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Original Research Paper

Valorizing corn stover waste into valuable bioproducts using subcritical water hydrolysis

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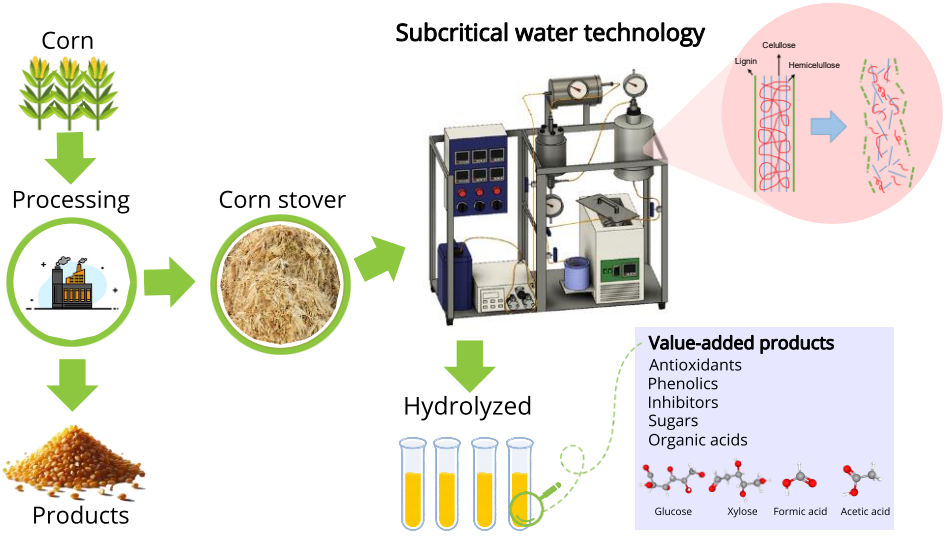
HIGHLIGHTS

- Subcritical water hydrolysis was used to obtain high-value compounds from corn stover.
- The highest amount of total phenolic compounds and the highest antioxidant activities were observed at pH 1 and a temperature of 170 °C.
- The highest sugar concentration (460.92 mg g^{-1}) was obtained at pH 1 and 170 °C.
- The highest organic acid concentration (1,157.19 mg g^{-1}) was obtained at pH 4.5 and 226 °C.

GRAPHICAL ABSTRACT



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ABSTRACT

This study examined the behavior of the semi-continuous hydrolysis process of corn stover (CS) in subcritical water, focusing on the effects of varying pH levels (1, 2, 4.5, 7, and 8) and temperatures (113, 130, 170, 210, and 226°C). The results showed that the process at 170°C and pH 1 was able to recover the highest amount of phenolic compounds (76.82 mg Gallic Acid Equivalents g^{-1}), consequently demonstrating the highest antioxidant activities by the Ferric Reducing Antioxidant Power (FRAP) (423.85 µMol TEAC g^{-1}) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (293.12 µMol TEAC g^{-1}) methods. Additionally, it was possible to obtain the highest concentration of sugars (cellobiose, glucose, and xylose) (460.92 mg g^{-1}). High temperatures (226°C and pH 4.5) produced the highest amount of organic acids (1,157.19 mg g^{-1}). The formation of inhibitors was observed only at temperatures of 170 and 210 °C at a pH of 1.0. The highest yields of sugars, organic acids, and inhibitors were 0.565, 1.123, and 0.665 mg g^{-1} of carbohydrates, respectively. The statistical analysis identified the optimal conditions for the recovery of various compounds: phenolic compounds at 190.7 °C and pH 1, soluble proteins at 187.4 °C and pH 1, sugars at 134.9 °C and pH 1, organic acids at 223.59 °C and pH 4.1, and for minimizing inhibitors at 114.95 °C and pH 7.3. EcoScale analysis identified subcritical water as the most sustainable and efficient method for CS hydrolysis. Subcritical water treatment effectively recovers valuable compounds from CS, promoting a circular economy by valorizing waste and reducing resource dependence.

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Contents

Abbreviations

5-HMF	5-Hydroxymethylfurfural
ANOVA	Analysis of Variance
CS	Corn Stover
CSH	Corn Stover Hydrolysate
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
DTG	Derivative Thermogram
FRAP	Ferric Reducing Antioxidant Power
GAE	Gallic Acid Equivalent
HPLC	High-Performance Liquid Chromatography
RID	Refractive Index Detector
RSM	Response Surface Methodology
SWH	Subcritical Water Hydrolysis
TEAC	Trolox Equivalent Antioxidant Capacity
TGA	Thermogravimetric Analysis
USD	United States Dollar

1. Introduction

The agricultural and food sectors annually generate large amounts of waste. Some of this waste is used for animal feed and soil biofertilizers, while the remaining waste must be treated to avoid environmental pollution (Caporusso et al., 2021; Astudillo et al., 2023). Agriculture alone generates around 140 billion tonnes of waste annually (Singh and Jana, 2023). A significant portion of this agricultural waste is either discarded in landfills or inadequately incinerated. These disposal methods cause environmental issues such as the release of dioxins, combustion gases, and the release of toxic byproducts that contaminate groundwater, as well as the emission of gases such as methane, hydrogen sulfide, and other corrosive compounds. Although food waste is often discarded without reuse or recycling, it has significant potential for the production of value-added products if processed using appropriate technological methods (da Rosa et al., 2023). Various valorization methods are being evaluated, with the most prominent being green and sustainable methods aimed at producing low-cost, high-value products from widely available agro-industrial waste. Since these agro-industrial wastes are sources of bioactive components, they support concepts such as the circular economy and biorefinery approaches. The adoption of innovative technologies for the valorization of agro-industrial waste is anticipated to play a more prominent role in the current agro-industrial landscape, aiming to mitigate the improper disposal of waste and promote sustainable practices.

Corn stover (CS) is agricultural residue from corn cultivation and is abundantly generated worldwide, primarily in major corn-producing countries such as the United States, China, and Brazil. Around 250 million tonnes are generated annually in the United States and 220 million tonnes in China (Khan et al., 2021). With a lignocellulosic composition, CS has a complex structure consisting of cellulose, lignin, and hemicellulose (Akhtar et al., 2016). Due to its composition, CS is highly recalcitrant, which hinders

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the effective production of value-added products (Khan et al., 2021; Wang et al., 2024a). Therefore, pretreatment methods are necessary to alter the structure of lignocellulosic biomass and increase its digestibility by enzymes and microorganisms, transforming it into fermentable sugars (Wang et al., 2015a). The optimal pretreatment method should be selected based on cost, energy efficiency, and the minimization of degradation products (Gao et al., 2012). Several studies have focused on pretreating CS to disrupt its lignocellulosic structure, employing various technologies, including acid treatment (Xie et al., 2021; Wang et al., 2022), alkali (Geng and Henderson, 2012; Wang et al., 2022), torrefaction (Li et al., 2022), and steam explosion (Wang et al., 2015b).

Subcritical water hydrolysis is an innovative technology. It has been employed as a pretreatment method for the recovery of value-added compounds from different plant matrices, such as jabuticaba peel (Barroso et al., 2022a and b), grape pomace (Castro et al., 2023), pitaya peel (Ferreira et al., 2023), brewers spent grain (Sganzerla et al., 2023), and onion skins (Benito-Román et al., 2022). This hydrolysis technology has gained recognition due to its action methods, which involve breaking down cell walls and releasing monomeric sugars. Subcritical hydrolysis has been utilized to tackle one of the key challenges in hydrolyzing plant biomass: the effective fractionation of hemicellulose and cellulose. This method facilitates the breakdown of these compounds, leading to the release of low-molecular-weight oligomers, which are then further hydrolyzed into simple sugars (Martins-Vieira et al., 2022). These products are of great interest for various applications, including bioprocesses for biofuel production (Jarunglumlert et al., 2022; Chen et al., 2023). In addition to its effective action method, the use of water as a solvent makes the process green and sustainable.

For water to be in the subcritical state, its temperature must range between 100°C and 374°C, and its pressure must be between 0.1 MPa and 22 MPa (Smith, 2002; Zhang et al., 2022). Under these conditions, hydrolysis, oxidation, and other chemical reaction rates increase significantly (Paini et al., 2021a). The dielectric constant (ε) of subcritical water is inversely proportional to temperature. In simpler terms, it boosts hydrogen bonds when temperatures are lower. Conversely, as the temperature is increased, the dielectric constant is decreased, leading to enhanced thermal vibrations among the molecules. This decrease in dielectric constant translates to a reduced polarity of water, promoting the solubility of hydrophobic organic substances like phenolic compounds (Carr et al., 2011). The dielectric constant (ε) of subcritical water decreases significantly with increasing temperature (ε = 80 at 25 °C and ε = 27 at 250 °C), bringing its polarity closer to that of typical organic solvents like methanol (ε = 33) and ethanol (ε = 24) (Ong et al., 2006; Paini et al., 2021a). Subcritical water offers a unique advantage due to its temperature-dependent tunable polarity. This characteristic enables the hydrolysis and extraction of diverse compounds within a single process, enhancing its versatility and efficiency (Ferrentino et al., 2018; Paini et al., 2021a). **Figure 1** represents the impact of subcritical water hydrolysis pretreatment on the structure of the cellulose-hemicellulose-lignin complex.

The application of subcritical technology to CS remains relatively underexplored, as evidenced by **Table 1**, which highlights various hydrolysis methods but identifies only one study using subcritical technology. Investigating this approach for breaking down the cellular structure of CS is highly innovative. It addresses key challenges, including the valorization of agro-industrial waste, the production of high-value-added products, and the generation of extracts for potential applications within a circular economy framework.

Building on these advantages, this study explored the subcritical hydrolysis of CS using response surface methodology (RSM) to optimize the process conditions. The experiment investigated the effects of subcritical water's hydrolysis temperature (130, 170, and 210 °C) and pH levels (2, 4.5, and 7) on the recovery of sugars and organic acids. This approach aimed to determine the optimal conditions for maximizing the yield of valuable products from CS.

2. Material and Methods

2.1. Raw material

CS was acquired from a rural property in western Santa Catarina (Itá, SC, Brazil). The samples were dried in an oven (Lucadema, model LUCA-82/250, São Paulo, SP, Brasil) at 60°C for 48 h with forced air circulation and ground in a forage crusher (TRAPP-2932416 TRF 400F 2CV, Jaraguá do Sul, SC, Brazil). CS characterization analyses were conducted in triplicate, and the results were presented in grams per 100 grams dry weight (g $100g^{-1}$ CS). The amounts of moisture and ash were determined according to the AOAC methodology (AOAC, 2023). Protein content was determined using the Kjeldahl method as described by de Souza et al. (2016). Lipid quantification was performed using the Bligh-Dyer method, following the procedure outlined by Smedes and Thomasen (1996). Carbohydrate content was calculated by subtracting the combined percentages of moisture, protein, lipids, and ash from 100. Cellulose, hemicellulose, and lignin contents were determined using the Van Soest and Wine method (Van Soest and Wine, 1968).

2.2. Subcritical water process to obtain corn stover hydrolysate

The hydrolysis experiment in subcritical water was carried out using equipment previously designed and described in studies by Barroso et al. (2022a and b). A laboratory-scale process was developed and used for the subcritical water hydrolysis (SWH) of BGP (**Fig. 1**). Water was introduced and pressurized in a reactor (Model 36, Apple Valley, MN, USA) using a high-pressure, double-piston liquid pump. The 110 mL reactor, made of 316

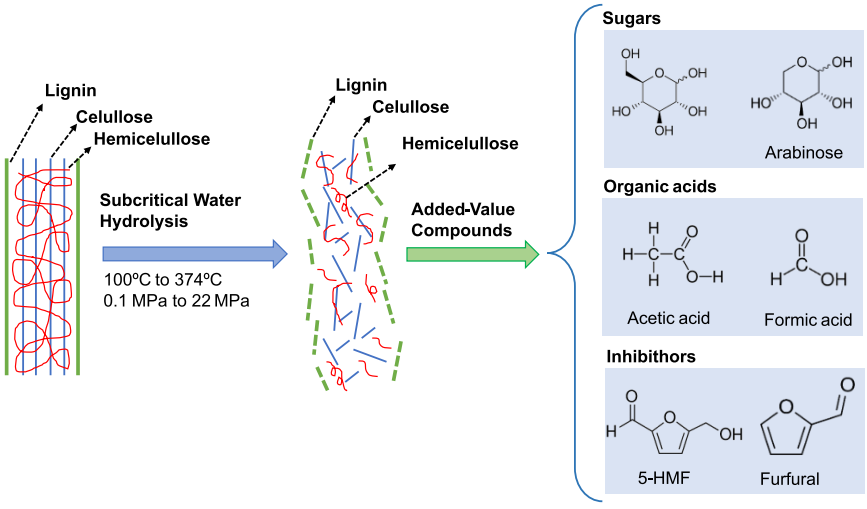

Fig. 1. Impact of subcritical water hydrolysis pretreatment on the structure of the cellulose-hemicellulose-lignin complex.

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Table 1.
Comparison of this study with the most relevant published articles on bioproduct production from CS valorization.

No.	Hydrolyzed	Compounds analyzed	Ref.
1	Enzymatic	Cellulose Hemicellulose Lignin	Zhang et al. (2023)
2	Enzymatic	Hemicellulose	Wu et al. (2021)
3	Enzymatic	Glucose Xylose	Li et al. (2019)
4	Enzymatic	Cellulose Hemicellulose Lignin Xylose	Wang et al. (2022)
5	Enzymatic	Lignin Hemicellulose Cellulose	Huang et al. (2019)
6	Ionic Liquids	Lignocellulose Sugars	Wang et al. (2024b)
7	Acid	Furfural, 5-Hydroxymethylfurfural acetic acid	Zhang et al. (2022a)
8	Acid	Biocarbon levulinic acid	Thakkar et al. (2021)
9	Acid	Lignin Lipid Sugars	Xu et al. (2019)
10	Alkaline	Phenolic compounds Lignin	Vazquez-Olivo et al. (2019)
11	Alkaline	p-Cumaric acid Ferulic acid	Yang See et al. (2021)
12	Supercritical	Large molecular mothballs, Phenanthrene, Pyrene	Zhang et al. (2024)
13	Supercritical	Lignocellulose	Zhao et al. (2019)
14	Supercritical	Lignin	Vincent Sahayaraj et al. (2023)
15	Subcritical water	Phenolic compounds, antioxidants, soluble proteins, sugars, organic acids, inhibitors, hemicellulose	**Present Study**

stainless steel, was heated by a thermal jacket insulated with ceramic fiber (RSA Equipment and Instrumentation, Campinas, SP, Brazil). To maintain the temperature, Type K thermocouples (RSA Equipment and Instrumentation, Campinas, SP, Brazil) were installed at both the reactor's inlet and outlet. The liquid product (hydrolysate) leaving the reactor was cooled in a heat exchanger using a thermostatic bath (Marconi, model MA184, São Paulo, SP, Brazil). A micrometer valve (Parker Autoclave Engineers, model 10VRMM2812, Erie, PA, USA) downstream of the heat exchanger regulated the pressure. Pressure gauges (0–50 MPa) from Wika (Klingenberg am Main, Bavaria, Germany), with an accuracy of 0.1%, were used to monitor the system's pressure. **Figure 2** presents a schematic diagram of the equipment utilized for the experiments.

For CS hydrolysis (CSH), 10 g of ground and dried CS was introduced into the reactor, which was then operated semi-continuously. The reactor was charged with water adjusted to different pH levels until the internal pressure reached 15 MPa (Barroso et al., 2022a and b), and the pressure was maintained constant in all experiments. The flow rate for the experiments was 10 mL min^{-1} for 45 min (da Rosa et al., 2023). The pH of the mobile phase used for hydrolysis was adjusted using H_2SO_4 (1 mol L^{-1}) or NaOH (1 mol L^{-1}). All the experiments had a solvent/feed ratio (S/F) equal to 45 g water g^{-1} CS; this parameter was chosen by analyzing previous studies (Barroso et al., 2022b; Castro et al., 2023).

When selecting the range for the temperature parameter, it is important to consider its significant influence on the subcritical hydrolysis process.

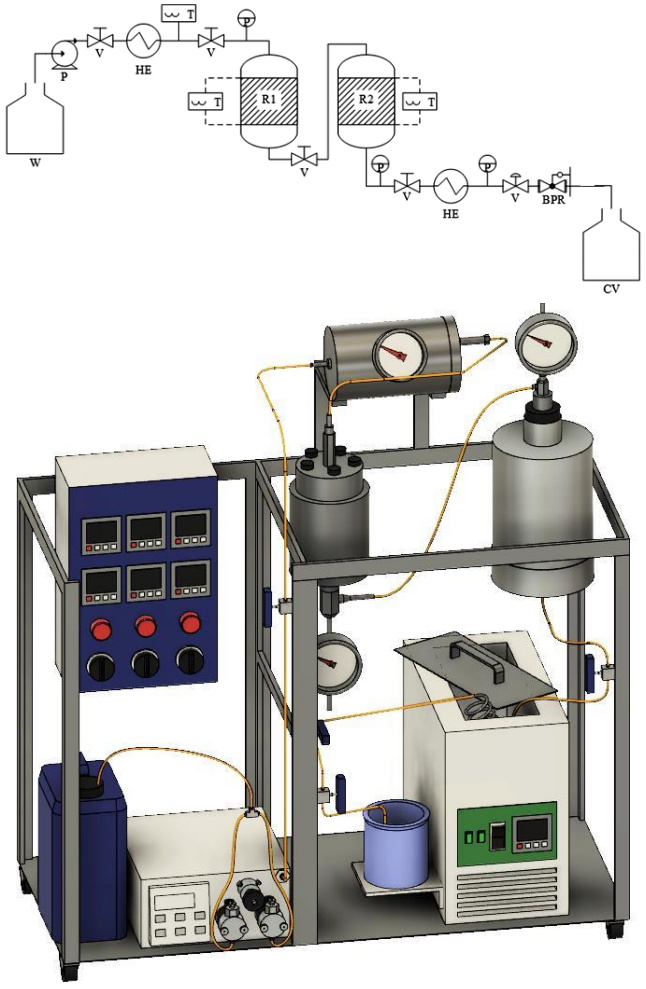

Fig. 2. Schematic diagram of the experimental apparatus for CS semi-continuous subcritical water hydrolysis. Reproduced from (Hydrothermal pretreatment based on semi-continuous flow-through sequential reactors for the recovery of bioproducts from jabuticaba (*Myrciaria cauliflora*) peel (Barroso et al., 2022a)), with permission from Elsevier. Label: W, water tank; P, high-pressure pump; V, block valves; P, manometer; T, thermocouples; R, subcritical water hydrolysis reactor; HE, heat exchanger; MV, micrometric valve; CV, collecting vessel.

Temperature variations impact key physicochemical properties, including the dielectric constant, diffusion rate, surface tension, and thermal energy, all of which play crucial roles in the efficiency and outcomes of the hydrolysis process (Costa et al., 2023). Additionally, hydrolysis in subcritical water at elevated temperatures significantly accelerates the reaction, reducing the required reaction time and enhancing process efficiency. However, excessively high temperatures can lead to the degradation of target compounds due to hydrolysis and oxidation reactions, potentially diminishing the yield of desired products (Watchararuji et al., 2008). Regarding the choice of the pH parameter, some studies have found that pH significantly affects hydrolysis in subcritical water, demonstrating the possibility of recovering several compounds from sweet blue lupin hulls (Ciftci and Saldaña, 2015), microalgal biomass (Phusunti et al., 2017) and grape pomace (Castro et al., 2023). Therefore, it is essential to evaluate whether the pH of the solution also influences the hydrolysis of CS in subcritical water, as pH may play a critical role in optimizing the reaction conditions and product yield.

This experiment studied the influence of the pH of the mobile phase (1, 2, 4.5, 7, and 8) and the temperature (113, 130, 170, 210, and 226 °C) on the CSH process using an RSM. A 2^2-response surface method design with replicates at the central point was developed for the study. The tests conducted are detailed in **Table 2**. CSH samples were collected every 5

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min during the 45-min subcritical water hydrolysis process. The collected liquid was stored at -18°C for subsequent analysis.

Table 2.
Experimental conditions for subcritical water hydrolysis of CS.

Treatment	Codified variables		Non-codified variables	
	pH (X_1)	Temperature (X_2)	pH	Temperature (°C)
CSH1	-1	-1	2	130
CSH2	-1	+1	2	210
CSH 3	+1	-1	7	130
CSH 4	+1	+1	7	210
CSH 5	-1.41	0	1	170
CSH 6	+1.41	0	8	170
CSH 7	0	-1.41	4.5	113
CSH 8	0	+1.41	4.5	226
CSH 9	0	0	4.5	170
CSH 10	0	0	4.5	170
CSH 11	0	0	4.5	170

2.3. Hydrolysis by conventional methods

Two conventional methods (acid hydrolysis and alkali hydrolysis) were carried out to compare the results and assess the efficiency of the CS hydrolysis process with subcritical water.

2.3.1. Acid hydrolysis

The acid hydrolysis method was adapted from Varilla-Mazaba et al. (2024). In this process, 3 g of CS (dry basis) was mixed with 45 mL of an acid solution (1% H_2SO_4, v/v). The mixture was placed in a vertical autoclave (Prismatec, Model: CSA-18, Itu, SP, Brazil) and subjected to hydrolysis for 30 min at 121°C. After hydrolysis, the resulting hydrolysate was centrifuged at 5,000 rpm for 10 min. The supernatant was then collected and stored at -18 °C for subsequent analysis.

2.3.2. Alkali hydrolysis

The alkali hydrolysis method was adapted from Ruan et al. (2024). In this procedure, 3 g of CS (dry basis) was mixed with 45 mL of an alkaline solution (1% NaOH, w/v). The mixture was placed in a vertical autoclave (Prismatec, Model: CSA-18, Itu, SP, Brazil) and subjected to hydrolysis for 30 min at 121°C. After hydrolysis, the resulting hydrolysate was centrifuged at 5,000 rpm for 10 min. The supernatant was collected and stored at -18 °C for subsequent analysis.

2.4. Characterization of hydrolysates

2.4.1. Color parameters

The colorimetric coordinates L* (brightness), a* (red/green value), and b* (blue/yellow value) of the CIELab system were obtained by measuring transmittance values every 5 nm between 340 and 830 nm on a UV-Vis spectrophotometer (Model UV-M51, Bell Photonics) (Otha and Robertson, 2005; Gilchrist and Nobbs, 2017).

2.4.2. pH

The pH of all samples obtained during hydrolysis in subcritical water was measured using a digital pH meter (IonLab, model THS-3E, New York, NY, USA). The pH meter was calibrated with standard solutions, and all measurements were taken at room temperature. The pH analysis was carried out in triplicate.

2.4.3. Sugars, organic acids and inhibitors

High-performance liquid chromatography (HPLC) equipment with a refractive index detector (RID) was used to quantify sugars, organic acids, and inhibitors. A Rezex™ column (Phenomenex, model ROA-Organic Acid H+ (8%), 8 µm, 300 × 7.8 mm, Torrance, CA, USA) was used to separate the compounds. The analysis methodology was described by Castro et al. (2023). The concentrations of cellobiose, glucose, xylose, arabinose, formic acid, acetic acid, furfural, and 5-hydroxymethylfurfural (5-HMF) were calculated from calibration curves of each standard with concentrations ranging from 0 to 1 g L^{-1}. All calibration curves showed high coefficients of determination ($R^2 = 0.999$). The analysis was carried out in triplicate, and the results were expressed in mg per gram of dry CS (mg g^{-1} CS).

2.4.4. Total phenolic compounds

Total phenolic compounds were determined using the Folin-Ciocalteu method (Singleton and Rossi, 1965). Gallic acid was used as a standard, and a seven-point calibration curve was constructed to calculate the results ($y = 0.0008x - 0.0227$; $R^2 = 0.994$). The analysis was conducted in triplicate, and the results were presented as milligrams of gallic acid equivalent per gram of dried CS (mg GAE g^{-1} CS).

2.4.5. Antioxidant activity (FRAP and DPPH)

Antioxidant activity was assessed using the ferric reducing antioxidant power (FRAP) method as described by Benzie and Strain (1996), with modifications based on Ferreira et al. (2023). Trolox was used as a standard ($y = 0.0015x + 0.0258$; $R^2 = 0.998$). The analysis was conducted in triplicate, and the results were presented as µMol of antioxidant capacity equivalent to Trolox per gram of dry CS (µMol TEAC g^{-1} CS).

The DPPH free radical inhibition method was used to verify the antioxidant activity *in vitro*. The methodology was described by Brand-Williams et al. (1995). Trolox was used as a standard ($y = -0.0006x + 0.5743$; $R^2 = 0.944$). The analysis was carried out in triplicate, and the results were presented as µMol of antioxidant capacity equivalent to Trolox per gram of dry CS (µMol TEAC g^{-1} CS).

2.4.6. Soluble proteins

The hydrolysates obtained were analyzed for soluble protein content using the Bradford method (Bradford, 1976), with modifications as described by Sganzerla et al. (2022b). A calibration curve was constructed to calculate the protein concentration. The curve was built with standard solutions of bovine serum albumin with a variation of 0.005 to 0.1 g L^{-1} ($y = 20.98x - 0,0045$; $R^2 = 0.995$). The analysis was carried out in triplicate, and the results were presented in g of albumin L^{-1} of hydrolysate (g L^{-1}).

2.4.7 Yield calculation

To calculate the yield of sugars (**Eq. 1**), organic acids (**Eq. 2**), and inhibitors (**Eq. 3**), the total amount of sugars (sum of cellobiose, glucose, xylose, and arabinose), organic acids (sum of formic acid and acetic acid), and inhibitors (sum of 5-HMF and furfural) present in the hydrolysate in g 100 g^{-1} and the amount of total carbohydrates in the CS (81.6 g 100 g^{-1}) was considered.

$$Yield\ of\ sugars\left(\frac{g_{sugars}}{g_{carbohydrates}}\right) = \frac{Sugars_{hydrolysate}}{Carbohydrates_{CS}} \quad \text{Eq. 1}$$

$$Yield\ of\ organic\ acids\left(\frac{g_{organic\ acids}}{g_{carbohydrates}}\right) = \frac{Organic\ acids_{hydrolysate}}{Carbohydrates_{CS}} \quad \text{Eq. 2}$$

$$Yield\ of\ inhibitors\left(\frac{g_{inhibitors}}{g_{carbohydrates}}\right) = \frac{Inhibitors_{hydrolysate}}{Carbohydrates_{CS}} \quad \text{Eq. 3}$$

2.4.8. Characterization of the solid material remaining after subcritical water hydrolysis

The final solid residues obtained at the end of each hydrolysis treatment with subcritical water were subjected to thermogravimetric analysis (TGA) in a simultaneous thermal analyzer (PerkinElmer, model STA6000, Akron, Ohio, EUA). The analysis methodology was described by Castro et al. (2023). To determine the composition of the lignocellulosic material,

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derivative thermogram (DTG) analysis was carried out, and TGA data were generated, considering the thermal decomposition of semi-volatiles (40–175 °C), hemicellulose (175–300 °C), cellulose (300–370 °C), lignin (370–550 °C), and char (550–700 °C) (Carrier et al., 2011).

2.5. Optimization, modeling, and statistical analysis

All results obtained were subjected to analysis of variance (ANOVA) to evaluate which factors were statistically significant and their interaction effects. Significant differences were determined using the Tukey test ($p \leq 0.05$). The experimental results for phenolic compounds, soluble proteins, sugars (sum of all sugars), organic acids (sum of all organic acids), and inhibitors (sum of all inhibitors) were analyzed using RSM following the method described by Castro et al. (2023). MiniTab® software (version 19, Minitab, LLC., State College, PA, USA) was used for all the statistical analyses.

2.5.1. Uncertainty analysis

The uncertainty analysis was conducted using the methodology described by Campana et al. (2023). To ensure accurate product yields, we report the weighted average of three replicate experiments. Each product exhibited slight variations (approximately ±0.5-1.5%) across these replicates.

2.6. EcoScale analysis

The EcoScale analysis evaluated the environmental impact of the biomass hydrolysis strategy for obtaining fermentable sugars. The assessment considered safety, solvent choice, cost and availability, technical configuration, temperature control, and work procedures. The EcoScale scores of comparable studies were also analyzed for performance comparison. These parameters were normalized by the relative sugar yield, with the highest experimental yield set at 100%. Results ranged from 1 to 100, where 1 indicated the least eco-friendly process, and 100 represented the most eco-friendly approach (Van Aken et al., 2006).

2.7. Investment analysis in capital recovery and payback time

An investment analysis of capital recovery and payback time was conducted to evaluate the use of CS as a raw material in a hydrolysis system, followed by a sugar purification system. The objective was to determine the project's economic return (payback) time. **Table 3** presents the assumptions used for dimensioning the hydrolysis system, where a scale-up involving three hydrolysis vessels, each with a capacity of 500 liters (3 x 500 L), was considered for sugar production with an accompanying purification system. The data were obtained from a hydrolysis system with subcritical water, which provided the data necessary for the scale-up (Sganzerla et al., 2021). Payback is a measure that indicates how long it will take for an investment to recover the capital (i.e., for the cash flows generated by the investment to be sufficient to cover the initial amount spent). The simple payback period was calculated using **Eq. 4**.

$$Simple\ payback = \frac{Initial\ investment}{Annual\ cash\ flow} \quad \text{Eq. 4}$$

where: Initial investment is the total amount invested in the project or asset, and Annual Cash Flow is the amount of money the investment generates per year.

3. Results and Discussion

3.1 Raw material

Table 4 displays the results of the CS composition analysis used for hydrolysis. The CS had a moisture content of 10.38 ± 1.44 g 100 g^{-1}, 2.68 ± 0.20 g ash 100 g^{-1}, 4.60 ± 0.8 g crude protein 100 g^{-1}, 0.76 ± 0.042 g lipids 100 g^{-1}, and 81.6 ± 0.81 g carbohydrates 100 g^{-1}. A previous study found values close to the moisture content (7.66 g 100 g^{-1}) and ash content (5.44 g 100 g^{-1}) of CS (Yu et al., 2007). It is worth noting that the physicochemical composition of plant raw materials varies greatly due to several factors, including harvest time, geographical location, climate change, and plant species variability (Barroso et al., 2022b). The low ash content is advantageous for the hydrolysis process. A high ash content hinders hydrolysis by forming a barrier around cellulose and hemicellulose, making it difficult for water to access these components (Abaide et al., 2019a). The results for cellulose (48.28 g 100 g^{-1}), hemicellulose (25.16 g 100 g^{-1}), and lignin (14.8 g 100 g^{-1}) demonstrate values close to those found in the literature (Kumar et al., 2008). Additionally, they show a high amount of hemicellulose, which, in hydrolysis processes with subcritical water, can be converted into monosaccharides (Sganzerla et al., 2022b).

Table 3.
Assumptions for sizing sugar production using subcritical water hydrolysis technology with a sugar purification system.

Parameters	Amount	Unit	Ref.
Subcritical Water Unit with Purification System (3 × 500 L)	8,276,785.09	USD	Sganzerla et al. (2021)
Approximate annual operating cost	2,111,000.00	USD	
Amount of CS processed	1,000.00	Kg day^{-1}	
Operating time	264	Day $year^{-1}$	*Estimated*
Cellobiose	70	USD kg^{-1}	
Glucose	1.2	USD kg^{-1}	
Xylose	9	USD kg^{-1}	Sganzerla et al. (2021)
Arabinose	59	USD kg^{-1}	

Table 4.
Characterization of CS (dry matter).

Parameters	Composition*	Unit
Moisture	10.36 ± 1.44	g 100 g^{-1}
Ashes	2.68 ± 0.20	g 100 g^{-1}
Crude protein	4.60 ± 0.8	g 100 g^{-1}
Lipids	0.76 ± 0.042	g 100 g^{-1}
Carbohydrates	81.6 ± 0.81	g 100 g^{-1}
Cellulose	48.28 ± 4.19	g 100 g^{-1}
Hemicellulose	25.16 ± 3.97	g 100 g^{-1}
Lignin	48.28 ± 2.11	g 100 g^{-1}

* The results are expressed as the mean ± standard deviation. Analysis conducted in triplicate (n=3).

3.2. Characterization of hydrolysates

3.2.1. Color parameters

The hydrolysates obtained were evaluated using the colorimetric parameters of the CIELab scale (L*, a*, and b*), and the results are presented in **Table 5**. The L* parameter refers to luminosity; that is, the closer it is to 0, the darker the sample, and the closer it is to 100, the lighter the sample. The L* values in the experiments ranged from 64.12 to 87.09. pH was observed to influence the L* values of the hydrolysates; the CSH1 and CSH3 treatments differed statistically from CSH2 and CSH4, respectively, having the same temperature but different pH. Treatments with lower pH had higher luminosity. Temperature also influenced luminosity, as treatments CSH7, CSH8, CSH9, CSH10, and CSH11 differed statistically while having the same pH but different temperatures.

Regarding the a* parameter, which verifies the variation in green (<0) and red (>0) colors, the values ranged from -0.90 to 20.10. The lowest value was observed in the CSH4 treatment, indicating a shift toward green, while the highest was in the CSH1 treatment. The b* parameter represents the color spectrum from blue (<0) to yellow (>0). In this parameter, the hydrolysates ranged from 46.42 to 78.78, indicating a predominantly yellow coloration, as expected, given the presence of yellow pigments in CS. The

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Table 5.
Color parameters of the hydrolysates obtained from CS semi-continuous subcritical water hydrolysis.

Treatment	L*	a	b
CSH1	78.35 ± 0.11^{de}	4.23 ± 0.54^{f}	46.42 ± 0.37^{d}
CSH2	80.11 ± 0.35^{bd}	0.23 ± 0.40^{c}	59.52 ± 0.30^{b}
CSH3	65.65 ± 0.14^{c}	8.47 ± 0.42^{de}	51.52 ± 0.28^{c}
CSH4	87.09 ± 0.49^{a}	-0.90 ± 0.85^{c}	53.41 ± 0.18^{c}
CSH5	81.39 ± 0.20^{b}	6.76 ± 0.88^{e}	71.85 ± 0.57^{e}
CSH6	78.73 ± 0.64^{de}	7.06 ± 0.92^{de}	72.29 ± 0.78^{e}
CSH7	78.30 ± 0.13^{e}	3.24 ± 0.47^{f}	47.48 ± 0.47^{d}
CSH8	84.87 ± 0.21^{a}	2.86 ± 0.49^{f}	60.90 ± 0.78^{b}
CSH9	78.54 ± 0.39^{de}	8.61 ± 0.83^{d}	72.26 ± 0.49^{e}
CSH10	77.34 ± 0.56^{de}	11.24 ± 0.88^{b}	78.78 ± 0.57^{a}
CSH11	64.12 ±0.53^{c}	20.10 ± 0.91^{a}	77.79 ± 0.42^{a}

* The results are expressed as the mean ± standard deviation. Analysis conducted in triplicate (n=3). Different letters in each column indicate significant differences by Tukey's test at $p \leq 0.05$.

predominance of yellow can be observed in **Figure 3.** Additionally, yellow coloration is a characteristic of hydrolysis processes and can indicate the occurrence of the Maillard reaction (Ferreira et al., 2023).

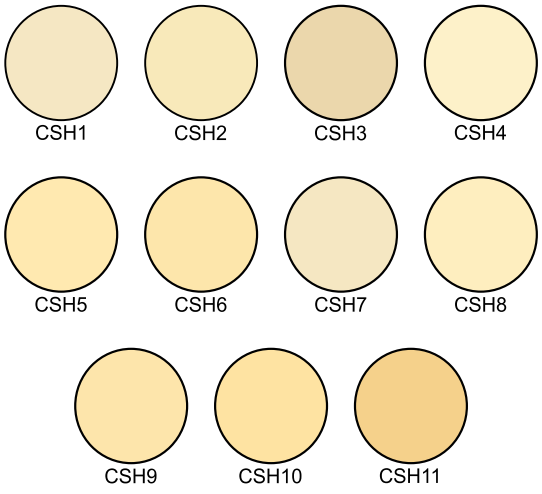

Fig. 3. Colorimetric representation of the hydrolysates obtained using subcritical water. Label: CSH1 (130 ºC, pH 2), CSH2 (210 ºC, pH 2), CSH3 (130 ºC, pH 7), CSH4 (210 ºC, pH 7), CSH5 (170 ºC, pH 1), CSH6 (170 ºC, pH 8), CSH7 (113 ºC, pH 4.5), CSH8 (226 ºC, pH 4.5), CSH9 (170 ºC, pH 4.5), CSH10 (170 ºC, pH 4.5), and CSH11 (170 ºC, pH 4.5).

The colorimetric parameters for the control experiments (acid hydrolysis and alkali hydrolysis) are presented in **Table 6**. The analysis revealed no statistically significant difference in the L* parameter between the acid and alkali treatments. However, the a* and b* parameters showed statistically significant differences. **Figure 4** illustrates the color representation of samples from acid and alkali hydrolysis. The control treatments exhibit a lighter and less yellow appearance compared to other samples. This may suggest that the hydrolysis process was not entirely successful. In contrast, the hydrolysates obtained by subcritical water hydrolysis have a more yellow color, with b* values ranging from 46.42 ± 0.37 (lowest) to 78.78 ± 0.57 (highest). The b* values for acid and alkaline hydrolysis (32.53 ± 0.27 and 39.59 ± 0.35, respectively) are significantly lower, indicating a less intense yellow coloration compared to the subcritical water hydrolysates.

Table 6.
Summary of the results obtained by applying conventional hydrolysis methods to CS.‡

Parameters	Acid hydrolysis	Alkali hydrolysis	Unit
Sugars			
Cellobiose	3.4 ± 0.05^{a}	1.68 ± 0.02^{b}	mg g^{-1}
Glucose	7.08 ± 0.05^{a}	2.89 ± 0.02^{b}	mg g^{-1}
Xylose	22.99 ± 0.10	-	mg g^{-1}
Arabinose	27.53 ± 0.13	-	mg g^{-1}
Organic acids			
Acetic acid	6.27 ± 0.05^{a}	18.96 ± 0.03 ^{b}	mg g^{-1}
Phenolic compounds	12.03 ± 1.38^{a}	11.78 ± 0.63^{a}	mg GAE g^{-1}
pH	1.57 ± 0.05	6.75 ± 0.06	–
Soluble protein	0.0048 ± 0.0001^{a}	0.0054 ± 0.0001^{a}	g L^{-1}
Antioxidant activity			
DPPH	3.35 ± 0.64^{a}	5.42 ± 0.15^{b}	µMol TEAC g^{-1}
FRAP	2.88 ± 0.60^{a}	1.94 ± 0.062^{b}	µMol TEAC g^{-1}
Color parameters			
L*	89.09 ± 0.38^{a}	86.61 ± 0.37^{a}	–
a*	1.26 ± 0.71^{a}	1.52 ± 0.32^{b}	–
b*	32.53 ± 0.27^{a}	39.59 ± 0.35^{b}	–

‡ The results are expressed as the mean ± standard deviation. Analysis conducted in triplicate (n=3). Different letters in each column indicate significant differences by Tukey's test at $p \leq 0.05$.

Fig. 4. Colorimetric representation of the hydrolysates obtained using conventional methods.

3.2.2. pH

The pH of the hydrolysates was monitored throughout the CSH process, and the corresponding kinetic profile is presented in **Figure 5**. In the milder temperature treatments (CSH1, CSH2, and CSH7), the initial pH of the hydrolysates was basic. A possible explanation for this is the hydrolysis of basic compounds. Over time, the hydrolysates gradually matched the pH of the initial mobile phase. In treatments conducted at temperatures exceeding 170 °C, the pH values showed a noticeable decrease. This result indicates that the hydrolysis process at higher temperatures can lead to the production of significant quantities of organic acids and inhibitory compounds, such as furfural and 5-HMF (Prado et al., 2014). Thus, pH can serve as an indicator of the catalytic conversion of sugars into acids (Sganzerla et al., 2022b). The highest pH level at the kinetic time of 45 min was observed in CSH6, while the lowest was in CSH5, with pH values of 8.95 and 1, respectively. The results demonstrate that pH variation significantly influences process yields, underscoring the importance of monitoring pH as a key variable in this study. A similar trend was observed in the hydrolysis of grape pomace, further validating the impact of pH on hydrolysis efficiency (Castro et al., 2023).

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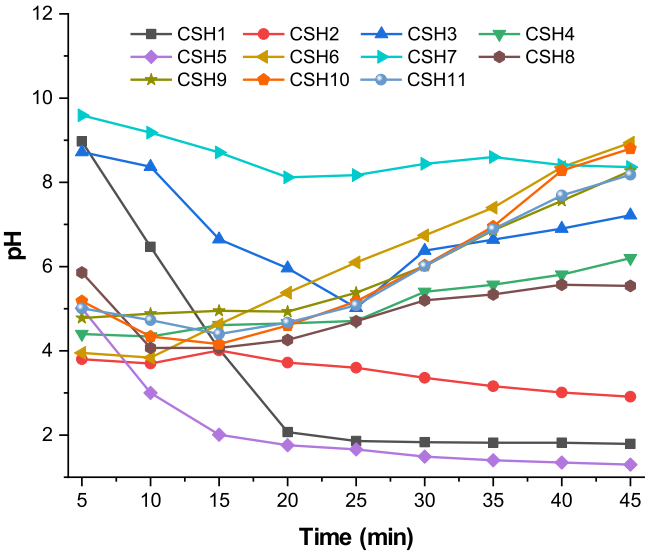

Fig. 5. Profile of pH during the subcritical water hydrolysis of CS. Label: CSH1 (130 ºC, pH 2), CSH2 (210 ºC, pH 2), CSH3 (130 ºC, pH 7), CSH4 (210 ºC, pH 7), CSH5 (170 ºC, pH 1), CSH6 (170 ºC, pH 8), CSH7 (113 ºC, pH 4.5), CSH8 (226 ºC, pH 4.5), CSH9 (170 ºC, pH 4.5), CSH10 (170 ºC, pH 4.5), and CSH11 (170 ºC, pH 4.5).

3.2.3. Total phenolic compounds

Studies on phenolic compounds are receiving increasing attention in academic research due to their remarkable antioxidant, anti-inflammatory, and antimicrobial properties. These compounds, derived from plants and their byproducts, are secondary metabolites with a diverse range of biological functions. They play a crucial role in protecting plants against ultraviolet radiation, predators, and pathogens, which contributes to their value in applications such as pharmaceuticals, food preservation, and cosmetics (Alara et al., 2021).

The total phenolic compounds present in the hydrolysates are shown in **Figure 6c**. The results indicate that the quantity of phenolic compounds was significantly affected by both temperature and pH, with values ranging from 16.06 to 76.82 mg GAE g^{-1} CS. Comparatively, a previous study reported a phenolic compound content of 12.76 mg GAE g^{-1} CS in dry CS obtained through acid hydrolysis, highlighting the potential of subcritical hydrolysis to enhance phenolic compound recovery under optimal conditions (Vazquez-Olivo et al., 2019). The process of hydrolysis in subcritical water can improve the extraction of phenolic compounds. The treatment yielding the highest amount of phenolic compounds was CSH5, conducted at a temperature of 170 °C and a pH of 1, achieving a total of 76.82 mg GAE g^{-1} CS. Conversely, the treatment with the lowest phenolic content was CSH7, which utilized a hydrolysis temperature of 113 °C and a pH of 4.5, resulting in 16.06 mg GAE g^{-1} CS. The results indicate that the highest concentrations of phenolic compounds were observed in treatments conducted at elevated temperatures (above 170 °C), with the CSH6 treatment yielding 54.65 mg GAE g^{-1} CS.

At temperatures ≤130 °C, as seen in the CSH1, CSH3, and CSH7 treatments, phenolic compound levels remained below 27.96 mg GAE g^{-1} CS. The trend of increased phenolic compound yield at higher temperatures has been observed in other studies (Barroso et al., 2022a; Ferreira et al., 2023). This phenomenon can be attributed to phenolic compounds often being bound to structural components of the plant cell wall or sugars. High temperatures are necessary to hydrolyze these bonds, facilitating the release of phenolic compounds.

Yields of phenolic compounds from the control treatments are presented in **Table 6** and illustrated in **Figure 7a**. The results confirm that conventional hydrolysis methods are inferior to subcritical water hydrolysis. Acid hydrolysis and alkali hydrolysis produced significantly lower yields (12.03 ± 1.38 mg GAE g^{-1} CS and 11.78 ± 0.63 mg GAE g^{-1} CS, respectively) than the yield obtained in the CSH7 treatment with subcritical

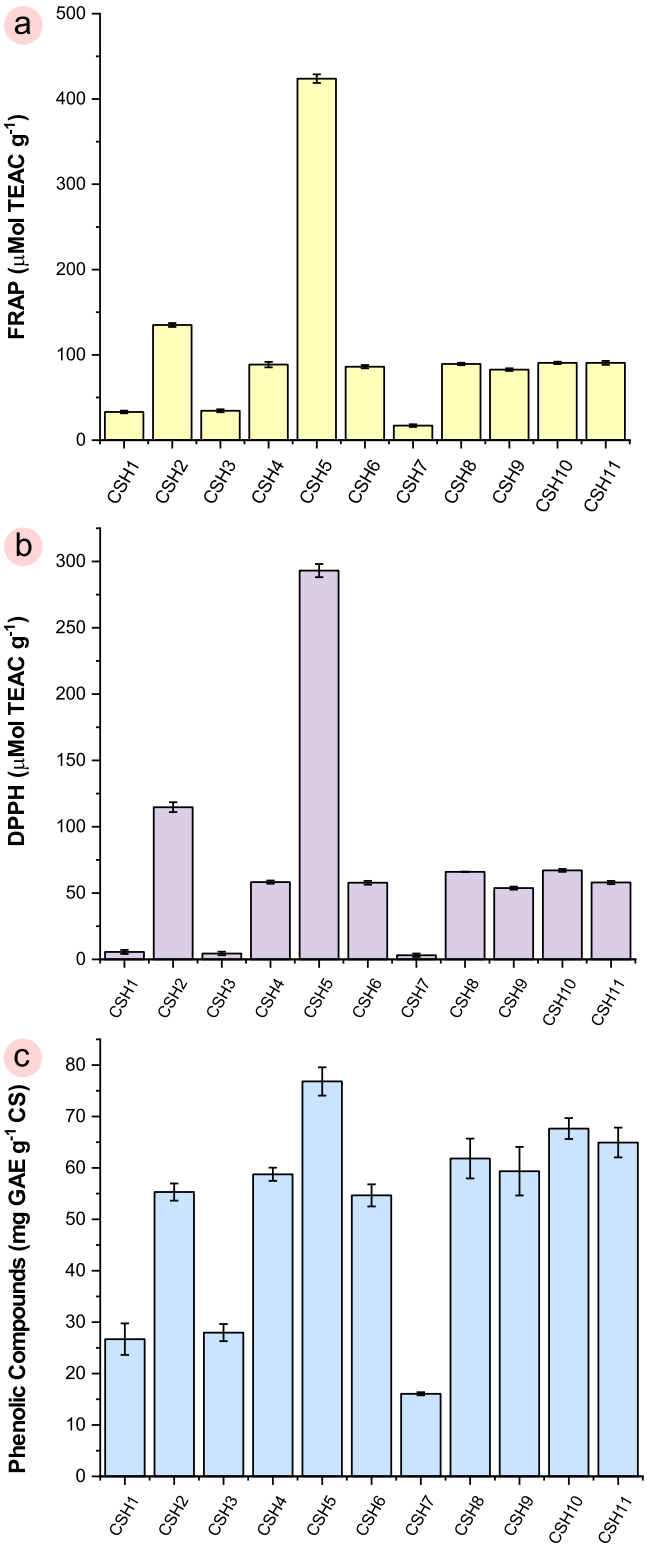

Fig. 6. The antioxidant activity of hydrolysates obtained from the CS hydrolysis process in subcritical water. **(a)** accumulated FRAP; **(b)** accumulated DPPH; and **(c)** phenolic compounds accumulated. Label: CSH1 (130 ºC, pH 2), CSH2 (210 ºC, pH 2), CSH3 (130 ºC, pH 7), CSH4 (210 ºC, pH 7), CSH5 (170 ºC, pH 1), CSH6 (170 ºC, pH 8), CSH7 (113 ºC, pH 4.5), CSH8 (226 ºC, pH 4.5), CSH9 (170 ºC, pH 4.5), CSH10 (170 ºC, pH 4.5), and CSH11 (170 ºC, pH 4.5).

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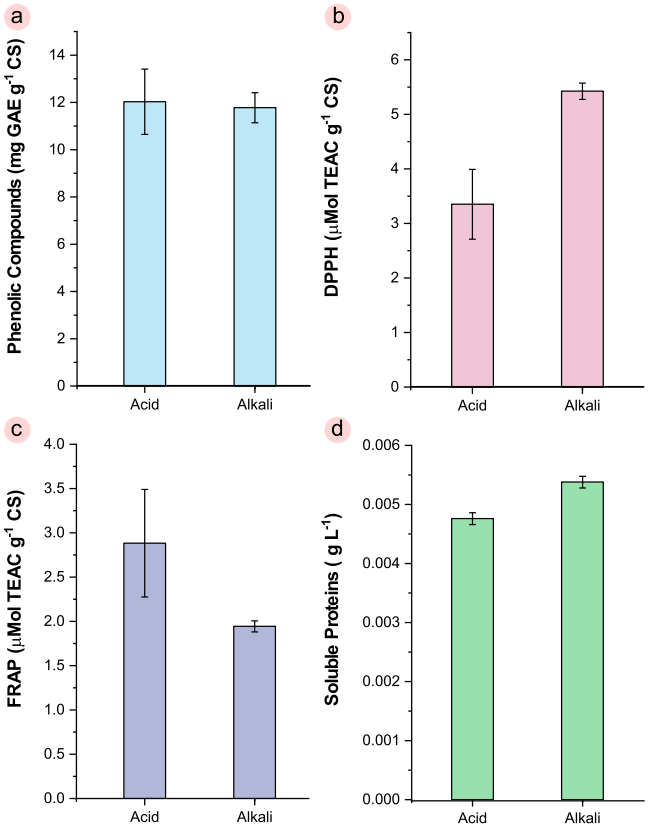

Fig. 7. Results obtained from the CS hydrolysis process using conventional methods. **(a)** phenolic compounds, **(b)** DPP, **(c)** FRAP, and **(d)** soluble proteins.

water hydrolysis (16.06 mg GAE g^{-1} CS). Moreover, the highest yield achieved with subcritical water hydrolysis (CSH5 treatment: 76.82 mg GAE g^{-1} CS) was 6.3 times higher than that of acid hydrolysis. These findings underscore the superiority of subcritical water hydrolysis over conventional methods for extracting phenolic compounds.

3.2.4. Antioxidant activity (FRAP and DPPH)

The results for antioxidant activity using the FRAP and DPPH assays are shown in **Figure 6a-b**. Antioxidant activity values obtained using the FRAP method ranged from 17.16 to 423.85 µmol TEAC g^{-1} CS, while those obtained using the DPPH method ranged from 3.13 to 293.12 µmol TEAC g^{-1} CS. The results show that antioxidant activity is directly linked to the number of phenolic compounds since the hydrolysate from the CSH5 treatment showed the highest antioxidant activities: 423.85 µmol TEAC g^{-1} CS for FRAP and 293.12 µmol TEAC g^{-1} CS for DPPH. In contrast, the hydrolysates from treatments CSH1, CSH3, and CSH7 showed the lowest antioxidant activity, with values of 5.66 µmol TEAC g^{-1} CS for DPPH and 33.55 µmol TEAC g^{-1} CS for FRAP.

The relationship between antioxidant activity and phenolic compound content observed in this study aligns with findings from previous research (Tang et al., 2021; Ferreira et al., 2023). For instance, one study assessed the FRAP antioxidant capacity of methanolic extracts of CS obtained through ultrasound-assisted extraction, demonstrating a similar correlation between phenolic content and antioxidant activity. They reported a value of 442.2 µmol TEAC g^{-1} CS (Đorđević et al., 2019), which is very close to the results obtained in this study. The FRAP method showed higher antioxidant activity than the DPPH method, which can be attributed to its greater effectiveness in reducing iron ions compared to the inhibition of DPPH free radicals. Additionally, in aqueous media, DPPH has reduced solubility, which limits the availability of the radical to react with antioxidant compounds (Oliveira, 2015; Angonese et al., 2021).

Table 6 and **Figure 7b-c** show the results of antioxidant activity for hydrolysates obtained through conventional methods. Both acid and alkali hydrolysis yielded FRAP and DPPH values lower than all subcritical water hydrolysis treatments. Acid hydrolysis yielded antioxidant activity values of 3.35 ± 0.64 µmol TEAC g^{-1} CS for DPPH and 5.41 ± 0.15 µmol TEAC g^{-1} CS for FRAP. Alkali hydrolysis yielded even lower values: 2.88 ± 0.60 µmol TEAC g^{-1} CS for DPPH and 1.94 ± 0.062 µmol TEAC g^{-1} CS for FRAP. In contrast, the subcritical water treatment (CSH5), which had the highest antioxidant activity, exhibited DPPH and FRAP values that were 87.5 and 78.2 times higher, respectively, than those obtained through conventional acid and alkali hydrolysis.

3.2.5. Soluble Proteins

Figure 8 shows the concentration of soluble proteins during the hydrolysis process in subcritical water. Protein content ranged from 0.00744 to 0.059 g L^{-1}, with the highest concentration observed in the CSH5 treatment. At the same time, the lowest concentration was found in the hydrolysate from the CSH1 treatment. Sganzerla et al. (2022b) used subcritical water hydrolysis at 180°C to pretreat brewers' spent grains (BSG) and obtained a soluble protein content of 5.01 g L^{-1}, a significantly higher result than in this study. This difference can be explained by the higher protein content of BSG (19.72%) compared to CS (4.60%). Another factor that may have contributed is the hydrolysis temperature; very low temperatures were unable to solubilize the protein, while very high temperatures caused protein degradation into secondary compounds such as carbonic acids, amines, and aldehydes (Marcet et al., 2016).

The acid and alkali hydrolysis methods produced soluble protein levels comparable to those obtained with subcritical water hydrolysis. **Table 6** and **Figure 7d** display the results. The acid method yielded 0.0048 ± 0.0001 g L^{-1}, while the alkali method yielded 0.0054 ± 0.0001 g L^{-1}, a value similar to that obtained in the CSH5 treatment (0.0059 ± 0.0002 g L^{-1}).

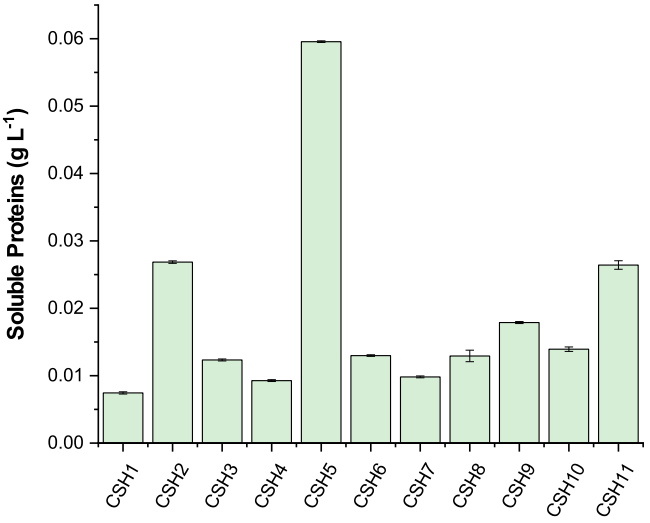

Fig. 8. Soluble proteins in hydrolysates obtained from the CS hydrolysis process in subcritical water. Label: CSH1 (130 °C, pH 2), CSH2 (210 °C, pH 2), CSH3 (130 °C, pH 7), CSH4 (210 °C, pH 7), CSH5 (170 °C, pH 1), CSH6 (170 °C, pH 8), CSH7 (113 °C, pH 4.5), CSH8 (226 °C, pH 4.5), CSH9 (170 °C, pH 4.5), CSH10 (170 °C, pH 4.5), and CSH11 (170 °C, pH 4.5).

3.2.6. Sugars

Figure 9 shows the results of the kinetic profile and the yield of individual sugars, while **Table 7** shows the total amount of sugars for each treatment studied.

The highest sugar yields were observed in the CSH5 treatment (460.92 ± 6.17 mg g^{-1}) and the CSH1 treatment (448.54 ± 39.21 mg g^{-1}). Specifically,

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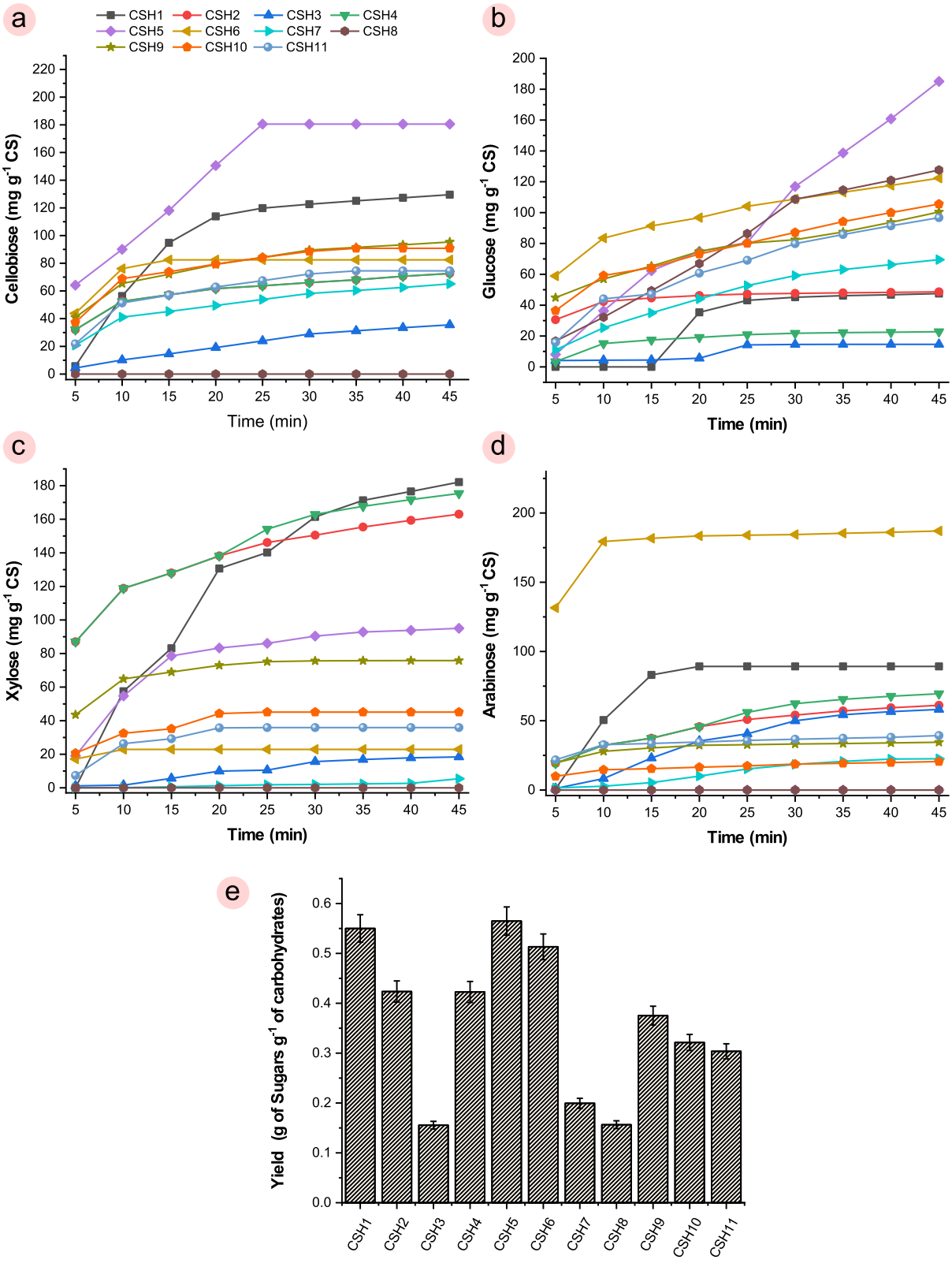

Fig. 9. Profile of sugars during the time of subcritical water hydrolysis of corn stover: **(a)** cellobiose; **(b)** glucose; **(c)** xylose; **(d)** arabinose; and **(e)** global sugars yield. Label: CSH1 (130 ºC, pH 2), CSH2 (210 ºC, pH 2), CSH3 (130 ºC, pH 7), CSH4 (210 ºC, pH 7), CSH5 (170 ºC, pH), CSH6 (170 ºC, pH 8), CSH7 (113 ºC, pH 4.5), CSH8 (226 ºC, pH 4.5), CSH9 (170 ºC, pH 4.5), CSH10 (170 ºC, pH 4.5), and CSH11 (170 ºC, pH 4.5).

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Table 7.
Sugar composition of the CS hydrolysates obtained from subcritical water hydrolysis.*

Compound	CSH1 130°C pH 2	CSH2 210°C pH 2	CSH3 130°C pH 7	CSH4 210°C pH 7	CSH5 170°C pH 1	CSH6 170°C pH 8	CSH7 113°C pH 4.5	CSH8 226°C pH 4.5	CSH9 170°C pH 4.5	CSH10 170°C pH 4.5	CSH11 170°C pH 4.5	Unit
Cellobiose	129.55 ± 3.54^{c}	72.63 ± 1.32^{de}	35.54 ± 0,41^{bd}	77.02 ± 1.18^{de}	180.56 ± 4.99^{a}	82.45 ± 3.17^{e}	65.1 ± 0.20^{de}	n.d	95.36 ± 2.57^{c}	90.81 ± 2.19^{ce}	74.53 ± 2.40^{de}	mg g^{-1}
Glucose	47.55 ± 3.69^{c}	48.64 ± 5.73^{bc}	14.62 ± 0.1^{d}	22.79 ± 0.12^{d}	185.06 ± 10.75^{a}	122.30 ± 0.42^{ef}	69.5 ± 1.77^{b}	127.06 ± 0.3^{e}	100.38 ± 1.26^{efg}	105.55 ± 10.05^{fg}	96.72 ± 7.65^{g}	mg g^{-1}
Xylose	182.1 ± 5.45^{b}	162.99 ± 13.3^{b}	18.43± 0.45^{cd}	175.38 ± 13.4^{b}	95.03 ± 0.42^{a}	26.68 ± 1.59^{cd}	5.43 ± 0.20^{d}	n.d	75.78 ± 9.89^{a}	45.11 ± 3.59^{c}	37.04 ± 1.55^{c}	mg g^{-1}
Arabinose	89.34 ± 1.13^{b}	61.17 ± 1.12^{cd}	58.20 ± 2.56^{cd}	69.49 ± 0.17^{bc}	n.d	187.06 ± 10.35^{a}	22.56 ± 1.07^{ef}	n.d	34.50 ± 5.67^{def}	20.59 ± 2.37^{ef}	39.29 ± 6.60^{de}	mg g^{-1}
Total	448.54 ± 39.21	345.43 ± 16.6	126.79 ± 2.42	344.68 ± 11.94	460.92 ± 6.17	418.49 ± 25.31	162.59 ± 2.62	127.06 ± 0.3	306.02 ± 14.25	262.01 ± 13.47	247.58 ± 0.18	mg g^{-1}

* The results are expressed as the mean ± standard deviation. Analysis conducted in triplicate (n=3). Different letters in each line indicate significant differences by Tukey's test at $p \leq 0.05$.

mild temperatures, the experimental design, and acidic pH favored hydrolysis, leading to enhanced sugar formation. Previous studies have also reported high sugar yields at temperatures below 180°C in subcritical water hydrolysis. For example, Castro et al. (2023) achieved the highest sugar concentrations from grape pomace hydrolysate at 150°C and 180°C. Similarly, Sganzerla et al. (2022b) reported the best sugar recovery from brewer's spent grains at 180°C, while Barroso et al. (2022a) found optimal sugar yields from jabuticaba peel at 135°C. These findings align with the results obtained in this study, further supporting the effectiveness of subcritical water hydrolysis at moderate temperatures for sugar recovery. The higher sugar production in this temperature range can be explained by more efficient hemicellulose hydrolysis into monomers, while these temperatures prevent significant sugar degradation into organic acids or inhibitors (Cocero et al., 2018).

The results showed that pH and temperature influenced sugar production, with treatment CSH1 producing approximately 72% more than CSH3, which used the same temperature but had a higher pH (7 *vs*. 2 in CSH1). Regarding temperature, CSH1 produced approximately 23% more sugars than CSH2, which had the same pH but a higher temperature (210 *vs*. 170°C in CSH1). Thus, using milder temperatures (±170°C) and a more acidic pH makes it possible to produce greater quantities of sugars. The kinetics of sugar recovery indicate that the highest sugar production occurs within the first 10–15 min, stabilizing by 30 min. Therefore, the hydrolysis process can be completed in 30 min, at which point 84.3% of the total sugar yield has been obtained. After this point, sugar production shows a significant decrease. This assessment is crucial for cost reduction in the process, as longer hydrolysis times entail greater energy expenditure, which may not always be compensated by the amount produced (Sganzerla et al., 2023). Some studies have found similar optimal times for terminating hydrolysis and maximizing sugar yields. For example, in the hydrolysis of grape pomace using subcritical water, Castro et al. (2023) determined that 30 min was the optimal time to halt the process, as sugar production had stabilized by that point. Similarly, in another study focusing on jabuticaba peel, Barroso et al. (2022a) found that the ideal time to stop the hydrolysis was 25 min, achieving a recovery rate of 73.20% for reducing sugars. These findings highlight the importance of identifying the optimal reaction time to maximize sugar yields during subcritical hydrolysis.

The composition of the hydrolysates showed the presence of cellobiose, glucose, xylose, and arabinose. The composition found was very similar to that reported in a study on liquor extraction using autohydrolysis (water + temperature), where glucose, xylose, and arabinose were detected (Egüés et al., 2012). The highest cellobiose content was found in CSH5, at 180.56 ± 4.99 mg g^{-1}. In subcritical water hydrolysis, cellobiose is derived from accessible cellulose, which undergoes degradation and deoxygenation reactions (Amadeus et al., 2007). Glucose reached its highest yield in the CSH5 treatment, at 185.06 ± 10.75 mg g^{-1}. Glucose is derived from hemicellulose and cellulose, which become accessible during hydrolysis (Prado et al., 2014). Xylose content was highest in the CSH1 treatment (182.1 ± 5.45 mg g^{-1}), while arabinose content peaked in the CSH6 treatment (187.06 ± 10.35 mg g^{-1}). Both arabinose and xylose are primary sugars derived from hemicellulose during hydrolysis (Cocero et al., 2018; Sganzerla et al., 2022b).

The sugar yield was calculated based on the amount of carbohydrates present in the CS, and the highest yield was observed in treatment CSH5 (170°C and pH 1), with a yield of 0.565 g sugars g^{-1} carbohydrates. The sugar yield results indicate that subcritical water hydrolysis of CS converted part of the lignocellulosic material into sugars. Thus, the hydrolysate obtained can be used in fermentation processes, for example, to produce second-generation (2G) ethanol (Oliveira et al., 2021).

Table 6 and **Figure 10a-b** present the sugar yields obtained through conventional hydrolysis methods. The results show that sugar yields from acid and alkali hydrolysis were notably low, with only 0.0745 g sugars g^{-1} carbohydrates and 0.0055 g sugars g^{-1} carbohydrates, respectively. These findings underscore the limitations of conventional methods for sugar recovery from CS. In comparison, the CSH5 treatment achieved a yield that was 7.6 times higher than that of acid hydrolysis and 99 times higher than that of alkali hydrolysis, demonstrating that subcritical water hydrolysis of CS is significantly more efficient for sugar production than conventional methods.

3.2.7. Organic acids

Figure 11 shows the results of the kinetic profile and the yield of individual organic acids, while **Table 8** shows the total amount of organic acid for each treatment studied.

The highest concentration of organic acids was observed in the CSH8 treatment (1,157.19 ± 17.77 mg g^{-1}) and CSH4 (780.54 ± 0.24 mg g^{-1}). The results showed that higher temperatures led to increased organic acid production. This finding can be explained by the fact that higher temperatures intensify hydrolysis reactions due to increased ionic strength, causing hydronium ions to act as catalysts, thereby favoring organic acid formation (Abaide et al., 2019a; Nejatie et al., 2022). Additionally, at temperatures above 200ºC, the hydrolytic breakdown of hemicellulose produces organic acids (Calvo and Vallejo, 2002; Santos et al., 2020). Previous studies have also reported the highest concentrations of organic acids at temperatures above 200ºC (Barroso et al., 2022a and b; Castro et al., 2023). Consistent with the results obtained in this study, pH also influenced organic acid production. The treatments conducted at the same temperature (170°C) but with a more acidic pH resulted in higher organic acid production: CSH5 (pH 1) produced 755.73 ± 2.50 mg g^{-1}, CSH6 (pH 8) produced 623.86 ± 25.09 mg g^{-1}, and CSH9 (pH 4.5) produced 778.1 ± 9.30 mg g^{-1}. This finding can be explained by the fact that lower pH values enhance catalytic activity, thereby favoring organic acid production (Asghari and Yoshida, 2006).

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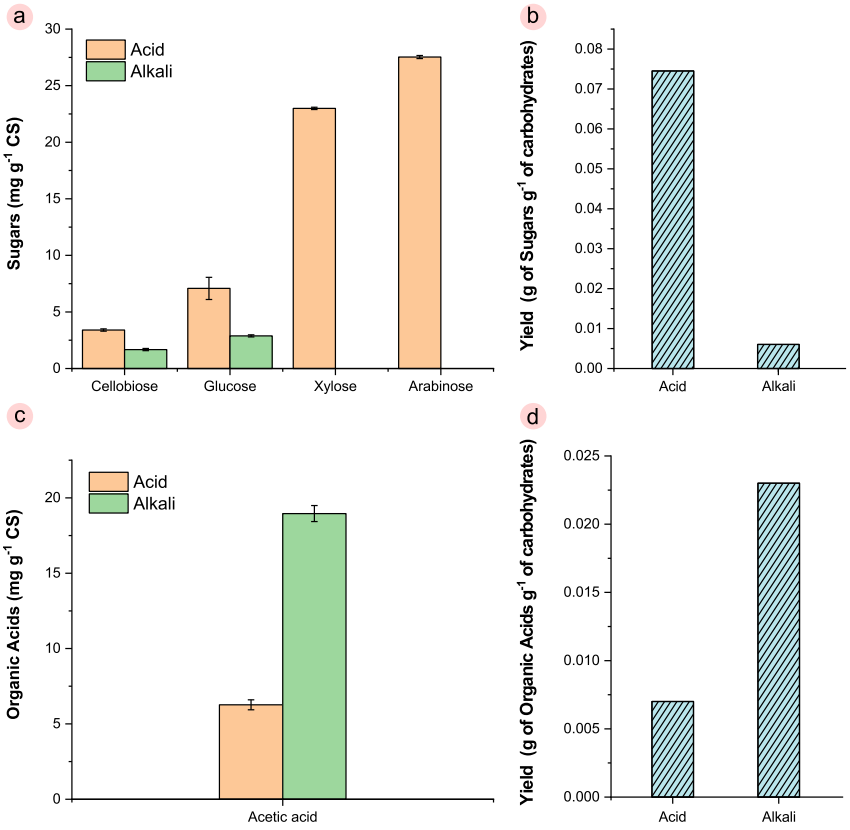

Fig. 10. Profile of sugars and organic acids obtained from the hydrolysis of corn stover by conventional methods: **(a)** sugars; **(b)** global sugar yield; **(c)** organic acids; and **(d)** global organic acids yield.

Table 8.
Sugar composition of the CS hydrolysates obtained from subcritical water hydrolysis.*

Compound	CSH1 130°C pH 2	CSH2 210°C pH 2	CSH3 130°C pH 7	CSH4 210°C pH 7	CSH5 170°C pH 1	CSH6 170°C pH 8	CSH7 113°C pH 4.5	CSH8 226°C pH 4.5	CSH9 170°C pH 4.5	CSH10 170°C pH 4.5	CSH11 170°C pH 4.5	Unit
Formic acid	n.d	596.73 ± 34.54^{c}	n.d	593.92 ± 2.22^{c}	569,11 ± 14.90^{c}	371.65 ± 11.42^{b}	n.d	736.54 ± 25.58^{a}	557.05 ± 7.66^{c}	564.26 ± 25.04^{c}	581.14 ± 1.86^{c}	mg g^{-1}
Acetic acid	103.05 ± 0.75^{e}	111.25 ± 3.28^{e}	103.36 ± 6.05^{e}	186.62 ± 1.95^{d}	349,78 ± 17.41^{b}	252.21 ± 36.52^{c}	84.45 ± 2.96^{e}	420.65 ± 7.80^{a}	221.05 ± 1.63^{cd}	207.56 ± 18.33^{cd}	184.41 ± 9.05^{d}	mg g^{-1}
Total	103.05 ± 0.75	707.98 ± 31.26	103.36 ± 6.05	780.54 ± 0.27	755.73 ± 2.50	623.86 ± 25.09	85.45 ± 2.96	1,157.19 ± 17.77	778.1 ± 9.30	771.82 ± 6.71	765.55 ± 10.91	mg g^{-1}

* The results are expressed as the mean ± standard deviation. Analysis conducted in triplicate (n=3). Different letters in each line indicate significant differences by Tukey's test at $p \leq 0.05$.

The composition of the hydrolysates revealed the presence of formic acid and acetic acid. The highest concentrations of formic acid (736.54 ± 25.58 mg g^{-1}) and acetic acid (420.65 ± 7.50 mg g^{-1}) were observed in the CSH8 treatment, which used the highest temperature (226°C) and pH 4.5. Acetic acid formation results from the degradation of the hemicellulose component of biomass and the monosaccharides present in the hydrolysate (Mohan et al., 2015; Ishak et al., 2019). Formic acid is derived from hemicellulose during the furfural formation process. Additionally, formic acid can originate from soluble fibers and xylose (Alvarez-Vasco and Zhang, 2013; Lamminpää et al., 2014; Huang et al., 2016; Yedro et al., 2017). The yield of organic acids was calculated based on the amount of carbohydrates present in the CS, with the highest yield observed in the CSH8 treatment (226°C and pH 4.5), reaching 1,123 mg organic acids g^{-1} carbohydrates. This finding demonstrates that organic acid formation increases at higher temperatures and more acidic pH conditions.

Table 6 and Figure 10c-d display the production and yield of organic acids achieved through conventional methods. The yields of organic acids obtained by acid and alkali hydrolysis were very low (0.0076 g organic acids g^{-1} carbohydrates and 0.0023 g organic acids g^{-1} carbohydrates, respectively). In comparison, the CSH8 treatment achieved a yield that was 147.7 times higher than that of acid hydrolysis and 48.08 times higher than that of alkali hydrolysis, demonstrating that subcritical water hydrolysis of CS is significantly more efficient for organic acid production than conventional hydrolysis methods.

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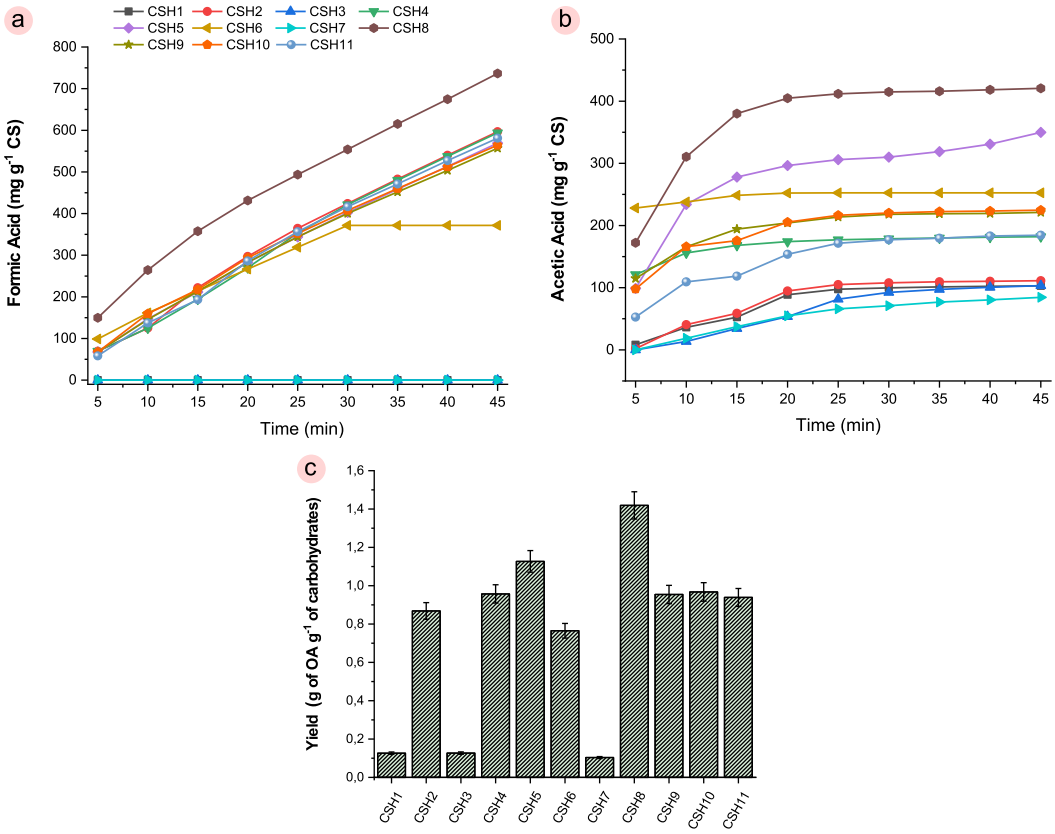

Fig. 11. Profile of organic acids during time of subcritical water hydrolysis of corn stover: **(a)** formic acid; **(b)** acetic acid; and **(c)** global organic acids yield. Label: CSH1 (130 ºC, pH 2), CSH2 (210 ºC, pH 2), CSH3 (130 ºC, pH 7), CSH4 (210 ºC, pH 7), CSH5 (170 ºC, pH 1), CSH6 (170 ºC, pH 8), CSH7 (113 ºC, pH 4.5), CSH8 (226 ºC, pH 4.5), CSH9 (170 ºC, pH 4.5), CSH10 (170 ºC, pH 4.5), and CSH11 (170 ºC, pH 4.5).

3.2.8. Inhibitors

Figure 12 shows the kinetic profile and yield of individual inhibitors, while Table 9 shows the total amount of inhibitors for each treatment studied. If the hydrolysate is intended for use in fermentation processes, it is crucial to minimize the presence of inhibitors. High levels of inhibitors, such as furfural and 5-HMF, can significantly impede the growth and activity of microorganisms, adversely affecting the efficiency and success of fermentation (Sganzerla et al., 2022a; Sillero et al., 2022). Only the treatments using temperatures above 210°C (CSH2, CSH4, and CSH8), as well as the treatment at 170°C and pH 1 (CSH5), produced inhibitors, demonstrating that temperature and pH influence inhibitor formation. A similar pattern of inhibitor formation was observed in a study where inhibitors were only produced at temperatures above 210°C (Castro et al., 2023).

The treatment with the highest inhibitor formation was CSH5, with a total yield of 542.54 ± 8.35 mg g^{-1}. This treatment was conducted at 170°C and pH 1, indicating that pH significantly influenced inhibitor formation. This finding can be explained by the fact that, at acidic pH levels, the higher concentration of hydrogen ions catalyzes hydrolysis, leading to increased inhibitor formation (Amarasekara et al., 2024). Additionally, high temperatures promote inhibitor formation by triggering hydrolytic reactions (Torres-Mayanga et al., 2019). The highest inhibitor formation occurred within the first 25 to 30 min, after which inhibitor formation declined significantly.

The composition of the hydrolysates revealed the presence of 5-hydroxymethylfurfural and furfural. The highest concentrations of 5-hydroxymethylfurfural (266.14 ± 4.07 mg g^{-1}) and furfural (276.40 ± 8.35 mg g^{-1}) were observed in the CSH5 hydrolysate. The production of 5-HMF results from the dehydration of hexose sugars (C6), such as glucose. Furfural is produced from the dehydration of pentose sugars (C5), such as arabinose and xylose (Lachos-Perez et al., 2017; Benito-Román et al., 2022).

The yield of inhibitors was calculated based on the carbohydrate content of CS. The highest inhibitor yield was observed in treatment CSH5 (170°C and pH 1) at 0.665 mg inhibitors g^{-1} carbohydrates. Notably, treatments with higher inhibitor yields had lower sugar yields. For instance, CSH5 had a sugar yield of 0.564 mg sugars g^{-1} carbohydrates, suggesting that some sugars degraded into inhibitors. This observation is consistent with literature findings indicating that inhibitors such as furfural and 5-HMF are produced from sugar degradation (Slak et al., 2022). The inhibitors present in hydrolysates can be effectively removed through purification methods, such as activated carbon, biosorbents, and other techniques. Once separated, these compounds can serve as valuable raw materials for producing fuels, resins, lubricants, and a variety of industrial products (Slak et al., 2022).

3.2.9. Optimization, modeling, and statistical analysis

To verify the influence of different reactor operating temperatures and pH levels, RSM was used, including replication at the central point. The statistical model used first- and second-order functions to evaluate all variables measured during the hydrolysis experiment, which were analyzed using analysis of variance (ANOVA). For all compounds studied, the variables temperature (x) and pH (y) had a statistically significant effect at the 5% significance level. These results are also evident in the ANOVA (Table S1).

Figure S1 of the Supplementary Material shows the Pareto charts for all response variables. The graphs indicate that temperature (x) and pH (y) significantly affected the results for phenolic compounds, soluble proteins, sugars, organic acids, and inhibitors. Figure S2 shows the effects of

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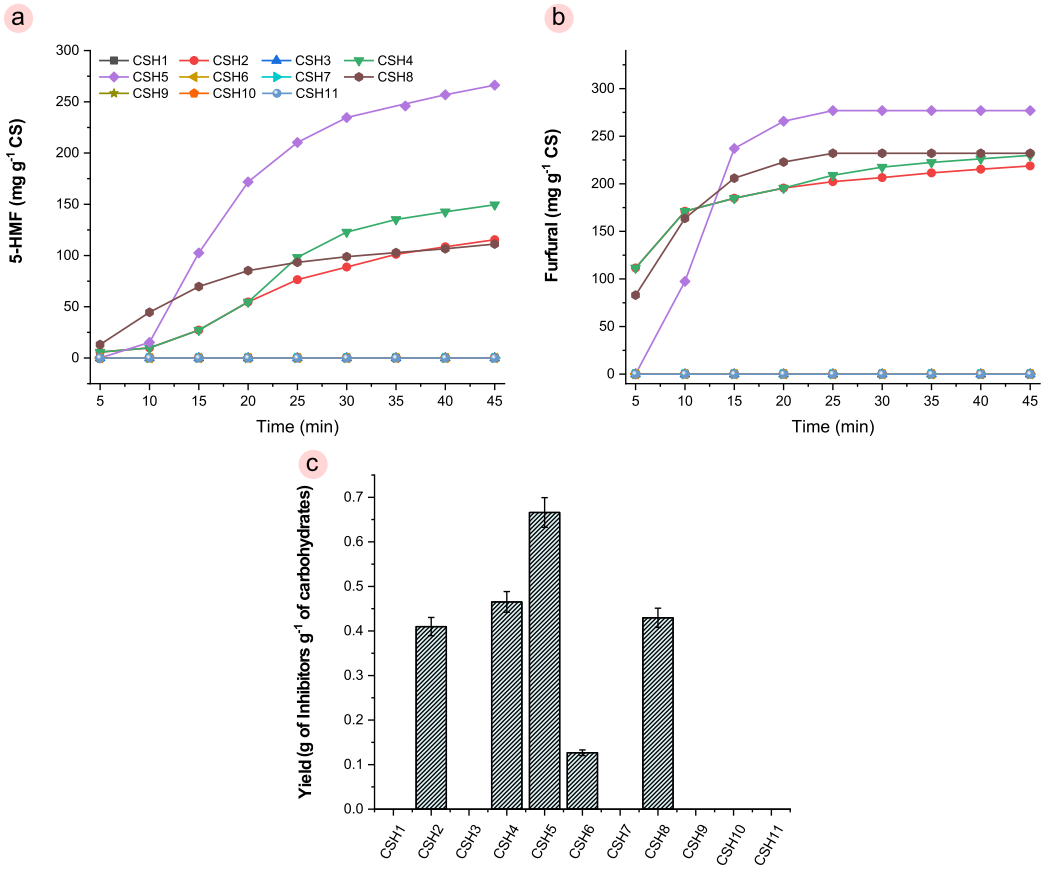

Fig. 12. Profile of Inhibitors during the time of subcritical water hydrolysis of Corn stover: **(a)** 5-HMF; **(b)** furfural; and **(c)** global inhibitors yield. Label: CSH1 (130 ºC, pH 2), CSH2 (210 ºC, pH 2), CSH3 (130 ºC, pH 7), CSH4 (210 ºC, pH 7), CSH5 (170 ºC, pH 1), CSH6 (170 ºC, pH 8), CSH7 (113 ºC, pH 4.5), CSH8 (226 ºC, pH 4.5), CSH9 (170 ºC, pH 4.5), CSH10 (170 ºC, pH 4.5), and CSH11 (170 ºC, pH 4.5).

Table 9.
Inhibitors composition of the CS hydrolysates obtained from subcritical water hydrolysis.*

Compound	CSH1 130°C pH 2	CSH2 210°C pH 2	CSH3 130°C pH 7	CSH4 210°C pH 7	CSH5 170°C pH 1	CSH6 170°C pH 8	CSH7 113°C pH 4,5	CSH8 226°C pH 4.5	CSH9 170°C pH 4.5	CSH10 170°C pH 4.5	CSH11 170°C pH 4.5	Unit
5-HMF	n.d	115.43 ± 4.09^{c}	n.d	149.52 ± 5.21^{b}	266.14 ± 4.07^{a}	n.d	n.d	111.21 ± 1.71^{c}	n.d	n.d	n.d	mg g^{-1}
Furfural	n.d	218.80 ± 11.96^{b}	n.d	229.83 ± 12.07^{b}	276.40 ± 4.27^{a}	n.d	n.d	239.10 ± 13.42^{b}	n.d	n.d	n.d	mg g^{-1}
Total	-	334.23 ± 16.05	-	379.35 ± 17.29	542.54 ± 8.35	-	-	350.31 ± 11.71	-	-	-	mg g^{-1}

* The results are expressed as the mean ± standard deviation. Analysis conducted in triplicate (n=3). Different letters in each line indicate significant differences by Tukey's test at $p \leq 0.05$.

temperature and pH on the response variables. The vertical axis displays compound concentration, while the horizontal axis represents temperature (x) and pH (y). **Figures S2a-d** indicate that pH had little effect on the recovery of phenolic compounds and organic acids. In contrast, temperature had the greatest influence, demonstrating that higher temperatures led to higher recovery of phenolic compounds and organic acids. For the other variables, temperature and pH significantly influenced the recovery of soluble proteins, sugars, and inhibitors.

Figure S3 illustrates the synergistic effects of the studied factors, where the vertical axis represents compound concentration, and the horizontal axis indicates hydrolysis temperature. The curves illustrate the interaction between pH and the variables under investigation. The most acidic pH (pH 2) demonstrated the best overall performance, as evidenced by the greater spacing of its curve from the others, signifying a stronger effect of pH. However, exceptions were noted for organic acid recovery (**Fig. S3d**), where pH 4.5 exhibited behavior nearly identical to that of pH 2, with overlapping curves, and for sugar recovery (**Fig. S3c**), where a neutral pH (pH 7) yielded higher sugar yields at temperatures above 180°C. These results emphasize the complex interplay between pH and temperature in the hydrolysis process and their distinct impacts on different compounds.

Two-dimensional response surface graphs were generated to understand better the effects of temperature and pH (**Fig. 13**). Phenolic compounds

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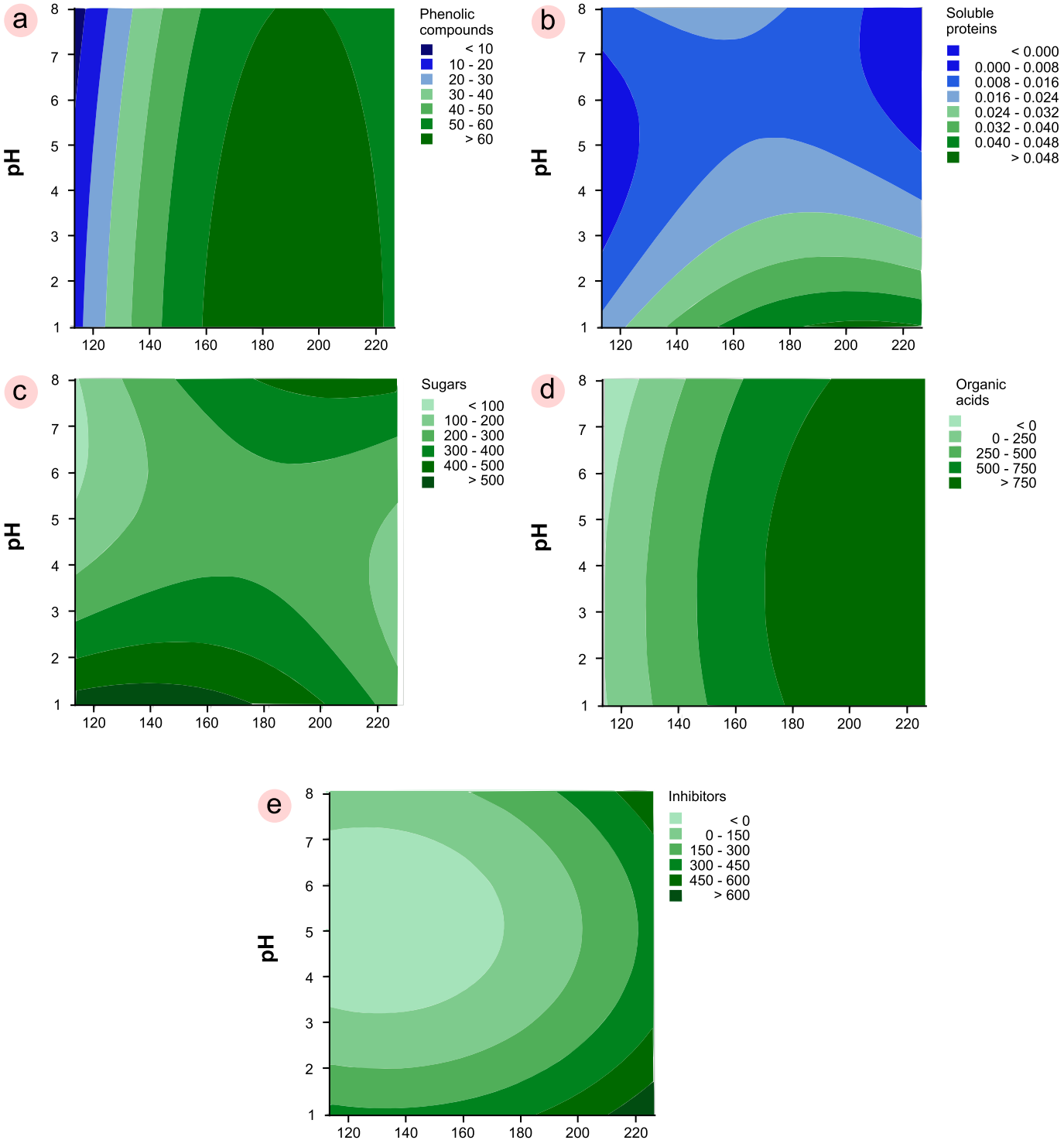

Fig. 13. Response surface plots (2D) of statistical analysis: **(a)** phenolic compounds; **(b)** soluble proteins; **(c)** sugars; **(d)** organic acids; and **(e)** inhibitors.

(Fig. 13a) reach maximum production within a high-temperature range (170–210°C), regardless of pH, corroborating Figure S2a, which shows that pH has little influence on bioactive compound recovery. The highest amounts of soluble proteins (Fig. 13b) are produced at high temperatures (170–220°C) and under highly acidic conditions (pH 1). Sugars (Fig. 13c) are more abundantly produced at milder temperatures (130–170°C) and acidic pH (pH 1–2) or higher temperatures (170–210°C) under neutral to alkaline pH (pH 7–8). For organic acids (Fig. 13d), the highest production

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occurs between 170 and 210°C, regardless of pH, confirming **Figure S2d**, which shows that pH has little influence on organic acid production. Minimal inhibitor production is desirable because excessive amounts can negatively affect fermentation processes when using the hydrolysate. Thus, the lowest inhibitor production occurs between 130 and 170°C and at higher pH values (4.5–7). Equations and coefficients of determination for the regression model fitted to second-order functions were derived for phenolic compounds, soluble proteins, sugars, organic acids, and inhibitors. These equations and their respective goodness-of-fit values are presented in **Table 10**, providing a comprehensive overview of the model's accuracy and predictive capabilities for each variable.

The models showed a good fit, with coefficients of determination (R^2) greater than 69.84%. The factorial design was also optimized to maximize or minimize the compounds of interest. The obtained models were evaluated using the Simplex method, with calculations performed using the Microsoft Excel® Solver tool. The optimization was subject to the temperature and pH constraints of the experimental design: temperature ($113°C \leq x \leq 226°C$) and pH ($1 \leq y \leq 8$). The following parameters were determined through maximization: for phenolic compounds (T = 190.7°C, pH = 1, and 69.29 mg GAE g^{-1}), soluble proteins (T = 187.4°C, pH = 1, and 0.036 g L^{-1}), sugars (T = 134.9°C, pH = 1, and 566.69 mg g^{-1}), and organic acids (T = 223.59°C, pH = 4.1, and 981.23 mg g^{-1}). For inhibitor minimization, the optimal parameters were T = 114.95°C, pH = 7.3, and 11.64 mg g^{-1}. These parameters are important, as experimental conditions can be adjusted based on the target compound to maximize or minimize its concentration.

In **Table S1**, the lack of fit for inhibitors is evidenced by a p-value below the significance level ($\alpha = 0.05$). This result may be due to the absence of inhibitor formation in some treatments, as their production is strongly dependent on high temperatures and very low pH, which were only observed experimentally in a few cases. Future studies should explore alternative models that could more accurately capture the dynamics and conditions governing inhibitor production.

3.2.10. Characterization of the solid material remaining after subcritical water hydrolysis

TGA and DTG were used to analyze the solid residues remaining after subcritical water hydrolysis to assess their lignocellulosic composition. The decomposition profile and derived thermogravimetric analysis are shown in **Figures 14a** and **14b**, respectively. The DTG results were used to determine the contributions of individual components through the integration method and normalized area approach (Carrier et al., 2011).

The first components to degrade are the semi-volatile compounds, which undergo degradation between 50 and 175°C. Semi-volatile compounds include residual water, extractable compounds, small hydrophilic molecules, and unstable proteins (Mosteiro-Romero et al., 2014; Sganzerla et al., 2022b). **Figure 14c** shows a reduction in semi-volatiles after hydrolysis (values below 4.4%) compared to the initial CS content (11.76%). This reduction was expected since subcritical water hydrolysis can extract various semi-volatile compounds, such as organic acids and lipids (Paini et al., 2021b).

Between 175 and 300°C, hemicellulose undergoes degradation. The most significant reduction occurred within this range, as the initial hemicellulose content in CS (25.16%) decreased to 5.46% in CSH5, which supports the high sugar yield observed in this treatment since hemicellulose breaks down into sugars. A similar trend was observed in a hydrolysis study of spent brewer's grains in subcritical water, where a 13% decrease in hemicellulose content was reported (Sganzerla et al., 2022b). Between 300 and 370°C, cellulose undergoes degradation. The results showed that the cellulose content remained largely unchanged, indicating that higher hydrolysis temperatures are necessary for cellulose degradation.

Lignin undergoes thermal degradation between 370 and 550°C, while char forms between 550 and 700°C. These two compounds accumulate at higher hydrolysis temperatures. These results are consistent with findings in the literature (Abaide et al., 2019b; Sganzerla et al., 2022b; Castro et al., 2023).

3.3. EcoScale analysis

The EcoScale tool was used to evaluate the environmental impact of the biomass hydrolysis method used for CS in this study. Four studies from the literature that used different methodologies for CS pretreatment were considered for comparison. **Table 11** shows that the subcritical water process in this study achieved a score of 93, which was higher than those of the other studies. Other studies showed either low yields of fermentable sugars or negative environmental impacts due to extensive solvent usage, which poses safety risks. For instance, Study 2 (**Fig. 15**) received a 10-point penalty in the EcoScale score due to extensive solvent usage. It is important to note that although the subcritical water method in this study received a 5-point penalty in the technical and setup evaluation for being pressurized and unconventional, this did not significantly affect the final score. The temperature and time penalties were quite similar among the studies since all studies relied on temperature as a crucial factor for hydrolysis, with variations in the required time, either below or above 1 h. In summary, subcritical water proved to be a more sustainable and efficient approach for CS biomass hydrolysis than the other evaluated methods.

3.4. Investment analysis in capital recovery and payback time

The payback calculation in this study must consider the initial investment. The result represents the number of years required to recoup the initial investment. This simplified formula works well when annual cash flows are constant. **Table 12** shows sugar production and expected payback. The two treatments that produced the largest sugar yields were analyzed: CSH1 (pH 2, 130°C) and CSH5 (pH 0.96, 170°C).

For all calculations, the parameters used in the experiment and the quantities of sugars produced were taken into account, and the scale-up was carried out proportionally. With a daily processing rate of 1,000 kg of CS, the CSH1 treatment yields 129.55 kg of cellobiose, 47.55 kg of glucose, 182.10 kg of xylose, and 89.34 kg of arabinose. In comparison, the CSH5 treatment yields 180.56 kg of cellobiose, 185.06 kg of glucose, and 95.03 kg of xylose.

Table 10.
Equations and the coefficients of determination of the regression model adjusted for the second-order function variables for phenolic compounds, soluble proteins, sugars, organic acids, and inhibitors.

Parameter	Equation	Coefficients of Determination
Phenolic compounds (mg GAE g^{-1})	$-254.1 + 3.398x - 0.98y - 0.008924x^2 - 0.142y^2 + 0.0054xy$	$(R2 = 95.33\%)$ $(R2adj = 93.47\%)$
Soluble protein (g L^{-1})	$-0.1018 + 0.001553x - 0.00392y - 0.000004x^2 + 0.001062y^2 - 0.000056xy$	$(R2 = 78.46\%)$ $(R2adj = 69.84\%)$
Sugars (mg g^{-1})	$142 + 9.34x - 281.2y - 0.03748x^2 + 13.97y^2 + 0.802xy$	$(R2 = 91.59\%)$ $(R2adj = 88.23\%)$
Organic acids (mg g^{-1})	$-3042 + 35.44x + 30.1y - 0.0809x^2 - 8.67y^2 + 0.181xy$	$(R2 = 94.30\%)$ $(R2adj = 92.02\%)$
Inhibitors (mg g^{-1})	$1388 - 12.52x - 261.6y + 0.0464x^2 + 23.73y^2 + 0.113xy$	$(R2 = 84.67\%)$ $(R2adj = 78.54\%)$

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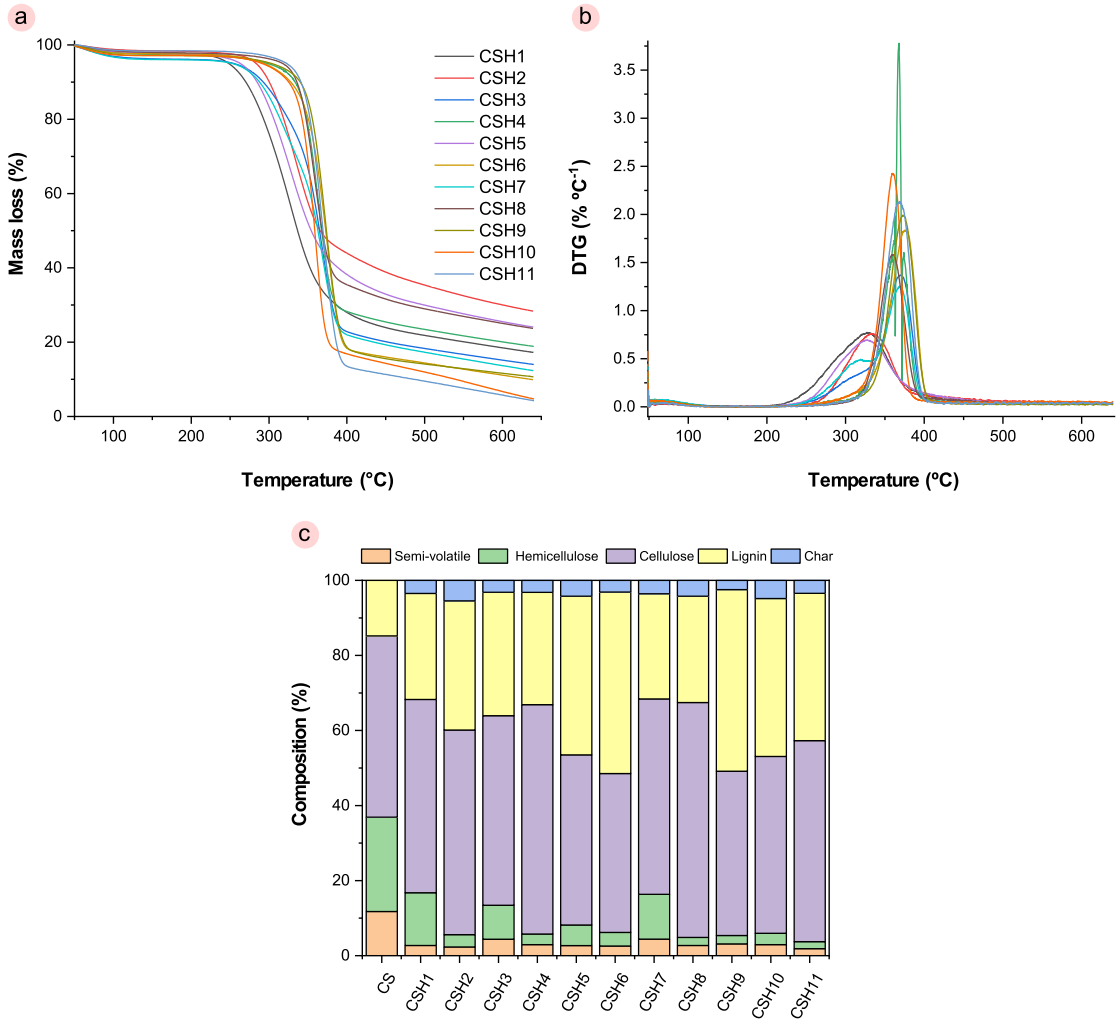

Fig. 14. Thermogravimetric analysis: **(a)** thermogram of residue after the subcritical water hydrolysis process; **(b)** derivative thermogravimetric analysis (DTG); and **(c)** lignocellulosic composition of corn stover and solid residue after the subcritical water hydrolysis process based on DTG. Label: CS (Corn Stover), CSH1 (130 ºC, pH 2), CSH2 (210 ºC, pH 2), CSH3 (130 ºC, pH 7), CSH4 (210 ºC, pH 7), CSH5 (170 ºC, pH 1), CSH6 (170 ºC, pH 8), CSH7 (113 ºC, pH 4.5), CSH8 (226 ºC, pH 4.5), CSH9 (170 ºC, pH 4.5), CSH10 (170 ºC, pH 4.5), and CSH11 (170 ºC, pH 4.5).

Table 11.
Attribution of points using the EcoScale methodology for the environmental analysis of CS hydrolysis with subcritical water. This table also compares these points to penalty points from other relevant studies with the same objective.

Ref.	Yield (mg $_{sugars}$ g $_{biomass}^{-1}$)	Relative yield (%)[a]	Extraction method	Reagents	Technical / Setup	Price / Availability	Safety	Temperature / Time	EcoScale
This Study	460.92	100	Subcritical water	Water, sulfuric acid.	-5 (pressure equipment, unconventional technique)	0	0	-2 (heating < 1 h)	93
P. Wang et al. (2015)	442.85	96.08	Alkali pretreatment, enzymatic hydrolysis	Water, Sodium hydroxide, Calcium oxide, Hydrogen peroxide, Water, enzymes (cellulase and xylanase)	-2 (instruments for controlled addition of chemicals, special glassware)	0	-10	-3 (heating > 1 h)	83.04
Wang et al. (2022)	247	53.59	Acid pretreatment	Water, sulfuric acid	-2 (instruments for controlled addition of chemicals, special glassware)	0	0	-3 (heating > 1 h)	71.79
Satarn et al. (2014)	79.6	17.27	Microwave, Acid pretreatment	Water, acetic acid	-2 (instruments for controlled addition of chemicals, special glassware)	0	0	-2 (heating < 1 h)	54.63
Katsimpouras et al. (2016)	370	80.27	Acid pretreatment	Water, sulfuric acid	-2 (instruments for controlled addition of chemicals, special glassware)	0	0	-3 (heating > 1 h)	85.13

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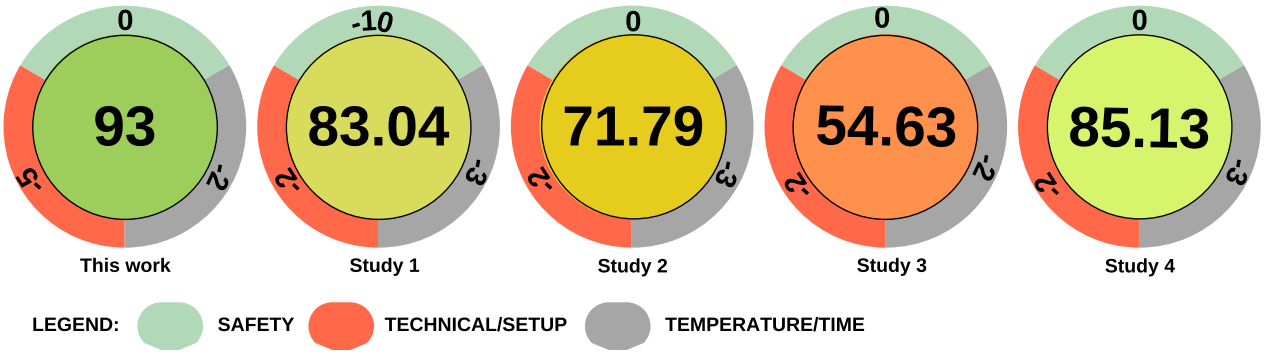

Fig. 15. Environmental EcoScale analysis scores of subcritical corn stover water hydrolysis and comparison studies.

Table 12.
Sugar Production and investment analysis in capital recovery and payback time.

Parameters	CSH1	CSH5	Unit
Cellobiose	2,394,084.00	3,336,748.80	USD yr^{-1}
Glucose	15,063.84	58.627.01	USD yr^{-1}
Xylose	432,669.60	225,791.28	USD yr^{-1}
Arabinose	1,391,559.84	-	USD yr^{-1}
Total	4,233,377.28	3,621,167.09	USD yr^{-1}
Payback	3.90	5.10	Yr

As shown in **Table 12**, and assuming an annual operating time of 264 days, the sales values per kilogram of each sugar, as detailed in the table, lead to an annual gross revenue of USD 4,233,377.28 for the CSH1 treatment and USD 3,621,167.09 for the CSH5 treatment. It should be noted that these figures represent gross revenues and do not account for operating costs, such as electricity, water, reagents, labor, and maintenance, which affect the net profitability of the process.

According to **Table 12**, the estimated annual operating cost for a unit with three hydrolysis vessels (500 liters each) is USD 2,111,000.00. Using these data, the payback period can be determined by comparing the annual net revenue (gross revenue minus operating costs) to the initial investment. The payback period represents the time required to recover the initial investment and for the system to reach profitability, marking the point when it begins generating a net profit (Du et al., 2024). The payback period is calculated based on the initial investment of USD 8,276,785.09 and the annual cash flow, which is the value derived from annual sugar production minus annual operating costs. The annual cash flow is USD 2,122,377.28 for the CSH1 treatment and USD 1,510,167.09 for the CSH5 treatment. Based on these calculations, the payback periods are 3.9 years for the CSH1 treatment and 5.1 years for the CSH5 treatment. In other words, scaling up using the CSH1 treatment parameters would yield a financial return sooner than the CSH5 treatment. In a techno-economic study on subcritical water hydrolysis to produce sugars from spent brewer's grains using the same scale-up (3 × 500 L), payback periods ranged from 0.85 to 3.86 years across eight scenarios with different parameters (Sganzerla et al., 2021).

This study concludes that a quick financial return is possible, with an estimated payback period of 3.9 years using the analyzed parameters. The research evaluated different investment and cash flow scenarios and concluded that optimizing key variables, such as efficiently allocating resources and selecting projects with consistent returns, may enable investors to recoup their initial capital in as little as four years. This result highlights the importance of careful strategic analysis, demonstrating that with the right parameters, payback can be achieved in a relatively short period while minimizing risks and enhancing investment attractiveness.

3.5. Practical implications of the study

The conditions employed in this study enabled the high recovery of sugars, organic acids, and inhibitors, each with significant industrial potential. Sugars, including cellobiose, glucose, xylose, and arabinose, are valuable for industrial applications such as fermentation and biofuel production. Organic acids, such as formic acid and acetic acid, have widespread applications in the food, chemical, and cosmetics industries. While inhibitors generated during the process are unsuitable for biological fermentation due to their antimicrobial properties, they hold considerable value in the chemical industry, where they serve as precursors for plastics, biofuels, and other industrial chemicals.

Subcritical water hydrolysis of CS produced a hydrolysate with strong antioxidant activity, highlighting the potential of this technology for extracting antioxidant compounds. However, further studies are needed to assess its efficacy in food and pharmaceutical applications. In support of this study, recent research has explored the technical and economic feasibility of subcritical water hydrolysis for producing sugars (Sganzerla et al., 2021) and xylooligosaccharides (Sganzerla et al., 2023) from spent brewer's grains. The techno-economic assessment for xylooligosaccharides showed strong profitability indicators across all processes studied, with the two-reactor system achieving the highest gross margin (63.28%) and a payback period of 1.84 years. The authors concluded that subcritical water hydrolysis for the production of xylooligosaccharides is an economically viable process. Similarly, sugar recovery via the simulated and extended technological routes was found to be feasible on an industrial scale.

Scaling up subcritical water hydrolysis presents a promising opportunity for industries that manage CS waste. Transforming CS, often regarded as a low-value byproduct with disposal costs, into valuable compounds can significantly enhance revenue. This study underscores the potential of subcritical water hydrolysis as a sustainable and efficient technology for converting CS into value-added products. The findings offer essential insights for optimizing process conditions to target specific end products, facilitating broader industrial adoption.

3.6. Limitations of the study

The conversion of lignocellulosic materials into value-added products is a major focus of global research. This study provided valuable insights into the subcritical water hydrolysis of CS; however, future investigations are needed to explore its potential limitations. A key limitation of this study was the use of a single solvent/feed ratio (45 mL of water per gram of CS). Investigating different solvent/feed ratios may enhance product recovery and process efficiency. Additionally, process scale-up must be evaluated, as the parameters and conditions optimized in this study may not directly translate to an industrial scale. Industrial-scale implementation may require adjustments to engineering parameters and operational conditions to maintain efficiency and cost-effectiveness. Further research on these aspects

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will enhance the understanding of the process and help overcome the identified limitations, ultimately paving the way for broader industrial applications.

4. Conclusions

This study evaluated the semi-continuous subcritical water hydrolysis of CS by varying hydrolysis temperature and pH. Using a temperature of 170 ºC and pH 1 (CSH5) resulted in the highest recovery of phenolic compounds (76.82 mg GAE g^{-1}), leading to the greatest antioxidant activities: FRAP (423.85 μMol TEAC g^{-1}) and DPPH (293.12 μMol TEAC g^{-1}). The highest recovery of soluble proteins (0.059 g L^{-1} of hydrolysate) was also obtained under these conditions. Additionally, the CSH5 treatment achieved the highest sugar yield (460.92 ± 6.17 mg g^{-1}), consisting of cellobiose (180.56 ± 4.99 mg g^{-1}), glucose (185.06 ± 10.75 mg g^{-1}), and xylose (95.03 ± 0.42 mg g^{-1}).

The highest organic acid recovery (1,157.19 ± 17.77 mg g^{-1}) was obtained at 226 ºC and pH 4.5 (CSH8), indicating that increased temperature and pH enhance organic acid production. Under these conditions, the organic acid composition consisted of formic acid (736.54 ± 25.85 mg g^{-1}) and acetic acid (420.65 ± 7.80 mg g^{-1}). Temperatures below 170 ºC and pH above 4.5 did not result in inhibitor formation (furfural and 5-HMF), demonstrating that elevated temperatures and extreme pH conditions promote inhibitor production.

Analysis of the solid residue confirmed the hydrolysis and solubilization of hemicellulose. Statistical modeling demonstrated that pH and temperature strongly influence the CS hydrolysis process in subcritical water, identifying optimal conditions for each target compound. The capital recovery and payback analysis indicated that scaling up the process with three hydrolysis vessels (500 liters each) could recover the initial investment in under four years.

Subcritical water technology is an economically viable solution for industrial applications, as demonstrated by techno-economic studies in the literature. The optimized conditions from these studies provide a foundation for designing and improving industrial hydrolysis systems tailored to specific product goals. As a sustainable alternative to conventional methods, subcritical water treatment eliminates the need for harsh chemicals while enabling efficient recovery of valuable bioproducts from CS, promoting a circular bioeconomy. Instead of being discarded as waste, CS is transformed into a renewable resource, aligning with sustainable development goals.

However, further research is needed to optimize reaction parameters for improved cost-effectiveness and scalability. Integrating subcritical hydrolysis with downstream conversion technologies is crucial for realizing a biorefinery approach and maximizing bioproduct utilization. This study lays the groundwork for a scalable and sustainable strategy to convert CS waste into valuable products. Industrial-scale implementation of this technology could advance the circular bioeconomy, reduce dependence on fossil fuels, and mitigate resource depletion, contributing to a more sustainable future.

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Supplementary Material

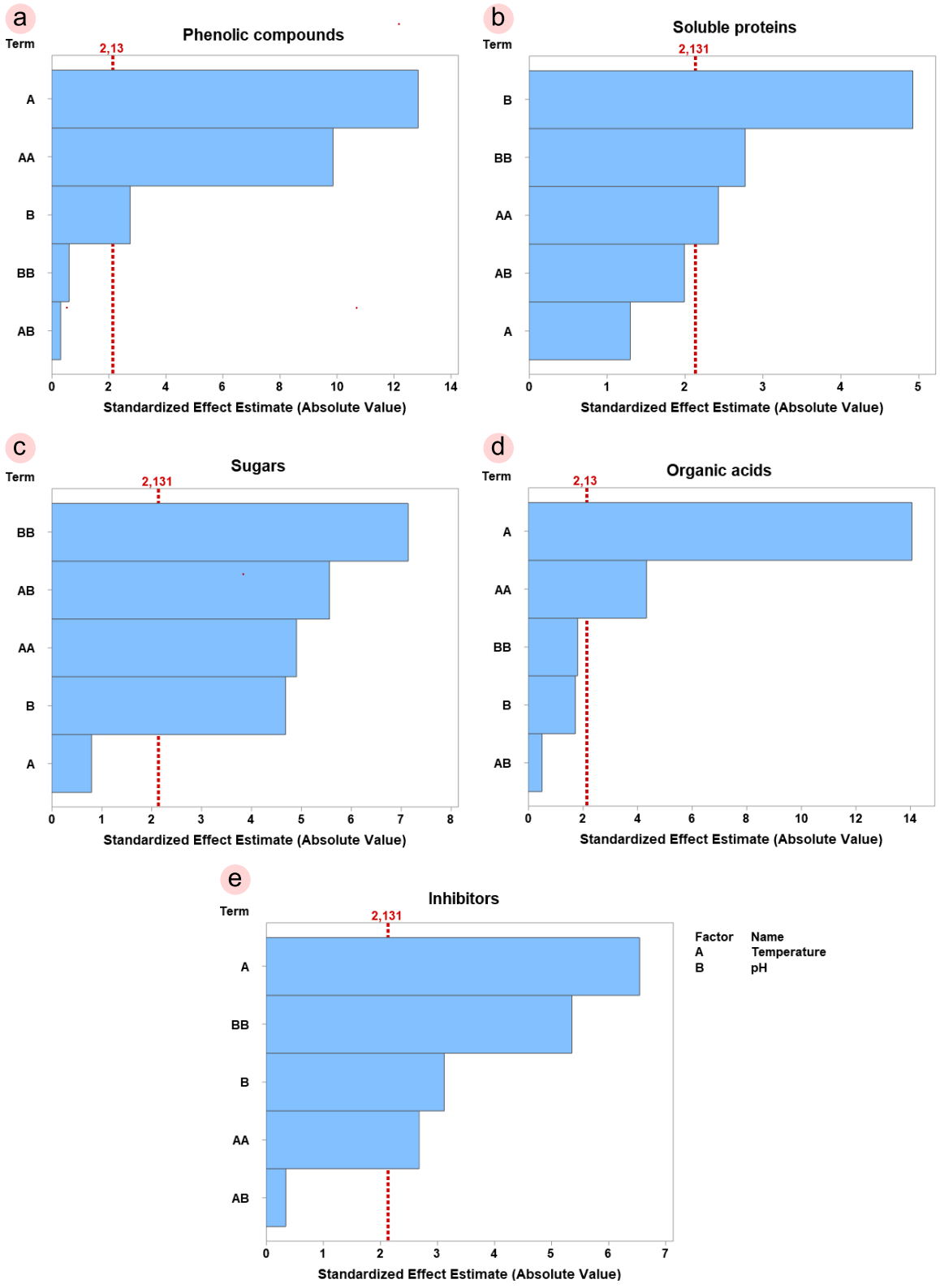

Fig. S1. Pareto chart for the RSM design response variables: **(a)** phenolic compounds; **(b)** soluble proteins; **(c)** sugars; **(d)** organic acids; and **(e)** inhibitors.

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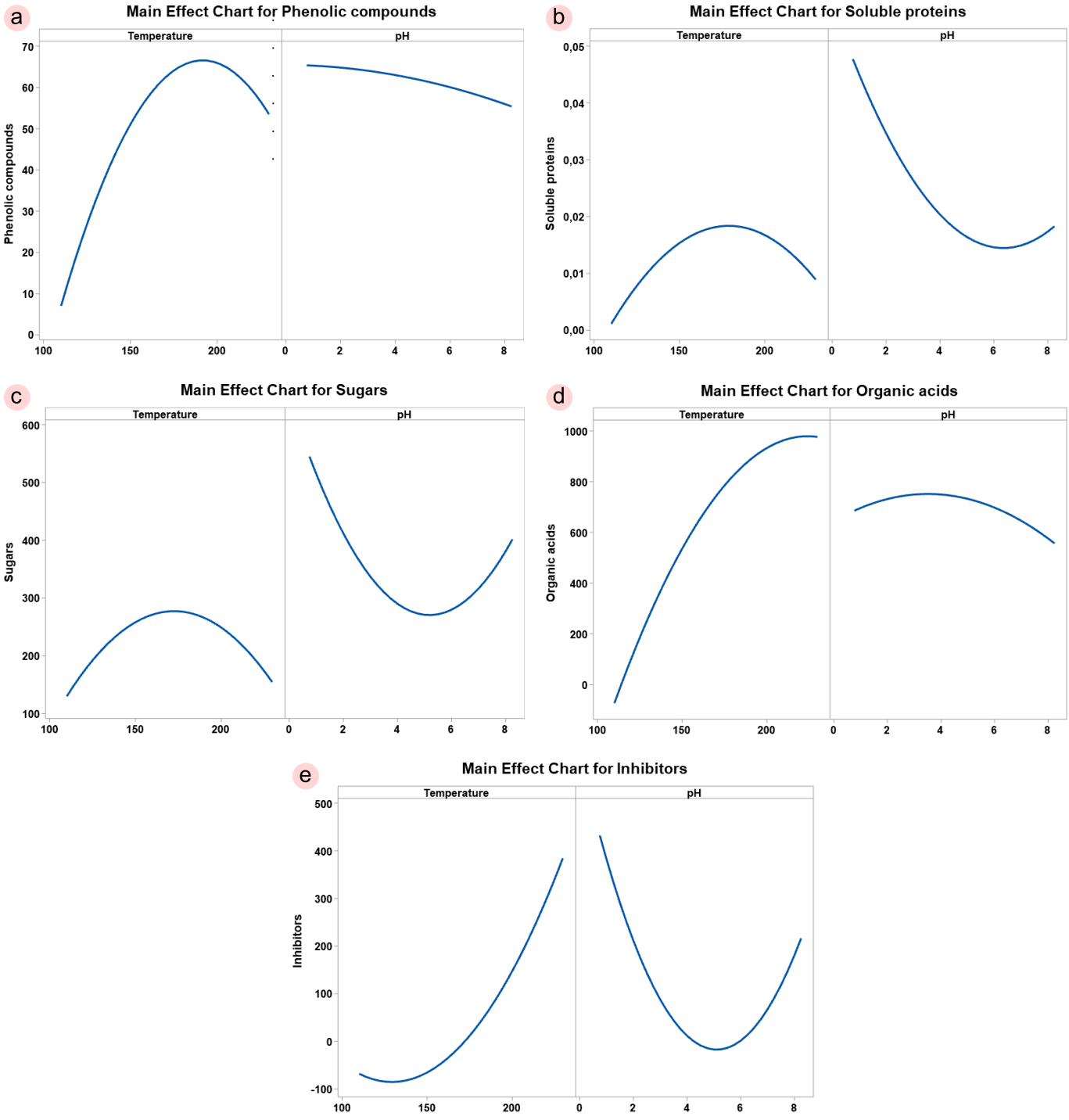

Fig. S2. Main effect chart for the RSM design response variables: **(a)** phenolic compounds; **(b)** soluble proteins; **(c)** sugars; **(d)** organic acids; and **(e)** inhibitors.

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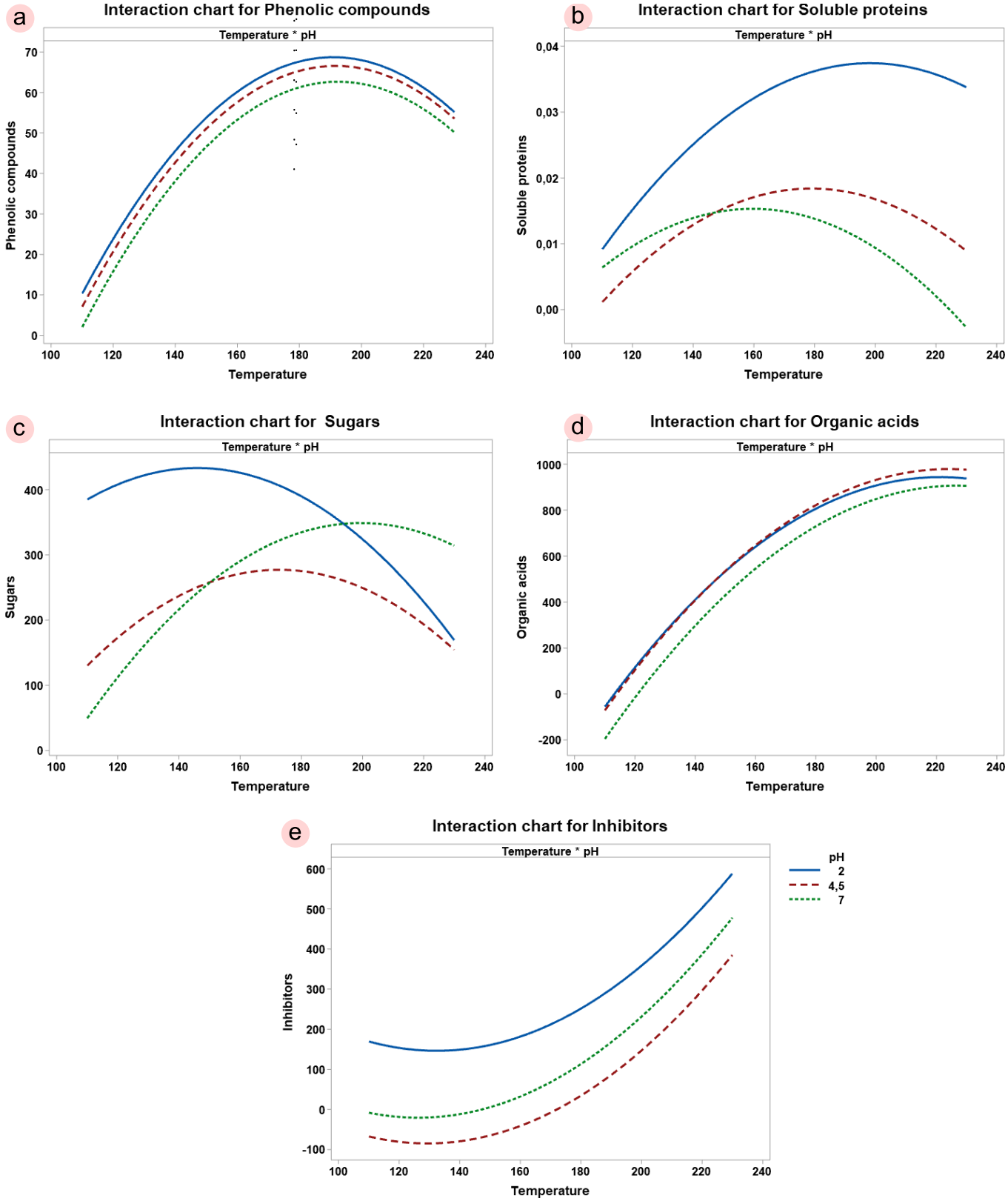

Fig. S3. Interaction chart for the RSM design response variables: **(a)** phenolic compounds; **(b)** soluble proteins; **(c)** sugars; **(d)** organic acids; and **(e)** inhibitors.

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Table S1.
ANOVA results for the RSM design for the response variable.

Source	DF*	Phenolic compounds				Soluble proteins				Sugars			
		SS	MS	F value	*p*-value	SS	MQ	F value	p-value	SS	MQ	F value	*p*-value
Temperature (°C)	1	3852.00	3852.00	164.99	*0.000*	0.000108	0.000108	1.69	*0.214*	1064.00	1064.40	0.64	*0.001*
Temperature² (°C)	1	2269.02	2269.02	97.19	*0.000*	0.000376	0.000376	5.90	*0.028*	40021.00	40020.70	24.03	*0.001*
pH	1	177.17	177.17	7.59	*0.015*	0.001543	0.001543	24.21	*0.000*	36507.00	36507.20	21.92	*0.082*
Temperature (°C)*pH	1	2.36	2.36	0.10	*0.755*	0.000253	0.000253	3.97	*0.065*	51513.00	51513.10	30.93	*0.082*
Residual	15	350.20	23.35	-	-	0.000956	0.000064	-	-	24979.00	1665.20	-	-
Lack-of-fit	3	342.82	114.27	185.77	*0.184*	0.000800	0.000267	20.52	*0.500*	24769.00	8256.20	471.92	*0.117*
Total	21	7503.56	-	-	-	0.004438	-	-	-	297186.00	-	-	-

Source	DF	Organic acids				Inhibitors			
		SS	MS	F value	*p*-value	SS	MQ	F value	*p*-value
Temperature (°C)	1	1958911.00	1958911.00	197.16	*0.000*	365427.00	365427.00	42.78	*0.000*
Temperature² (°C)	1	186533.00	186533.00	18.77	*0.001*	61461.00	61461.00	7.19	*0.017*
pH	1	29649.00	29649.00	2.98	*0.105*	83165.00	83165.00	9.74	*0.007*
Temperature (°C)*pH	1	2610.00	2610.00	0.26	*0.616*	1018.00	1018.00	0.12	*0.735*
Residual	15	149032.00	9935.00	-	-	128135.00	8542.00	-	-
Lack-of-fit	3	148483.00	49494.00	1082.45	*0.077*	128135.00	42712.00	0.00	*0.001*
Total	21	2614331.00	-	-	-	836027.00	-	-	-

* Abbreviations: DF, degrees of freedom; SS, sum of squares; MQ, mean square.Legend: DF, degrees of freedom; SS, sum of squares; MQ, mean square.

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