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Optimizing autohydrolysis conditions for production of ferulic and *p*-coumaric acids with antioxidant activity from rice husk

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Abstract

Uruguay generates approximately 275 thousand tons of rice husk (RH) annually as a by-product of rice production. RH, a lignocellulosic material rich in cellulose, hemicelluloses and lignin, is typically burned or landfilled, leading to environmental concerns. This study aimed to optimize the obtention of phenolic acids with antioxidant properties, particularly ferulic and *p*-coumaric acids, through hydrothermal treatment (autohydrolysis) of RH. Response surface methodology (RSM) was employed to assess the influence of temperature, liquid/solid (L/S) ratio, and hydrolysis time on autohydrolysis yield, total phenolic content (TPC), antioxidant activity, and phenolic acids concentrations. The optimal conditions for production of ferulic and *p*-coumaric acids were determined to be 190 °C, 46.8 mL/g, and 9.5 minutes, yielding 24.5 mg/100 g RH on a dry basis (d.b.) of ferulic acid and 188.0 mg/100 g RH d.b. of *p*-coumaric acid. The hydrolysates also exhibited significant antioxidant activity and TPC. Compared to previous studies, our findings indicate enhanced hydrolysis yield, highlighting autohydrolysis as a promising, environmentally friendly method for valorization of RH. These results contribute to the development of sustainable biorefinery processes for agricultural waste valorization.

Keywords: antioxidants, autohydrolysis, ferulic acid, rice husk, *p*-coumaric acid

Optimización de las condiciones de autohidrólisis para la producción de ácidos ferúlico y *p*-cumárico con actividad antioxidante a partir de cáscara de arroz

Resumen

Uruguay genera aproximadamente 275 mil toneladas de cáscara de arroz (CA) anualmente como subproducto de la producción de arroz. La CA, un material lignocelulósico rico en celulosa, hemicelulosas y lignina, suele ser quemada o depositada en terrenos, lo que genera preocupaciones ambientales. Este estudio tuvo como objetivo optimizar la obtención de ácidos fenólicos con propiedades antioxidantes, en particular los ácidos ferúlico y *p*-cumárico, mediante tratamiento hidrotérmico (autohidrólisis) de la CA. Se empleó la metodología de superficie de respuesta (RSM) para evaluar la influencia de la temperatura, la relación líquido/sólido (L/S) y el tiempo de hidrólisis en el rendimiento de autohidrólisis, el contenido fenólico total (CFT), la actividad antioxidante y las concentraciones de ácidos fenólicos. Las condiciones óptimas para la producción de ácidos ferúlico y *p*-cumárico se determinaron en 190 °C, 46,8 mL/g y 9,5 minutos,

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obteniéndose 24,5 mg/100 g de CA en base seca (b.s.) de ácido ferúlico y 188,0 mg/100 g CA b.s. de ácido *p*-cumárico. Los hidrolizados también mostraron una actividad antioxidante y un CFT significativos. En comparación con estudios previos, nuestros hallazgos indican un mayor rendimiento de hidrólisis, destacando la autohidrólisis como un método prometedor y ambientalmente amigable para la valorización la CA. Estos resultados contribuyen al desarrollo de procesos sostenibles de biorrefinería para la utilización de residuos agrícolas.

Palabras clave: antioxidantes, autohidrólisis, ácido ferúlico, cáscara de arroz, ácido *p*-cumárico

Otimização das condições de autohidrólise para a produção de ácidos ferúlico e *p*-cumárico com atividade antioxidante a partir da casca de arroz

Resumo

O Uruguai gera aproximadamente 275 mil toneladas de casca de arroz (CA) anualmente como subproduto da produção de arroz. A CA, um material lignocelulósico rico em celulose, hemiceluloses e lignina, é geralmente queimada ou descartada em aterros, levando a preocupações ambientais. Este estudo teve como objetivo otimizar a obtenção de ácidos fenólicos com propriedades antioxidantes, especialmente os ácidos ferúlico e *p*-cumárico, através do tratamento hidrotérmico (autohidrólise) da CA. A metodologia de superfície de resposta (RSM) foi utilizada para avaliar a influência da temperatura, relação líquido/sólido (L/S) e tempo de hidrólise no rendimento da autohidrólise, no conteúdo fenólico total (CFT), atividade antioxidante e concentrações de ácidos fenólicos. As condições ideais para a produção de ácidos ferúlico e *p*-cumárico foram determinadas em 190 °C, 46.8 mL/g e 9.5 minutos, resultando em 24.5 mg/100 g de CA em base seca (b.s.) de ácido ferúlico e 188.0 mg/100 g CA b.s. de ácido *p*-cumárico. Os hidrolisados também apresentaram atividade antioxidante significativa e alto CFT. Comparado a estudos anteriores, nossos resultados indicam uma maior eficiência da hidrólise, destacando a autohidrólise como um método promissor e ecologicamente correto para valorizar a CA. Esses resultados contribuem para o desenvolvimento de processos sustentáveis de biorrefinaria para a utilização de resíduos agrícolas.

Palavras-chave: antioxidantes, autohidrólise, ácido ferúlico, casca de arroz, ácido *p*-cumárico

1. Introduction

Uruguay is a country with significant agricultural and agro-industrial activity, with rice being one of the highest-yielding crops. During the 2022/23 harvest season, 1.37 million tons of rice were produced, where approximately 20% of the weight corresponds to rice husk (RH)[(1)]. Therefore, approximately 275 thousand tons of this byproduct are generated annually. RH is generally used as fuel for direct combustion to generate electricity or it is disposed of by open-field burning and landfilling, leading to significant environmental impact[(2)]. Additionally, the ashes generated during the burning of RH constitute another waste product that must be managed. For these reasons, the investigation of alternatives for the use of RH is ongoing, making the valorization of this byproduct of interest to Uruguay. RH is a lignocellulosic material that could be used as a raw material for obtaining high value-added compounds under the biorefinery concept. RH is composed of cellulose (29-43%), hemicelluloses (22-30%), lignin (19-24%), and ash (17-20%)[(3)]. The hemicelluloses in RH consist mainly of xylans, which can give rise to xylo-oligosaccharides (XOS) and xylose. Use of lignocellulosic biomass for the obtention of fermentable sugars from both cellulosic and hemicellulosic fraction to obtain bioethanol and other chemical products has been widely reported in the last decade and it is well known[(4)]. On the other hand, there is still a scarce knowledge regarding the full exploitation of the lignin fraction, which is needed for any biorefinery to succeed, by producing high value-added products from it and thus lowering the operational costs[(4)]. Lignin is the most abundant aromatic heterogeneous polymer on the planet, synthesized from three precursors: *p*-coumaryl, coniferyl, and sinapyl alcohols[(5)]. The structure of this polymer suggests that it is a valuable source of phenolic

compounds (PCs). Solubilization and decomposition of lignin can lead to low molecular weight PCs such as phenolic acids. Lignin is always associated with hemicelluloses, not only through physical bonds but also through covalent ester linkages[5]. Ferulic acid and *p*-coumaric acid, the most abundant phenolic acids in RH, are also found to be esterified to the cell wall polysaccharides[7]. Obtention of XOS with esterified ferulic acid (so called feruloylated oligosaccharides) has been reported for byproducts such as corn fiber, maize bran, wheat bran, etc[8].

Given the recalcitrant nature of RH, it is necessary to subject it to some treatment to obtain the various compounds of interest. There are various procedures to separate lignocellulosic materials into their components, which can be physical, chemical, or a combination of both[8]. A promising technology for biomass fractionation is hydrothermal treatment or autohydrolysis. This process involves contacting the biomass with liquid water at high temperatures (usually in the range of 140-200 °C). During this process, hydronium ions generated from the autoionization of water promote the hydrolytic degradation of the cell wall constituents, acting as catalysts. Additionally, hydronium ions are also generated from organic acids, primarily acetic acid derived from the acetyl groups of hemicelluloses. Autohydrolysis is considered an economical and effective method, with one of its greatest advantages being that it does not require the addition of solvents or chemical reagents other than water, making it an environmentally friendly technology[9].

The main effect of autohydrolysis, though not the only one, is the solubilization of the hemicellulosic fraction. The resulting liquors or hydrolysates may contain oligosaccharides and monosaccharides (mainly XOS and xylose, respectively) in higher proportions, sugar degradation products (furfural or hydroxymethylfurfural (HMF)), organic acids, extractives, and PCs. XOS can be used as prebiotics in food formulations, while xylose serves as a carbon source in the fermentative production of ethanol and other chemical products[10]. On the other hand, the PCs present in the hydrolysates originate from the partial depolymerization of lignin and the solubilization of lignin-hemicellulose ester bonds. These low molecular weight PCs are an attractive source of natural antioxidants, with potential applications in the food, pharmaceutical, and cosmetic industries, as crude extracts of naturally sourced PCs have shown antioxidant activity comparable to that of synthetic antioxidants[11].

The present study aimed to optimize the obtention of ferulic and *p*-coumaric acids with antioxidant activity through autohydrolysis of RH by means of response surface methodology (RSM). To achieve this goal, a central composite design (CCD) was implemented, where the effect of temperature, liquid/solid (L/S) ratio and hydrolysis time on the following dependent variables was assessed: hydrolysis yield, total phenolic content (TPC), antioxidant activity, and the content of ferulic and *p*-coumaric acids in the hydrolysates. Additionally, the hydrolysate obtained under optimal conditions was characterized in terms of sugar and sugar degradation products composition.

2. Materials and methods

2.1 Chemicals

Ferulic acid (CAS 1135–24–6), *p*-coumaric acid (CAS 501–98–4), Trolox ((±)−6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, CAS 53188–07–1), ABTS (2,2'-Azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid, CAS 30931–67–0), TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine, CAS 3682–35–7), D(+)-glucose (CAS 50-99-7), D(+)-xylose (CAS 58-86-6), formic acid (CAS 64-18-6), furfural (CAS 98-01-1) and HMF (5-Hydroxymethyl-2-furaldehyde, CAS 67-47-0) were purchased from Sigma-Aldrich (Steinheim, Germany). Gallic acid (CAS 149–91–7), sodium carbonate (CAS 497–19–8), L-ascorbic acid (CAS 50–81–7), anhydrous ethanol (CAS 64–17–5), acetonitrile (CAS 75–05–8) and Folin-Ciocalteu's reagent were supplied by Merck (Darmstadt, Germany). Hydrochloric acid (CAS 7647–01–0), potassium persulfate (CAS 7727–21–1), acetic acid (CAS 64–19–7), ferric chloride hexahydrate (CAS 7705–08–0), sulfuric acid (CAS 7664–93–9) and sodium acetate anhydrous (CAS 127–09–3) were purchased from Panreac (Barcelona, Spain). All reagents were analytical or HPLC grade.

2.2 Raw material and autohydrolysis reaction set-up

RH samples were received from Arrozur S. A., a local rice producer, air-dried until it reached its equilibrium moisture content (8.68 ± 0.04% on a wet basis) and stored at room temperature. Moisture content was determined by subjecting the sample to oven-drying at 105 ºC until constant weight. The RH particle size was 10% between 2 and 3.4 mm, 76% between 1 and 2 mm, 12% between 0.5 and 1 mm, and 2% under 0.5 mm. Additionally, Fourier-transform infrared spectroscopy (FTIR) was performed to characterize the RH sample using a Shimadzu IRAffinity-1S spectrometer (Kyoto, Japan) with a resolution of 4 cm^{-1}.

The RH was subjected to autohydrolysis using a reactor from Amar Equipment (Mumbai, India), consisting of a 2 L stainless steel vessel equipped with an electric heating system, paddle agitator and an internal serpentine coil. A schematic diagram of the system is shown in **Figure 1**.

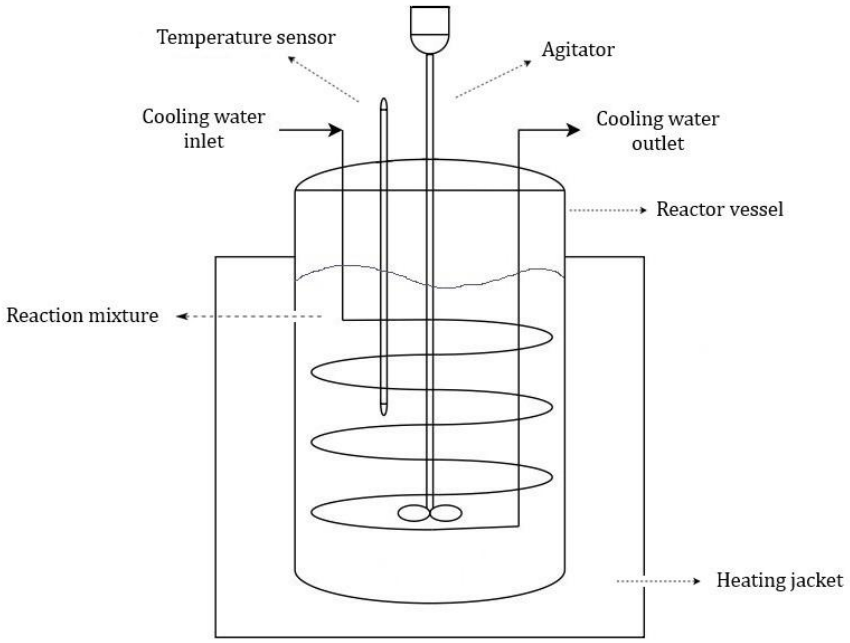

Figure 1. Diagram of the hydrothermal reactor used in the present study

The reactor was loaded with RH and distilled water in the specific ratios outlined by the experimental design (refer to the section below). RH was used as received, since no significant differences were observed on the hydrolysate properties when reducing the sample's particle size by milling (data not shown). The temperature of the reaction mixture was monitored using a Pt100 RTD sensor with thermowell (supplied with the reactor) and maintained at the desired level by a PID controller. The reaction mixture was heated from room temperature to the target temperature, with the hydrolysis time starting once the desired temperature was reached. Heating took between 20 and 30 minutes, depending on the target temperature. During all experiments, the agitation speed was maintained at 230 rpm. Upon completion of the specified reaction time, the vessel was removed from the heating jacket and cooling water was charged through the serpentine coil, reducing the temperature to 30 °C within approximately 10 minutes. Then, the liquid phase (or hydrolysate) was separated from the solid residue by filtration and kept at 4 ºC for subsequent analysis.

The autohydrolysis yield (AY) was quantified by measuring the content of non-volatile compounds (NVC) in hydrolysates by oven-drying at 105 ºC until constant weight. The yield was expressed as g of NVC/100 g of RH on a dry basis (d.b.).

2.3 Experimental design and modelling

A central composite design (CCD) consisting of fourteen experiments, including six replicates at the central point to estimate the experimental error, was conducted to evaluate the influence of temperature (x_1), L/S ratio (x_2) and hydrolysis time (x_3) on the AY and the following properties of the hydrolysates: TPC, antioxidant capacity and content of ferulic and *p*-coumaric acids, as shown in **Table 1**. The tested levels were 136.4, 150, 170, 190 and 203.6 ºC for temperature, whereas 13.2, 20, 30, 40 and 46.8 mL of water per gram of RH (d.b.) for the L/S ratio, and 9.5, 30, 60, 90 and 110.5 minutes for hydrolysis time. Experimental results for the dependent variables were fitted to a second-order polynomial model:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} {x_i}^2 + \sum_{i=1}^{2} \sum_{j=2}^{3} \beta_{ij} x_i x_j \quad (1)$$

where $j>i$, β stands for each regression coefficient, Y is the experimental value for each response variable and x_i and x_j are the independent variables coded at the following levels: low (-1), high (1), medium (0) and star points (-1.68 and 1.68). The star points correspond to an alpha value for rotatability in a design with three factors.

The models were statistically analyzed through analysis of variance (ANOVA). Both the regression coefficients R^2 and adjusted R^2 were calculated. The significance of each model and its regression coefficients were evaluated at a 95% confidence level using the InfoStat software 2013 version.

2.4 Optimization and model validation

Response surface contour plots were generated for each response variable using the programming language Python. The models were analyzed aiming to find the operating condition allowing maximum production of ferulic and *p*-coumaric acids in the hydrolysate, as well as high yield and antioxidant activity. These operating conditions were established through the desirability function methodology using GNU Octave software[12]. This approach calculates a desirability value for each of the n responses, which reflects how closely the predicted value matches the target value under the optimal factors setting. The desirability function ranges from zero (indicating a completely undesirable outcome) to one (representing a fully desirable result). For each considered response, the minimum and maximum acceptable values were set as the minimum and maximum obtained experimental values, respectively. The predicted values from the response model (Y_n) were converted into desirability values (d_n) using the following equation:

$$d_n = \begin{cases} 0, & Y_n \leq {Y_n}^{min} \\ \frac{Y_n - {Y_n}^{min}}{{Y_n}^{max} - {Y_n}^{min}}, & {Y_n}^{min} < Y_n < {Y_n}^{max} \\ 1, & Y_n \geq {Y_n}^{max} \end{cases} \quad (2)$$

Where ${Y_n}^{min}$ and ${Y_n}^{max}$ correspond to the minimum and maximum acceptable values of the n response, respectively.

When optimizing multiple variables simultaneously, the individual desirability functions (d_n) can be combined into an overall desirability function (D) for a set of variables, defined as the geometric mean of the individual desirability functions for the considered response variables. Since the aim of this work was to simultaneously maximize both ferulic and *p*-coumaric acid content, the overall desirability function used is described by equation **(3)**.

$$D = \sqrt{d_f d_c} \quad (3)$$

Where d_f and d_c represent the individual desirability functions for ferulic and *p*-coumaric acids, respectively.

An experiment under the selected optimal condition was carried out in triplicate aiming to validate the models, by comparison between experimental and predicted results.

2.5 Analytical methods

2.5.1 Total Phenolic Content (TPC)

The TPC was assessed using a modified version of the Folin-Ciocalteu method described by Singleton and Rossi[13]. In summary, 0.5 mL of the hydrolysate was added to a test tube, followed by the addition of 2.5 mL of a diluted Folin-Ciocalteu reagent (diluted 1:10 with water) and 2 mL of sodium carbonate solution (7.5% w/v). The mixture was incubated in a water bath at 50 ºC for 5 minutes. After reaching room temperature, absorbance readings were taken at 760 nm using a Shimadzu UVmini-1240 spectrophotometer (Kyoto, Japan). TPC was reported as mg of gallic acid equivalents (GAE) per gram of RH d.b.

2.5.2 Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay was carried out following the method outlined by Szöllösi and Szöllösi-Varga[14]. To prepare the FRAP reagent, 2.5 mL of a 10 mM TPTZ solution was combined with 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ and 25 mL of a 300 mM acetate buffer (pH 3.6). Then, 0.1 mL of hydrolysate was mixed with 3 mL of the FRAP reagent and kept at 25 ºC for 5 minutes, and absorbance was measured at 593 nm. Results were expressed as mmol of ascorbic acid equivalents (AAE) per 100 g of RH d.b.

2.5.3 ABTS Radical Scavenging Assay

The ABTS assay was performed to measure the antioxidant capacity according to the method by Re and others[15] with minor adjustments. In summary, the ABTS radical cation ($ABTS^{\cdot+}$) solution was generated by reacting a 7 mM ABTS solution with 2.45 mM potassium persulfate. The mixture was stored in the dark at 4 ºC and then diluted with distilled water to reach an absorbance of 0.700 at 734 nm, between 14-48 hours after its preparation. An aliquot of 2.5 mL of the reagent was then mixed with 25 µL of hydrolysate, and absorbance was measured at 734 nm. Results were reported as mmol of Trolox equivalents (TE) per 100 g of RH d.b.

2.5.4 Ferulic and p-coumaric acids quantification

High-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) was employed to quantify the levels of ferulic acid and *p*-coumaric acid on the hydrolysates. Prior to analysis, hydrolysate samples were filtered through 0.22 µm syringe filters. A C18 column (5 µm, 4.6 × 250 mm, Waters Spherisorb) with reversed-phase and a diode array detector (DAD) set at 320 nm were used. The elution was performed isocratically at a flow rate of 1 mL/min at 25 ºC, with a mobile phase consisting of 20% (v/v) acetonitrile and 80% acidified water (1% acetic acid). Results were expressed as mg of ferulic or *p*-coumaric acid per 100 g of RH d.b.

2.5.5 Carbohydrates and organic acids determination

The content of sugars and organic acids was carried out over the hydrolysate obtained under the defined optimal condition. Glucose, xylose, acetic acid and formic acid were quantified by HPLC (Shimadzu, Kyoto, Japan) using an Aminex HPX-87 H column (9 µm, 7.8 × 300 mm, Bio-Rad Laboratories Ltd., USA) at 45 °C with refractive index (RI) and UV-Vis spectrophotometric (SP) detectors. H_2SO_4 (5 mM) was used as mobile phase at 0.6 mL/min under isocratic conditions. Hydrolysate samples were subjected to post-hydrolysis with 72% (w/w) H_2SO_4 and autoclaved (Astori Tecnica, Brescia, Italy) for 1 hour at 121 ºC to quantify the content of oligosaccharides according to the National Renewable Energy Laboratory (NREL) procedures[16]. Both hydrolysate and post-hydrolysis samples were filtered through 0.22 µm filters prior to analysis.

2.5.6 Statistical analysis

All analyses were performed in triplicate. The mean values of the triplicates were presented with the corresponding confidence intervals at a 95% significance level.

3. Results and discussion

3.1 Autohydrolysis and central composite design

Table 1 presents the experimental conditions for all assays and the values obtained for the response variables. Autohydrolysis of RH yielded hydrolysates containing 7.40 – 26.16 g NVC/100 g RH d.b. The hydrolysates exhibited TPC ranging from 6.8 to 27.9 mg GAE/g RH d.b., antioxidant activities of 3.15 – 12.09 mmol AAE/100 g RH d.b. and 3.89 – 14.40 mmol TE/100 g RH d.b., and phenolic acids with concentrations of 7.4 – 25.1 mg/100 g RH d.b. (ferulic acid) and 66.4 – 167.7 mg/100 g RH d.b. (*p*-coumaric acid). Experimental data were fitted to the proposed models, as shown in **Table 2**, which includes the regression coefficients and the corresponding statistical parameters. All models were statistically significant ($p < 0.05$), and the response surface contour plots are illustrated in **Figure 2**, **Figure 3**, **Figure 4**, **Figure 5**, **Figure 6** and **Figure 7**.

In the case of AY, the most significant coefficients were the linear and quadratic terms of temperature, followed by the interaction term between temperature and time, the quadratic time term, and the linear term for the L/S ratio. It was observed that an increase in temperature initially favored the yield up to a certain point, after which the yield began to decline. This is likely due to the increased solubility of cell wall components at higher temperatures, but beyond a certain threshold, these compounds may decompose into gaseous products, or secondary reactions involving the formation of non-soluble solids (such as char) may occur, leading to a reduction in soluble compounds and, consequently, a decrease in yield[17][18][19]. Additionally, the maximum AY values at each time point, as shown in the response surface plots (see **Figure 2**), were attained with the highest L/S ratio tested in our experiments. This is consistent with the understanding that a higher L/S ratio (more diluted conditions) enhances mass transfer potential and reduces saturation effects by providing a greater amount of solvent, which promotes the solubilization of more compounds. On the other hand, **Figure 2** shows that AY increased when hydrolysis time was extended from 30 to 60 minutes. However, further prolongation of time caused a decline in yield. Several studies have reported that prolonged exposure to high temperatures accelerates the degradation of these compounds[17][20]. This observation aligns with the negative values for the quadratic time coefficient and the interaction term between time and temperature in the model.

Table 1. CCD and results obtained for autohydrolysis of RH

	x_1	x_2	x_3	Temperature (ºC)	L/S ratio (mL/g)	Time (min)	AY (g NVC/100 g RH d.b.)	TPC (mg GAE/g RH d.b.)	FRAP (mmol AAE/100 g RH d.b.)	ABTS (mmol TE/100 g RH d.b.)	Ferulic acid (mg/100 g RH d.b.)	*p*-Coumaric acid (mg/100 g RH d.b.)
1	-1	-1	-1	150	20	30	7.45 ± 0.22	7.8 ± 0.1	4.45 ± 0.10	4.73	7.6 ± 0.7	66.4 ± 0.9
2	-1	1	1	150	40	90	15.61 ± 1.18	12.8 ± 1.0	7.02 ± 0.17	11.03	12.5 ± 0.4	122.1 ± 0.2
3	1	-1	1	190	20	90	13.70 ± 0.46	20.5 ± 0.7	9.84 ± 0.77	8.57	10.3 ± 2.8	69.7 ± 3.4
4	1	1	-1	190	40	30	26.16 ± 0.36	27.8 ± 2.0	11.88 ± 0.10	14.40	25.1 ± 1.5	167.7 ± 6.7
5	0	0	0	170	30	60	23.39 ± 0.70	17.8 ± 1.1	8.65 ± 0.37	13.45	15.4 ± 0.5	130.9 ± 8.8
6	0	0	0	170	30	60	23.34 ± 0.66	18.2 ± 1.1	7.18 ± 0.14	10.62	16.3 ± 0.6	134.2 ± 9.8
7	-1	-1	1	150	20	90	12.57 ± 0.23	10.1 ± 0.8	4.35 ± 0.15	6.45	10.3 ± 0.6	95.1 ± 5.8
8	-1	1	-1	150	40	30	10.81 ± 0.82	10.0 ± 0.5	4.66 ± 0.39	7.07	8.2 ± 0.3	92.1 ± 4.6
9	1	-1	-1	190	20	30	21.17 ± 0.73	20.1 ± 1.3	9.18 ± 0.74	9.29	19.3 ± 0.8	131.9 ± 5.2
10	1	1	1	190	40	90	17.43 ± 0.27	27.9 ± 1.5	11.42 ± 1.08	13.29	13.8 ± 0.9	101.3 ± 0.3
11	0	0	0	170	30	60	24.20 ± 0.51	17.4 ± 0.6	8.46 ± 0.53	10.06	15.0 ± 0.0	145.6 ± 1.0
12	0	0	0	170	30	60	22.86 ± 0.49	16.9 ± 0.8	7.44 ± 0.88	9.27	13.8 ± 0.2	137.1 ± 1.4
13	-1.68	0	0	136.4	30	60	7.40 ± 0.12	6.8 ± 0.2	3.15 ± 0.32	3.89	7.4 ± 0.1	75.0 ± 1.1
14	1.68	0	0	203.6	30	60	13.45 ± 0.28	27.8 ± 1.7	12.09 ± 0.30	10.61	10.4 ± 2.2	73.1 ± 1.8
15	0	-1.68	0	170	13.2	60	20.86 ± 0.17	14.8 ± 0.3	4.74 ± 0.27	7.25	11.5 ± 2.0	117.3 ± 4.0
16	0	1.68	0	170	46.8	60	24.57 ± 1.57	14.9 ± 0.9	9.06 ± 0.76	13.38	16.8 ± 0.9	157.4 ± 9.8
17	0	0	-1.68	170	30	9.5	11.36 ± 0.80	9.8 ± 0.8	5.39 ± 0.24	9.69	12.6 ± 2.7	113.2 ± 3.1
18	0	0	1.68	170	30	110.5	22.16 ± 0.28	12.7 ± 0.2	7.83 ± 0.23	10.43	17.0 ± 3.8	128.8 ± 5.0
19	0	0	0	170	30	60	22.72 ± 0.82	17.3 ± 0.2	8.18 ± 0.61	10.02	14.2 ± 0.3	140.8 ± 0.4
20	0	0	0	170	30	60	22.52 ± 1.80	13.4 ± 0.1	7.84 ± 0.38	10.35	13.9 ± 0.1	136.8 ± 1.1

x_1, temperature; x_2, L/S ratio; x_3, time. Response variables are presented as mean ± uncertainty for three analysis replicates. Uncertainties were calculated with the *t*-Student value for a 95% confidence level. Standard uncertainties (u) are u(T) = ± 0.1 ºC, u(w) = ± 0.01 g, u(t) = ± 1 s. The experimental error of L/S ratio was estimated from the variation coefficient (0.06%).

Table 2. Regression coefficients for the models and statistical parameters

	AY (g NVC/100 g RH d.b.)			TPC (mg GAE/g RH d.b.)			FRAP (mmol AAE/100 g RH d.b.)			ABTS (mmol TE/100 g RH d.b.)			Ferulic acid (mg/100 g RH d.b.)			*p*-Coumaric acid (mg/100 g RH d.b.)		
	RC	ES	S	RC	ES	S	RC	ES	S	RC	ES	S	RC	ES	S	RC	ES	S
β_0	22.952	0.840	0.000	17.223	0.689	0.000	7.641	0.173	0.000	10.462	0.285	0.000	14.870	0.645	0.000	137.03	3.52	0.000
β_1	3.090	0.657	0.000	6.654	0.652	0.000	2.700	0.209	0.000	2.019	0.270	0.000	2.554	0.610	0.001	6.71	2.75	0.030
β_2	1.564	0.657	0.033	1.478	0.652	0.039	1.056	0.209	0.000	1.981	0.270	0.000	1.540	0.610	0.024	13.73	2.75	0.000
β_3	0.871	0.657	NS	0.777	0.652	NS	0.479	0.209	0.036	0.373	0.270	NS	-0.428	0.610	NS	-3.19	2.75	NS
β_{11}	-4.609	0.636	0.000	-	-	NS	-	-	NS	-1.127	0.260	0.001	-1.903	0.588	0.006	-23.10	2.67	0.000
β_{22}	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-	-	NS
β_{33}	-2.369	0.636	0.003	-1.441	0.629	0.037	-	-	NS	-	-	NS	-	-	NS	-6.48	2.67	0.030
β_{12}	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-	-	NS
β_{13}	-3.265	0.858	0.002	-	-	NS	-	-	NS	-0.937	0.352	0.019	-3.403	0.797	0.001	-23.40	3.60	0.000
β_{23}	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-	-	NS
R^2		0.8908			0.8853			0.9252			0.9078			0.7914			0.9213	
R^2 a.		0.8404			0.8548			0.9112			0.8748			0.7168			0.8850	
ES		2.427			2.411			0.772			0.997			2.254			10.176	
S		0.000			0.000			0.000			0.000			0.000			0.000	

RC, regression coefficient; ES, error standard; S, significance; NS, not significant; R^2 a., adjusted R^2. (Level of significance: $p < 0.05$).

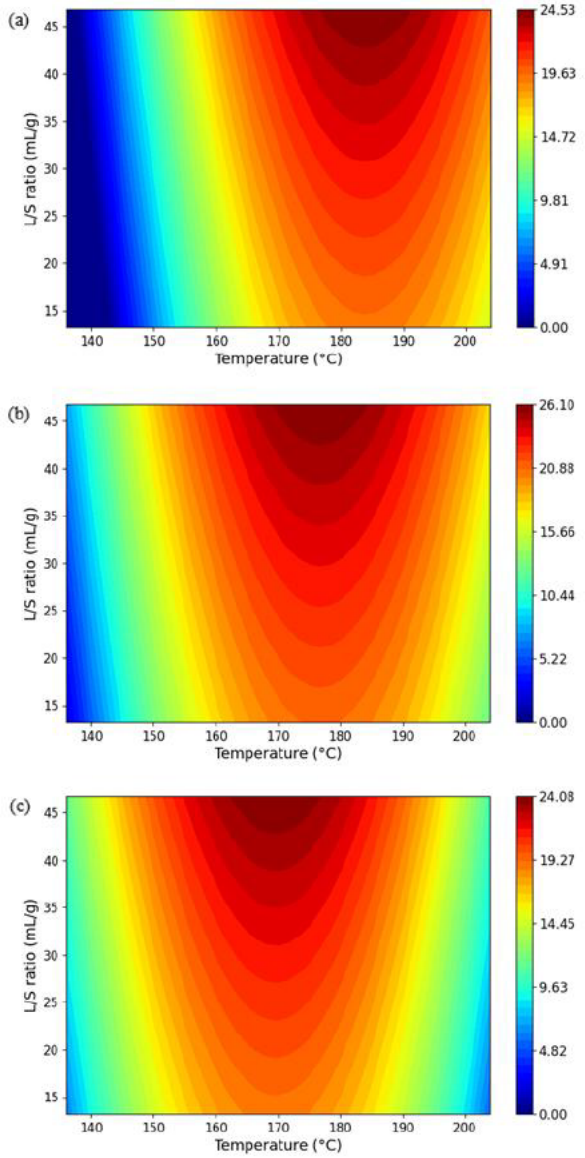

Figure 2. Response surface plots for autohydrolysis yield (g NVC/100 g RH d.b.) under different hydrolysis times. a. 30 minutes; b. 60 minutes; c. 90 minutes

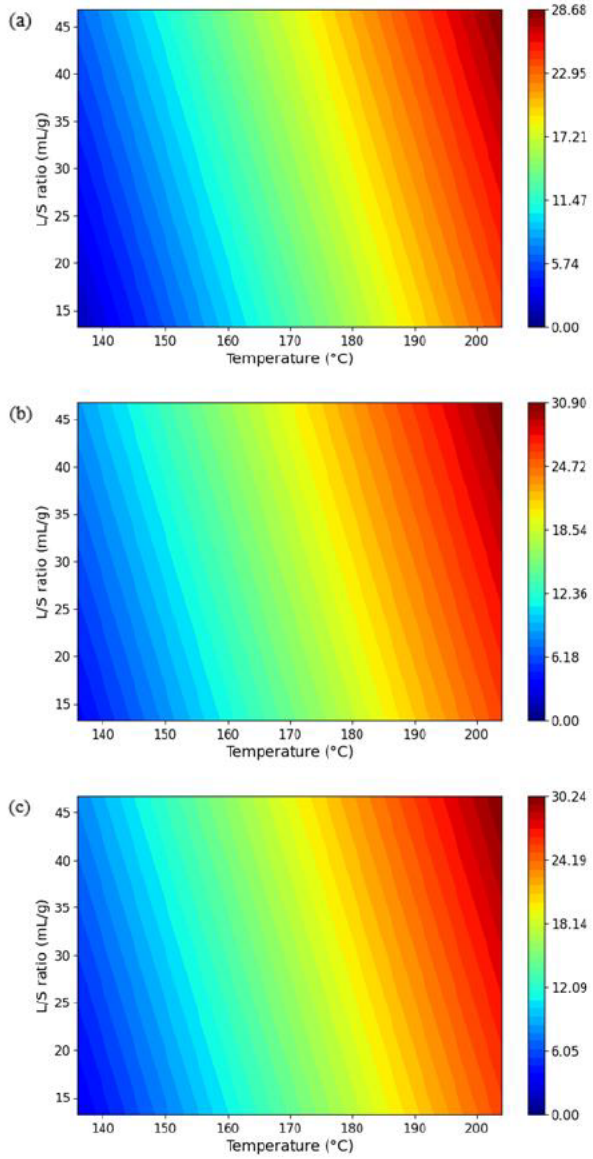

Figure 3. Response surface plots for total phenolic content (mg GAE/g RH d.b.) under different hydrolysis times. a. 30 minutes; b. 60 minutes; c. 90 minutes

A similar pattern was observed for TPC and FRAP, where the maximum values for these response variables were achieved at the highest temperature and L/S ratio (**Figure 3** and **Figure 4**, respectively). However, the models for these variables exhibit different functionalities. In the case of TPC, the model includes all linear terms and the quadratic dependence with time, being the linear term of temperature the most significant, followed by the quadratic dependence with time and the linear term for the L/S ratio. On the other hand, the FRAP model was primarily built from linear terms, with temperature and L/S ratio being the most significant, followed by the time factor. Notably, the TPC model exhibits behavior analogous to AY when considering the effect of time: both models have negative coefficients for the quadratic time term. Similar to AY, TPC initially increases with longer reaction times but decreases beyond a certain point (see **Figure 3**). However, this decrease is less pronounced compared to AY, a difference that can be attributed to the more negative quadratic time coefficient in the AY model.

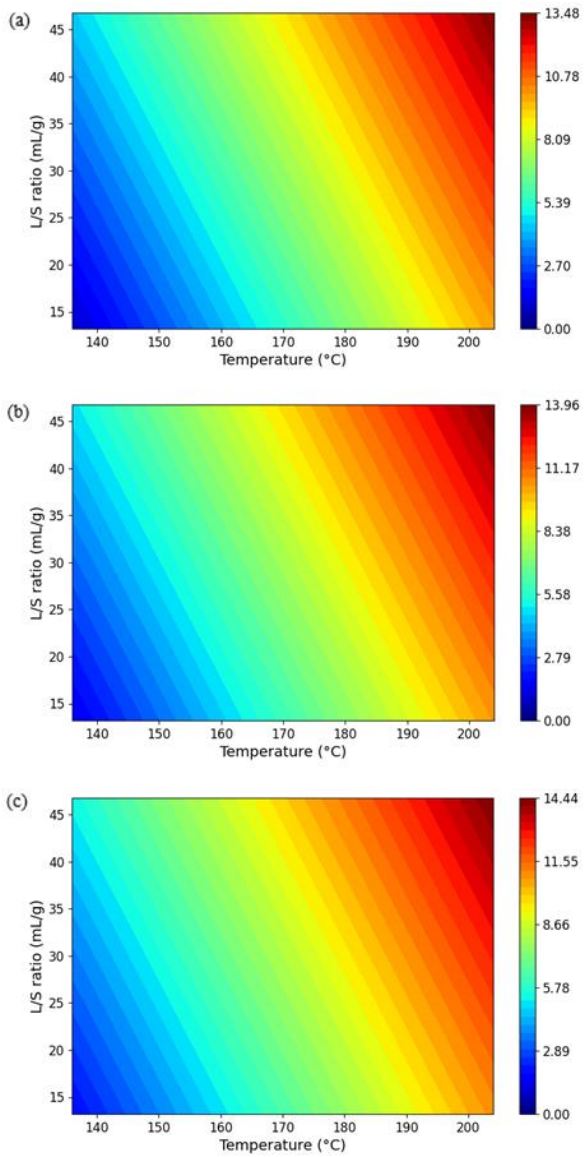

Figure 4. Response surface plots for FRAP (mmol AAE/100 g RH d.b.) under different hydrolysis times. a. 30 minutes; b. 60 minutes; c. 90 minutes

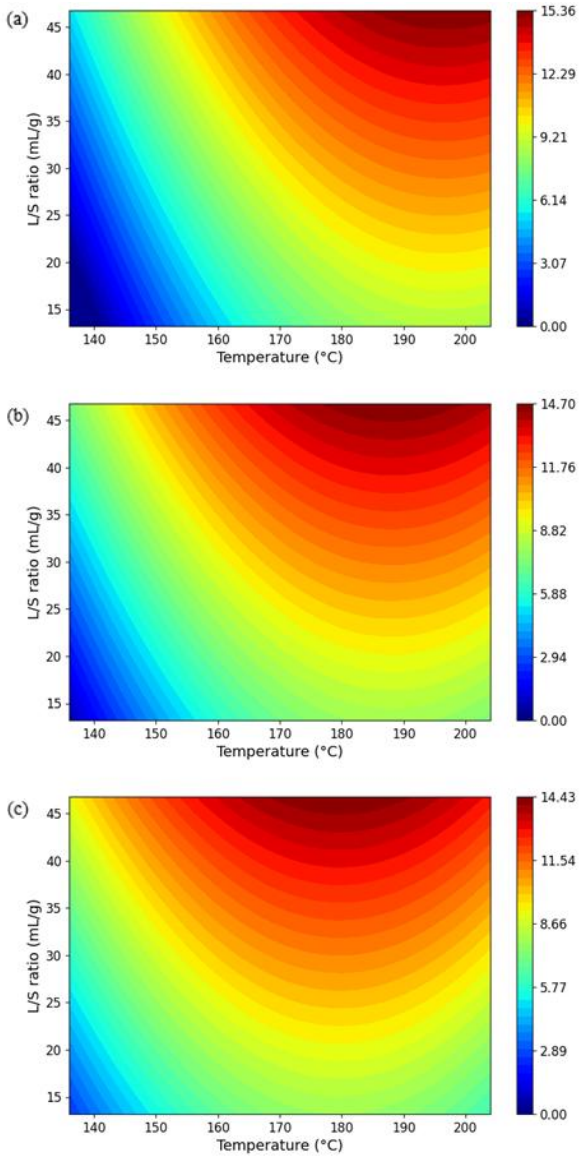

Figure 5. Response surface plots for ABTS (mmol TE/100 g RH d.b.) under different hydrolysis times. a. 30 minutes; b. 60 minutes; c. 90 minutes

Moreover, the ABTS and ferulic acid models exhibited similar behavior, as both share the same functionality. In the ABTS model, the most significant coefficients were the linear terms of temperature and L/S ratio, followed by the quadratic temperature term and the temperature-time interaction term. For the ferulic acid model, the most significant coefficients were the linear term of temperature and the temperature-time interaction term, followed by the quadratic temperature term and the linear L/S ratio term. In the case of *p*-coumaric acid, the model included the quadratic time term as an additional coefficient compared to the ABTS and ferulic acid models. The most significant factors were the linear term of L/S ratio, the quadratic dependence on temperature, and the temperature-time interaction, followed by the linear dependence on temperature and the quadratic dependence on time. Analysis of **Figure 5**, **Figure 6** and **Figure 7** revealed that the maximum values for ABTS, ferulic acid, and *p*-coumaric acid were reached at lower temperatures when the hydrolysis time was extended. However, these maximum values decreased, which is consistent with the fact that prolonged exposure of these compounds to harsh conditions can lead to their decomposition[17][20], resulting in lower concentrations and reduced antioxidant capacity.

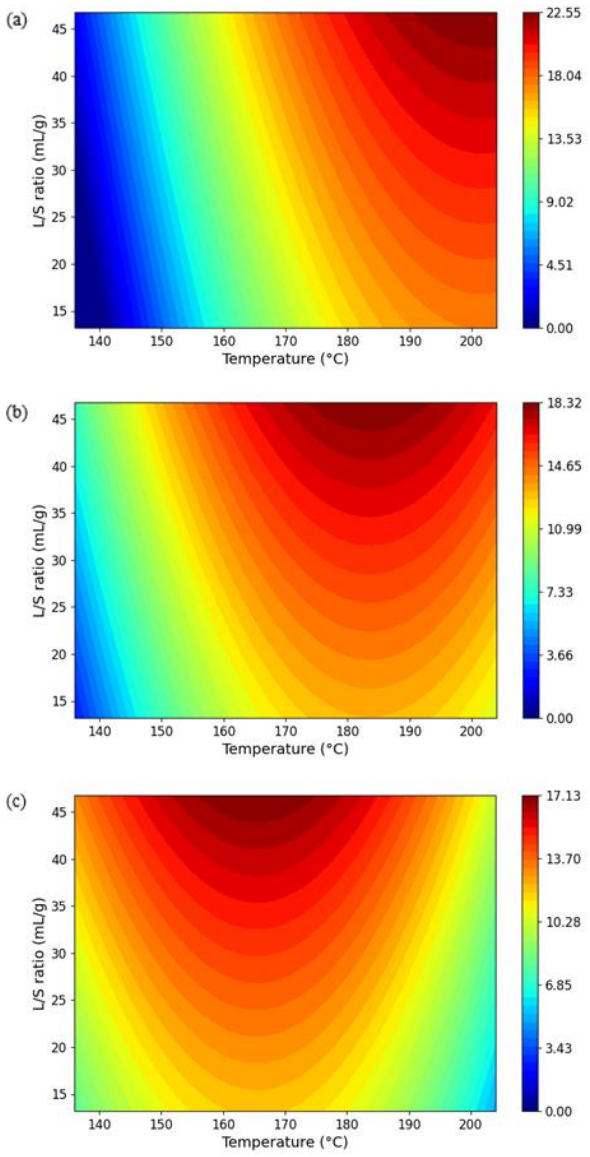

Figure 6. Response surface plots for ferulic acid (mg/100 g RH d.b.) under different hydrolysis times. a. 30 minutes; b. 60 minutes; c. 90 minutes

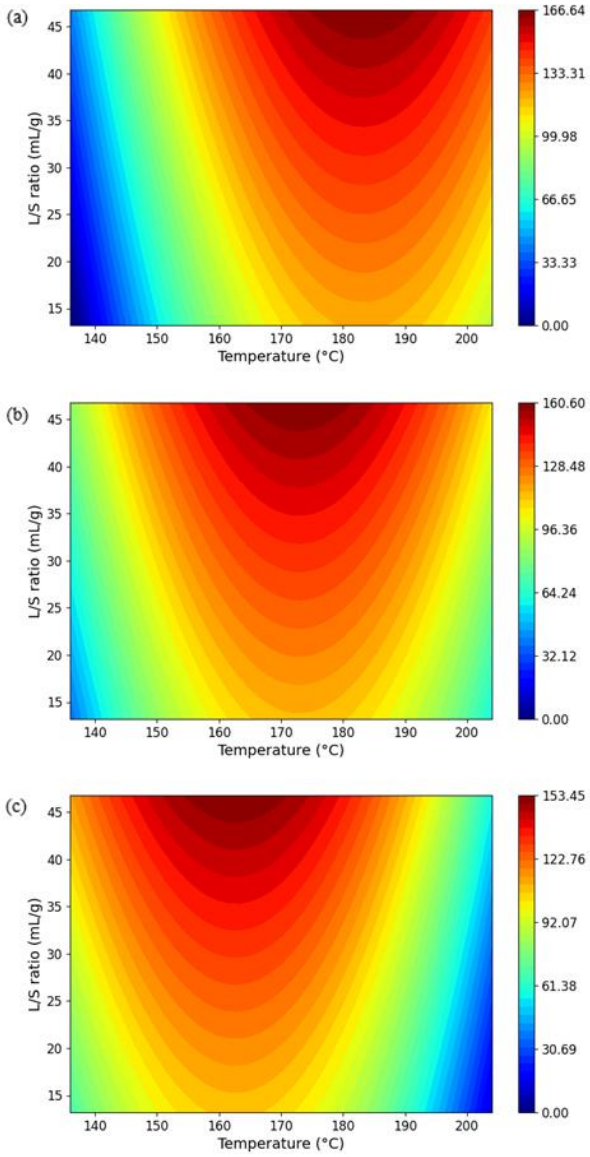

Figure 7. Response surface plots for *p*-coumaric acid (mg/100 g RH d.b.) under different hydrolysis times. a. 30 minutes; b. 60 minutes; c. 90 minutes

3.2 Optimal conditions for production of phenolic acids

The models predict that the maximum ferulic and *p*-coumaric acids concentrations are achieved under different conditions, as illustrated in **Table 3**. It is interesting to point out that the only difference between these conditions was the temperature, while L/S ratio and hydrolysis time remained constant. Optimal temperature for ferulic acid production was 203.6 ºC, while maximum *p*-coumaric acid content was attained at 190 ºC. The temperature that maximizes both phenolic acids simultaneously was found to be 192.8 ºC. Since the phenolic acid contents were statistically equivalent across the three conditions, with overlapping confidence intervals for both ferulic and *p*-coumaric acids, the optimal condition was selected as 190 ºC, 46.8 mL/g, and 9.5 minutes for economic reasons. Additionally, the other response variables did not show significant differences among the three conditions. A verification experiment was conducted in triplicate under the selected optimal condition, and the experimental results (**Table 3**) were consistent with the predicted values, confirming the accuracy of the models in representing the experimental data. **Figure 8** features the chromatogram obtained with the hydrolysate sample under optimal condition.

Table 3. Predicted values for response variables under different optimal conditions and verification experiment results

Response variable	Optimal condition			Verification experiment
	Ferulic acid	Both phenolic acids simultaneously	*p*-Coumaric acid	
	203.6 °C 46.8 mL/g 9.5 min	192.8 °C 46.8 mL/g 9.5 min	190.0 °C 46.8 mL/g 9.5 min	190.0 °C 46.8 mL/g 9.5 min
AY (g NVC/100 g RH d.b.)	18.8 ± 8.1	21.2 ± 6.1	21.4 ± 5.8	29.0 ± 2.5
TPC (mg GAE/g RH d.b.)	25.5 ± 5.1	21.9 ± 4.8	21.0 ± 4.7	27.4 ± 2.0
FRAP (mmol AAE/100 g RH d.b.)	13.2 ± 1.3	11.7 ± 1.2	11.3 ± 1.2	10.7 ± 2.3
ABTS (mmol TE/100 g RH d.b.)	16.0 ± 3.0	15.8 ± 2.2	15.6 ± 2.0	16.9 ± 1.6
Ferulic acid (mg/100 g RH d.b.)	26.7 ± 6.8	25.1 ± 4.9	24.6 ± 4.6	24.5 ± 3.0
p-Coumaric acid (mg/100 g RH d.b.)	159.3 ± 33.8	169.6 ± 25.8	170.1 ± 24.3	188.0 ± 18.6

Predicted and experimental values are presented with a confidence interval (95% confidence level)

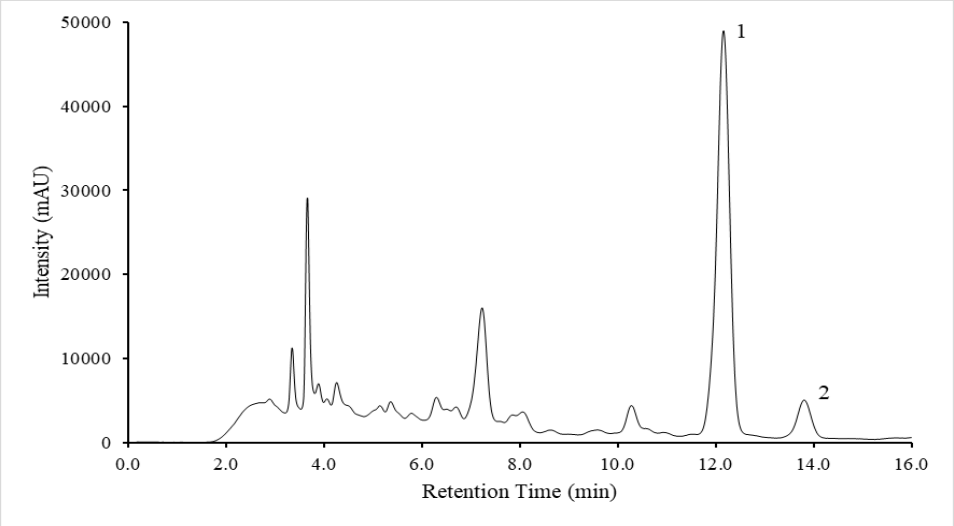

Figure 8. Chromatogram for the hydrolysate sample obtained under optimal condition for *p*-coumaric (peak number 1) and ferulic acid (peak number 2) quantification

Rivas and others[7] conducted autohydrolysis of RH in a Parr reactor operating in batch mode at 185 °C, using a L/S ratio of 8 g/g for 20 minutes. They reported ferulic and *p*-coumaric acids concentrations of 0.155 and 1.10 g/100 g NVC, respectively, which were higher than those found in our study (0.0845 ± 0.0055 and 0.647 ± 0.031 g/100 g NVC for ferulic and *p*-coumaric acids, respectively). However, the ratio of *p*-coumaric to ferulic acid was quite similar in both studies (7.1 vs. 7.7). The lower concentrations of phenolic acids in our study could be explained by the higher L/S ratio used, which likely resulted in a greater amount of NVC in the hydrolysates, reducing the concentration of phenolic acids when expressed per 100 grams of NVC. Additionally, differences may also arise from variability in the RH samples, as the composition of the raw material can vary depending on factors such as weather and soil conditions during cultivation[21]. This variability is an important consideration that complicates direct comparisons between studies. On the other hand, Rivas and others[7] reported a lower TPC compared to our findings (5.0 vs. 9.4 ± 1.1 g GAE/100 g NVC), although both values are of the same order of magnitude. Their FRAP values ranged from 0 to 2.5 mM $FeSO_4$, whereas our study recorded a value of 2.64 ± 0.57 mM $FeSO_4$. Park and Lee[22] performed hydrothermal treatment of RH using an autoclave at temperatures ranging from 105 to 121 °C, with a L/S ratio of 10 mL/g and hydrolysis times from 15 to 60 minutes. They obtained

TPC values between 4.35 and 8.4 mg GAE/g RH d.b., which are significantly lower than our TPC value of 27.4 mg GAE/g RH d.b.

Regarding AY, our result align with that reported by Gullón and others[23] (18.4 g NVC/100 g RH d.b.) for auto-hydrolysis at 185 ºC with a L/S ratio of 8 g/g and 20 minutes, as well as with that reported by Vegas and others[24] (21.02 g NVC/100 g RH d.b.), who performed autohydrolysis under non-isothermal conditions using the fastest heating profile of the reactor to achieve 205 °C and L/S ratio of 8 g/g. Both studies carried out the reaction in batch mode using a Parr reactor.

Comparing these results with those from our previous work using the same batch of RH, we found that extraction with a mixture of 70% EtOH (v/v) and water at 60 ºC for 60 minutes, with a L/S ratio of 10 mL/g, yielded 1.33 g NVC/100 g RH d.b. with 1.19 mg GAE/g RH d.b. of TPC, and antioxidant activities of 0.45 mmol AAE (FRAP) and 0.81 mmol TE (ABTS) per 100 grams of RH d.b. The contents of ferulic and *p*-coumaric acids were 0.31 and 2.91 mg/100 g RH d.b., respectively, which are significantly lower than those in the current study. This is expected since only free PCs are extracted under these conditions, while bound phenolics remain in the plant matrix. On the other hand, alkaline hydrolysis of RH using 2 N NaOH at 120 ºC for 90 minutes with a L/S ratio of 30 mL/g resulted in higher values: 55.2 g NVC/100 g RH d.b., 41.6 mg GAE/g RH d.b., 16.2 mmol AAE/100 g RH d.b., 23.9 mmol TE/100 g RH d.b., and ferulic and *p*-coumaric acids concentrations of 345 and 1100 mg/100 g RH d.b., respectively[25]. These values are reasonable, given that lignin is depolymerized and solubilized more extensively under alkaline conditions compared to other treatments[26].

3.3 Hemicelluloses-derived products

Table 4 features the monosaccharides, oligosaccharides and organic acids content in the hydrolysate obtained under optimal condition. No furfural or HMF were detected under these autohydrolysis conditions. These results effectively show that carbohydrates are present in higher proportions than phenolic acids due to extensive hemicelluloses dissolution.

Table 4. Carbohydrate and organic acids content of the hydrolysate obtained under the optimal condition

Component	Concentration	
	mg/g RH d.b.	g/g NVC
Xylose	6.3 ± 1.3	0.022 ± 0.003
Xylo-oligosaccharides (as xylose)	85.6 ± 3.3	0.295 ± 0.012
Total xylose	91.9 ± 7.0	0.317 ± 0.021
Glucose	21.8 ± 2.9	0.075 ± 0.012
Gluco-oligosaccharides (as glucose)	5.2 ± 1.8	0.018 ± 0.004
Total glucose	27.2 ± 5.2	0.095 ± 0.019
Formic acid	19.5 ± 3.6	0.067 ± 0.015
Acetic acid	29.3 ± 6.9	0.101 ± 0.027

Concentrations are presented with a confidence interval (95% confidence level)

These findings are consistent with those reported by Rivas and others[7], who under similar autohydrolysis conditions (outlined in the previous section) obtained 0.036, 0.445, 0.016, 0.067, and 0.027 g/g NVC of xylose, XOS, glucose, gluco-oligosaccharides (GOS), and acetic acid, respectively. Our results are of the same order of magnitude, though it is notable that glucose was produced in greater amounts compared to GOS in our study, whereas Rivas and others[7] observed the opposite trend. Additionally, their acetic acid concentration was lower than ours.

Similarly, Gullón and others[23] obtained 0.029, 0.426, 0.007, 0.089, and 0.029 g/g NVC of xylose, XOS, glucose, GOS, and acetic acid, respectively, showing trends comparable to those of Rivas and others[7], while Vegas and

others[24]. reported values of 0.0252, 0.4247, 0.0084, 0.1131, and 0.0288 g/g NVC for the same compounds, respectively, further supporting these trends.

3.4 FTIR analysis

Figure 9 features the FTIR spectrum for the RH sample. The broad band between 3500 and 3000 cm^{-1} indicates the presence of OH groups belonging to the Si-OH bonding. On the other hand, the high intensity peak around 1030 cm^{-1} indicates the presence of Si-O-Si bonding, while Si-H presence can be related to the medium and high intensity peaks appearing at around 780 and 440 cm^{-1}, respectively[27]. The presence of lignin is associated with peaks in the range of 1600-1300 cm^{-1}, where characteristic vibrations related to aromatic structures are observed, due to the stretching of C=O and C=C bonds[28]. **Figure 10** provides an amplified view of the spectrum in the previously mentioned range. Signals around 1740 and 1630 cm^{-1}, corresponding to the C=O stretching and O-H bending bands from ester and acetyl groups in hemicellulosic polysaccharides, can also be observed[28].

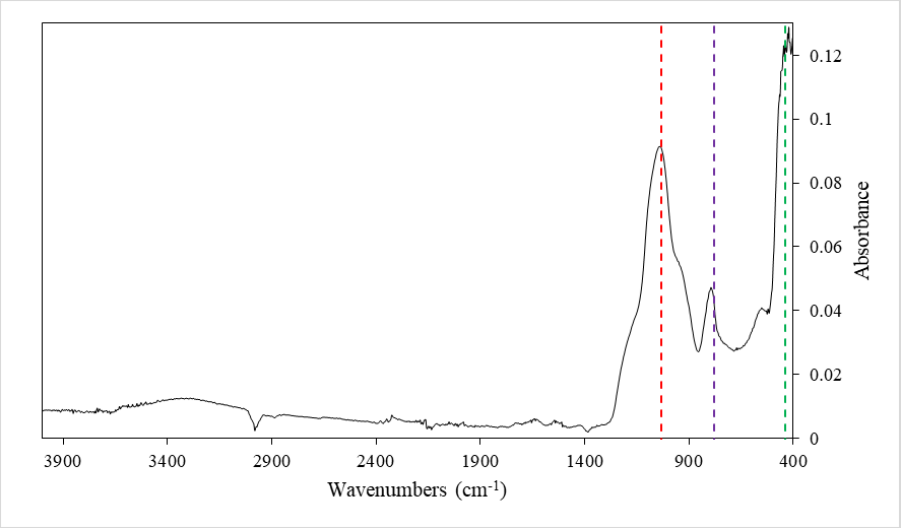

Figure 9. RH FTIR spectrum. Dashed lines colored as red, purple and green indicate wave numbers of 1030, 780 and 440 cm^{-1}, respectively

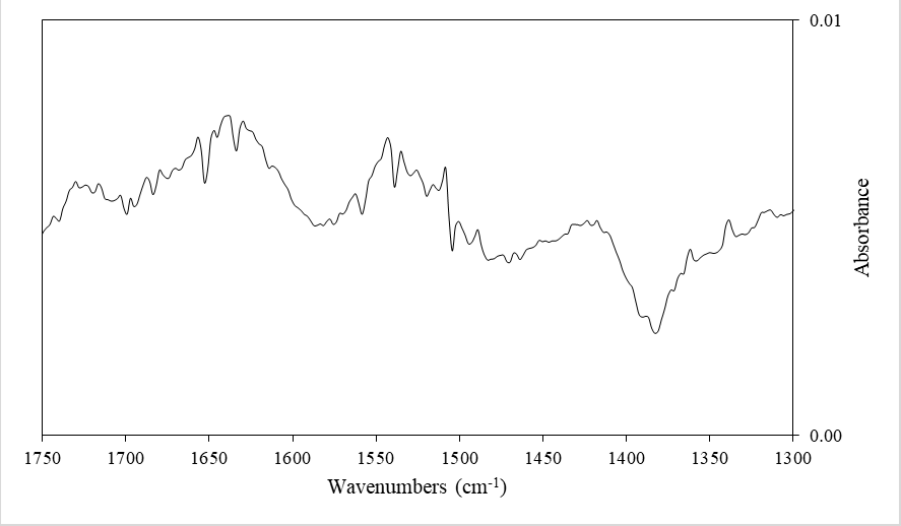

Figure 10. Amplified RH FTIR spectrum in the range 1750-1300 cm^{-1}

4. Conclusions

Under the evaluated conditions, autohydrolysis was shown to be effective in releasing ferulic and *p*-coumaric acids with antioxidant activity from RH. Several operation conditions were studied and optimized, such as temperature, L/S ratio and hydrolysis time, allowing to develop a promising process for RH valorization. These results are promising as they demonstrate the potential to produce compounds with antioxidant activity from a byproduct generated in large quantities using a relatively simple and environmentally friendly technology, without the use of chemical reagents. Future research should focus on optimizing the separation of phenolic acids from sugars. This could lead to the development of extracts with enhanced antioxidant activity and a liquor with a higher purity of XOS, which is necessary for producing food-grade prebiotics. Additionally, removing PCs could create a xylose-rich medium free from substances that might inhibit fermentation processes, thereby improving the production of other chemical compounds under a biorefinery approach.

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Transparency of data

Available data: The entire data set that supports the results of this study was published in the article itself.

Author contribution statement

Mauro Rocha: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing.

Lucía Xavier: Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision, Project administration.

Berta Zecchi: Conceptualization, Writing – review & editing, Funding acquisition, Supervision, Project administration.

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