

Aqueous two-phase systems based on cholinium ionic liquids for the recovery of ferulic and *p*-coumaric acids from rice husk hydrolysate

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ABSTRACT

Rice husk (RH) is an abundant agro-industrial residue in Uruguay, being the disposal of it an environmental problem because of the pollution it generates. This work had as objective the obtention of phenolic compounds from RH by alkaline hydrolysis and their separation by means of aqueous two-phase systems (ATPS). An experimental design was implemented for the alkaline delignification process, studying the influence of temperature, solid/liquid (S/L) ratio and NaOH concentration on the hydrolysis yield (HY), total phenolic content (TPC), the antioxidant capacity (FRAP and TEAC) and the ferulic and *p*-coumaric acids content of the hydrolysates. The optimal conditions were 120 °C, S/L ratio 1/30 g/mL and 2 N NaOH. Subsequently, ATPS based on choline chloride and different surfactants, Tween 20 (Tw20) and Tween 80 (Tw80), were used to separate the phenolic compounds from the hydrolysate. The influence of the tie-line length (TLL), volume ratio (V_R) and temperature were studied. Ferulic acid and *p*-coumaric acid migrated to the surfactant rich-phase in all cases. Systems containing Tw80 turned out to have better separation yields (SY), greater than 90%, for both phenolic acids. SY was not affected by use of different TLL and temperatures, although it was affected by changes on V_R .

1. Introduction

Rice is one of the highest-production cereals in the world, having 503 million tons generated worldwide in the 2022/23 harvest, according to the United States Department of Agriculture (2023). Meanwhile, rice production reached 1.37 million tons in Uruguay during the same time frame (Ministerio de Ganadería, Agricultura y Pesca, 2023). An important by-product from rice industry is the rice husk (RH), which accounts about a 20 % of rice's weight and it is directly collected in the production process without additional costs (Barana et al., 2016). In this context, waste valorization constitutes nowadays an important challenge for any industry, not only from an environmental point of view, but also from an economic point of view. RH is a waste that is generated in large quantities in Uruguay and worldwide. Currently, it is disposed on lands or used for a combustion process to generate electrical energy (Dagnino et al., 2018; Offei et al., 2021). However, both strategies have associated problems. The disposal of RH on land causes its accumulation in the environment since it has low biodegradability and, given that it has a low bulk density, it occupies large spaces when piled up. This can cause problems such as contamination of soils and water sources. On the other hand, direct combustion of RH has its limitations due to its

abrasive nature and its inherent resistance to combustion. Furthermore, ashes generated in large quantities are composed of 90 % silica and represents another waste to be treated (Umasabor & Okovido, 2018; Zou & Yang, 2019). These reasons lead to the need of looking for alternatives for RH management and valorization.

In the last decade, lignocellulosic biomasses have been highlighted as renewable resources to obtain high value-added products such as chemicals, biofuels and biomaterials (Van Heiningen et al., 2011). Having an annually large stock of RH in our country opens opportunities to study its potential as phytochemicals source, which remains not fully investigated nowadays.

RH is composed of 48.7 % carbohydrates, 17.2 % acid-insoluble lignin, 1.8 % lignin soluble in acid, 15 % inorganics and 8 % extractives in water-ethanol. Fractionation of the RH into lignin, cellulose and hemicelluloses has already been reported (Dagnino et al., 2013). The use of cellulose to produce biofuels is an objective that has been increased in recent years. However, the use of lignin to produce high value compounds through an efficient process is nowadays one of the challenges in the area. Lignin is an aromatic polymer made up from three main monomers, all derived from phenylpropane: *p*-coumaryl, coniferyl and sinapyl alcohols (Santos et al., 2017). It can be dissolved in a process

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known as alkaline delignification in order to isolate phenolic compounds (vanillic acid, ferulic acid, *p*-coumaric acid, gallic acid, hydroxybenzoic acid, syringic acid) with antioxidant properties (Akpınar & Usal, 2015; Mussatto et al., 2007). There are also other methodologies for that purpose reported in literature such as extraction in acid conditions, sulfite and organosolv processes (Strassberger et al., 2014). Alkaline treatment has been highlighted as one of the most effective methods since a selective and effective delignification can be achieved with mild operation conditions, along with the possibility of recovery and reuse of reagents like NaOH (Kim et al., 2016). Although the alkaline treatment has been reported for the release of phenolic acids from RH, studies on their separation from the hydrolysate for possible use have not yet been carried out, to the best of our knowledge.

Phenolic compounds are of great interest due to their well-known properties such as antioxidant, antimicrobial, anti-inflammatory, anticancer, among others (Lima et al., 2023; Mussatto et al., 2007; Xavier et al., 2021). Hence, RH can be used as raw material to obtain bioactive compounds with considerable application in the food and pharmaceutical industry. However, phenolic compounds must be isolated from the liquor obtained in the delignification process prior to use them in foods, pharmaceuticals, cosmetics, etc.

Among several known separation techniques, liquid-liquid extraction remains nowadays as one of the most used processes in the industry, commonly employing organic solvents where most of them are toxic and flammable. In that sense, researchers around the world have been lately trying to develop environmentally friendly liquid-liquid extraction systems, where aqueous two-phase systems (ATPS) have started to gain popularity since they are greener than traditional liquid-liquid systems (Silva et al., 2019; Xavier et al., 2017). ATPS are ternary systems able to form two immiscible phases coexisting in equilibrium under certain conditions of temperature, pressure and composition.

The most common ATPS are composed by polyethylene glycol (PEG)-dextran or PEG-salt, although recently, ionic liquids (ILs), alcohols or surfactants have also been investigated (Abolghasembeyk et al., 2017; Ulloa et al., 2012). Use of surfactants promote phase segregation at short times and offer systems with low interfacial tension, and those of the Triton X family had been widely reported in literature to obtain ATPS, along with surfactants classified as food additives in many countries like Tween (Tw) 20 and Tw 80 (Álvarez et al., 2015).

Many studies have been conducted on imidazolium-based ILs, but limitations have been encountered due to their price, toxicity and biodegradability. Among ILs, cholinium-based ones have been proposed as a new alternative to the traditional ILs, due to the low toxicity of the cation, its biodegradability and its lower price (Abolghasembeyk et al., 2017; Souza et al., 2015). In particular, choline chloride has advantages such as easy preparation, high stability, it can be used as a pharmaceutical ingredient and it also has lower viscosity than other choline-based salts (Abolghasembeyk et al., 2017; Shahriari et al., 2013). ATPS based on choline chloride have been used for separation of several products: antibiotics (Álvarez et al., 2015; Shahriari et al., 2013), stevioside (Abolghasembeyk et al., 2017), pigments and proteins (Suarez Ruiz et al., 2020), dyes (Escudero et al., 2019), among others.

The aim of this work was to obtain phenolic compounds with antioxidant activity from RH, paying particular attention to the extraction and separation of ferulic acid and *p*-coumaric acid.

The effect of alkaline hydrolysis operational conditions (temperature, solid/liquid (S/L) ratio and NaOH concentration) on the hydrolysis yield (HY), total phenolic content (TPC), the antioxidant capacity and the ferulic and *p*-coumaric acids content of the hydrolysates was studied. Furthermore, the potential of ATPS based on choline chloride and different nonionic surfactants was explored for the separation of ferulic and *p*-coumaric acids from the hydrolysate. Additionally, the influence of the tie-line length (TLL), volume ratio (V_R) and temperature on the separation yield was studied.

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 53,188–07–1), ABTS (2,2'-Azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid)) (CAS 30,931–67–0), TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine) (CAS 3682–35–7), choline chloride (CAS 67–48–1), Tw20 (CAS 9005–64–5) and Tw80 (CAS 9005–65–6) 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), sodium hydroxide (CAS 1310–73–2), 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 ethanol pretreatment

RH samples were provided by Arrozur S.A. (a local rice producer), with particles size between 0.5 and 2.4 mm. Later, in the laboratory, the RH was air-dried until equilibrium moisture content was reached (~10.5% wet basis). Moisture content of the sample was determined according to Mansaray and Ghaly (Mansaray & Ghaly, 1997).

Then, the RH was subjected to a pretreatment with EtOH to extract the free phenolic compounds present in it. RH was used as received, since no significant differences were observed on the extract properties when reducing the sample's particle size by milling (data not shown). The extraction was carried out with 70% (v/v) ethanol at 60 °C, using a S/L ratio of 1/10 g/mL. These conditions were selected through a design of experiments carried out previously (unpublished results). Extractions were performed at 200 rpm for 60 min. Both extract and solid residue were separated by vacuum filtration and stored in the dark at 4 °C for further analysis. The analysis carried out over the extracts were extraction yield (EY), TPC, antioxidant activities (FRAP and TEAC) and content of ferulic and *p*-coumaric acids.

The EY was calculated by subjecting the extract to oven drying until a constant weight was achieved, and then it was expressed as grams of solid extract per 100 g of RH on a dry basis (d.b.).

2.3. Alkaline hydrolysis

The solid residues obtained from the pretreatment stage with EtOH were subjected to hydrolysis with NaOH to release the bound phenolic compounds present in the RH lignin. All assays were performed for 90 min in a shaking bath. The solid residue was separated by vacuum filtration and then, the hydrolysate was neutralized using H₂SO₄ prior to analysis.

The HY was calculated as:

$$HY \left(\frac{g}{100 \text{ g RH d.b.}} \right) = \frac{m_{BH}(d.b.) - m_{AH}(d.b.)}{m_{BH}(d.b.)} \times 100 \quad (1)$$

where m_{BH} and m_{AH} are the dry weight of RH before and after hydrolysis, respectively.

An experimental design was conducted to evaluate the influence of temperature (x_1 , 40–80–120 °C), S/L ratio (x_2 , 1/10–1/20–1/30 g/mL) and NaOH concentration (x_3 , 0.5–2–3.5 N) on the HY, TPC, the antioxidant capacity (FRAP and TEAC) and the ferulic and *p*-coumaric acid content of the hydrolysates. An incomplete 3³ factorial design (Box Behnken) consisting of 12 assays with three replicates at the center point to estimate the experimental error was conducted (Table 1).

Table 1
Experimental design and the results obtained for the alkaline hydrolysis of RH at 101 kPa.

| x_1 | x_2 | x_3 | Temperature (°C) | S/L ratio (g/mL) | NaOH concentration (N) | HY (g/100 g RH d.b.) | TPC (mg GAE/g RH d.b.) | FRAP (mmol AAE/100 g RH d.b.) | TEAC (mmol TE/100 g RH d.b.) | Ferulic acid content (mg/g RH d.b.) | <i>p</i> -coumaric acid content (mg/g RH d.b.) |
|-------|-------|-------|---------------------|---------------------|---------------------------|-------------------------|---------------------------|----------------------------------|---------------------------------|--|---|
| 1 | -1 | -1 | 0 | 1/10 | 2 | 36.90 ± 0.19 | 16.16 ± 0.58 | 2.67 ± 0.03 | 12.44 | 1.14 ± 0.03 | 6.24 ± 0.23 |
| 2 | -1 | 0 | -1 | 1/20 | 0.5 | 30.34 ± 0.26 | 11.28 ± 0.12 | 2.57 ± 0.05 | 8.96 | 0.82 ± 0.02 | 3.59 ± 0.05 |
| 3 | -1 | 0 | 1 | 1/20 | 3.5 | 35.24 ± 0.36 | 16.91 ± 0.54 | 3.28 ± 0.05 | 12.68 | 1.31 ± 0.03 | 6.78 ± 0.23 |
| 4 | -1 | 1 | 0 | 1/30 | 2 | 37.61 ± 0.07 | 18.30 ± 0.55 | 3.42 ± 0.16 | 14.30 | 1.47 ± 0.11 | 5.93 ± 0.30 |
| 5 | 0 | -1 | -1 | 1/10 | 0.5 | 40.27 ± 0.03 | 20.18 ± 0.42 | 3.68 ± 0.09 | 15.65 | 1.10 ± 0.03 | 6.23 ± 0.05 |
| 6 | 0 | -1 | 1 | 1/10 | 3.5 | 50.95 ± 0.13 | 19.88 ± 0.27 | 3.87 ± 0.13 | 11.66 | 1.36 ± 0.06 | 7.71 ± 0.13 |
| 7 | 0 | 1 | -1 | 1/30 | 0.5 | 40.53 ± 0.23 | 27.33 ± 1.09 | 4.34 ± 0.08 | 20.58 | 1.25 ± 0.01 | 8.21 ± 0.12 |
| 8 | 0 | 1 | 1 | 1/30 | 3.5 | 47.84 ± 0.37 | 26.86 ± 0.79 | 5.08 ± 0.15 | 14.81 | 1.24 ± 0.01 | 8.75 ± 0.10 |
| 9 | 1 | -1 | 0 | 1/10 | 2 | 57.72 ± 0.02 | 34.85 ± 1.03 | 10.17 ± 0.95 | 18.35 | 2.37 ± 0.08 | 9.84 ± 0.35 |
| 10 | 1 | 0 | -1 | 1/20 | 0.5 | 48.12 ± 0.04 | 41.54 ± 0.46 | 9.18 ± 0.77 | 24.81 | 2.22 ± 0.17 | 10.67 ± 0.24 |
| 11 | 1 | 0 | 1 | 1/20 | 3.5 | 58.61 ± 0.01 | 35.23 ± 0.79 | 10.97 ± 0.64 | 15.89 | 2.57 ± 0.05 | 10.34 ± 0.42 |
| 12 | 1 | 1 | 0 | 1/30 | 2 | 53.33 ± 0.34 | 42.75 ± 1.31 | 16.75 ± 1.22 | 22.81 | 3.71 ± 0.10 | 11.62 ± 0.34 |
| 13 | 0 | 0 | 0 | 1/20 | 2 | 49.42 ± 0.11 | 25.05 ± 1.66 | 4.10 ± 0.09 | 21.52 | 1.43 ± 0.06 | 9.36 ± 0.34 |
| 14 | 0 | 0 | 0 | 1/20 | 2 | 47.08 ± 0.06 | 27.07 ± 0.91 | 4.36 ± 0.07 | 20.66 | 1.41 ± 0.02 | 8.96 ± 0.16 |
| 15 | 0 | 0 | 0 | 1/20 | 2 | 46.75 ± 0.20 | 24.87 ± 1.64 | 4.23 ± 0.08 | 20.90 | 1.37 ± 0.01 | 8.88 ± 0.15 |

x_1 , temperature; x_2 , S/L ratio; x_3 , NaOH concentration. Dependent variables are presented as mean ± standard deviation (SD) for three analysis replicates. Standard uncertainties (u) are $u(T) = \pm 0.1$ °C and $u(P) = \pm 10$ kPa. The experimental error for both S/L ratio and the soda concentration was estimated from the variation coefficient (0.81 and 0.21%, respectively).

Experimental results for the dependent variables were fitted to an empirical second-order polynomial model applying the backward elimination method:

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

where $j > i$, β represents each regression coefficient, Y is the experimental value for each response variable and x_i and x_j are the independent variables, that are coded at three levels: low (−1), high (1) and medium (0).

The conditions leading to the maximum value for each dependent variable were obtained by means of response surface methodology, within the experimental region. Taking these conditions into account, a working point was selected prioritizing the ferulic and *p*-coumaric acid content. A verification experiment at the selected condition was carried out in triplicate aiming to validate the model.

2.4. Separation of phenolic compounds with ATPS

The partition behavior of ferulic and *p*-coumaric acids present in the hydrolysate obtained on 2.3 was studied. ATPS based on different surfactants (Tw20 and Tw80) and choline chloride were prepared using the equilibria data found in the literature (Álvarez et al., 2015).

These systems were selected because their components are biodegradable and have low toxicity. In addition, Tw20 and Tw80 are considered GRAS, and can be used in cosmetic, food and pharmaceutical applications (Álvarez et al., 2015; Suarez Ruiz et al., 2020).

In a first stage, influence of the tie-line length (TLL) on the separation of ferulic and *p*-coumaric acids was investigated at 25 °C and atmospheric pressure. Two tie-lines were selected for each system (Tw20-choline chloride and Tw80-choline chloride) based on the equilibrium data at 25 °C reported by Álvarez et al. (Álvarez et al., 2015). Table 2 shows the global compositions for each ATPS. These compositions were selected in order to obtain a volume ratio (V_R) close to one in all cases. The latter was experimentally measured and defined as follows:

$$V_R = \frac{V_T}{V_B} \tag{3}$$

where V_T and V_B are the volume of the top and bottom phase, respectively.

Among the evaluated ATPS, the one presenting better separation performance was selected to investigate the influence of temperature and V_R on the partition of phenolic acids. Assays at 40 and 50 °C were carried out using the same global composition than the system at 25 °C. It should be noted that at these temperatures we are not working on the same tie-line, since equilibrium conditions changes with temperature.

Table 2
Global composition, V_R and TLL for the tested ATPS at 25.0 °C and 101 kPa.

| System | Surfactant | X_s (%) w/w) | X_{cc} (%) w/w) | X_w (%) w/w) | V_R | TLL (%) w/w) |
|--------|------------|-------------------|----------------------|-------------------|--------------|-----------------|
| 1 | Tw20 | 55.02 | 23.00 | 21.97 | 1.6 ± 0.0 | 94.68 |
| 2 | | 35.01 | 30.81 | 34.18 | 0.9 ± 0.0 | 70.83 |
| 3 | Tw80 | 47.72 | 34.83 | 17.45 | 1.4 ± 0.0 | 116.67 |
| 4 | | 44.98 | 31.07 | 23.95 | 1.2 ± 0.1 | 102.03 |

X_s is the concentration of surfactant in% (w/w). X_{cc} is the concentration of choline chloride in% (w/w). X_w is the concentration of water in% (w/w). V_R is the volume ratio. TLL is the tie-line length. Each TLL reported here was taken from literature (Álvarez et al., 2015). Standard uncertainties (u) are $u(T) = \pm 0.1$ °C, $u(w) = \pm 0.01\%$ and $u(P) = \pm 10$ kPa.

The total mass for each system was fixed at 20 g. The proper amount of surfactant and ionic liquid was mixed and then, 2 g of hydrolysate obtained at the selected working condition was added to each system, followed by water addition to reach the required global compositions (Table 2). The systems were prepared by mixing the components in centrifuge tubes. Afterwards, the phase separation was performed in an incubator with temperature control for 24 h at the corresponding temperature. After equilibrium conditions were achieved, aliquots from the bottom phase were taken and analyzed by HPLC as described later.

All the experiments were carried out in triplicate. The partition coefficient, K , of the phenolic acid of interest i (ferulic or p -coumaric acid) was quantified as follows (Suarez Ruiz et al., 2018):

$$K = \frac{C_{i,T}}{C_{i,B}} \quad (4)$$

where $C_{i,T}$ and $C_{i,B}$ are the concentration of phenolic acid i in the top and bottom phase respectively. $C_{i,B}$ was experimentally measured, while $C_{i,T}$ was obtained by a mass balance knowing the amount of phenolic acids added to the system.

The separation yield (SY) is defined as the percentage of the phenolic acid i in the top phase over the total existing in the biphasic system (Mendes et al., 2023).

$$SY (\%) = \frac{C_{i,T} V_T}{C_{i,T} V_T + C_{i,B} V_B} \times 100 \quad (5)$$

2.5. Analytical methods

2.5.1. Total phenolic content

The TPC was determined following the Folin-Ciocalteu's method according to Singleton et al. (1965) with some modifications. Briefly, 0.5 mL of the hydrolysate was placed into a test tube, then 2.5 mL of dilute Folin-Ciocalteu reagent (1/10 in water) was added, along with 2.0 mL of sodium carbonate (7.5% w/v). The mixture was placed into a 50 °C water bath for 5 min. The absorbance was measured at 760 nm using a Shimadzu UVmini-1240 spectrophotometer. All assays were performed in triplicate and the TPC was expressed as mg of gallic acid equivalents (GAE)/g RH d.b.

2.5.2. Ferric reducing antioxidant power (FRAP)

The reducing power of the extract was determined according to the method proposed by Szöllösi and Szöllösi-Varga (2002). The FRAP reagent was prepared by mixing 2.5 mL of 10 mM TPTZ solution and 2.5 mL of 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ with 25 mL of 300 mM acetate buffer (pH 3.6). 0.1 mL of hydrolysate was mixed with 3 mL of FRAP reagent at 25 °C for 5 min and the absorbance was measured at 593 nm. The results were expressed as mmol of ascorbic acid equivalents (AAE)/100 g RH d.b.

2.5.3. Trolox equivalent antioxidant capacity (TEAC)

The assay was performed as proposed by Re et al. (1999) with slight modifications. Briefly, the ABTS radical cation ($\text{ABTS}^{+\bullet}$) solution was prepared by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate. The mixture was stored in the dark at 4 °C for 40 h and then diluted with distilled water to reach an absorbance of 0.700 at 734 nm. An aliquot of 2.5 mL of the previous reagent was mixed with 25 μL of the hydrolysate and the absorbance readings were taken at 734 nm. The results were expressed as mmol of trolox equivalents (TE)/100 g RH d.b.

2.5.4. High performance liquid chromatography (HPLC)

The HPLC was used to determine the content of ferulic and p -coumaric acids. The chromatographic conditions and equipment were the ones described by Xavier et al. (2017). A C-18 column (5 μm , 4.6 mm \times 250 mm) with reversed phase and a diode array detector at 320 nm were used. The elution was carried out under an isocratic flow of 1 mL/min at 25 °C, using as mobile phase a solution composed of 20 % (v/v)

acetonitrile and 80 % (v/v) water (1 % acetic acid); with an injection volume of 20 μL . The results were expressed as mg of ferulic or p -coumaric acid/g RH d.b.

2.5.5. Statistical analysis

Statistical analysis of the models was carried out by means of analysis of variance (ANOVA). Significance of the model and regression coefficients were established with a 90 % confidence level using software Infostat version 2013 (Universidad Nacional de Córdoba, Argentina, <http://www.infostat.com.ar>). To determine significant differences (95% confidence level) within the ATPS results, ANOVA and Tukey test were performed also using InfoStat software.

3. Results and discussion

3.1. Ethanol pretreatment of RH

The results of the pretreatment with ethanol are shown in Table 3. As can be seen, the presence of ferulic and p -coumaric acid was detected, as well as antioxidant activity in the extract.

These results are in concordance with those reported by Butsat and Siriamornpun (2010). They performed an extraction with methanol to release the free phenolic compounds of different varieties of RH, obtaining extracts with TPC ranging from 0.69 to 2.56 mg GAE/g RH, ferulic acid content from 0.53 to 5.57 mg/100 g RH and p -coumaric acid content from 0.72 to 6.93 mg/100 g RH (Butsat & Siriamornpun, 2010). Irakli et al. (2018) treated the RH with 41 % ethanol at 40 °C in addition to applying ultrasound and obtained higher values: TPC (1.68 mg GAE/g RH d.b.), FRAP (1.92 mmol AAE/100 g RH d.b.), TEAC (0.94 mmol TE/100 g RH d.b.), ferulic acid content (5.2 mg/100 g RH d.b.) and p -coumaric acid content (14.2 mg/100 g RH d.b.).

3.2. Alkaline hydrolysis

Table 1 presents the conditions of the experiments, the design matrix, their coding, and the results of the dependent variables: HY, TPC, FRAP, TEAC, ferulic acid and p -coumaric acid. Table 4 shows the coefficients, the statistical parameters, and the significance of the model. As can be seen, not all the parameters of the adjustment equation (Eq. (2)) were used to build the models for each dependent variable, since they did not turn out to be significant ($p < 0.10$).

The experimental data was satisfactory fitted to the proposed models, proven by the high values of R^2 (from 0.879 to 0.992). Response surfaces plots are shown in Figs. 1 to 6.

In Fig. 1, the HY increases with the temperature and with the NaOH concentration, while the S/L ratio did not influence this variable. The linear effect of temperature and NaOH (β_1, β_3) were the most significant variables on HY, followed by the square term of the NaOH concentration (β_{33}). The maximum performance (58.14 g extract/100 g RH d.b.) was obtained at 120 °C with a NaOH concentration of 3.05 N. It is already widely reported that the HY is favored by increasing the temperature, since it increases the solubility and facilitates the migration of phenolic compounds from the plant matrix into the solvent (Dai & Mumper, 2010; Irakli et al., 2018).

Both the TPC and the antioxidant activity by the FRAP method

Table 3
Experimental results for the ethanol pretreatment stage.

| Extract property | Experimental value |
|---|--------------------|
| EY (g /100 g RH d.b.) | 1.33 \pm 0.09 |
| TPC (mg GAE/g RH d.b.) | 1.19 \pm 0.04 |
| FRAP (mmol AAE/100 g RH d.b.) | 0.45 \pm 0.04 |
| TEAC (mmol TE/100 g RH d.b.) | 0.81 \pm 0.12 |
| Ferulic acid content (mg/100 g RH d.b.) | 0.31 \pm 0.05 |
| p -coumaric acid content (mg/100 g RH d.b.) | 2.91 \pm 0.01 |

Experimental values are presented as mean \pm standard deviation (SD).

Table 4
Regression coefficients for the models and statistical parameters.

| | HY (g/100 g RH d.b.) | | | TPC (mg GAE/g RH d.b.) | | | FRAP (mmol AAE/100 g RH d.b.) | | | TEAC (mmol TE/100 g RH d.b.) | | | Ferulic acid content (mg/g RH d.b.) | | | p-coumaric acid content (mg/g RH d.b.) | | |
|--------------|----------------------|-------|-------|------------------------|-------|-------|-------------------------------|-------|-------|------------------------------|-------|-------|-------------------------------------|-------|-------|--|-------|-------|
| | RC | ES | S | RC | ES | S | RC | ES | S | RC | ES | S | RC | ES | S | RC | ES | S |
| β_0 | 46.973 | 0.772 | 0 | 25.565 | 0.513 | 0 | 4.237 | 0.498 | 0 | 21.027 | 0.638 | 0 | 1.488 | 0.149 | 0 | 8.690 | 0.176 | 0 |
| β_1 | 9.711 | 0.675 | 0 | 11.464 | 0.378 | 0 | 4.390 | 0.466 | 0 | 4.185 | 0.391 | 0 | 0.766 | 0.110 | 0 | 2.491 | 0.165 | 0 |
| β_2 | -0.816 | - | NS | 3.023 | 0.378 | 0 | 1.150 | 0.466 | 0.033 | 1.801 | 0.391 | 0.002 | 0.213 | 0.110 | 0.081 | 0.561 | 0.165 | 0.009 |
| β_3 | 4.173 | 0.675 | 0 | -0.181 | - | NS | 0.428 | - | NS | -1.869 | 0.391 | 0.002 | 0.136 | - | NS | 0.610 | 0.165 | 0.006 |
| β_{11} | -1.590 | - | NS | 2.524 | 0.554 | 0.002 | 3.140 | 0.682 | 0.001 | -2.070 | 0.575 | 0.009 | 0.620 | 0.161 | 0.003 | -0.270 | - | NS |
| β_{22} | 0.230 | - | NS | -0.166 | - | NS | 0.881 | - | NS | -1.982 | 0.575 | 0.011 | 0.138 | - | NS | -0.390 | - | NS |
| β_{33} | -2.985 | 0.988 | 0.012 | -1.926 | 0.554 | 0.008 | -0.869 | - | NS | -3.372 | 0.575 | 0.001 | -0.315 | 0.161 | 0.079 | -0.905 | 0.242 | 0.006 |
| β_{12} | -1.275 | - | NS | 1.443 | 0.534 | 0.027 | 1.458 | 0.659 | 0.051 | 0.650 | - | NS | 0.253 | - | NS | 0.523 | 0.233 | 0.056 |
| β_{13} | 1.398 | - | NS | -2.985 | 0.534 | 0.001 | 0.273 | - | NS | -3.160 | 0.553 | 0.001 | -0.035 | - | NS | -0.880 | 0.233 | 0.005 |
| β_{23} | -0.843 | - | NS | -0.043 | - | NS | 0.138 | - | NS | -0.448 | - | NS | -0.068 | - | NS | -0.235 | - | NS |
| R^2 | 0.959 | | | 0.992 | | | 0.924 | | | 0.972 | | | 0.879 | | | 0.973 | | |
| R^2 adj. | 0.947 | | | 0.987 | | | 0.893 | | | 0.944 | | | 0.830 | | | 0.952 | | |
| ES | 1.909 | | | 1.068 | | | 1.318 | | | 1.106 | | | 0.310 | | | 0.467 | | |
| S | 0 | | | 0 | | | 0 | | | 0 | | | 0 | | | 0 | | |

RC, regression coefficient; ES, error standard; S, significance; NS, not significant. (Level of significance: $p < 0.10$).

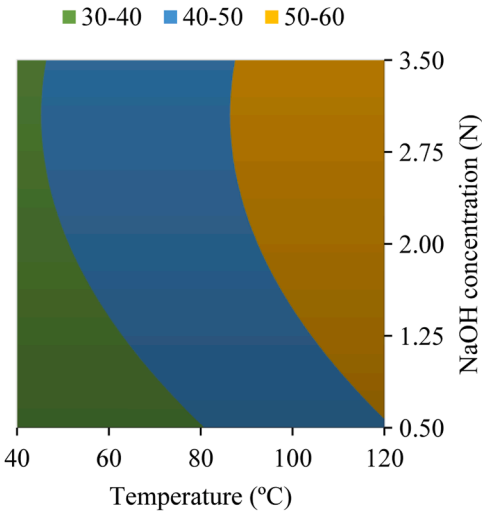


Fig. 1. Response surface for hydrolysis yield (HY), expressed as g/100 g RH d. b. The plot is the same one for all S/L ratios.

increased with temperature and the S/L ratio (See Fig. 2-3). It can be seen on Table 4 that responses were strongly affected positively by the linear term of temperature and S/L ratio (β_1, β_2). The linear term of the NaOH concentration (β_3) affected negatively the TPC, while in the case of the FRAP, it was not significant. The maximum TPC (45.17 mg GAE/g RH d.b.) was obtained at 120 °C, with a S/L ratio of 1/30 g/mL and a NaOH concentration of 0.8 N, while, for FRAP, the maximum (14.37 mmol AAE/100 g RH d.b.) was obtained at the same temperature and S/L ratio, but the concentration of NaOH did not significantly affect it. The TPC concentration obtained in this work was higher than that determined by Irakli et al. (2018) and Gao et al. (2018) in the extraction of RH phenolic compounds with NaOH (6.96 and 13.7 mg GAE/g RH d.b. respectively). Also, the FRAP value obtained for this RH was higher than that obtained by Butsat and Siriamornpun (2010) (10 – 20 μ mol FeSO₄/g RH).

In the case of the TEAC (Fig. 4), the most significant terms were all the linear terms ($\beta_1, \beta_2, \beta_3$), the quadratic term of the NaOH concentration (β_{33}) and the temperature-NaOH interaction (β_{13}). The maximum (25.21 mmol TE/100 g RH d.b.) was obtained at 120 °C with an S/L ratio of 1/20 g/mL and an NaOH concentration of 0.5 N.

Regarding the response surfaces obtained for ferulic acid (Fig. 5) and p-coumaric acid (Fig. 6), a similar behavior was found, since the concentration of both acids increased with increasing temperature and increasing the S/L ratio. The quadratic term of the NaOH concentration (β_{33}) turned out to have a negative effect on the response for both acids, which is why a maximum around 2 N is observed in both Figs. (5 and 6). These results of ferulic and p-coumaric acids were higher than the ones reported by Butsat and Siriamornpun (2010) (0.14–0.25 mg/g RH for ferulic acid; 1.60–3.90 mg/g RH for p-coumaric acid), who studied the alkaline hydrolysis of RH from different rice varieties using 2 N NaOH, S/L ratio of 1/20 at 25 °C for 60 min. It must be considered in comparisons with other studies, that there are differences in cultivation and extraction methods that makes comparison difficult (Akpınar et al., 2012). However, we can attribute this difference to the higher temperature used in our experiences, although hydrolysis time might also be a key factor (which is also higher in our work).

Through analysis of the response surfaces obtained for each dependent variables, the working condition was selected. Since maximum values for all dependent variables are obtained at different conditions, the working point was selected prioritizing the highest content of ferulic and p-coumaric acids. The selected working condition was 120 °C, 1/30 g/mL and 2 N NaOH. It was also verified that high values of HY, TPC and antioxidant activities (close to the maximum ones) are obtained.

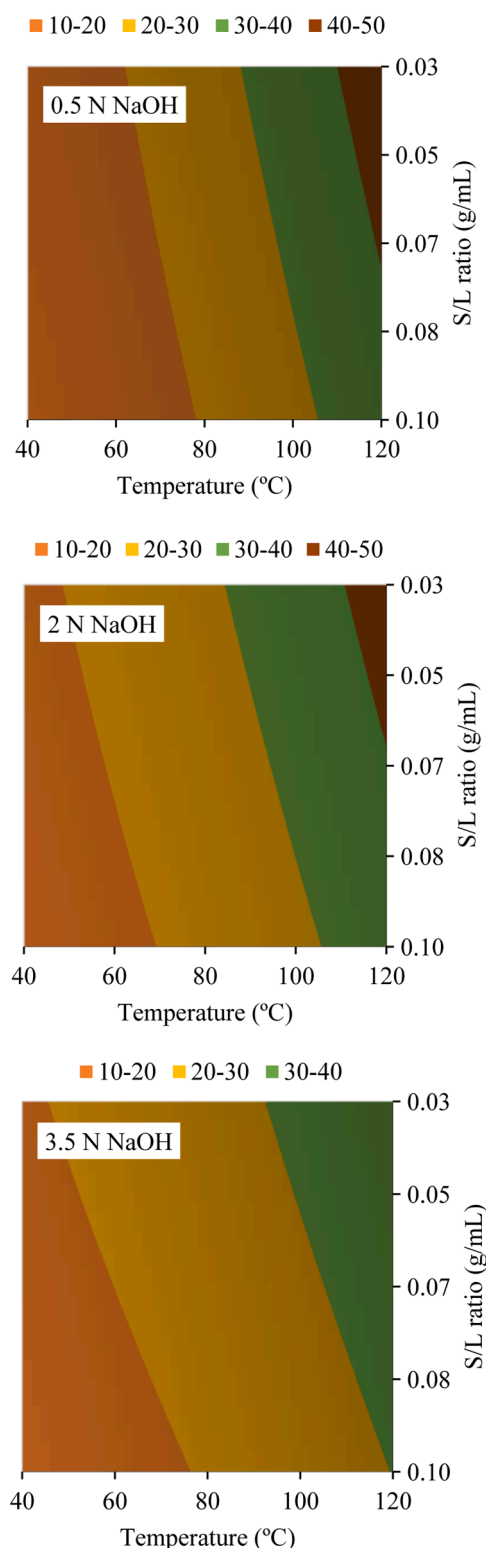


Fig. 2. Response surface plots for total phenolic content (TPC), expressed as mg GAE/g RH d.b.

Comparison between predicted and experimental values on the selected condition is shown in Table 5. The results confirmed the applicability of the model for the extraction of phenolic compounds by treatment with NaOH.

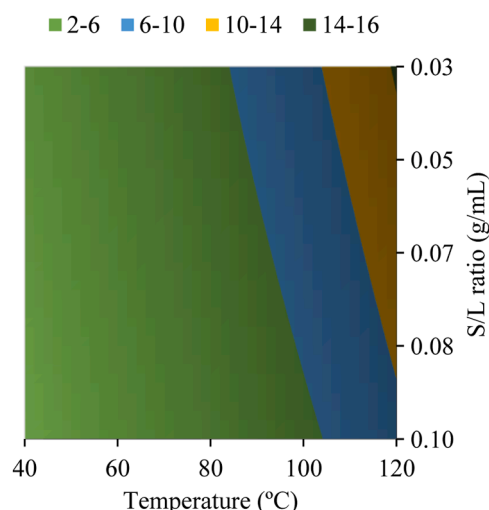


Fig. 3. Response surface for FRAP, expressed as mmol AAE/100 g RH d.b. The plot is the same one for all NaOH concentrations.

3.3. Separation of phenolic acids with ATPS

To evaluate the separation capacity of phenolic acids from the RH hydrolysate, different ATPS based on choline chloride and different surfactants were studied (Table 6).

In a first stage of the study, a screening of the systems was carried out, analyzing K and SY for ferulic acid and *p*-coumaric acid. The V_R used was close to 1. In all cases was observed that both ferulic and *p*-coumaric acids were mostly transferred to the surfactant-rich phase, since SY were higher than 60%. This seems feasible because the phenolic acids present in the hydrolysate are both amphiphilic compounds, being *p*-coumaric acid the most hydrophobic one (Xavier et al., 2017). This agrees with previous work where ferulic and *p*-coumaric acids from wheat straw hydrolysate migrated to the more hydrophobic phase with efficiencies in the range of 70–90% using ATPS formed by Tw20 and choline dihydrogen citrate (Xavier et al., 2017). Furthermore, it can be observed that SY for the systems formed by Tw80 are ~ 90 % and significantly higher ($p < 0.05$) than for the systems formed by Tw20. The same trend was observed with the K values for both acids.

The reason why these phenolic acids migrated to the Tw80-rich phase in a greater extent may lie on the hydrophobicity of this molecule. A useful parameter to quantify the degree of hydrophobicity for a given surfactant is the Hydrophilic-Lipophilic Balance (HLB). This unitless parameter ranges from 0 to 20, being a more hydrophobic compound when closer to 0 is this value. Tw80 and Tw20 have HLB values of 15.0 and 16.7, respectively (Escudero et al., 2019). The more hydrophobic nature of Tw80 might be correlated with the greater K and SY obtained in the systems containing it. In addition, the systems formed by Tw80 have less amount of water, which favors the hydrophobic interactions between the compounds of the system and the solutes to be separated (Simental-Martínez et al., 2014). It is interesting to point out that use of different TLL for a given system did not affect SY significantly. Since yields obtained in systems 3 and 4 are statistically the same, system 4 was chosen as the more suitable one to continue experimentation since less quantities of reactants are required.

Table 7 shows the effect of temperature on the partition of phenolic acids for system 4. Rising the temperature did not affect the SY for *p*-coumaric acid. On the other hand, there was a slight improvement on SY for ferulic acid since values at 40 and 50 °C were a bit higher than the one obtained at 25 °C. Influence of V_R on system 4 is summarized in Table 8. The study was conducted at 25 °C since use of higher temperatures does not improve the separation process, as mentioned above. Values of V_R higher than one implies that V_T is higher than V_B . Increasing the volume of top phase led to higher separation efficiencies

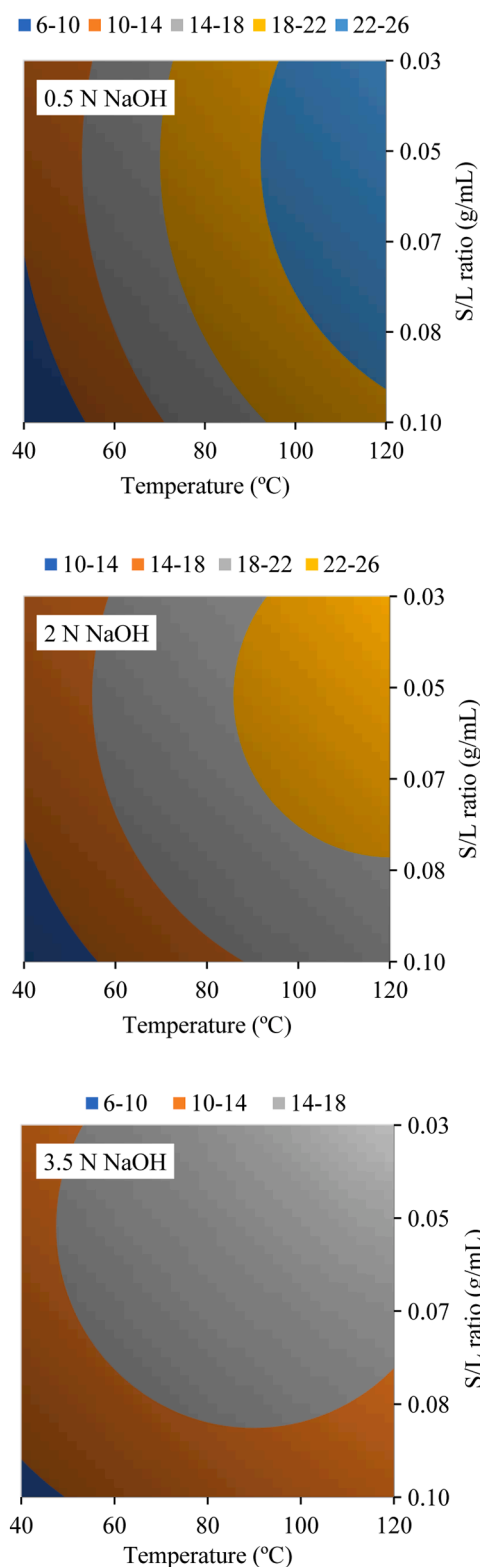


Fig. 4. Response surface plots for TEAC, expressed as mmol TE/100 g RH d.b.

since it helps to overcome saturation issues. The opposite happened when V_R was lower than one, meaning V_T is lower than V_B . Since volume of top phase is lowered, the mass of phenolic acid in that phase is also lowered due to the saturation effect, thus the separation efficiency diminishes (Xavier et al., 2017).

One of the main technical difficulties existing when ATPS are used to separate compounds are the protocols to recover these compounds from

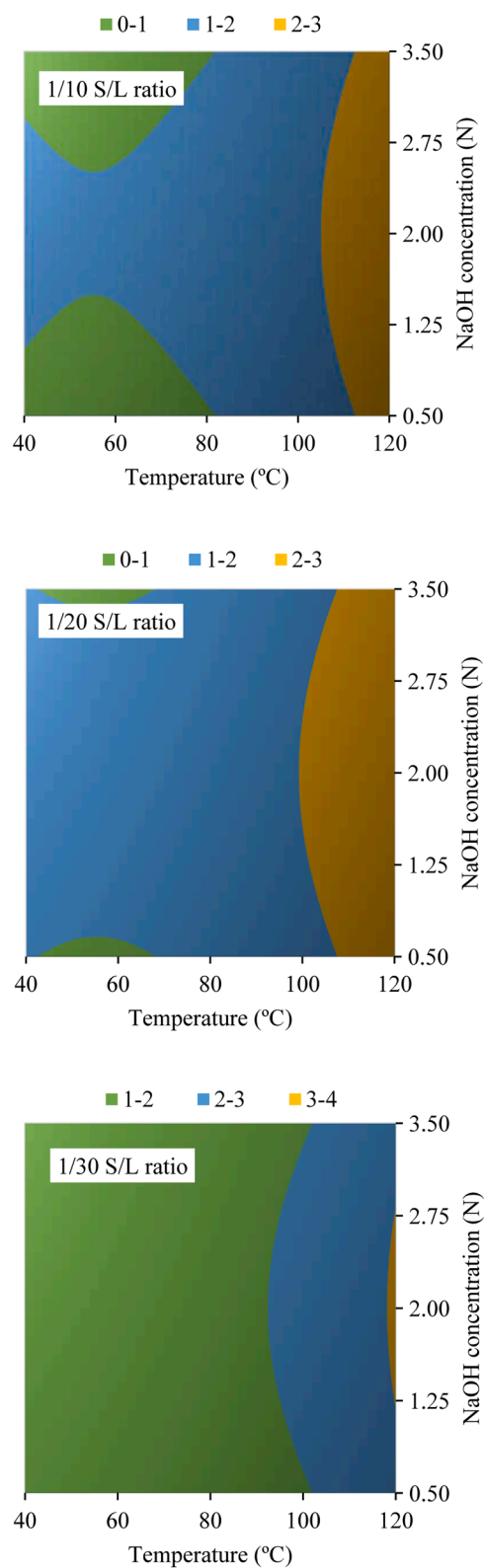


Fig. 5. Response surface plots for ferulic acid content, expressed as mg/g RH d.b.

the phase they migrated to. Another difficulty is the need to reuse the components to minimize the cost of the process (Simental-Martínez et al., 2014; Suarez Ruiz et al., 2020). Although it already exists international evidence of the reuse of the components that make up the ATPS, more studies are still needed to optimize these types of approaches

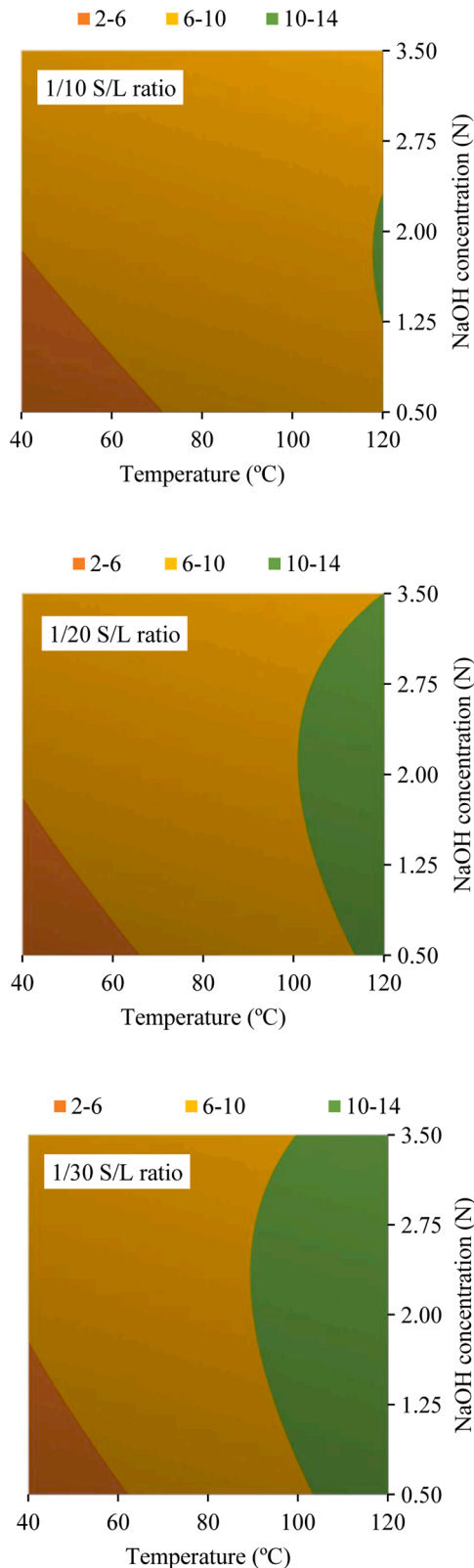


Fig. 6. Response surface plots for *p*-coumaric acid content, expressed as mg/g RH d.b.

(Álvarez et al., 2016). These compounds obtained in the surfactant-rich phase could be recovered, for example, with another solvent (back extraction), and then the surfactant can be reused in another process. However, it should be noted that since Tw80 is considered GRAS,

Table 5
Verification experiment results on the working condition.

| Dependent variable | Predicted value | Observed value |
|--|-----------------|----------------|
| HY (g/100 g RH d.b.) | 56.68 ± 2.11 | 55.23 ± 3.86 |
| TPC (mg GAE/g RH d.b.) | 44.02 ± 2.51 | 41.61 ± 2.95 |
| FRAP (mmol AAE/100 g RH d.b.) | 14.38 ± 1.89 | 16.17 ± 1.15 |
| TEAC (mmol TE/100 g RH d.b.) | 22.96 ± 1.86 | 23.89 ± 2.59 |
| Ferulic acid content (mg/g RH d.b.) | 3.09 ± 0.41 | 3.45 ± 0.35 |
| <i>p</i> -coumaric acid content (mg/g RH d.b.) | 12.27 ± 0.84 | 11.00 ± 0.66 |

Observed values are presented as mean ± standard deviation (SD) of three replicates. Predicted values are presented with a confidence interval (95% confidence level).

Table 6
Experimental results for the tested ATPS at 25.0 °C and 101 kPa.

| System | TLL (w/w %) | V _R | K _f | K _c | SY _f (%) | SY _c (%) |
|--------|-------------|----------------|--------------------------|--------------------------|-------------------------|-------------------------|
| 1 | 94.68 | 1.6 ± 0.0 | 1.32 ± 0.07 ^a | 0.98 ± 0.04 ^a | 67.6 ± 0.8 ^a | 60.5 ± 0.6 ^a |
| 2 | 70.83 | 0.9 ± 0.0 | 2.54 ± 0.16 ^b | 1.69 ± 0.10 ^a | 69.5 ± 1.4 ^a | 60.3 ± 1.4 ^a |
| 3 | 116.67 | 1.4 ± 0.0 | 6.61 ± 0.27 ^c | 7.96 ± 0.31 ^b | 90.0 ± 0.2 ^b | 91.5 ± 0.1 ^b |
| 4 | 102.03 | 1.2 ± 0.1 | 7.55 ± 0.43 ^d | 9.37 ± 0.78 ^c | 89.5 ± 0.2 ^b | 91.4 ± 0.2 ^b |

TLL, tie-line length; V_R, volume ratio; K_f, partition coefficient of ferulic acid; K_c, partition coefficient of *p*-coumaric acid; SY_f, separation yield of ferulic acid; SY_c, separation yield of *p*-coumaric acid. Each TLL reported here was taken from literature (Álvarez et al., 2015). Values with different letters within a column are significantly different (*p* < 0.05). Standard uncertainties (u) are u(T) = ± 0.1 °C, u(w) = ± 0.01% and u(P) = ± 10 kPa.

Table 7
Effect of temperature on System 4 at 101 kPa.

| T (°C) | V _R | K _f | K _c | SY _f (%) | SY _c (%) |
|--------|----------------|--------------------------|--------------------------|-------------------------|-------------------------|
| 25 | 1.2 ± 0.1 | 7.55 ± 0.43 ^a | 9.37 ± 0.78 ^a | 89.5 ± 0.2 ^a | 91.4 ± 0.2 ^a |
| 40 | 1.6 ± 0.0 | 7.46 ± 0.39 ^a | 6.39 ± 0.13 ^b | 92.1 ± 0.4 ^b | 90.9 ± 0.2 ^a |
| 50 | 1.5 ± 0.0 | 7.83 ± 0.41 ^a | 6.15 ± 0.54 ^b | 92.3 ± 0.4 ^b | 90.3 ± 0.8 ^a |

V_R, volume ratio; K_f, partition coefficient of ferulic acid; K_c, partition coefficient of *p*-coumaric acid; SY_f, separation yield of ferulic acid; SY_c, separation yield of *p*-coumaric acid. Values with different letters within a column are significantly different (*p* < 0.05). Standard uncertainties (u) are u(T) = ± 0.1 °C, u(w) = ± 0.01% and u(P) = ± 10 kPa.

phenolic acids could be recovered together with Tw80 and used together for other applications.

4. Conclusions

Through RH hydrolysis, high concentration of phenolic acids with antioxidant activity were obtained (3.45 and 11.00 mg/ g RH d.b. of ferulic and *p*-coumaric acid, respectively). The condition for the alkaline hydrolysis leading to the highest concentration of both phenolic acids was 120 °C, 1/30 g/mL S/L ratio and 2 N NaOH concentration. In addition, high levels of K and SY (greater than 90 %) of ferulic and *p*-coumaric acids from the RH hydrolysates were obtained with ATPS based on choline chloride and Tw80. It was shown that the separation of ferulic and *p*-coumaric acids depends on the selected surfactant and that it is essential to choose an adequate V_R. These results are promising, because very good separations of phenolic acids were achieved, without the use of energy and with a separation system with biodegradable, low-cost and GRAS components. Future studies should be conducted to evaluate different approaches to the reuse of ATPS components, in addition to characterizing the impurities obtained in the surfactant-rich phase and the techno-economic analysis of the process.

Table 8

Effect of global composition on system 4 at 25.0 °C and 101 kPa.

| X_s (%w/w) | X_{cc} (%w/w) | X_w (%w/w) | TLL (% w/w) | V_R | K_f | K_c | SY_f (%) | SY_c (%) |
|--------------|-----------------|--------------|-------------|-----------|--------------------------|--------------------------|-------------------------|-------------------------|
| 44.98 | 31.07 | 23.95 | 102.03 | 1.2 ± 0.1 | 7.55 ± 0.43 ^a | 9.37 ± 0.78 ^a | 89.5 ± 0.2 ^a | 91.4 ± 0.2 ^a |
| 23.01 | 45.51 | 31.49 | | 0.4 ± 0.0 | 8.86 ± 0.24 ^b | 8.38 ± 0.19 ^b | 78.5 ± 0.5 ^b | 77.5 ± 0.4 ^b |
| 65.00 | 17.93 | 17.06 | | 2.8 ± 0.0 | 4.47 ± 0.03 ^c | 5.82 ± 0.07 ^b | 92.7 ± 0.0 ^c | 94.3 ± 0.1 ^c |

X_s , mass fraction of surfactant in% (w/w); X_{cc} , mass fraction of choline chloride in% (w/w). X_w mass fraction of water + hydrolysate in% (w/w). TLL, tie-line length; V_R , volume ratio; K_f , partition coefficient of ferulic acid; K_c , partition coefficient of *p*-coumaric acid; SY_f , separation yield of ferulic acid; SY_c , separation yield of *p*-coumaric acid. TLL reported here was taken from literature (Álvarez et al., 2015). Values with different letters within a column are significantly different ($p < 0.05$). Standard uncertainties (u) are $u(T) = \pm 0.1$ °C, $u(w) = \pm 0.01\%$ and $u(P) = \pm 10$ kPa.

CRediT authorship contribution statement

Lucía Xavier: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervision. **Mauro Rocha:** Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Justina Pisani:** Conceptualization, Formal analysis, Investigation, Writing – review & editing. **Berta Zecchi:** Methodology, Writing – review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors have no competing interests to declare.

Data availability

No data was used for the research described in the article.

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Ethical statement

The authors declare that they have not carried out any studies with animals or involving humans.

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