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RESEARCH

Cascade Valorization of Okara by Subcritical Water Hydrolysis: Assessment of Protein and Amino Acid Profile in Batch and Semi-Continuous Systems

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Abstract

This study assesses subcritical water hydrolysis (SWH) as a sustainable approach for protein recovery from okara, following isoflavone valorization. The initial extract, obtained at 120 °C, contained over 1200 µg of isoflavones per gram of dry okara. The solid residue underwent a second protein extraction step in both batch and semi-continuous modes. The batch extraction was conducted at 180 °C for 3 h at 5 MPa in a 0.5 L reactor. Under these conditions, total amino acid recovery reached 81 ± 3% (12.6 g/L), with 20 ± 1% of free amino acids, mainly glutamic acid, and a favorable profile of essential amino acids. These results were comparable to protein extraction from untreated okara, indicating that the prior isoflavone extraction did not hinder protein hydrolysis, but rather contributed positively within the cascade framework. Hydrolysis was also performed in a semi-continuous mode in a 2.3 L reactor, with a residence time of 2.75 min, in a two-stage temperature process (180 °C and 270 °C). The second stage yielded a significantly higher extraction than the first one, achieving a total 70.4% protein recovery (1.22 g/L) with 10% of free amino acids, with glutamic acid as the dominant one. Carbohydrate analysis revealed high hydrolysis yields for galactans, arabinans, and xylans, but these exhibited lower thermal stability compared to proteins. Okara emerges as a promising by-product for a bio-cascade process, combining a mild first stage for isoflavone extraction with a more severe second stage for protein extraction/hydrolysis.

Keywords Subcritical water · Okara · Isoflavones · Protein · Amino acid profile · Scaling-up process

Introduction

Okara is the primary by-product of soybean (*Glycine max*) processing, generated during the production of soymilk and tofu in the filtration phase. As the most widely cultivated and consumed legume worldwide, soybean plays a central role in global food systems. The demand for soybean-based products grows yearly, guided by an increasing awareness of their nutritional benefits, the increasingly extended use of soybean oil and the rising adoption of plant-based diets as alternatives. Soymilk and tofu, in whose production okara results as a by-product, are among the greatest substitutes for cow's milk and animal meat, respectively (Colletti et al., 2020).

World production of soybean in 2023 reached 378 Mt (million tons), a 5.1% increase from 2022. The leading producer is Brazil (152 Mt) followed by the US and Argentina. In Spain, production reached 7670 t (FAO, 2025a). Global production is estimated to increase up to 398 Mt in 2023/24, with forecasts predicting a continued annual growth of 6.1%,

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reaching approximately 423 Mt in 2024/25 and predicted to keep increasing for 2025/26 and upcoming years (FAO, 2025b). For every ton of soybean processed, approximately 1.1 tons of wet okara are generated as a by-product (Barea et al., 2025a). Therefore, the global production of wet okara can be estimated around 465 million tons only in 2025.

Okara has been extensively studied for its isoflavone content, with numerous works focusing on the hydrolysis and concentration of these compounds. Isoflavones are the most distinctive phytochemicals in soybean, present in high concentrations and known for their functional properties and health benefits due to their estrogen-like structure (Hsiao et al., 2020). Beyond isoflavones, one of the most valuable compounds in okara is protein, being present in high concentrations and offering a complete amino acid profile with all essential amino acids (Colletti et al., 2020). However, the sequential process for extraction of isoflavones followed by proteins has received far less attention, particularly when using subcritical water hydrolysis as an extraction technique. Additionally, detailed amino acid distribution analysis of these extracts is essential for evaluating their potential applications; but it is rarely reported.

In previous studies, isoflavone extraction from okara has been optimized using various techniques. Among them, subcritical water hydrolysis (SWH) under mild conditions (30-min heating up to 120 °C and 5 MPa) stands out as the most effective method (Barea et al., 2025a). In this work, SWH is also proposed here as a novel green method for a complete okara valorization including protein extraction from the protein-rich solid residue remaining after isoflavone extraction, which has demonstrated efficiency in recovering valuable compounds like proteins from various food by-products. SW uses pressurized liquid water at temperatures between 100 and 374 °C. These conditions make water act as both an acid and a base catalyst, due to a decrease in its dielectric constant and an increase in the ionic product (K_w), enhancing the hydrolysis of proteins into smaller peptides and free amino acids without requiring any added chemicals or enzymes (Barea et al., 2024; Haq et al., 2025).

Compared to enzymatic hydrolysis, which is the most commonly reported method for protein extraction/hydrolysis from okara (de Figueiredo et al., 2018; Ningrum et al., 2025), SWH offers significant advantages, such as shorter reaction times, higher degrees of hydrolysis, and greater release of free amino groups (Barea et al., 2023). SWH not only maximizes protein hydrolytic efficiency, but also enhances bioactive properties of hydrolysates. SWH at 180 °C proved highly effective in protein hydrolysis, avoiding unwanted residues like salts. All these factors make it a highly promising method for sustainably valorizing protein-rich by-products (Haq et al., 2025).

Peptides exhibit enhanced bioactive properties that are inactive in their native protein structure until released through hydrolysis. Several of these bioactive peptides, including soybean-derived peptides, have been associated with antidiabetic, antihypertensive (ACE-inhibition), antithrombotic or mineral-binding effects, besides antioxidant capacity (Haq et al., 2025; Marcet et al., 2016). High concentrations of low-molecular-weight peptides and free amino acids are associated with enhanced bioactivities. In addition to bioactive properties, hydrolyzed peptides also possess valuable techno-functional properties such as emulsifying and foaming capacity. Techno-functional and bioactive properties are closely related to the physicochemical characteristics of the hydrolysates, particularly peptide size and amino acid profile. Consequently, protein hydrolysates rich in these functional and bioactive molecules are considered promising ingredients for applications in nutraceuticals, functional foods, pet feed, and clinical nutrition (Barea et al., 2024; Marcet et al., 2016).

The aim of this study is to evaluate SWH efficiency as a green technology for protein hydrolysis from okara residue after isoflavone extraction, using batch and semi-continuous configurations within a sequential extraction approach. In the semi-continuous configuration, the biomass is retained in the extractor while pressurized hot water is pumped through it. This setup allows solvent (water) residence time to be shorter than the total extraction time, helping to preserve the integrity of the recovered fractions, which are continuously removed, being this configuration most preferred against batch configurations (Pedras et al., 2019; Ruiz et al., 2020). Furthermore, this configuration enables the recovery of extracts with different properties in a single assay, by simply modifying the water properties at the reactor inlet (Pedras et al., 2019). Results were also compared with those obtained using untreated okara to assess the advantages of performing a cascade valorization strategy. The hydrolysis efficiency of the carbohydrate fraction was also followed during treatment.

Materials and Methods

Raw Materials

Okara was supplied by *Frías Nutrición S.A.U.* (Burgos, Spain). This by-product presents 82.5% moisture, and it will be referred from now on as OK-1. OK-1 was preserved frozen at – 18 °C in individual plastic bags. For each experiment, the required amount was thawed and used without any prior drying or additional treatment. To facilitate comparison with other studies, all results were expressed on a dry basis. Characterization of the raw material was performed according to the methodology detailed in the "Analysis" section.

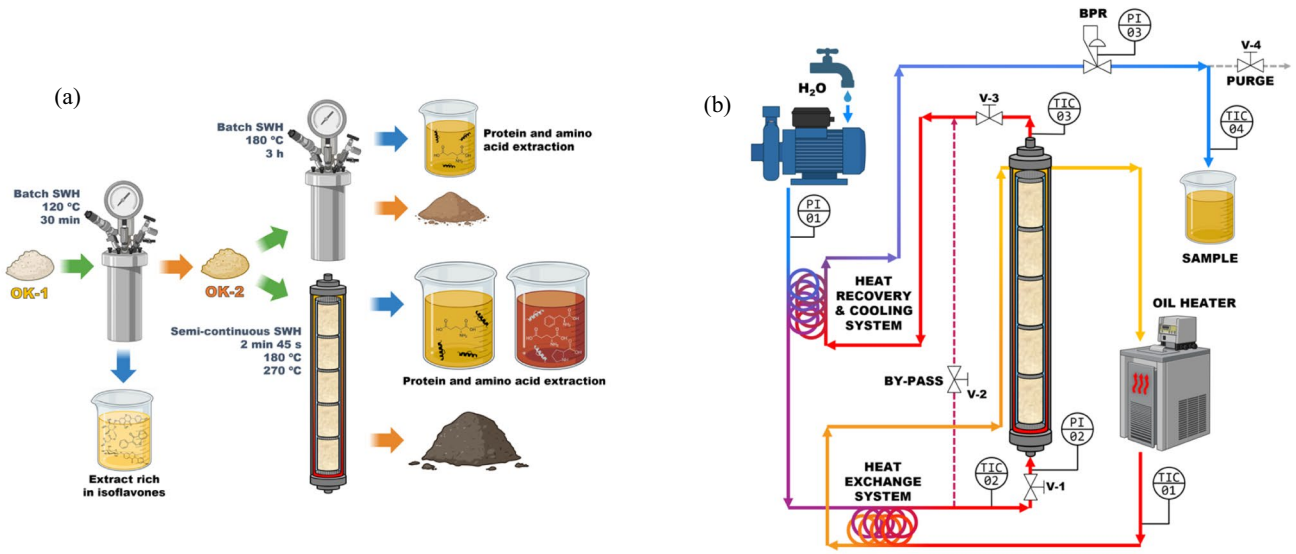


Fig. 1 **a** Schematic representation of the cascade valorization approach of okara by SWH. First step: isoflavone extraction from OK-1 at 120 °C; second step: protein extraction from OK-2. **b** Details of the semi-continuous SW configuration. BPR, system back-pressure regulator; V-1, reactor inlet valve; V-2, by-pass valve; V-3, reactor outlet valve; V-4, purge valve; TIC-01, heating fluid temperature; TIC-02, reactor inlet temperature; TIC-03, reactor outlet temperature; TIC-04, system outlet temperature (sampling); PI-01, system inlet pressure; PI-02, reactor inlet pressure; PI-03, system (regulated by BPR) pressure

Subcritical Water Process for Okara Valorization

In this study, a cascade approach was proposed to selectively fractionate compounds of high added value present in the OK-1 using SW, as illustrated in Fig. 1a. Initially, mild conditions (120 °C and 30 min) were selected based on previous studies to extract the isoflavone fraction present in the crude okara (OK-1) using a batch reactor (Barea et al., 2025a). Following this initial step, a liquid extract rich in bioactive compounds was generated. This optimized isoflavone extraction process also generated a solid residue (OK-2), whose subsequent valorization constitutes the main objective of the present work.

Pre-extracted okara was subjected to more severe conditions, ranging from 180 to 270 °C, to extract/hydrolyze the protein fraction and other structural components. This second step was conducted in both batch and semi-continuous configurations (Fig. 1a) for comparison.

Batch SWH reactor for protein extraction from pre-extracted okara.

The batch SW experiments were carried out in a lab-built batch reactor system with a total internal volume of 0.5 L. The reactor was externally heated using a ceramic heating jacket (230 V, 4000 W, Ø 95 mm, 160 mm height). A Pt100 temperature sensor placed inside the reactor was connected to a PID controller to regulate and monitor the temperature during the reaction. A needle valve connected to a cooling system enabled sample collection during hydrolysis.

In a typical run, 10 g of dry biomass (considering the moisture content) and 200 mL of distilled water were loaded into the reactor (5 wt.% of biomass loading on a dry basis). Operating pressure was fixed at 5 MPa by using nitrogen as pressurization agent, this pressure being enough to keep water in its liquid state at the testing operating temperatures of this work in the batch configuration. Two sequential batches were performed. In the first step, crude okara (OK-1) was charged into the reactor and treated at 120 °C to obtain an extract rich in isoflavones with low amounts of N solubilized at this extraction condition (Barea et al., 2025a). This extraction was repeated several times to accumulate enough solid residue (OK-2). In the second step, this solid residue was subjected to SWH at a higher temperature of 180 °C, which has been reported in the literature to be sufficient to promote protein hydrolysis from various sources (Barea et al., 2023; Trigueros et al., 2023). After each treatment step, the reactor was cooled and depressurized when the internal temperature dropped below 90 °C. The hydrolysates were filtered to separate solids and then stored at – 18 °C until analysis.

Semi-Continuous SWH Reactor for Protein Extraction from Pre-Extracted Okara

Semi-continuous SW experiments were carried out in a pilot-scale installation (*Eurotechnica GmbH*, Germany) equipped with a reactor of 2.3 L (*Sitec*, Germany) and a plunger-pump (NP25/12—500, *Speck*, Germany), allowing

a maximum operating temperature and pressure of 300 °C and 20 MPa, respectively (Fig. 1b). This semi-continuous extractor was built and designed in collaboration with *Eurotechnica*, which designs and manufactures high-pressure custom-made equipment. A tube with five baskets was fitted into the reactor, which made it easier to introduce and distribute the sample evenly along the reactor, avoiding agglomeration in the bottom. The tube is closed at the top and bottom with metallic filters, preventing the passage of any solid into the different connections of the equipment. The heating of the jacketed reactor is achieved by circulating the heating fluid through the jacket. Until the desired temperature was achieved, water was circulated through a bypass circuit, avoiding extraction during heating. The temperature of the heating fluid is regulated by a smart controller integrated into the heater unit (Oil Advanced D1-300-9-50, 380–415 V, 50 Hz, *Single*, *Group GmbH*), able to reach temperatures up to 300 °C. In a typical run, 100 g on a dry basis (considering the humidity content) were loaded into the reactor by conveniently placing 20 g on a dry basis in each one of the vessels previously described. Two different temperatures were sequentially applied, starting from 180 °C and going further to 270 °C. This two-step approach is designed to achieve high protein recovery in a shorter time frame, avoiding the need for extended treatment.

The pump frequency was fixed at 5 Hz providing a water flux of 0.84 L/min and the system was pressurized to 9 MPa to keep water in its liquid state when working at the highest temperature. Initially, water was recirculated through the bypass line. Once the desired water temperature was reached for the first extraction step, 180 °C, the bypass valve was closed and water began to flow through the biomass in the reactor. The effluent was collected continuously, and samples were taken every 1–2 L for analysis. After this first extraction step at 180 °C, the bypass valve was opened again and pre-heating water up to 270 °C was achieved in the bypass circuit, repeating the process. After completion of the whole process and cooling of the equipment to below 60 °C (as recommended by the manufacturer), the reactor was opened, and the remaining solid was recovered. Aliquots from each collected liquid fraction were filtered to remove suspended solids and stored at − 18 °C until further analysis.

Comparison of the results obtained in SW in both configurations will be compared by evaluating the severity factor. This factor is a dimensionless parameter that integrates time and temperature to assess the intensity of hydrothermal treatments and not only considering the operating temperature. It was evaluated through Eq. 1:

$$Severity\ factor = log(R_0); R_0 = t \cdot \exp\left(\frac{T-100}{14.75}\right) \quad (1)$$

where t is the residence time (min) and T the temperature (°C). The constant 14.75 reflects the activation energy for glycosidic bond cleavage (first-order kinetics), while 100 °C is the reference temperature for hemicellulose depolymerization (Illera et al., 2025). Furthermore, the severity factor has been defined as an optimum parameter in the scaling-up process to apply in the engineering of hydrothermal processes for the biorefinery concept and circular bioeconomy (Ruiz et al., 2021).

Analysis

All biomass characterization analyses were performed on extractive-free samples. Extractives were determined sequentially by H_2O extraction followed by ethanol extraction, according to the NREL protocols. Extractive-free biomass was dried and quantified gravimetrically for subsequent characterization.

Analysis of Saccharides

The polysaccharide fraction and derived products were analyzed by high-performance liquid chromatography (HPLC) following the National Renewable Energy Laboratory (NREL) protocols (Sluiter et al., 2008). The HPLC system was equipped with a Bio-Rad Aminex HPX-87H column and its corresponding pre-column, using 0.005 M H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. Detection was performed with both a refractive index detector and a variable wavelength detector (VWD) set at 210 nm; both detectors and the column were maintained at 40 °C. A sample volume of 10 µL was injected after filtration through 0.22-µm syringe filters (Scharlab).

Briefly, extract-free solid samples were subjected to two-step acid hydrolysis, according to the NREL protocol: The acid insoluble residue (AIR), ash, insoluble lignin, and polysaccharide determination were determined as described by Sluiter et al. (2008). Monomers and sugar-derived compounds were directly measured in liquid extracts and quantifying its yield considering biomass total sugars (Eq. 2). Oligomeric structural sugars in liquid hydrolysates were quantified by comparing monomer concentrations before and after acid autoclave hydrolysis (Eq. 3).

$$Monomer\ yield(\%) = \frac{(Monomeric\ sugar)_{hydrolysate}}{(Total\ sugar)_{initial\ biomass}} \times 100 \quad (2)$$

$$Oligomer\ yield(\%) = \frac{(Total\ sugar - Monomeric\ sugar)_{hydrolysate}}{(Total\ sugar)_{initial\ biomass}} \times 100 \quad (3)$$

The total yield of each sugar was calculated as the sum of its monomeric and oligomeric fractions.

Compounds were identified by comparing their retention times with pure analytical-grade standards. Xylose and galactose were determined by enzymatic spectrophotometric kits, due to the fact that both sugars elute at the same time in the current HPLC setup. L-Arabinose/D-Galactose Assay Kit (700,004,264, Megazyme) and D-Xylose Assay Kit (700,004,355, Megazyme) were used to determine the concentration of both monomers.

Lipid Content

The oil content of okara was determined by Soxhlet extraction (Buchi B-811) using hexane for non-polar lipids. Total lipids (polar + non-polar) were measured following the Bligh and Dyer (1959) method, and polar lipids were calculated as the difference between total and non-polar lipids.

Protein Content

Protein in the samples was estimated from the nitrogen content present in the samples as measured by the elemental analysis. The *N*-factor was calculated from the total amino acid profile (free + bound amino acids) and elemental nitrogen data, according to the NREL standard protocols. The purity of the protein hydrolysate was calculated by considering the total soluble solids as shown in Eq. 4:

$$Purity(\%) = \frac{(Protein)_{hydrolysate}}{(Total\ soluble\ solids)_{hydrolysate}} \times 100 \quad (4)$$

Elemental Analysis

Elemental composition (C, H, N, S) of raw material and the remaining solids was determined by an elemental microanalyzer EA Flash 2000 apparatus (Thermo Scientific, USA) with a thermal conductivity detector (TCD) integrated. Temperature in the oven and gas flow were set, respectively, at 900 °C and 250 mL/min for oxygen and 140 mL/min for helium, serving the latter as the carrier gas. An additional helium flow of 100 mL/min is used as reference for the detector. Calibration was performed with 4-aminobenzenesulfonamide as standard.

Total Soluble Solids

Aliquots of 1 mL of the final hydrolysate were filtered through a 0.22 µm syringe filter and dried in an oven at 105 ± 1 °C until constant weight, allowing to calculate the percentage of soluble solids in each liquid extract.

Isoflavone determination

The method for the determination of isoflavones has been described in detail elsewhere in the literature (Barea P. et al., 2025a). Briefly, isoflavones in the extract were analyzed by HPLC–DAD using an Agilent 1260 Infinity II/HP 1100 system equipped with a Phenomenex precolumn and a Kinetex biphenyl column (4.6 × 250 mm, 5 µm). Samples were filtered (0.22 µm) prior to analysis, and the column temperature was set at 25 °C. Separation was performed using a gradient of (A) 1% acetic acid in 0.005 M ammonium acetate (aqueous) and (B) 1% acetic acid in 0.005 M ammonium acetate (in acetonitrile), at a flow rate of 0.8 mL/min, from 98 to 20% A over 90 min. Detection was carried out at 254 nm, with additional monitoring at 280, 330, and 370 nm, and full spectra (190–950 nm) were recorded for peak identification. Quantification and identification were based on calibration with eight isoflavone standards (daidzin (D) and genistin (G) from *TargetMol*; glycitin (GY) and malonyl-genistin (MG) from *ChemCruz*; daidzein (DE) from *Thermo Scientific*; glycitein (GYE) from *Chengdu Biopurify Phytochemicals*; genistein (GE) from *Fluorochem*; and malonyl-daidzin (MD) from *ChemFaces*.

Amino Acid Profile and Total Quantification

The amino acids were analyzed by gas chromatography (Hewlett-Packard, 6890 series) with flame ionization detection (GC-FID). Derivatization of amino acids is required prior to analysis to increase their volatility, allowing them to be analyzed by GC without requiring excessively high temperatures that would otherwise lead to their thermal degradation. The derivatization was carried out following the method detailed by Barea et al. (2025b) based on the method reported by Hušek (1991), which involves a mixture of ethanol/pyridine, Norvaline as internal standard, propylchloroformate (PCF) as derivatization agent and chloroform with 1% PCF as organic phase. Four microliters of the organic phase were injected at a 1:15 split ratio at 250 °C into a ZB-AAA column (10 m × 0.25 mm I.D., *Phenomenex Inc.*). The initial temperature of 110 °C was maintained for 1 min; then, the oven temperature was programmed from 110 to 320 °C at 32 °C/min. Helium was used as a carrier gas at 60 kPa and nitrogen was used as a make-up gas. The detector temperature was set at 320 °C. Amino acids were identified using pure standards for the different amino acids and with norvaline as internal standard.

Amino acid profile of structural amino acids from biomasses, hydrolysates or residual solids was determined after acid hydrolysis using 6 N HCl at 100 °C during 24 h and further derivatization. Tryptophan, cysteine and methionine are lost by acid hydrolysis, so an alkaline hydrolysis (4.2 M

NaOH at 100 °C during 24 h) was also carried out to determine these amino acids. Glutamine and asparagine can be underestimated being considered as glutamic acid and aspartic acid respectively, due to deamination; however, they have been reported accordingly in the results.

In the liquid hydrolysates, free amino acids were quantified without previous acid/alkaline hydrolysis, while total amino acids were determined after complete acid/alkaline hydrolysis which would yield to the medium the free and bound amino acids forms. Free amino acids percentage was calculated as shown in Eq. 5. Bound amino acids (in peptides and proteins) were calculated as the difference between total and free amino acids (Eq. 6):

$$Free\ aa(\%) = \frac{(Free\ aa)_{hydrolysate}}{(Total\ aa)_{initial\ biomass}} \times 100 \quad (5)$$

$$Bound\ aa(\%) = \frac{(Total\ aa - Free\ aa)_{hydrolysate}}{(Total\ aa)_{initial\ biomass}} \times 100 \quad (6)$$

The hydrolysis yield of each amino acid in the semi-continuous SWH configuration was calculated as the ratio between the cumulative amount of free amino acid released during each interval and the initial total content of that amino acid in the raw biomass.

Total Nitrogen

Total nitrogen (TN) in the hydrolysates was quantified using a total organic carbon analyzer (Shimadzu TOC-V CSN). Nitrogen-containing compounds were oxidized by catalytic thermal decomposition during combustion, producing nitrogen oxides (NO_X) which can be detected by a chemiluminescence detector integrated in the instrument. Potassium nitrate (KNO_3) was used as the calibration standard.

The nitrogen content of each hydrolysate, together with its corresponding amino acid profile, was used to calculate the specific nitrogen-to-protein conversion factor (N factor), which in turn was applied to estimate accurately the protein content of each hydrolysate.

Statistical Analysis

All the samples were produced and analyzed at least two times and expressed as mean ± standard deviation of the replicates. Data was analyzed using the software Statgraphics19 X64, and the significance of the differences between sample results was determined based on an analysis of the variance with the Fisher's Least Significant Difference (LSD test) at p-value ≤ 0.05.

Results and discussion

Characterization of Okara

The chemical composition on a dry basis of the original okara (OK-1) is shown in Table 1. The protein content was 29.7 ± 0.5% (w/w).

The amino acid distribution of okara is presented in Table 2. Glutamic acid was the most abundant amino acid (57 ± 4 mg/$g_{dryOK\text{-}1}$), followed by aspartic acid (23 ± 1 mg/$g_{dryOK\text{-}1}$), representing 28% and 11% of the amino acids, respectively. These values are consistent with previous reports in the literature, although some differences may exist depending on the crop origin and cultivar (Colletti et al., 2020; Kumar et al., 2016). OK-1 exhibits a great amino acid profile with a potentially high nutritional value in its hydrolysates, owning 38% of essential amino acids (EAA) in its profile (78 ± 2 mg/$g_{dryOK\text{-}1}$). Lysine (15 ± 1 mg/$g_{dryOK\text{-}1}$), phenylalanine (14.6 ± 0.8 mg/$g_{dryOK\text{-}1}$), isoleucine (12.4 ± 0.3 mg/$g_{dryOK\text{-}1}$), leucine (11.4 ± 0.7 mg/$g_{dryOK\text{-}1}$), and valine (9.3 ± 0.4 mg/$g_{dryOK\text{-}1}$) were the most prevalent EAAs. The EAA profile was consistent with other values reported in the literature. For example, Colletti et al. (2020) reported the same dominant EAAs, although leucine was the most abundant (phenylalanine was reported together with tyrosine). Kumar et al. (2016) also found leucine as most abundant, followed by phenylalanine, while maintaining the same top five EAAs.

Extractives in water were 12.2 ± 0.5% (w/w), which included 3.3 g/100 g $_{dryOK\text{-}1}$ of water-soluble protein. Ash and lipid content was 3.3 ± 0.1% (w/w) and 9.5 ± 0.4% (w/w), respectively. The major sugars present in the OK-1 were galactans and glucans, with values of 12 ± 1% (w/w) and 10 ± 1% (w/w), respectively. Acid insoluble lignin accounted for 8.3 ± 0.4% (w/w). Data more detailed can be found in Table 1. The elemental analysis composition of OK-1 yielded values of 49.9 ± 0.4% (w/w) of C, 5.9 ± 0.2% (w/w) of N and 8.0 ± 0.1% (w/w) of H.

Composition values were consistent with those reported by O'Toole (1999) for three different soybean cultivars, which showed protein contents ranging from 25.4 to 28.4%, lipid contents from 9.3 to 10.9%, insoluble fiber between 40.2 and 43.6% containing 11.7 ± 1.4% of lignin. Insoluble fiber values subtracting its lignin content correspond to approximately 30%, a value that agrees with actual insoluble saccharide content in OK-1.

Other studies, such as those from Li et al. (2012) and Vong and Liu (2016), report several values and wider ranges for okara biomolecule contents, with protein between 15.2 and 33.4%, fat between 8.3 and 10.9%, total fiber between

Table 1 Detailed composition (on a dry basis) of raw material (OK-1), solid residue after SW batch treatment at 120 °C (OK-2), solid residue after SW batch treatment and solid residue after SW 2-step semi-continuous treatment

Compound	OK-1, %	OK-2, %	Batch (OK-2) residue, %	Semi-continuous (OK-2) residue, %
Saccharides	32 ± 4	29 ± 2	44 ± 2	3.1 ± 0.2
Glucans	10 ± 1	15.9 ± 0.7	38 ± 2	0.91 ± 0.01
Galactans	12 ± 1	6.2 ± 0.3	4.1 ± 0.2	-
Xylans	2.0 ± 0.3	1.4 ± 0.1	0.07 ± 0.01	-
Arabinans	3.9 ± 0.9	2.3 ± 0.2	0.032 ± 0.003	-
Uronic acids	3.9 ± 0.5	2.9 ± 0.4	0.22 ± 0.02	2.2 ± 0.2
Extractives in H_2O	12.2 ± 0.5	n.d.	n.d.	n.d.
Extractives in EtOH	5.3 ± 0.5	n.d.	n.d.	n.d.
Protein	29.7 ± 0.5	32.8 ± 0.6	8.3 ± 0.4	10.5 ± 0.4
Lipid	9.5 ± 0.4	n.d.	n.d.	n.d.
polar	1.8 ± 0.3	*n.d.*	*n.d.*	*n.d.*
non-polar	7.8 ± 0.2	*n.d.*	*n.d.*	*n.d.*
Lignin	16 ± 1	31.7 ± 0.3	48 ± 3	83 ± 2
soluble	8 ± 1	6.1 ± 0.1	3.0 ± 0.2	2.5 ± 0.1
non-soluble	8.3 ± 0.4	25.6 ± 0.3	45 ± 3	81 ± 2
Ash	3.3 ± 0.1	4.0 ± 0.1	5.5 ± 0.2	6.1 ± 0.4
Elemental C, wt.%	49.9 ± 0.4	53.9 ± 0.3	60.1 ± 0.7	68.1 ± 0.2
Elemental N, wt.%	5.9 ± 0.2	7.0 ± 0.3	1.70 ± 0.08	2.7 ± 0.6
Elemental O, wt.%	32.9 ± 0.5	26.6 ± 0.4	24 ± 1	14 ± 1
Elemental H, wt.%	8.0 ± 0.1	8.5 ± 0.1	9.0 ± 0.1	9.3 ± 0.3
N:C molar ratio	0.10 ± 0.004	0.11 ± 0.005	0.024 ± 0.001	0.034 ± 0.008
O:C molar ratio	0.49 ± 0.01	0.37 ± 0.01	0.36 ± 0.01	0.22 ± 0.01
H:C molar ratio	1.92 ± 0.03	1.89 ± 0.02	1.80 ± 0.03	1.64 ± 0.05

n.d.: not determined

42.4 and 58.1%, and ash between 3.0 and 4.5%. The values obtained in the present work are within the mid-range of these reported values. The total fiber values reported in these studies, which do not separate lignin, are in agreement with the sum of saccharides and lignin determined in the present work, 48 ± 5%.

First Valorization Step: Isoflavone Extraction

OK-1 was subjected to a first valorization step (Fig. 1a) according to the experimental conditions optimized by Barea et al. (2025a): 120 °C and 30 min of SW treatment. After treatment a liquid extract was obtained with a high content of isoflavones, more than 1200 µg of isoflavones per g of dry OK-1, with a great isoflavone profile ([β-glycoside + aglycone] to [malonyl-glycoside] ratio of 4.09). More details of the isoflavones profile can be found in previous work (Barea et al., 2025a).

The solid residue generated after treatment will be referred to as OK-2. Chemical characterization of this solid is performed and detailed in Table 1, together with the data of OK-1 for a better comparison. Due to the solubilization and extraction of the easily extractive compounds in this first step, concentration for some of the insoluble compounds increased in the OK-2, especially insoluble acid lignin increasing its value up to 25.6 ± 0.3% (w/w). Glucans content also increased compared to OK-1, 15.9 ± 0.7% (w/w), while galactans decreased to 6.2 ± 0.3% (w/w). The protein content is assessed by the total nitrogen content determined in the elemental analysis and the amino acid profile of the OK-2 reported in Table 2. The total protein content was also higher in the OK-2 compared to OK-1, 32.8 ± 0.6% (w/w) according to the higher N content of the residue OK-2 determined by elemental analysis (53.9 ± 0.3% (w/w) of C, 7.0 ± 0.3% (w/w) of N and 8.5 ± 0.1% (w/w) of H). However, there were no significant differences in the total amino acid (TAA) content between OK-1 and OK-2 with a similar amino acid profile for both, being the major amino acids glutamic and aspartic acids, although slightly lower values were determined for glutamic acid in the OK-2 probably due to its high solubility in polar solvents. Similar content of EAA was found in the OK-2 (37.4%) compared to OK-1. The same five EAA as in OK-1 were predominant in OK-2 (Lys, Phe, Ile, Leu, and Val at 12.1 ± 0.8, 13 ± 1, 10.5 ± 0.4, 12 ± 1, and 9 ± 1 mg/$g_{dryOK-2}$, respectively, maintaining their relative order). The high protein content of the solid residue

Table 2 Amino acid profile of OK-1, OK-2, and OK-2 extract obtained after SW at 180 °C for 3 h at 5% wt. of OK-2, charged on a dry basis (percentage of free (Eq. 5), bound (Eq. 6), and total amino acids)

Amino acid	Initial aa, mg/$g_{dryOK-1}$	Initial aa, mg/$g_{dryOK-2}$	Liquid hydrolysate from OK-2			Solid residue from OK-2	
			Free aa, %	Bound aa, %	Total aa, %	Remaining aa, %	Remaining aa, mg/$g_{drySolid}$
Ala	7.9 ± 0.4[a]	8.9 ± 0.8[a]	18 ± 2	68 ± 11	86 ± 13	23 ± 2	2.02 ± 0.08
Gly	10 ± 1[a]	9.7 ± 0.9[a]	17 ± 2	54 ± 6	71 ± 8	24 ± 3	2.3 ± 0.1
Val	9.3 ± 0.4[a]	9 ± 1[a]	4.8 ± 0.5	75 ± 8	80 ± 9	22 ± 3	1.94 ± 0.09
Leu	11.4 ± 0.7[a]	12 ± 1[a]	4.0 ± 0.4	79 ± 7	83 ± 7	23 ± 3	2.7 ± 0.2
Ile	12.4 ± 0.3[b]	10.5 ± 0.4[a]	3.9 ± 0.2	68 ± 6	72 ± 6	20 ± 1	2.09 ± 0.09
Thr	1.2 ± 0.1[a]	1.4 ± 0.1[a]	7 ± 2	81 ± 11	88 ± 13	15 ± 3	0.21 ± 0.03
Ser	8.0 ± 0.1[b]	6.4 ± 0.1[a]	6 ± 2	65 ± 2	71 ± 4	20 ± 3	1.3 ± 0.2
Pro	11.0 ± 0.5[a]	11 ± 1[a]	7.4 ± 0.7	73 ± 15	80 ± 16	18 ± 2	1.9 ± 0.1
Asn	1.3 ± 0.2[a]	0.81 ± 0.04[a]	12.3 ± 0.9	64 ± 12	76 ± 13	26 ± 1	0.21 ± 0.01
Asp	23 ± 1[a]	27 ± 2[a]	26 ± 2	66 ± 5	92 ± 7	2.6 ± 0.5	0.7 ± 0.1
Met	4.8 ± 0.2[a]	4.1 ± 0.2[a]	29 ± 1	16 ± 4	45 ± 5	53 ± 5	2.2 ± 0.2
Hyp	0.81 ± 0.04[b]	3.1 ± 0.2[a]	13.2 ± 0.9	72 ± 5	85 ± 6	23 ± 2	0.70 ± 0.02
Glu	57 ± 4[b]	43.1 ± 0.8[a]	46 ± 2	37 ± 4	83 ± 6	12.2 ± 0.5	5.3 ± 0.2
Phe	14.6 ± 0.8[a]	13 ± 1[a]	8.0 ± 0.6	72 ± 14	80 ± 15	21 ± 2	2.8 ± 0.2
Cys	1.0 ± 0.1[a]	0.9 ± 0.1[a]	4.9 ± 0.8	85 ± 13	90 ± 14	7 ± 2	0.06 ± 0.01
Gln	0.44 ± 0.04[a]	0.4 ± 0.1[a]	65 ± 12	14 ± 3	79 ± 15	15 ± 3	0.064 ± 0.001
Lys	15 ± 1[a]	12.1 ± 0.8[a]	4.5 ± 0.7	74 ± 5	79 ± 6	14 ± 3	1.7 ± 0.4
His	6.1 ± 0.5[a]	5.1 ± 0.6[a]	19 ± 3	65 ± 8	84 ± 11	14 ± 3	0.7 ± 0.1
Tyr	5.0 ± 0.1[a]	5.4 ± 0.7[a]	9 ± 2	80 ± 13	89 ± 15	18 ± 3	0.96 ± 0.08
Hyl	1.34 ± 0.03[b]	0.94 ± 0.03[a]	11 ± 2	79 ± 3	90 ± 5	12 ± 4	0.11 ± 0.04
Trp	2.6 ± 0.1[a]	3.0 ± 0.1[b]	4.0 ± 0.5	85 ± 1	89 ± 2	19 ± 1	0.57 ± 0.02
TAA	204 ± 5[a]	187 ± 3[a]	20 ± 1	61 ± 2	81 ± 3	16.3 ± 0.4	30.5 ± 0.6
EAA	78 ± 2[a]	70 ± 2[a]	7.6 ± 0.3	70 ± 3	78 ± 3	21 ± 1	14.9 ± 0.5

Ala alanine, glycine, *Val* valine, *Leu* leucine, *Ile* isoleucine, *Thr* threonine, *Ser* serine, *Pro* proline, *Asn* asparagine, *Asp* aspartic acid, *Met* methionine, *Hyp* hydroxyproline, *Glu* glutamic acid, *Phe* phenylalanine, *Cys* cysteine, *Gln* glutamine, *Lys* lysine, *His* histidine, *Tyr* tyrosine, *Hyl* hydroxylysine, *Trp* tryptophan, *TAA* total amino acids, *EAA* total essential amino acids. Values with different letters (a, b) in "initial aa" columns are significantly different when applying the LSD method at p-value ≤ 0.05

offers a great potential for further valorization to extract/hydrolyze its protein content adding value to the cascade valorization.

Second Valorization Step: Protein Extraction

Batch Configuration

Protein Extraction from Pretreated Okara (OK-2)

The solid residue generated after the SW treatment contains a considerable protein content (32.8 ± 0.6%) with a valuable amino acid profile (Table 2). This solid residue (OK-2) was subjected to a second SW fractionation at 180 °C for 3 h (5% wt. of dry biomass loading into the extractor) to analyze the extraction/hydrolysis of protein fraction.

Table 2 reports the quantification of individual free (Eq. 5) and bound (Eq. 6) amino acids in the liquid hydrolysate, as well as the amino acid profile of the remaining protein fraction in the solid residue after treatment. SW treatment yielded more than 80% (81 ± 3%) of total amino acid extraction (or total protein extraction), indicating a highly effective hydrolysis under subcritical water conditions. In the liquid hydrolysate, 20 ± 1% were determined as free amino acids and 61 ± 2% as bound amino acids in small peptides released to the extraction medium, referring these percentages to the total initial individual amino acids determined in the OK-2.

Glutamic acid reached 1.70 ± 0.07 mg/mL in the hydrolysate, 83 ± 7% of initial total in OK-2. Glutamic acid accounted for 24% of the total amino acids extracted in OK-2 hydrolysate. Focusing on the free amino acid fraction, glutamic acid represents more than half, 53% (noticeable in Fig. 2). This composition offers a great potential in different

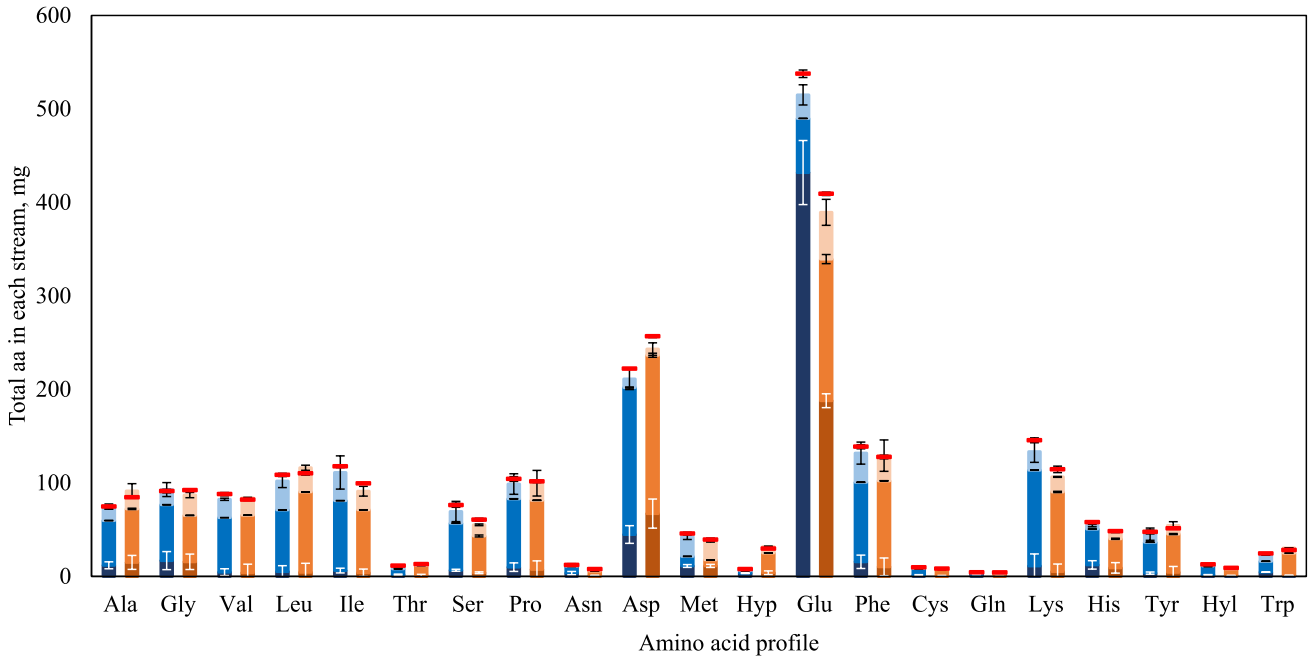


Fig. 2 Amino acid profile distribution after SW treatment at 180 °C for 3 h from OK-1 and OK-2 hydrolysates: Free aa: OK-1 ■, OK-2 ■ ; bound aa: OK-1 ■, OK-2 ■; remaining on solid: OK-1 ■, OK-2 ■; initial total mg of amino acids (considering the okara charged into the reactor) ■. Ala: alanine, Gly: glycine, Val: valine, Leu: leucine, Ile: isoleucine, Thr: threonine, Ser: serine, Pro: proline, Asn: asparagine, Asp: aspartic acid, Met: methionine, Hyp: hydroxyproline, Glu: glutamic acid, Phe: phenylalanine, Cys: cysteine, Gln: glutamine, Lys: lysine, His: histidine, Tyr: tyrosine, Hyl: hydroxylysine, Trp: tryptophan

food applications. Despite not being an EAA, glutamic acid has an important role as a neurotransmitter; besides, it is commonly used to produce glutamate and also contributes to the synthesis of antioxidant compounds such as glutathione (Sano, 2009). Glutamate is known as the main compound behind the umami taste and acts as a natural flavor enhancer; it can improve the taste of foods, reducing salt levels, helping promote healthier eating without raising fat or calorie intake. It is also a versatile amino acid that plays roles in flavor perception, metabolic pathways, and neural signaling (Jinap & Hajeb, 2010).

Other prevalent amino acids in the OK-2 hydrolysate, such as aspartic acid, have applications in pharmaceutical, chemical, and cosmetic industries as precursors for active compounds. Meanwhile, essential amino acids like isoleucine are primarily used as supplements in food and feed industries, and others (including the rest of more prevalent EAA: lysine, leucine, phenylalanine, and valine) are commonly used in both sectors (Madhariya et al., 2023).

After this second SW step, a solid residue was generated whose elemental composition is also presented in Table 1. The percentage of this solid residue accounted for 54% of the OK-2 charged into the extractor. According to the high protein extraction/hydrolysis as determined in the liquid stream with free and bound amino acids profile, the nitrogen content on the solid (Table 1) was only 1.70 ± 0.08% (w/w) while the C content increased up to 60.1 ± 0.7% (w/w). The N/C molar ratio (Table 1) decreases markedly from 0.11 in OK-2 to 0.02 in the corresponding residue after treatment, confirming the substantial hydrolysis of nitrogen-containing compounds. This is consistent with a relative concentration of saccharides and lignin, which are rich in carbon. In contrast, the slight decrease in O/C and H/C molar ratios (from 0.37 to 0.36 and from 1.89 to 1.80, respectively) suggests that the levels of oxygen and hydrogen bound to the remaining carbon-rich structures were mainly preserved during the process.

The amino acid profile of this residual solid is presented in Table 2. Analyzing the individual amino acid composition, it is observed that 16.3 ± 0.4% of the total initial amino acid content in the OK-2 introduced into the reactor remains in the final solid fraction. Glutamic acid remains the predominant amino acid in this solid, with a concentration of 5.3 ± 0.2 mg/g dry solid (Table 2), although only 12.2 ± 0.5% of the initial amount persists, followed by phenylalanine and leucine, with concentrations of 2.8 ± 0.2 and 2.7 ± 0.2 mg/g dry solid, respectively, representing 21 ± 2% and 23 ± 3% of their initial quantities.

Comparison of these data with previously published studies remains challenging due to the limited availability of detailed amino acid distribution data in okara hydrolysates, as noted by Kumar et al. (2016). This highlights

Table 3 Severity factor evaluated according to Eq. 1 calculated for each one of the treatments carried out in the present work, in addition to isoflavone optimum extraction from previous studies (Barea et al., 2025a)

	Isoflavones from OK-1	Protein from OK-2		
	Batch	Batch	Semi-continuous	
Temperature, °C	**120**	**180**	**180**	**270**
Time, min	30	180	2.75*	2.75*
Severity factor	2.07	4.61	2.79	5.44
Free aa recovery, %	n.d	20 ± 1	1.7	11.1
Protein recovery, %	n.d	81 ± 3	18.3	52.2

n.d. not determined

*Residence time for the extract in the semi-continuous reactor (τ = V/F)

the importance of this study, which proposes a two-step valorization process to extract both bioactive compounds, isoflavones, and proteins (focusing on detailed amino acid profiles).

Wiboonsirikul et al. (2013) showed protein extraction from okara by using SWH concluding that high temperatures must be used for an effective protein extraction, with values of 7 g/L of extracted protein when working at 240 °C for 5 min (corresponding to a severity factor of 4.82, Eq. 1). These authors showed slower protein extraction at 170 °C with increasing protein extraction from 5 to 40 min with values of 2.5 g/L to 4.0 g/L of protein, respectively. No results are shown after that time, but the trend presented by these authors suggests that the extraction would likely continue increasing. In this work, considering the OK-2 charged into the reactor and the percentage of amino acids (both free and bound) in the liquid hydrolysate, a total amino acid concentration of 7.2 g/L was obtained (log R_0 = 4.61, Table 3). However, a direct comparison of extraction yield is not possible, as the proportion of initial protein recovered was not reported.

In comparison with other studies that employed more severe extraction conditions, it is noteworthy that, in terms of the severity factor for amino acid extraction, Quitain et al. (2001) and Rogalinski et al. (2005) reported achieving their highest amino acid yields at considerably higher severity values. Specifically, Quitain et al. (2001) research identified optimal yields at severity factors of 5.6, 5.9, and 6.2 (250 °C for 15–60 min), whereas Rogalinski et al. (2005) observed maximum extraction at severity factors of 5.6 and 5.7 (290 °C for 1.1–1.3 min). These values are clearly higher than the severity factor applied in the present study (log R_0 = 4.61; Table 3), suggesting that more severe processing conditions are generally required to maximize amino acid release, particularly in their free form.

Several studies in the literature have focused on protein recovery from okara using conventional alkaline extraction. For example, Chan and Ma (1999) achieved a 53% protein recovery by adjusting the pH to 9.0 with NaOH at 80 °C, followed by isoelectric precipitation, obtaining a final extract with over 80% protein purity. Similarly, Tao et al. (2019) used "extreme alkaline" conditions (up to pH 12.0) in combination with pre-treatments such as steam-cooking, ultrasonication, or pressurization at 50 MPa. Among these, steam-cooking resulted in the highest protein recovery level (63 ± 2%) but lower than those reported in the present study (81 ± 3%). Despite the high purities obtained by alkaline methods, they involve the use of strong chemicals and achieved lower recovery rates than the subcritical water hydrolysates presented from OK-2.

Enzymatic methods have also been widely explored as greener alternatives. de Figueiredo et al. (2018) used Viscozyme as a pre-treatment and obtained an extract with 56% protein purity. Although this purity surpasses the protein purity obtained for OK-2 hydrolysate (28.2%, Eq. 4), the recovery yield was substantially lower, reaching only 29.8% of the initial protein, much less than the 81 ± 3% of total amino acid hydrolysis obtained for the present OK-2 extraction.

Effect of Isoflavone Pre-Extraction on Protein Extraction

To check if the initial treatment at 120 °C to extract the isoflavones could have an effect on protein extraction from the solid residue generated (OK-2), a similar treatment was conducted for OK-1 at 180 °C to analyze the extraction/hydrolysis from the crude okara.

The amino acid profiles of the liquid hydrolysates of OK-1 and OK-2 at 180 °C for 3 h, as free and bound amino acids expressed in mass, are plotted together in Fig. 2, allowing a direct comparison. For each amino acid, the bars represent the distribution into free amino acids in the hydrolysate, bound amino acids in the hydrolysate, and remaining amino acids in the solid residue after treatment. This representation helps to confirm that the sum of the different amino acid fractions closely approximates the total content initially present for each amino acid.

The subcritical water hydrolysis of both samples, OK-1 and OK-2, resulted in substantial and comparable amino acid extraction efficiencies, with yields of 81 ± 2% and 81 ± 3% for OK-1 and OK-2, respectively (Table 2 and Fig. 2). However, the proportion of free amino acids was higher in the fraction extracted from OK-1 (31 ± 1%) compared to OK-2 (20 ± 1%). This discrepancy is likely attributable to the loss of easily extractable amino acids during the isoflavone extraction process that produces OK-2. The hydrolysate of

OK-2 exhibited a higher percentage of bound amino acids (61 ± 2% compared to 50 ± 1%).

In any case, it can be concluded that OK-2 hydrolysates do not present disadvantages as a functional ingredient compared to the original by-product OK-1, regarding protein extraction/hydrolysis, considering also a higher extraction of the existing EAA (78 ± 3% over 71 ± 4% for OK-2 and OK-1, respectively) and a higher bound-to-free amino acid ratio. Furthermore, bioactive properties are attributed to peptides obtained in SWH hydrolysates (bound aa in these hydrolysates), as attributed antioxidant, antihypertensive, and antidiabetic activities, additionally to foaming and emulsifying properties (Marcet et al., 2016). Besides, since OK-2 had been previously subjected to isoflavone extraction, its valorization gains additional relevance.

To assess the scalability and potential improvements of this approach, a semi-continuous SWH scaled-up system was subsequently investigated.

Subcritical Water Semi-Continuous Extraction

Subcritical water hydrolysis was also performed using a semi-continuous, scaled-up pilot plant to compare protein extraction/hydrolysis from this biomass with previous results presented in a batch configuration.

Semi-continuous SW treatment was performed at different temperature steps: the first at 180 °C and the second at higher temperature, 270 °C, intended to maximize remaining protein and amino acid extraction. Pump flow rate was 0.84 L/min, leading to a residence time of 2.75 min.

Protein Extraction Assessment

Figure 3a shows the protein concentration in the 16 collected samples, eight for each temperature, 180 °C and 270 °C. Protein concentrations were much higher at 270 °C than at 180 °C. In both intervals, a peak concentration was observed followed by a decline. The highest value was 2331 mg/L in the 12th sample (4th sample at 270 °C), while at 180 °C the maximum was 407 mg/L in the 5th sample, 5.7 times lower. This highlights 270 °C as a much more effective temperature for achieving protein hydrolysis in short residence times under the current configuration (severity factor of 5.44).

Figure 3b represents the accumulation of extracted protein as a function of time. Protein showed moderate accumulated protein productivity (g/min) in the samples recovered during the first interval at 180 °C (moderate initial slope). According to the values presented in Fig. 3a, the extraction leveled off in the final three sampling points. The second interval at 270 °C showed higher extraction and therefore higher accumulated productivity, with an initial slope of 0.032 in this interval, almost five times higher than in the first case (Fig. 3b). Figure 3c shows the percentage of protein hydrolyzed in the liquid effluent as a function of time, which is calculated on the basis of the grams of protein extracted on each sample relative to the initial protein content. During the first interval at 180 °C, 18.3% of the total initial protein content was hydrolyzed (Fig. 3c). In the second interval at 270 °C, an additional 52.1% of total protein was further hydrolyzed. A total of 70.4% of the total initial protein content in the whole process was recovered (Fig. 3c). No specific studies were found on semi-continuous SW extraction of proteins from okara, as existing literature mainly focuses on batch systems (Wiboonsirikul et al., 2013).

The total residual solid after treatment represented only 12.8% of the initial biomass. The saccharide content was only 3.1 ± 0.2% and proteins 10.5 ± 0.4%, while acid-insoluble lignin increased up to 81 ± 2%, becoming the dominant fraction (Table 1). Elemental ratios also changed accordingly, N/C decreased to values of 0.03, confirming removal of nitrogenous compounds through protein and peptide hydrolysis; O/C and H/C also decreased, due to an increase in the C content of this residue, suggesting partial dehydration and deoxygenation reactions, due to thermal degradation and condensation of saccharides and lignin enrichment (Alonso-Riaño et al., 2021).

Free Amino Acid Profile Assessment

Free amino acids were quantified in each collected sample from the semi-continuous process to evaluate the degree of protein breakdown into individual amino acids.

The first interval of the treatment at 180 °C accumulated a total of 358.6 mg of free amino acids, which represents 60 mg of free amino acids per g of protein extracted. The distribution of individual amino acids (Fig. 4a) shows a high proportion of glutamic acid (42.3%), followed by aspartic acid (16.2%), consistent with previous batch results at the same temperature. Among the essential amino acids, valine (11.3%) and methionine (10.1%) were the most abundant in the extract.

In the second interval at 270 °C, 2080.2 mg of free amino acids were accumulated, 5.8 times higher than during the first interval. This corresponds to 121.7 mg of free amino acids per g of protein extracted. The distribution of individual amino acids (Fig. 4b) again shows a strong dominance of glutamic acid (52.1%), although the amino acid profile distribution was wider than at 180 °C.

Figure 5 shows the hydrolysis yield (calculated as defined in the "Isoflavone determination" section) for each individual free amino acid after both intervals. Asparagine and glutamine appear at 100%, likely due to their deamidation into aspartic acid and glutamic acid during hydrolysis, being also the least abundant amino acids in the original biomass. Excluding these, the highest yields were observed for hydroxyproline (37.9%), cysteine (33.1%), and

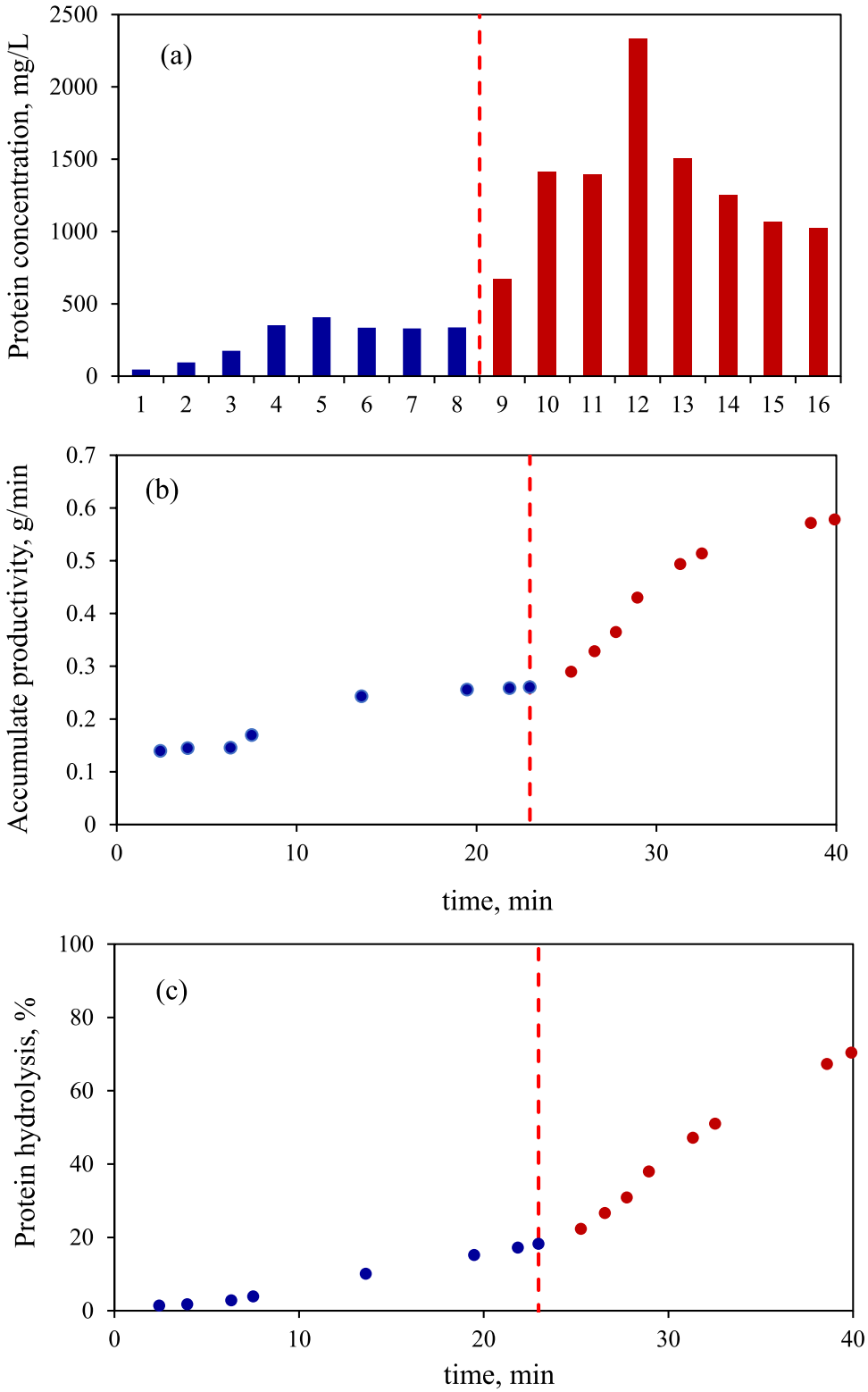


Fig. 3 **(a)** Protein concentration (mg/L) in each extract 1–16, each number corresponding to sequential samples collected (samples 1–8 from 180 °C interval (23 min) and 9–16 from 270 °C interval (17 min). (**b**) Accumulated protein extraction productivity (g/min) as a function of time. (**c**) Accumulated protein degree of hydrolysis (%). Semi-continuous SWH carried out in a 2.3 L reactor (residence time 2.75 min) from OK-2: 180 ºC interval, ■, ●; 270 ºC interval, ■, ●; Temperature interval division, **- -**

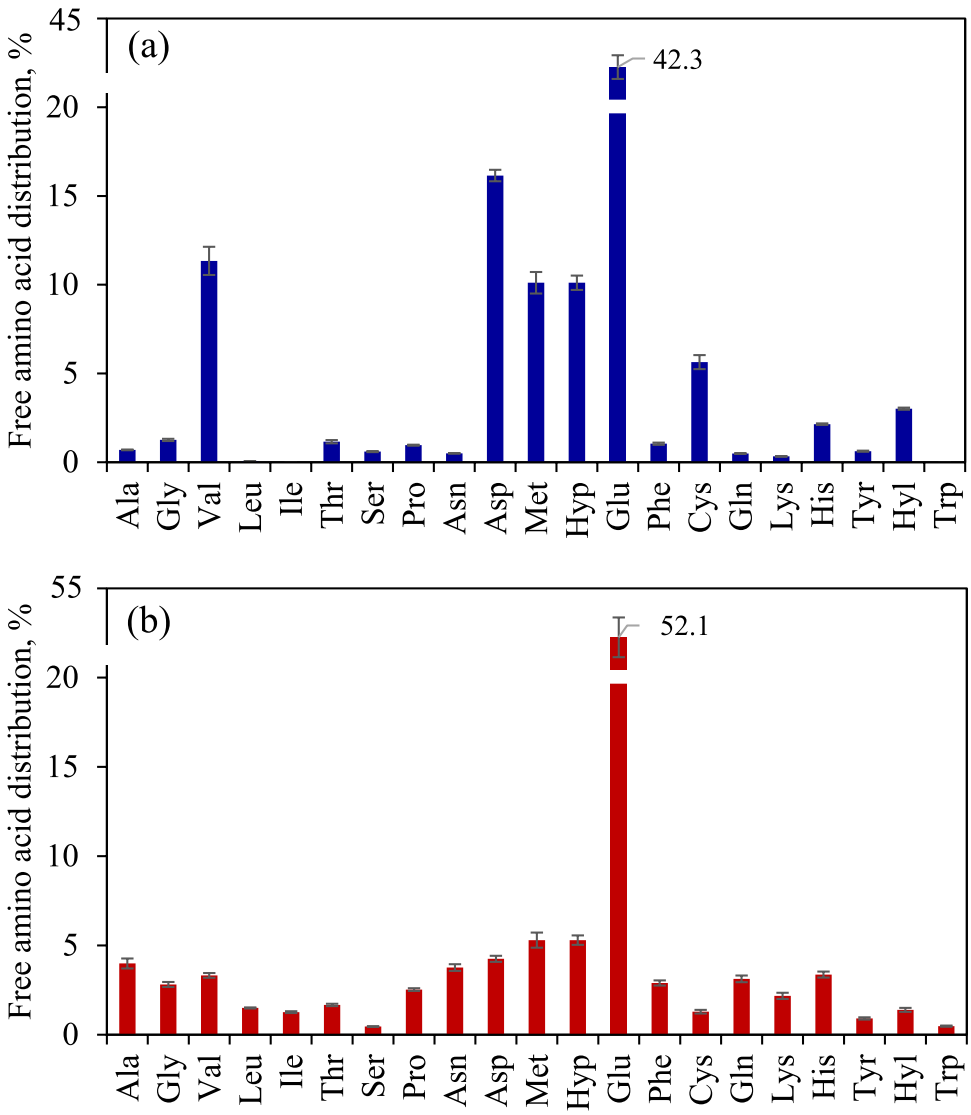


Fig. 4 Free amino acid distribution (%) respect the total amino acids extracted by semi-continuous SWH. **(a)** First interval at 180 °C (■). **(b)** Second interval at 270°C (■)

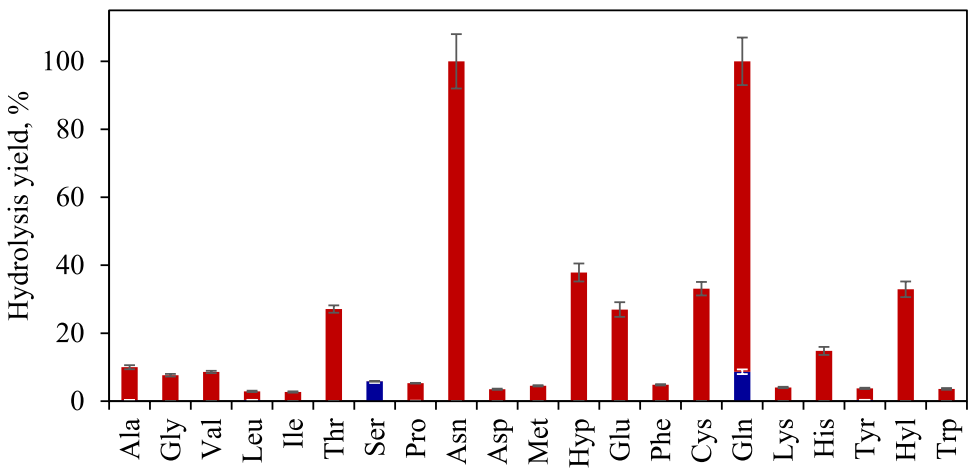
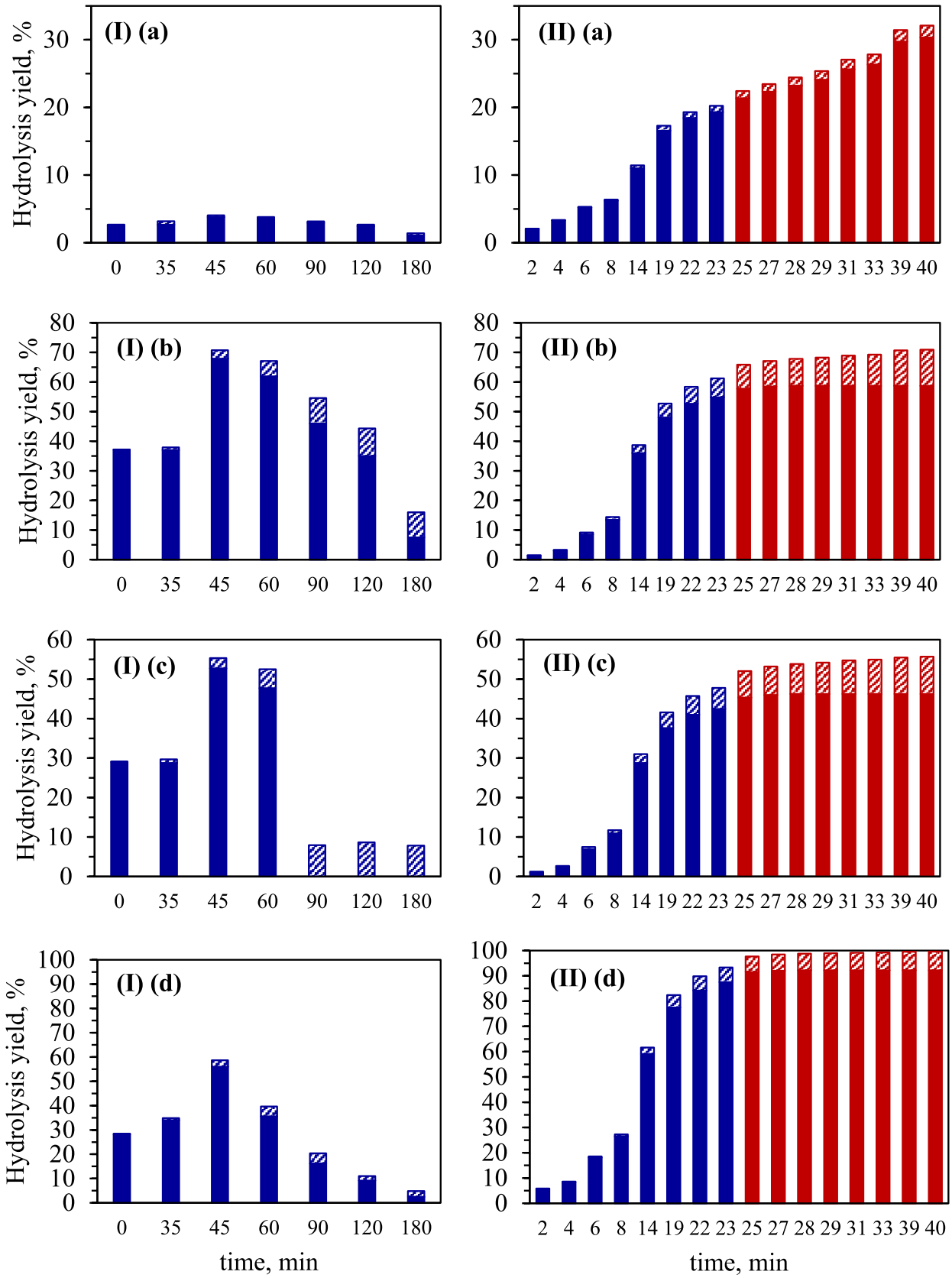


Fig. 5 Hydrolysis yield (%) of each amino acid after two intervals of semi-continuous SWH extraction from OK-2, represented in blue (■)180°C first interval and in red (■)270°C second interval


(I) (a)
(II) (a)
(I) (b)
(II) (b)
(I) (c)
(II) (c)
(I) (d)
(II) (d)
Hydrolysis yield, %
time, min
0 35 45 60 90 120 180
2 4 6 8 14 19 22 23 25 27 28 29 31 33 39 40

◂**Fig. 6** Degree of hydrolysis (%) of each structural sugar: **(I)** after batch SWH (180 min at 180 °C [**blue**]);**(II)** after semi-continuous SWH (including 23min at 180°C [**blue**] and 17 min at 270 °C [**red**]). **(a)** Glucans, **(b)** Galactans, **(c)** Xylans and **(d)** Arabinans, divided into oligomeric forms (■, ■) and monomeric forms (▨, ▨)

hydroxylysine (32.9%), followed by threonine (27.1%) and glutamic acid (27.0%).

Comparison of Batch and Semi-Continuous Results

Total accumulated protein productivity of the semi-continuous process resulted in 0.58 g/min (Fig. 3b). This value was more than 40 times higher than the one obtained in the experiment in the batch reactor, 0.014 g/min. However, due to the larger water volume used, the semi-continuous system produced much more diluted extracts. The final protein concentration in the batch configuration reached 12.6 g/L, whereas the maximum value in the semi-continuous process was 2.3 g/L, more than five times lower (12th sample at 270 °C, Fig. 3a).

The yield of free amino acids was lower in the semi-continuous configuration. For a better comparison in terms of time and temperature applied to each process, the severity factors of the different processes are listed in Table 3: log R_0 = 4.61 for the batch process, while in the semi-continuous log R_0 = 2.79 and 5.44, at 180 °C and 270 °C periods.

The lower severity factor at 180 °C applied in the semi-continuous configuration can explain the low percentage of free amino acids released. The higher severity at 270 °C (log R_0 = 5.44) led to higher release of free amino acids, but it could also promote partial degradation of thermally sensitive amino acids compared to batch processes at 180 °C (log R_0 = 4.61, Table 3). In any case, in the literature, higher severity factors in the order of 5.6–6.2 have been reported as optimal extraction conditions for free amino acids (Quitain et al., 2001; Rogalinski et al., 2005).

The amino acid distribution in the semi-continuous configuration also differed from that of the batch reactor. Although glutamic acid remained dominant, the batch configuration showed higher levels of aspartic acid and several essential amino acids (lysine, phenylalanine, valine, leucine, and isoleucine).

Notably, glutamic acid appeared in high concentrations in all experiments, despite being considered thermolabile. Together with cysteine, it is among the most thermally sensitive amino acids, whereas glycine and valine are more thermally resistant (Abdelmoez et al., 2007). It has been reported that under subcritical water conditions, glutamic acid can cyclize to pyroglutamic acid (PGA) at 230–290 °C within approximately 1 min and remain stable for at least 20 min (Abdelmoez et al., 2010). PGA may therefore be detected as glutamic acid in the analysis; however, this transformation was not directly confirmed in the present study. That potential transformation may open up alternative valorization pathways, as PGA has shown cosmetic and pharmacological applications and as an intermediate in peptide and pharmaceutical synthesis (de Mello et al., 1995), although its presence was not directly identified.

Structural Saccharides Hydrolysis

Saccharide fraction corresponds to 29 ± 2% (w/w) in OK-2 on a dry basis. Most abundant structural sugars on OK-2 are glucans, with 15.9 ± 0.7% (w/w) of total biomass followed by galactans, 6.2 ± 0.3% (w/w) (Table 1). Saccharide hydrolysis was evaluated through the degree of hydrolysis, calculated by quantifying the total dissolved structural sugars (free and oligomeric) relative to their initial content in the biomass. Figure 6 presents the degree of hydrolysis of the four main sugars under batch and semi-continuous SWH conditions, distinguishing between monomeric sugars (Eq. 2) and oligomeric sugars (Eq. 3).

Under batch SWH (Fig. 6I), a maximum hydrolysis yield for all sugars was observed at 45 min. Beyond this point, sugar concentrations in solution markedly decreased, particularly in the oligomeric fraction, indicating progressive degradation during prolonged treatment at this temperature. In contrast, in semi-continuous SWH (Fig. 6II), degradation was minimized due to the short residence time of the extracts in the reactor (2.75 min).

Glucans (Fig. 6a) showed the lowest hydrolysis in both configurations: 4% at 45 min in batch and nearly 30% at end of the treatment in semi-continuous, with oligomers predominating and minor monomer glucose formation. Conversely, the minor structural sugars (galactans, xylans, and arabinans; Fig. 6Ib–d) were extensively hydrolyzed under batch configuration, reaching maximum hydrolysis degrees of 71%, 55%, and 59%, respectively, at 45 min. Beyond this point, monomer-to-oligomer ratios increased, reflecting oligomer hydrolysis. In the semi-continuous configuration, hydrolysis degree increased at 180 °C compared to batch configuration (Fig. 6IIb–d), mainly as oligomers, reaching 61% for galactans, 48% for xylans, and 93% for arabinans. The lower severity of the process in the semi-continuous configuration (Table 3) led to higher final hydrolysis yield, avoiding oligomers degradation to monomers and further dehydration products formation. The semi-continuous SWH maintained oligomers as the dominant fraction (89.3%), with monomers accounting for only 10.7%.

Based on the carbohydrate and protein release profile in the semi-continuous configuration, an additional fractionation step is proposed: the first step at 180 °C will preferentially release carbohydrates, while the second step at 270 °C will focus on protein release.

Comparison with previous studies on okara SWH indicates both similarities and differences. Sun et al. (2018) reported in batch conditions decreased carbohydrate extraction with increasing temperature (160–230 °C for 10 min), while Wiboonsirikul et al. (2013) observed, also in a batch vessel, reduced carbohydrate recovery at higher temperatures and longer treatment times (170–260 °C for 2–120 min).

Analysis of the final solids confirmed these differences. Batch SWH extracted 37 ± 2% of structural sugars, leaving mainly glucans (91 ± 3%), followed by galactans (24.7 ± 0.1%), xylans (19.3 ± 0.2%), and arabinans (0.53 ± 0.02%). The semi-continuous process removed 99.5 ± 0.1% of sugars, with only 0.73 ± 0.01% of the initial glucans remaining in the solid and no detectable galactans, xylans, or arabinans. This near-complete removal was not fully reflected in the hydrolysates, likely due to degradation of remaining saccharides at 270 °C into secondary products rather than measurable sugars. In this regard, the Maillard reaction (MR) involves the condensation between a carbonyl group of reducing sugars and an amine group (such as amino acids and peptides). Therefore, in the SW reactive medium, MR can be initiated due to the presence of a reducing end carbonyl group from the hydrolysis of the polysaccharide fraction, and a free primary amine group from free amino acids and small peptides in the SW reaction medium, leading to a further reduction of their content and the formation of new MR products, including dark-brown polymeric compounds (Alonso-Riaño et al., 2024).

Conclusions

Subcritical water hydrolysis as a green and novel process provides a great protein extraction from okara, yielding high protein recovery and free and bound amino acid profiles. SW treatment in batch configuration (180 °C for 3 h) extracted 81% of the total amino acids in OK-2, with a purity of 28.2%. Comparing results with non-treated okara (OK-1), pre-extracted okara (OK-2) showed a higher amount of extracted protein. The semi-continuous SWH system showed markedly higher extraction at 270 °C (second interval) than at 180 °C (first interval). However, the batch configuration outperformed in terms of protein concentration, free amino acid hydrolysis, overall productivity, and total hydrolysis yield.

Carbohydrate extraction with short residence time of semi-continuous SWH maximizes sugar recovery at 180 °C while limiting degradation and preserving oligomeric structures compared to batch processing. Although the semi-continuous SWH configuration did not achieve the highest protein extraction efficiency, it presents substantial promise for industrial implementation due to its continuous operation, improved process control, and scalability within integrated biorefinery systems. Additionally, it enables a fractionated extraction of carbohydrates and proteins at different temperatures.

Overall, despite further techno-economic and energy assessments being necessary before up-scaling these results to the industry, this work supports a two-step green valorization of okara, maximizing its potential as a sustainable source of free amino acids and bioactive peptides under SWH conditions, particularly under the batch configuration. The combination of waste reduction, solvent-free processing, and recovery of value-added compounds indicates that the present SW process is a promising strategy for the industrial valorization of okara.

Author Contribution P. Barea methodology, M.T. Sanz contributes to conceptualization, H. Candela, A.E. Illera, P. Barea, R. Melgosa and O. Benito Roman contribute to data curation and formal analysis. P.B. and M.T. wrote the main manuscript. All the authors reviewed the manuscript.

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Data Availability Data supporting the reported results can be found on request to the authors.

Declarations

Conflict of Interest The authors declare no competing interests.

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