ranging from slightly polar to nonpolar (Ali et al, 2025).

Figure 1a shows the total isoflavone extraction kinetics at selected temperatures. The kinetics of total isoflavone extraction include the time required to reach the operating temperature (initiated immediately after mixing), which was approximately 30 min. At the lowest temperatures tested (110 °C to 140 °C), a maximum was observed in the early stages of the extraction process, with the highest initial extraction rate and maximum

329 occurring at 120 °C, which resulted in a maximum yield of 526.4 µg isoflavone /g dry
330 okara after 30 min of treatment, 19.9 % higher than the yield obtained with a 50 % ethanol
331 solution in a conventional extraction, (439 ± 10 µg /g dry okara). After this maximum,
332 the isoflavone content decreased because of degradation. The rate of isoflavone
333 degradation increased with an increase in temperature. At the highest temperature assayed
334 (160 °C), a continuous decrease in total isoflavone content was observed from the
335 beginning due to a faster degradation rate than the extraction rate.

336 Figure 1a shows the isoflavone extraction kinetics at 90 °C. Although water at 90 °C and
337 atmospheric pressure is not under subcritical conditions, this temperature was included in
338 the study because of the rapid extraction rates observed under mild subcritical water
339 conditions. Notably, for the treatment conducted at 90 °C, okara was added to the reactor
340 only after the water temperature reached 90 °C because no pressurization was necessary
341 at this temperature; the working temperature was attained within the first 5 min. At 90
342 °C, the isoflavone extraction also showed a maximum, followed by a decrease in the total
343 isoflavone content over time.

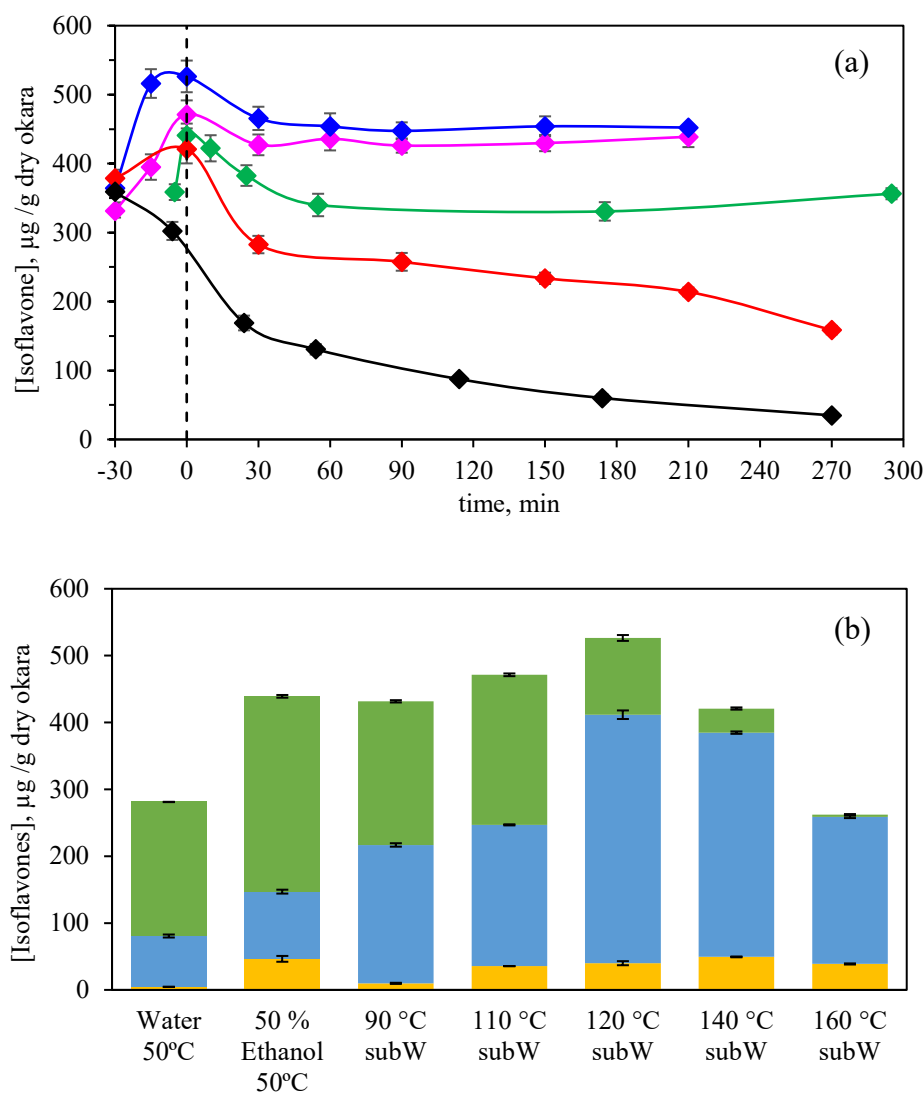


Figure 1. (a) Total isoflavone extraction ($\mu\text{g/g}$ dry okara) by subW treatment for OKW, as a function of time (min), at different temperatures: 90 °C (◆); 110 °C (◆); 120 °C (◆); 140 °C (◆); 160 °C (◆). **(b)** Isoflavone profile ($\mu\text{g/g}$ dry okara) after 30 min of water and 50 % ethanol extraction at 50 °C and for subW extractions at different temperatures. Malonyl-glycosides (■), β -glycosides (■), aglycones (■).

346 The higher isoflavone extraction yield observed could be attributed to the tunable
 347 properties of water under subcritical water conditions, as previously discussed. The

dielectric constant decreased with temperature, being similar to that of a 50 % ethanol aqueous mixture. However, at 120 °C, where maximum extraction was achieved (Figure 1), the extraction of isoflavones was higher when using subW than when using a 50 % ethanol aqueous mixture. This may indicate that this reaction medium promotes the extraction of isoflavones. A similar trend was observed in the extraction of bioactive compounds from onion skin waste when subW was used, in the temperature range from 105 to 180 °C, when compared with ethanolic aqueous mixtures, although they exhibited similar dielectric constant values (Benito Roman, 2020). Therefore, other properties might play an important role, such as the ionic product of water, which increases with increasing temperature, resulting in higher concentrations of hydronium and hydroxide ions in the medium, favouring a hydrolytic medium that might lead to a higher extraction of isoflavones.

Furthermore, in the literature it has been reported that isoflavones and proteins interact strongly via non-covalent interactions, mainly hydrogen bonds and hydrophobic interactions (Jankowiak et al., 2014; Le Bourvellec & Renard, 2012; Speroni et al., 2010). The use of subcritical water (subW) could reduce protein-isoflavone interactions due to its higher ionic product (K_w), which, among other effects, leads to more acidic conditions that could potentially facilitate the hydrolysis of the protein fraction. To elucidate the relationship between the hydrolysis of the protein fraction and the increase in isoflavone extraction, the total nitrogen content in the final extracts was determined (Table 2S). It can be clearly observed that the protein content (N content) in the final extracts was higher for the subW extracts than for the conventional water and 50 % ethanol extracts. Although

temperatures above 140 °C promoted higher protein hydrolysis in the extracts, degradation of the isoflavones occurred rapidly.

For comparison, the isoflavone profile at the point of achieving the operating temperature (approximately 30 min for all subcritical water (subW) extractions) is presented in Figure 1b, which also includes the isoflavone profile for extractions at 50 °C using pure water and a 50 % ethanol aqueous solution as solvents. A clear increase in the total isoflavone content in the extraction medium was observed during the heating stage at temperatures lower than 120°C, along with shifts in the proportion of each isoflavone group. For instance, the malonyl form percentage decreased from 47.6 % at 110 °C to 21.8 % at 120 °C, further reducing to just 1.3% at 160 °C. In contrast, the β -glycoside form increased from 44.8 % at 110 °C to 70.6 % at 120 °C and reached 83.9 % at 160 °C of total isoflavone content. These findings are consistent with those of previous studies, such as (Rostagno et al., 2004), which reported that malonyl-glycosides degrade at 100 °C, converting to β -glycosides due to thermal decomposition at these or higher temperatures. Conventional extractions with water and 50 % ethanol showed higher malonyl form percentages (71.3 % and 66.5 %, respectively) and lower β -glycoside content than any subW hydrolysis method (27.0 % and 22.9 %, respectively). Among all extractions, subW at 120 °C yielded the highest total isoflavone concentration (526.4 $\mu\text{g/g}$), with the highest combined content of β -glycoside and aglycone (411.6 $\mu\text{g/g}$, 78.2%), the two isoflavone groups of particular interest due to their bioactive properties.

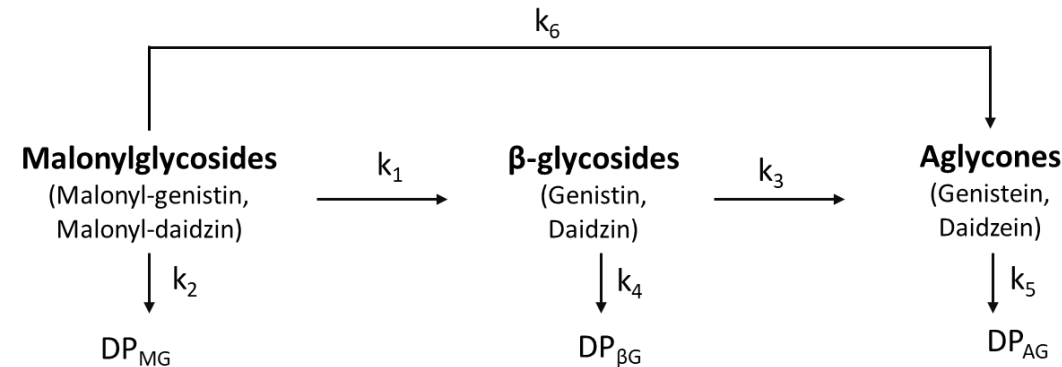
Therefore, subcritical water treatment clearly modified the isoflavone profile due to chemical transformations, as observed during the non-isothermal heating period. An in-depth kinetic study of the interconversion between isoflavone forms at different

temperatures was conducted during the isothermal period. Considering the isoflavones identified in this study, Figure 2 shows the kinetic profiles for the different forms of isoflavones within the genistein, daidzein, and glycitein families, in mole concentration ($\mu\text{mol/g}$ dry okara). Among these, the genistein family was the most abundant group, followed by the daidzein family with the lowest quantities determined in the glycitein family (see Figure 2), which was approximately ten times lower than for the genistein family. Among all isoflavone groups, malonyl glycosides were found to be the most thermosensitive forms, with concentrations decreasing consistently at all working temperatures, while temperatures exceeding $140\text{ }^{\circ}\text{C}$ were necessary to observe β -glycoside degradation. Aglycones were found in low amounts, with daidzein showing the highest concentration.

Chien et al. (2005) also reported that malonyl-glycosides, the predominant form in nature, are easily degraded under moderate conditions leading to the formation of β -glycosides, which have been reported to be more bioavailable than malonyl-forms. The transition to aglycones, the least hydrophilic form of isoflavones, is less common in nature, but the most bioavailable form requires harsh conditions to remove the glycoside group. Similarly, Moras et al. (2017) reported an interchange between the highly thermolabile malonyl-glycosides and β -glycosides in the study of the extraction of isoflavones from soybean flour and soybean protein isolate using pressurized water extraction at temperatures exceeding $80\text{ }^{\circ}\text{C}$, with complete degradation at higher temperatures. Moras et al. (2017) predicted optimal isoflavone extraction through subcritical water hydrolysis, obtaining a 61.0% yield at $114\text{ }^{\circ}\text{C}$ for 2 min with soybean protein isolate, and an 85.1% yield at $122\text{ }^{\circ}\text{C}$ for 14 min with soybean flour. These optimal temperatures align with the

subW extractions in this study, although they require longer times, likely due to the use of soybean by-products rather than soybean flour or protein isolates, which contain higher and more easily extractable concentrations of these bioactive compounds. By contrast, Nkurunziza et al. (Nkurunziza, Pendleton, & Chun, 2019), reported optimal isoflavone extraction at 146.23 °C and 3.98 MPa for 213.5 min, differing from most studies and the present results; however, their work did not account for malonyl-glycosides, which represent the most significant fraction of total soybean isoflavones.

To further analyze the results presented in Figure 2, the interconversion pathways proposed among the three identified isoflavone forms are presented in the following scheme:



Scheme 1. Representation of the interconversion pathways between the three forms of each isoflavone analyzed in this study.

First-order reaction kinetics have frequently been used to analyze food components, and have been proven effective in describing the interconversion and degradation behaviors of isoflavones (Chien et al., 2005). Scheme 1 illustrates six different conversion

pathways, each with an assigned reaction rate constant k . This model does not consider acetyl forms, as these were not determined in the present study:

$$\frac{dC_{MG}}{dt} = -(k_1 + k_2 + k_6) C_{MG} \quad [1]$$

$$\frac{dC_{\beta G}}{dt} = k_1 C_{MG} - (k_3 + k_4) C_{\beta G} \quad [2]$$

$$\frac{dC_{Ag}}{dt} = k_3 C_{\beta G} + k_6 C_{MG} - k_5 C_{Ag} \quad [3]$$

where k_1 is the kinetic rate constant for the formation of β -glycoside from malonyl forms and k_3 and k_6 are the kinetic rate constants for the formation of aglycones (Ag) from β -glycoside (βG) and malonyl glycosides (MG) forms, respectively. k_2 , k_4 , and k_5 are the degradation kinetic constants of the malonyl, β -glycoside, and aglycone forms, respectively.

The rate constants for the proposed kinetic equations were obtained by simultaneously solving the set of differential equations for each model. These differential equations were solved numerically using a fourth-order Runge-Kutta method, and the parameters were obtained by minimizing the following objective function (O.F.) using the simplex Nelder–Mead method:

$$O.F. = \sqrt{\frac{\sum_{all\ compounds} \sum_{i=1}^n abs(C_{i,exp} - C_{i,calc})}{n}} \quad [4]$$

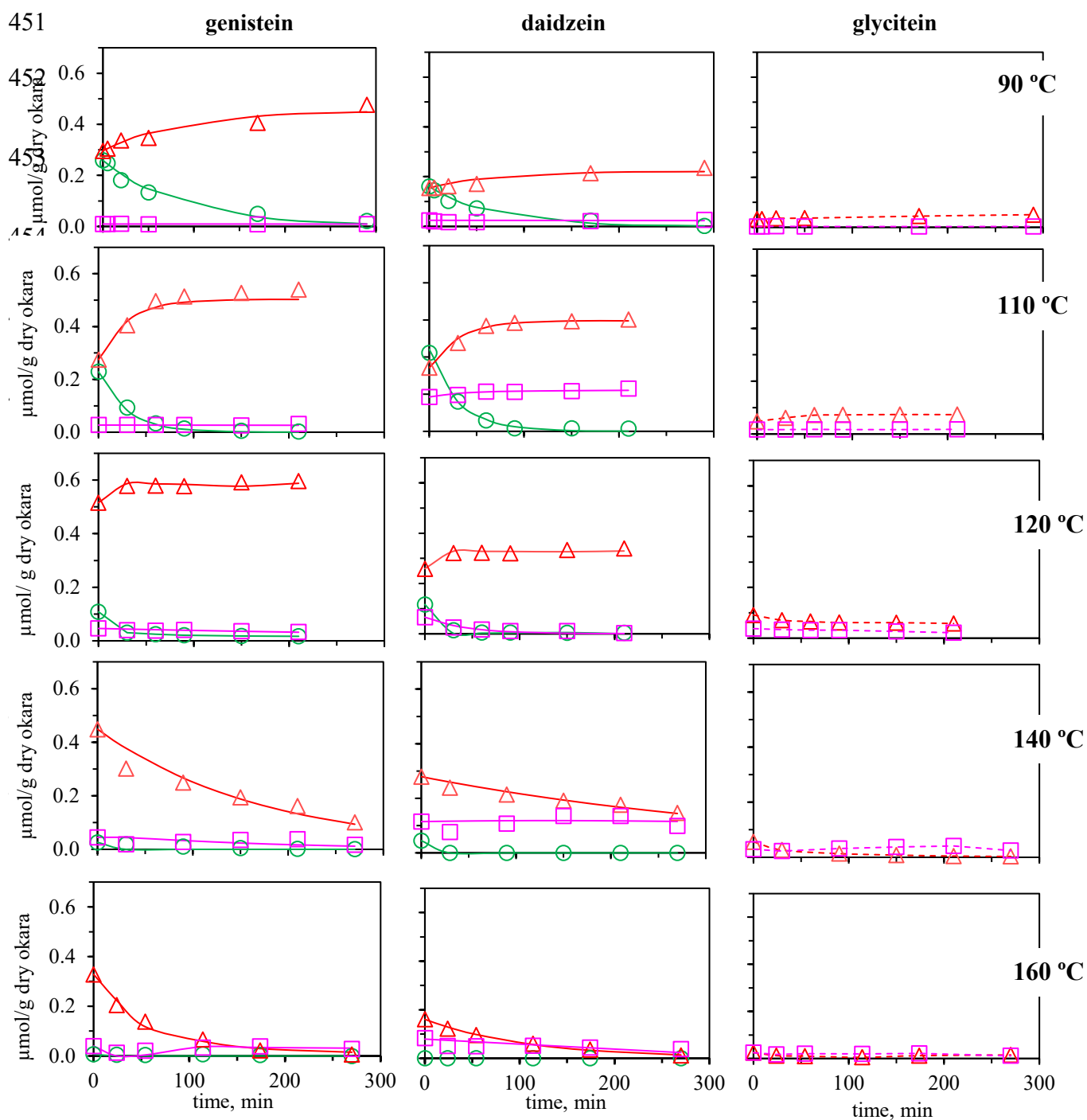


Figure 2. Kinetic curves of malonyl-glycosides (○), β-glycosides (△), and aglycones (□) of genistein (first column), daidzein (second column), and glycitein (third column) families at different temperatures (90 °C, 110 °C, 120 °C, 140 °C, and 160 °C from the first to the fifth row. Continuous lines represent the kinetic model of Eq 1-3 (parameters listed in Table 3S and 4S), and discontinuous lines for glycitein are used to guide the eye.

The kinetic parameters for the genistein and daidzein families are presented in Tables 3S and 4S. Due to the absence of a malonyl-glycitin standard in this study, kinetic parameters were not obtained for the glycitein group. Nevertheless, as previously noted, glycitein isoflavones constituted less than 3–10% of the total extracted mass of isoflavones, varying with extraction temperature. The temperature dependence of the reaction rate constants is described by the Arrhenius equation:

$$k = k_o \cdot \exp(E_a/RT) \quad [5]$$

Although the temperature-dependent trends for isoflavone form interconversions are not always fully consistent, several general conclusions can be drawn. Generally, the degradation rate constants for all isoflavone forms increased with temperature, with malonyl glycosides exhibiting the highest values. A deeper analysis of the isoflavone profiles of the genistein and daidzein families yielded similar conclusions, based on the kinetic rate constants presented in Tables 3S and 4S. The ratio of the reaction rate constants for malonyl-glycoside interconversion (for both genistein and daidzein family) to the β -glycoside and aglycone forms versus degradation to other degradation products, $(k_1+k_6)/k_2$, suggests that interconversion to other isoflavone forms occurs more rapidly than degradation to other compounds, particularly at low temperatures. Furthermore, a $k_1/(k_3 + k_4)$ ratio greater than one indicates that the formation of β -glycosides from malonyl-glycosides is faster than their conversion to aglycones or degradation into other products. Similarly, a value greater than one for the ratio k_6/k_3 suggests that aglycone formation from malonyl glycosides is faster than that from β -glycosides. Tables 3S and 4S also include the activation energy values for each interconversion step, showing generally high activation energies for all the steps, revealing high temperature sensitivity

of all isoflavone forms in the subcritical water reaction medium. For both isoflavone families (genistein and daidzein), the most temperature-dependent interconversion was observed in the conversion of β -glycosides to aglycones (k_3) or other degradation products (k_4). The continuous lines shown in Figure 2 represent the kinetic model proposed, with the corresponding kinetic parameters listed in Tables 3S and 4S.

Comparisons with literature are somewhat challenging due to variability in reported values, and most studies have reported changes in the isoflavone profile of soybean (no kinetics the by-product okara) subjected to different heating procedures, focusing on the genistein family. An et al. (2023) investigated the interconversion kinetics for the genistein family in isoflavone extraction from soybean in subW, at temperatures ranging from 100 °C to 180 °C over 3 to 30 min. Similar to the findings of this study, they observed a general trend of increasing degradation rate constants with increasing temperature, with malonyl-genistein being the most thermosensitive. A detailed analysis of their kinetic rate constants also suggests that genistin formation occurs faster than its consumption due to genistein formation or its degradation into other products. Additionally, they observed that genistein forms more rapidly from the acetyl form (as an intermediate of the malonyl group) than from the β -glycoside form. Regarding activation energies for the different steps, these authors (An et al., 2023) identified the degradation of genistein to unknown compounds as the most sensitive process with an activation energy of 1086 kcal/mol (4539.5 kJ/mol). This is an exceptionally high value compared to the data in this study or any other reported activation energies for kinetic processes.

Other kinetic studies in the literature also focus on the interconversion and degradation of isoflavone forms in soybean, but in media other than subW. Chien et al. (2005)

analyzed the interconversion and degradation of pure isoflavones during dry or moist heating at 100, 150 and 200 °C. Isoflavone standards (malonyl-genistin, acetyl-genistin, genistin, and genistein) were dissolved in methanol, and then the solution was placed in sealed ampoules that were immersed in an oil bath preheated to the selected temperature. On the other hand, Niamnuy et al. (2012), studied the drying kinetics of soybean, along with the inter-conversion and degradation of soy isoflavones, using gas-fired infrared combined with hot air vibrating drying at various temperatures (50, 70, 130 and 150 °C). Zhang & Chang (2016) treated soymilk, obtained by soaking and blanched soybean at 80 °C for 2 min, by UHT (ultra-high temperature) at 135 to 150 °C for 10 to 50 s with a pilot plant-scale Microthermics processor. In any case, in these studies, first-order kinetic models effectively described the changes among the isoflavone forms during heating, with the malonyl glycosides showing the highest degradation rate constants. Therefore, it can be concluded that the isoflavone profile, particularly malonyl glycosides, is greatly affected by the heating process.

3.1.2.1. TPC and antioxidant activity of OKW subW extractions

Figures 3a and 3b show the total polyphenol content (TPC) and reducing capacity, according to the FRAP assay, in the OKW subW extracts. Both parameters followed a similar trend, with the highest TPC and reducing capacity observed at 160 °C, while much lower values were recorded at lower temperatures of 110 °C and 120 °C. This pattern contrasts with the isoflavone content, which was higher at lower temperatures, whereas significant degradation was noted at higher temperatures.

These high TPC and reducing capacity values are likely due to browning reactions that occur at elevated temperatures, producing antioxidant compounds independent of

isoflavone concentration (Rimbach et al., 2003). Maillard Reaction Products (MRP), formed between sugars and amino groups during the treatment of okara at high temperatures, significantly influence the antioxidant capacity of these extracts. MRP are known for their ability to scavenge oxygen radicals and chelate metals (Li et al., 2012; Yilmaz & Toledo, 2005). Given these insights, it is evident that the high antioxidant activity observed at higher temperatures results from the degradation of compounds with strong antioxidant properties. These samples displayed more intense browning at elevated temperatures owing to MRP formation.

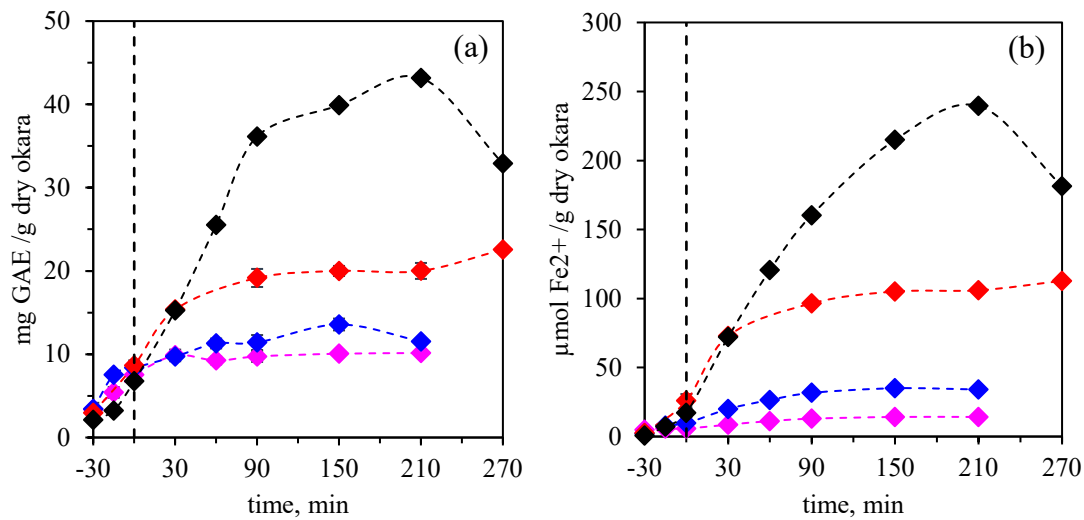


Figure 3. (a) Profile of total polyphenol content from OKW extracts by subW extraction (mg GAE /g dry okara). **(b):** Antioxidant activity of OKW subW extracts measured by FRAP (μmol Fe²⁺ /g dry okara), as a function of time (min): 110 °C (◆); 120 °C (◆); 140 °C (◆); 160 °C (◆)

Regarding the correlation between these two assays, it can be concluded that TPC and antioxidant capacity are directly correlated. However, isoflavone content does not show such a strong correlation due to the much higher antioxidant properties of other compounds generated during processing, primarily MRP.

3.2. Isoflavones extraction on crude okara (OKC)

3.2.1. Extraction by subcritical water

OKC has a high moisture content, exceeding 80 % w/w. During the beverage and solid residue separation process in soymilk production, a substantial amount of isoflavones remained in the retained solution. In the washing procedure of OKC to produce the OKW, the washing water contained approximately 40-55 µg of isoflavones/mL. This significant loss of isoflavones during the washing and drying processes makes OKC a promising candidate for further exploration to recover valuable isoflavones. Furthermore, the drying process is energy-intensive, and in the case of okara, it is particularly challenging to dry because of its tendency to spoil quickly during the drying processes unless it was not previously washed.

To explore OKC as a direct source of isoflavones, an extraction process was conducted using subcritical water (subW) at the optimal extraction temperature of 120 °C, previously determined using OKW as a reference, and at the same ratio of dry okara/solvent, 4.75 % (w/v) in a dry basis. For comparative purposes, the total isoflavone content during subW extraction at 120 °C for both OKW and OKC is plotted in Figure 4. The most notable difference observed was in the initial isoflavone content at the beginning of the extraction, with values of 364 and 716 µg isoflavone/g of dry okara for OKW and OKC, respectively. As previously noted, the higher initial isoflavone content in OKC was attributed to the isoflavones retained in the solution from okara and the beverage separation process. For both OKW and OKC, isoflavone content initially increased, reaching a maximum at 30 – 35 min, and then decreased due to isoflavone degradation at long treatment times. After 60 min, the levels stabilized. As described in

Figure 2, in the early stages of the treatment, malonyl forms were partially degraded but converted into more thermally stable β -glucoside forms; this plateau was observed after 60 min.

At the maximum, isoflavone content reached 1229 and 526.4 $\mu\text{g/g}$ dry okara, for OKC and OKW, respectively, with OKC yielding over twice as much isoflavone content as OKW. The initial isoflavones extraction rate, evaluated up to the maximum, was also higher for OKC, 13.7 $\mu\text{g/g}\cdot\text{min}$; nearly twice that of OKW (7.4 $\mu\text{g/g}\cdot\text{min}$). The slower extraction rate in the OKW may have resulted from thermal drying. In Figure 1S of the supplementary data, an HPLC chromatograms of the isoflavones identified in this work for OKC was plotted after 30 min and 60 min of treatment to observe the interchange between isoflavone forms as well as its degradation at longer operation times.

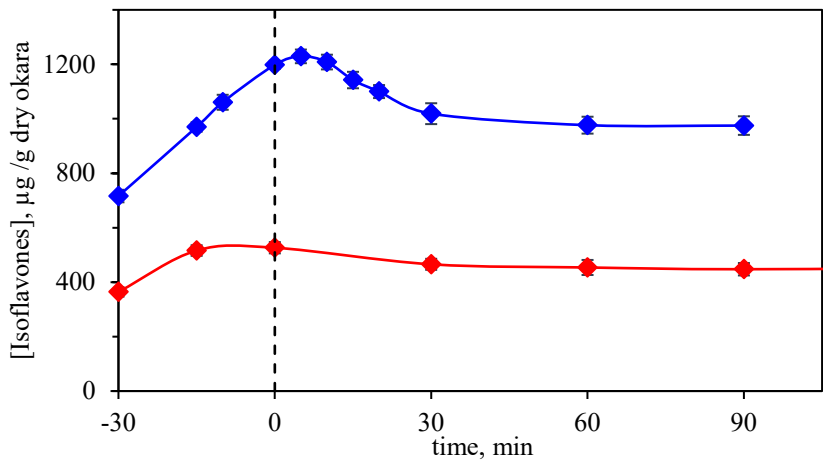


Figure 4. Isoflavone extraction ($\mu\text{g/g}$ dry okara) by subW treatment under the same conditions (120 $^{\circ}\text{C}$, 5 MPa) as a function of time (min). OKW ($\blacklozenge$); OKC ($\blacklozenge$).

The isoflavone extraction yield from OKC exceeds previous findings in the literature. Jankowiak et al. (2014) reported yields below 600 µg isoflavones/g okara, when using water as solvent, with a biomass concentration of 5 % dry okara at temperatures ranging from 20 °C to 95 °C, which resulted in higher aglycone concentration due to lab-scale production of okara, as previously indicated. Even with changes in solvent, as reported by Jankowiak et al. (2014), such as adjusting the pH (yielding 911 µg/g at pH 10) or adding ethanol (1018 µg/g, 70% ethanol), the extraction yields were lower than those obtained in this study, which used only pressurized water under mild conditions as a green solvent.

Isoflavone extraction from OKC was also conducted using the optimal hydroalcoholic mixture composition determined for OKW in conventional extractions (50% ethanol-water) in a pressurized vessel at 5 MPa and 120 °C. The highest extraction yield was achieved 30 min after initiating the heating process, with a value of 1216.6 µg/g OKC, which was similar to the value obtained using water alone. These results demonstrate that the use of pressurized fluids facilitates high isoflavone extraction yields when employing only water as the solvent, thereby eliminating the need for additional organic solvents when compared to conventional solvent extraction.

3.2.2. Comparison of isoflavones extraction by other methods

The results of isoflavone extraction from OKC were compared with those of other innovative and emerging extraction methods, including microwave assisted extraction (MAE) and ultrasound assisted extraction (UAE). The experiments were performed using OKC with 4.75 % dry okara in water.

3.2.2.1. *Microwave assisted extraction*

Figure 5a shows the extraction kinetics of total isoflavones from OKC using MAE at temperatures ranging from 110 to 140 °C. Experiments were not conducted at 160 °C because of the fast degradation of isoflavones observed at this temperature in previous subW treatments. At all the tested temperatures, an initial increase in isoflavone content was observed in the medium, except at 140 °C, where the degradation rate of isoflavones was faster than their extraction rate. The highest yields were achieved at lower temperatures of 110, 115, and 120 °C after 5 min of treatment, reaching similar values of 895, 885, and 888 µg/g dry okara, respectively. After reaching these peaks, the degradation rates increased at higher temperatures.

The profiles of the individual isoflavones at different temperatures are plotted in Figure 2S. It can be observed that at any higher temperature or time tested, the destruction of the malonyl forms is more notable than the formation or extraction of β-glycosides or aglycones, as the global recount of isoflavones is much lower. Treatments at 115 °C and 120 °C showed values similar to those at 110 °C, as the more thermolabile forms were not so affected in short times. In contrast, from 130 to 140 °C, the malonyl form degradation was more notable, while the β-glycoside concentration increased, but at a lower rate, notably decreasing the global quantity of isoflavones in the final extracts.

Few studies have addressed isoflavone extraction from okara using MAE. Tsubaki et al. (2009), reported the highest isoflavone yield from okara using water as solvent at 120 °C, with a 2-min heating period followed by 5 min of treatment, achieving 2191 µg/g dry okara. This value decreases as the temperature increases above 120 °C. At a low temperature of 60 °C, Tsubaki et al. (2009) achieved a yield of 2159 µg/g of dry okara,

but consisting of 59 % malonyl forms and 34 % β -glycosides. In contrast, at 120 °C, the proportion shifted to 22 % for malonyl and 69 % for β -glycosides. In any case, these values are much higher than the values usually reported for isoflavone extraction from okara (Jankowiak et al., 2014; Nkurunziza, Pendleton, & Chun, 2019).

3.2.2.2. Ultrasound assisted extraction

Isoflavone extraction from OKC was performed using UAE with a similar dry solid content of 4.75 % in water as the extraction solvent at 50 °C. The remaining experimental conditions are detailed in Section 2.6. The UAE results are shown in Fig. 5b. After 10 min of treatment, the isoflavone concentration reached a maximum of 928 $\mu\text{g/g}$ dry okara. From that point until the end of the process (30 min), a slight decrease was observed owing to isoflavone degradation.

Regarding the isoflavone forms, under the selected UAE conditions, slight degradation of malonyl glycosides was observed, with the highest value of 509 $\mu\text{g/g}$ of dry okara decreasing to 471–485 $\mu\text{g/g}$ after 20–30 min of extraction. Similarly, β -glycosides reached a peak of 401 $\mu\text{g/g}$ in dry okara and then decreased to 353 $\mu\text{g/g}$ after 30 min of UAE. The total aglycone content increased from 8 $\mu\text{g/g}$ to 29 $\mu\text{g/g}$ during the extraction period.

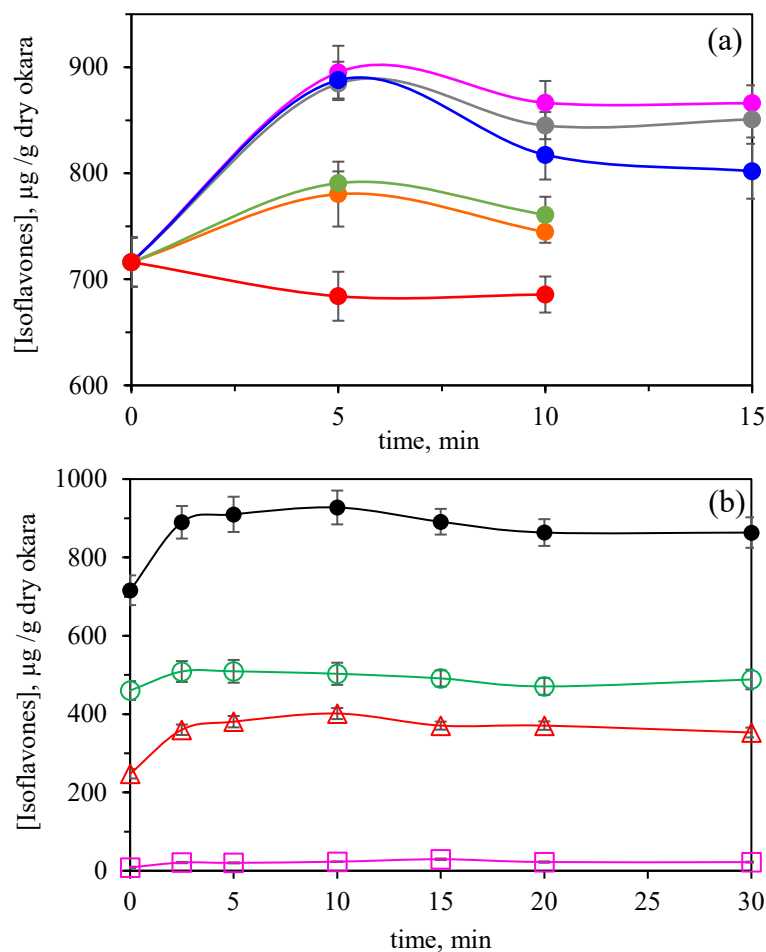


Figure 5. Total isoflavone extraction from OKC ($\mu\text{g/g dry okara}$) (a) by MAE treatment, as a function of time (min), at different temperatures. 110 °C (●); 115 °C (●); 120 °C (●); 130 °C (●); 135 °C (●); 140 °C (●) (b) by UAE treatment at 50 °C, as a function of time (min). Total isoflavones (●), subdivided in: Malonyl-glycosides (○), β -glycosides (Δ) and aglycones ($\square$).

3.2.2.3. Comparison of extraction technologies

Figure 6 and Table 2 compare the optimal extraction experiment for each technology applied to isoflavone extraction from OKC, as well as the best subW result for OKW. SubW achieved the highest isoflavone yield (1229 $\mu\text{g isoflavone/g dry okara}$) at 120 °C, 5 MPa, and 30 min of treatment. MAE and UAE technologies achieved 72.8 % and

75.4 % of the extraction efficiency of subW, respectively, with values of 895 and 928 μg isoflavone/g dry okara, respectively. The differences in the distribution of each isoflavone form among the four extraction technologies must be highlighted. The highest ratio of [β -glycoside + aglycone] to [malonyl-glycoside] was determined in the subW extracts. According to Scheme 1, subW treatment modifies the isoflavone profile owing to chemical transformations, and malonyl glycosides are easily degraded under moderate conditions in subW, leading to the formation of β -glycosides.

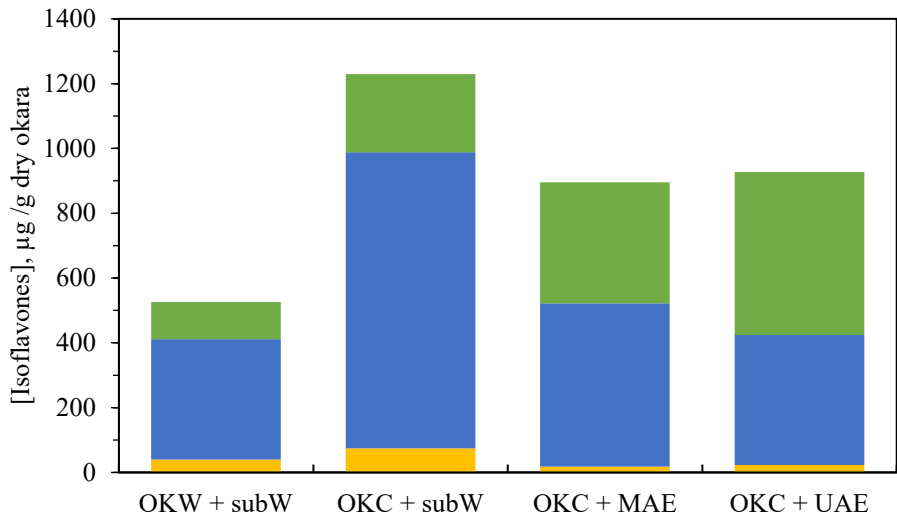


Figure 6. Total isoflavone extraction ($\mu\text{g/g}$ dry okara) and distribution of each isoflavone form (■ malonylglycosides-, ■ β -glycosides, ■ Aglycone) for the best extraction results obtained from each of the three methods studied (MAE at 110 °C for 5 min, UAE at 50 °C for 10 min, and subW at 120 °C for 30 min).

Because the maximum extraction yield was achieved at different treatment times, at the maximum isoflavone extraction yield, the productivity was evaluated considering time for comparison purposes, with values of 41.0, 82.5 and 92.8 μg isoflavone /g dry okara min for subW, MAE, and UAE, respectively. Although the lower productivity values

were achieved by subW extraction, when considering the distribution of different isoflavone forms, subW exhibited a significantly higher ratio of [β -glycoside + aglycone] to [malonyl-glycoside] at 4.09, compared to 1.40 and 0.84 for MAE and UAE, respectively, being these two isoflavones forms more noteworthy in terms of their functional properties (Hsiao et al., 2020). Thus, it can be concluded that subW treatment promoted the transformation of malonyl-glycoside into β -glycoside and aglycone forms, as shown in Figure 2 and Table 2. Table 2 also presents the total extraction yield along with the corresponding percentage of isoflavone content in the dry extracts (g isoflavones/g of total dry extract $\cdot$ 100), showing higher isoflavone concentrations in the dry extracts obtained from the subW and MAE extractions.

Table 2. Parameters for comparison of isoflavone extraction from crude okara (OKC) by subW, MAE, and UAE.

	Total ISF ($\mu\text{g/g}$ okara)	Ratio ISF ([β +A]/[M])	Productivity ($\mu\text{g ISF/g}\cdot\text{min}$)	% dry extract	% ISF in dry extract	Energy, J/ $\mu\text{g ISF}$
subW	1229	4.09	41	2.05	0.30	88
MAE	895	1.40	83	1.46	0.29	213
UAE	928	0.84	93	2.24	0.20	52

Further comparisons of the three technologies were performed in terms of energy consumption (see Table 2). UAE exhibited the lowest energy consumption per μg of extracted isoflavone, 52 J/ μg isoflavone, followed by subW at 88 J/ μg isoflavone, with MAE having the highest energy consumption at 213 J/ μg isoflavone. Ultrasound-assisted extraction achieved the highest productivity, while maintaining the lowest energy

consumption. This efficiency could be attributed to the cavitation effect generated in the solvent by ultrasonic waves, which allows the solvent to rapidly and effectively penetrate most matrices (Bustamante-Rangel et al., 2018). However, it is important to highlight the significantly more favorable [β -glycoside + aglycone] to [malonyl-glycoside] ratio achieved with subW compared with UAE, which recorded the lowest ratio among the three extraction techniques studied in this work (Table 2).

4. Conclusions

This work represents the first stage of a broader project aimed at the complete valorization of okara focusing on the obtention of isoflavones by water-based sustainable methods. Two types of okara were tested: (1) washed and dried okara and (2) untreated okara. Untreated okara resulted in higher isoflavone yields (1229.2 μg isoflavones /g dry okara, at 120 °C for 30 min) and energy savings by avoiding the drying stage.

A kinetic study revealed that subW promoted the interconversion between isoflavone forms and increased beta-glucosides. MAE and UAE technologies reached 72.8 and 75.4 % of subW extraction efficiency. UAE exhibited the highest productivity and lowest energy consumption. However, subcritical water extraction produced a significantly more favorable [β -glycoside + aglycone] to [malonyl-glycoside] ratio, as these two forms more bioavailable fractions.

These technologies offer potential for industrial-scale applications, providing improved efficiency, reduced processing times, and environmentally friendly alternatives to conventional extraction methods.

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