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Recovering phytochemicals from a brewery by-product: a sustainable reuse proposal using a lactic acid-based deep eutectic solvent

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Hot trub (HT), a nutrient-rich by-product of beer production, remains underutilized despite its potential for resource recovery. This study explores the valorization of HT, producing two phytochemical-rich extracts with potential functional applications and a protein-rich solid residue that can be repurposed as a food ingredient. The extraction of phytochemicals from HT, particularly xanthohumol (XN), was evaluated through a sequential extraction process employing a deep eutectic solvent (DES) composed of choline chloride (ChCl) and lactic acid (Lac) in a 1 : 2 molar ratio, followed by ethanol-based solvent extraction. The influence of temperature and DES-to-HT ratios was evaluated, as defined by the experimental design. DESs selectively extracted XN, reaching a maximum yield of 36 μg g^{-1} at 50 °C with 8 g of DES and 4 g of HT. However, sequential extraction with ethanol on DES-treated solid residues at 50 °C and equal DES-to-HT mass ratios yielded a higher XN extraction (~114 μg g^{-1}), emphasizing the use of DES as an effective pretreatment. The temperature influenced the extraction of other phytochemicals, including desmethylxanthohumol and phenolic acids, contributing to enriched antioxidant activity. Structural analysis of HT after extraction revealed modifications, such as shifts and loss of functional chemical groups induced by DESa, improving phytochemical accessibility for the ethanol extraction step. The analysis of the proposed process using the *Path2Green* metric demonstrated a strong alignment with the principles of green chemistry and the circular bioeconomy.

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Sustainability spotlight

This article demonstrates how beer residue can be valorized as a whole, serving as an alternative source of economically important phytochemicals, such as bitter acids that are primarily responsible for the bitter taste of beer, as well as indirectly acting as a raw material for plant-based proteins. To achieve this goal, we have developed an extraction process utilizing green solvents (ethanol and deep eutectic solvents), which enables the targeted extraction of phytochemicals and eliminates the need for high-cost processes that can fractionate compounds. Our work contributes to the implementation of the UN Sustainable Development Goals, particularly SDG 12 and SDG 15, by promoting efficient methods to reduce waste and minimize environmental impact through recycling for the production of food ingredients.

1. Introduction

Beer is the most consumed alcoholic beverage in the world, with approximately 189 million kiloliters produced in 2022.[1] Consequently, tons of food by-products, such as brewer's spent grains, hot trub (HT), spent hops, and brewer's spent yeast, were produced along with beer manufacturing steps per year. These by-products present significant nutritional properties, offering an excellent source of protein, fiber, and phytochemicals with therapeutic properties.[2] Among brewing by-products, HT has been underutilized as a food source for human consumption and is typically used to produce animal feed or discarded as fertilizer. HT is a by-product generated during the wort boiling stage of beer production, formed by the combination of spent hops and the agglomeration of high-molecular-weight proteins with a wide range of phytochemicals. After proteins, phytochemicals are the most abundant components in HT and have garnered increasing interest because they can serve as active

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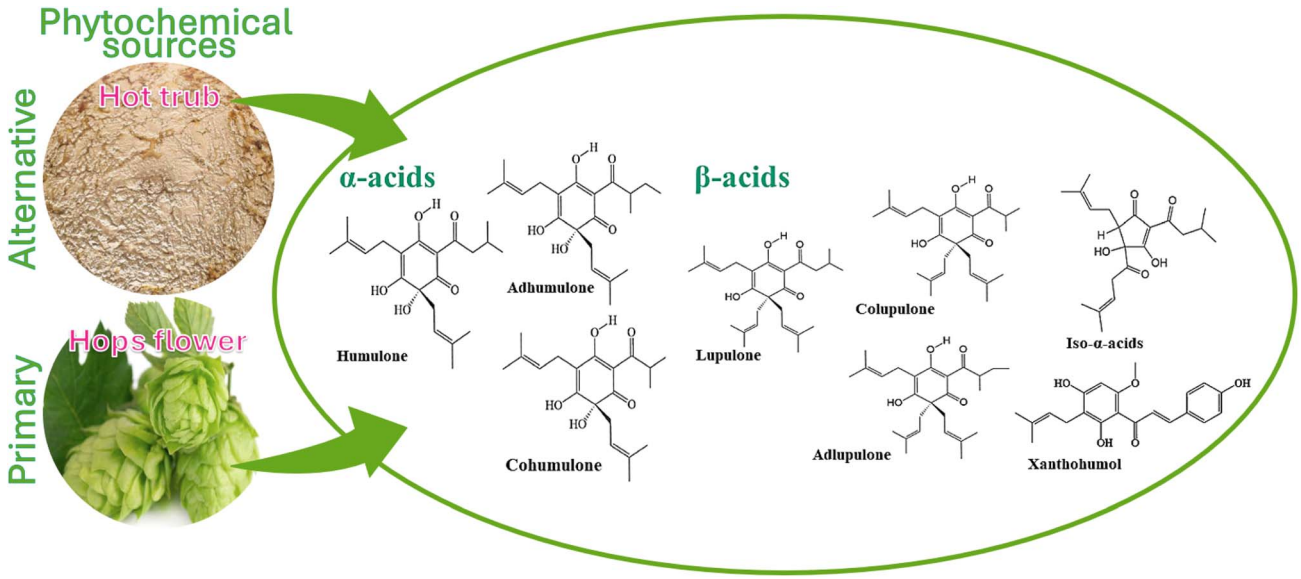

Fig. 1 The scheme illustrates the resources of bitter acids and XN in relation to their molecular structure.

compounds for creating healthier and cosmetic products.[3,4] The phytochemical market was valued at $1.6 billion in 2021 and is expected to increase at a compound annual growth rate of 7.4% from 2022 to 2030, demonstrating the economic potential of the HT use approach.[5]

XN and bitter acids (Fig. 1) are the most commercially important phytochemicals found in HT due to their anticarcinogenic, antioxidant, anti-aging, and flavor properties.[6,7] Contrasting bitter acids, XN has low water solubility, improving the cost-effectiveness of extraction in the hops industry. They have used solvents recognized as safe (GRAS) for XN extraction *via* a combination of supercritical CO_2 extraction and adsorption techniques.[8] When entering the XN extraction, it is important to acknowledge the main challenge: how to avoid the co-extraction of bitter acids, as reported by Silva *et al.*[4] The co-extraction of a broad spectrum of phytochemicals is not essentially a big issue, but when aiming to produce an isolated commercial product, the use of a selective extraction medium can significantly reduce separation and purification costs. In this context, the use of Deep Eutectic Solvents (DESs) can be a strategy to enhance XN content in a selective way. Unlike other solvents, DESs are not pure substances but rather mixtures of compounds with tailored properties that can serve diverse purposes.[9,10] Introduced by Abbott *et al.*,[11] such systems are prepared by mixing a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA), with chloride being the most commonly used HBA due to its low price, biocompatibility, low volatility, ease of preparation, non-flammability, miscibility with water, and high solvation.[12–16]

Furthermore, DESs are designed to be recyclable and reusable, representing a commitment to sustainability in biorefinery processes *via* reducing toxic solvent consumption.[17] Owing to these advantages, DES was used for XN extraction from spent hops and hop flowers, respectively, *via* combinations of alcohol-based[18] and lactic acid-based DES (Lac-based DES),[19] with the latter being the most reported approach.[20] Extracts obtained using Lac-based DES can be directly applied in various fields without solvent removal, due to their low cytotoxicity. Specifically, these DESs exhibit the lowest cytotoxicity among chloride-based DESs containing organic acids when evaluated against HT-29, Caco-2, MCF-7, and MRC-5 cell lines, with cytotoxic effects shown to be concentration-dependent.[21] Regarding their applications, DESs composed of ChCl and organic acids have demonstrated antimicrobial activity, due to the low pH of the system, which disrupts cell membranes and denatures membrane-associated proteins, leading to microbial death.[22]

Therefore, HT extracts obtained *via* the DES route can enhance product functionality while protecting against microbial activity. The novelty of this study lies in evaluating Lac-based DES extraction as a strategy to minimize the co-extraction of bitter acids during XN recovery, beyond the use of HT as an alternative source of hop-derived products. To this end, an experimental design approach was employed, as it allows the investigation of multiple variables with a limited number of experiments and the assessment of interaction effects on the response.[23] Thus, as a first step, the effects of the DES ratio and temperature were examined to evaluate XN extraction and the co-extraction of other phytochemicals from wet HT. The second step consisted of recovering the remaining phytochemicals *via* ethanol extraction to maximize resource utilization. Along with evaluating the chemical profile and antioxidant activity of the extracts from both steps, the study also investigated how the extraction process modified the residual solids. Finally, the sustainability of the process was assessed using the *Path2Green* metric proposed by Souza Mesquita *et al.*[24]

2. Materials and methods

2.1. Materials and chemicals

The HT (corresponding to Pilsen beer formulated with Magnum, Tettnanger, and Hallertau tradition hops) was donated by the Cevada Pura brewery located in Piracicaba/São

Paulo (Brazil). The HT material was stored at −22 °C and thawed as required for each assay. Its composition on a dry basis was 86.83 ± 0.03% of moisture, 18.2 ± 0.6% of total protein; 5.3 ± 0.3% of total fat; 1.65 ± 0.03% of ash; 74.85% of total carbohydrate (calculated by differences), with a particle size ($D_{[43]}$) of 132.4 ± 6.7 μm measured *via* the laser diffraction method (Mastersizer 2000, Malvern, United Kingdom). It is necessary to highlight that HT was used in its wet form, as received by the brewing industry, throughout this study, from raw material characterization to the extraction processes. The characterization of ChCl (≥98%, Sigma-Aldrich, USA) showed a water content of 1.61 ± 0.38% (by the Karl Fischer method). The DES mixture in the ratio of 1 part ChCl to 2 parts Lac (prepared according to Section 2.2) had a density of 1.15 ± 0.15 g cm^{-3} and a viscosity of 0.095 ± 0.002 mPa s.

2.1.1. Analytical standard. Mixtures of α-acids and β-acids [International Calibration Extract-4 (ICEF-4); 10.98% cohumulone, 31.60% *n*-+adhumulone (humulone + adhumulone)], 13.02% colupulone, and 13.52% *n*-+adlupulone (lupulone + adlupulone) were purchased from the American Society of Brewing Chemists (USA). The commercial XN extract (Xantho-Flav; 65%, HPLC) was donated by the Hopsteiner company (USA). Methanol and formic acid of HPLC grade were provided by Merck (Germany) and J. T. Baker (USA), respectively, while 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) and Trolox were from Sigma-Aldrich (USA). Lac, glacial acetic acid, and ethanol were purchased from Anidrol (Brazil). Water was provided by using a Milli-Q® water purification system (<18 μS cm^{-1}, Millipore Corp., USA).

2.2. DES preparation

According to Zhang *et al.*,[25] the DES was prepared by mixing 50 g of ChCl with 100 g of Lac, and then stirring under magnetic agitation at 60 °C until a clear liquid was obtained. After preparation, the solvent was stored at room temperature for 24 h to ensure the formation of a translucent, solid-free liquid.

2.3. Extraction process of polyphenol and bitter acids

The extraction process was carried out by utilizing two successive extraction steps on the wet form of HT. The first extraction (step 1) involved applying DES, while the subsequent process (step 2) used ethanol to extract any remaining phytochemicals into the solid residue. It is important to emphasize that the second extraction was performed to maximize resource utilization and to determine how much XN remains in the solid residue after the DES extraction step. For step 1, an experimental design was applied in the first extraction to optimize the extraction parameters, considering the DES-to-HT mass (grams) and temperature (°C). A thermos-shaker (KASVI, K80-200 model, China) was used for the extraction process, with conditioning for 3 h at 800 rpm, as described by de Almeida Pontes *et al.*[26]

2.3.1. Experimental design for the DES extraction step. The experimental design was used to optimize the parameters of DES extraction (step 1), with XN content and antioxidant capacity (FRAP method) as responses. The influence of two independent variables (factors), temperature (X_1) and DES proportion to HT mass (X_2), was analyzed using a Central Composite Rotatable Design (CCRD) with Response Surface Methodology (RSM). The factorial CCRD considered two factors (X_1 and X_2) with two levels (−1 and +1), with three repetitions at the central point (0) and axial conditions (−1.41, +1.41), totaling 11 runs. Table 1 presents the temperature and DES-to-HT mass parameters for all the levels investigated. To facilitate experimental reproduction, the HT mass was fixed at 4 g, while the DES mass varied from 4 to 8.82 g.

Table 1 Independent variables and levels of factorial design 2^2 with axial points

		Levels				
Independent variables		−1.41	−1	0	+1	+1.41
Temperature (°C)	X_1	25.9	30.0	40.0	50.0	54.1
DES to HT mass ratio (g)	X_2	(3.18 : 4)	(4 : 4)	(6 : 4)	(8 : 4)	(8.82 : 4)

After step 1, the DES extracts were analyzed for XN content (Y_1) and antioxidant capacity (Y_2), and the experimental design responses were obtained. The solid residue remaining from step 1 led to step 2. Curvatures were investigated using a first-order model (eqn (1)) to explain the extraction pattern.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{\substack{i=1 \\ j>i}}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad (1)$$

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{\substack{i=1 \\ j>i}}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad (2)$$

For both equations, $i \neq j$; k is the number of independent variables; Y is the dependent variable (response), X_i and X_j are the independent variables (extraction parameters), and β_0, β_i, and β_{ij} are the regression coefficients for intercept, linear, and interaction parameters, respectively. For eqn (2), the quadratic parameters are represented by β_{ii}. It is noteworthy that if significant curvature is detected, axial points will be included to adjust the quadratic model's parameters.

2.3.2. Ethanolic extraction. Seeking to extract the remaining phytochemicals from the solid residue of HT (raffinate from step 1), a second extraction (step 2) was carried out, as illustrated in Fig. 2. The second extraction was conducted in a thermo-shaker (KASVI, K80-200 model, China) under the fixed conditions of time (15 min), temperature (25 °C), solid-to-solvent ratio (100 mg mL^{-1}), and agitation speed (800 rpm) established in preliminary trials for all runs from the first extraction step and the second extraction was performed with ethanol at 100% (v/v).

2.3.3. Ethanol content in water. After selecting the extraction sequence (DES + ethanol) that yielded the highest XN, a scan of ethanol concentrations (40, 55, 70, and 100%; v/v) was conducted to minimize the volume of organic solvent while

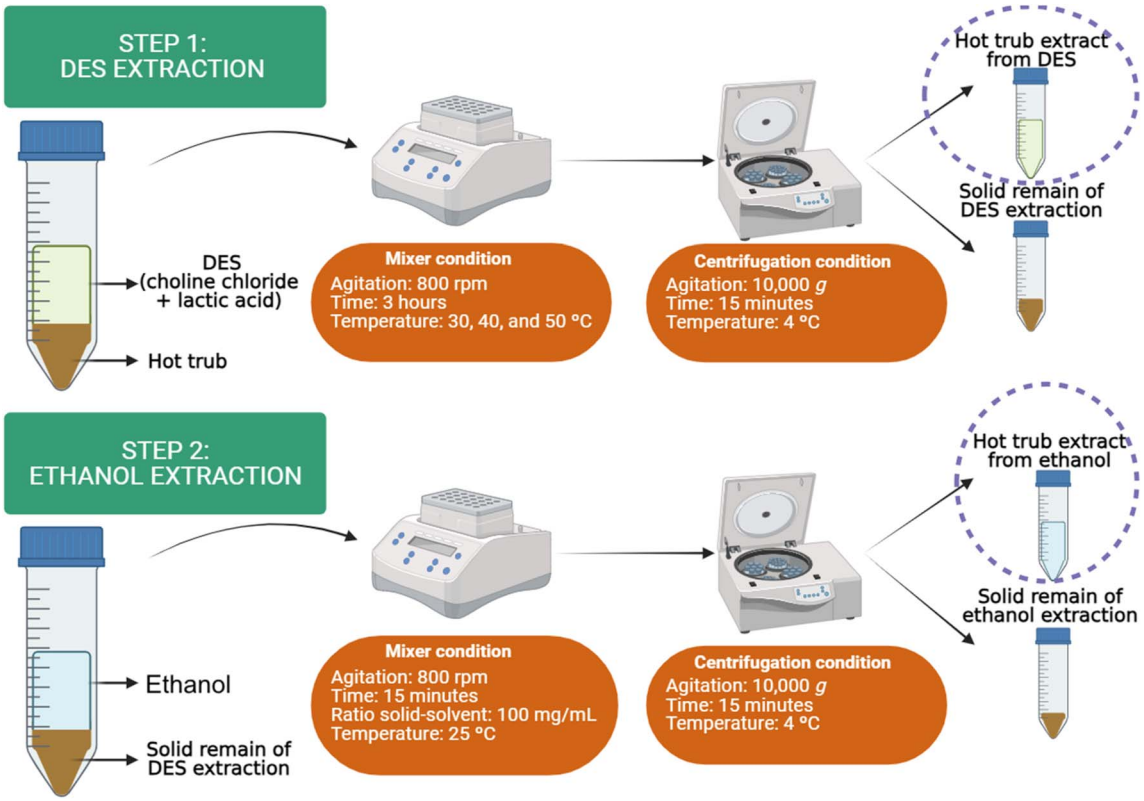

Fig. 2 Schematic diagram illustrating the two extraction steps.

maintaining extraction yields. Furthermore, this approach allows evaluation of the impact of increased water content in an ethanolic environment on phytochemical extraction.

The extraction protocol was the same as that employed with ethanol: in a thermo-shaker (KASVI, K80-200 model, China) under the fixed conditions of time (15 min), temperature (25 °C), solid-to-solvent ratio (100 mg mL^{-1}), and agitation speed (800 rpm). Extracts and solid residues were stored in a freezer (−21 °C) until the subsequent analysis. The solid residues from all extraction steps were subjected to freeze-drying (LS 3000, Terroni Equipamentos Científicos, Brazil) at −40 °C and 100 μmHg for 48 h. After this, the samples were stored in desiccators.

2.4. Extract characterization

2.4.1. Bitter acid and XN quantification *via* high-performance liquid chromatography (HPLC-PDA). The quantification method for bitter acids and XN was adapted from De Keukeleire *et al.*[27] Analysis was conducted on a Liquid Chromatograph (LC Standard Ultimate 3000, Dionex, USA) equipped with a photodiode array sensor (PAD-3000, Dionex, USA), with monitoring of the wavelength at 314 nm (the maximum absorption of bitter acids) and 370 nm (the maximum absorption of bitter acids). Compound separation was performed using a C18 reversed-phase column (Poroshell 120, 100 mm × 4.6 mm × 2.7 μm) at a temperature of 35 °C, a flow rate of 0.5 mL min^{-1}, and an injection volume of 8 μL. The mobile phase consisted of two solutions: A, water acidified with 0.025% (v/v) formic acid, and B, methanol acidified with 0.025% (v/v) formic acid. The gradient profile used was as follows: 0–3 min, 55% A: 45% B; 3–30 min, 5% A: 95% B; 30–38 min, 5% A: 95% B; 38–45 min, 55% A: 45% B; 45–47 min, 55% A: 45% B. All analyses were performed in triplicate.

2.4.2. Polyphenol identification *via* ultra-high performance liquid chromatography (UHPLC-PDA/MS-ESI). The phytochemical identifications were conducted by using an UHPLC-MS (Waters Acquity SQD/UPLC System, United States) system equipped with a photodiode array detector, and a single-quadrupole mass detector including an electrospray ionization camera, an automatic injector, a quaternary pump, and a column oven. Compound separation was performed using a C18 reverse-phase column (Poroshell 120, 100 mm × 4.6 mm × 2.7 μm) at a temperature of 35 °C, a flow rate of 0.5 mL min^{-1}, and an injection volume of 8.0 μL. The mobile phase consisted of two solutions: A, water acidified with 0.025% (v/v) formic acid, and B, methanol acidified with 0.025% (v/v) formic acid. The gradient profile used was as follows: 0–3 min, 55% A: 45% B; 3–30 min, 5% A: 95% B; 30–38 min, 5% A: 95% B; 38–45 min, 55% A: 45% B; 45–47 min, 55% A: 45% B. Data were acquired and processed using MassLynx software (Waters, United States).

The mass detector was operated in the positive mode, ranging from 130 to 600 *m*/*z* (mass/charge ratio), under fixed conditions: source temperature of 100 °C, desolvation temperature of 300 °C, desolvation gas flow of 600 L h^{-1}, cone gas flow of 60 L h^{-1}, capillary voltage of 4 kV, extractor voltage of 5 V, RF lens of 0.7 V, and cone voltage ranging from 10 to 100 V.

2.4.3. Ferric reducing antioxidant power (FRAP). The assay measured the antioxidant potential of the HT extracts (DES and ethanol) by quantifying the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by the antioxidants present in the samples. The FRAP methodology was based on Benzie and Strain[28] with modifications. The FRAP reagent consisted of 25 mL of 0.3 M acetate buffer, 2.5 mL of 10 mM TPTZ solution, and 2.5 mL of 20 mM ferric chloride aqueous solution. A volume of 90 μL of the HT extract was then added to the test tubes under dark conditions. Then, a dilution solution consisting of 270 μL of water was added to 2.7 mL of the prepared FRAP reagent. The resultant mixture was maintained in a water bath at 37 °C for 30 min. As a reference, a blank consisting solely of the FRAP reagent was included, and the absorbance of the samples was measured at a wavelength of 595 nm using a UV/vis spectrophotometer (BEL Engineering®, Italy). Trolox was employed at 54 and 1000 μM concentrations to construct the standard curve.

2.5. Thermo-shaking *versus* magnetic mixing

A comparative analysis was proposed between thermo-shaking and magnetic mixing, focusing on evaluating the impact of these two mixing strategies on phytochemical yields and on the characterization of solid residues. This evaluation was conducted as a preliminary test to understand pattern extraction across different equipment, providing a foundation for future studies on the operational aspects of scale-up. Magnetic mixing (model Hei-Tec, Heidolph, Germany) was performed under the same operating conditions as thermo-shaking (as described in Sections 2.3 and 2.3.2), including temperature, processing time, DES-to-HT mass ratio, and ethanolic solution at 70%. For magnetic mixing, the DES extraction was conducted at 30 °C with agitation at 300 rpm for 3 h. Subsequently, the second step involved mixing a 70% (v/v) ethanolic solution with the solid residue at 25 °C for 15 min, maintaining agitation at 300 rpm. All the solid residues were dried *via* lyophilization operation for evaluation in an attempt to track microstructure alteration, while the extract was stored under refrigerator (−10 °C).

2.5.1. Fourier transform infrared spectroscopy (FTIR). The dried solid residues were incorporated into dry potassium bromide and pulverized to a fine powder in an agate mortar. The resulting powder was then palletized in a hydraulic press. Subsequently, the samples were analyzed using an infrared spectrophotometer (IRPrestige-21, Shimadzu, Japan). The infrared spectra were recorded at a controlled temperature of 25 ± 1 °C, employing a data-acquisition process with a minimum of 10 scans over the spectral range of 400 to 4000 cm^{-1}.

2.5.2. Scanning electron microscopy (SEM). The dried solid residues were examined using a scanning electron microscope with energy dispersive X-ray detector, LEO 440i—6070 (LEO Electron Microscopy/Oxford, England). The microscope operated at 5 kV and 50 pA. The samples were affixed to stubs with carbon tape and metalized with a thin layer of gold using a sputter coater EMITECH K450 (EMITECH, United Kingdom). The samples were observed under 150×, 500×, and 1000× magnifications.

2.6. Extraction process sustainability evaluation by *Path2Green*

The *Path2Green* metric was used to verify the sustainability score of the proposed extractive process, focusing on its effects on environmental, social, and economic aspects. This analysis evaluates biomass, transport, pretreatment, solvent, scaling, purification, yield, post-treatment, energy, application, repurposing, and waste management for the extraction process.

2.7. Statistical analysis

In step 1, the experimental design, analysis of variance (ANOVA), and RSM were evaluated using TIBCO Statistica Software Inc., version 14 (2020). The results of step 2 were statistically analyzed using one-way ANOVA in Minitab® 16.0 (USA). Mean analysis was performed using Tukey's procedure at a p-value ≤ 0.05.

3. Results and discussion

3.1. Polyphenol compound identification

To evaluate the effect of DES, an ethanol extract (100% v/v) was used as a control to identify compounds present in the HT by chromatography (Fig. 3A). In this extract, the target compounds, XN and bitter acids, showed significant peaks. However, the DES extracts showed no peaks corresponding to bitter acids, suggesting a pronounced effect of extraction selectivity. In the chromatogram (Fig. 3A), two unidentified peaks were observed between 20 and 24 min. To address this, a phytochemical identification strategy was implemented using an UHPLC-PDA/MS-ESI, which served as a scanning method to determine the mass-to-charge ratio (m/z). To identify the unknown compounds, an extensive inventory of molecules commonly found in hops and beer products was compiled based on the studies of Wannenmacher *et al.*,[29] and Bravi *et al.*[30] Table S1 presents the twenty-nine molecular masses used, including the respective names, precise relative molecular masses (m/z; $M^+H]^+$), and corresponding molecular formulae. The runs 01 (−1; −1), 10 (0, 0 – central point), and 04 (+1, +1) of experimental design for the two extraction steps (DES and ethanol) were conducted for this examination.

In general, the extraction patterns of phytochemicals were mainly influenced by temperature and solvent, as indicated by the examination of total ion current chromatograms in Fig. S1–S6. The traces of tyrosol, quercetin/morin, and coumaric acid derivatives were only noticed in the DES extraction at the minimum point. This result suggests that DESs modified the raw material during treatments at temperatures above 30 °C, potentially leading to its degradation. On the other hand, increasing the temperature facilitated the extraction of ferulic and sinapic acids in both extraction steps. The concentrations of both compounds increased by approximately 11% when operated at 50 °C with DES as the solvent (the maximum point) compared to the treatment at 40 °C (the central point). Furthermore, the temperature also promoted increased extraction of chlorogenic acid in DES environments.

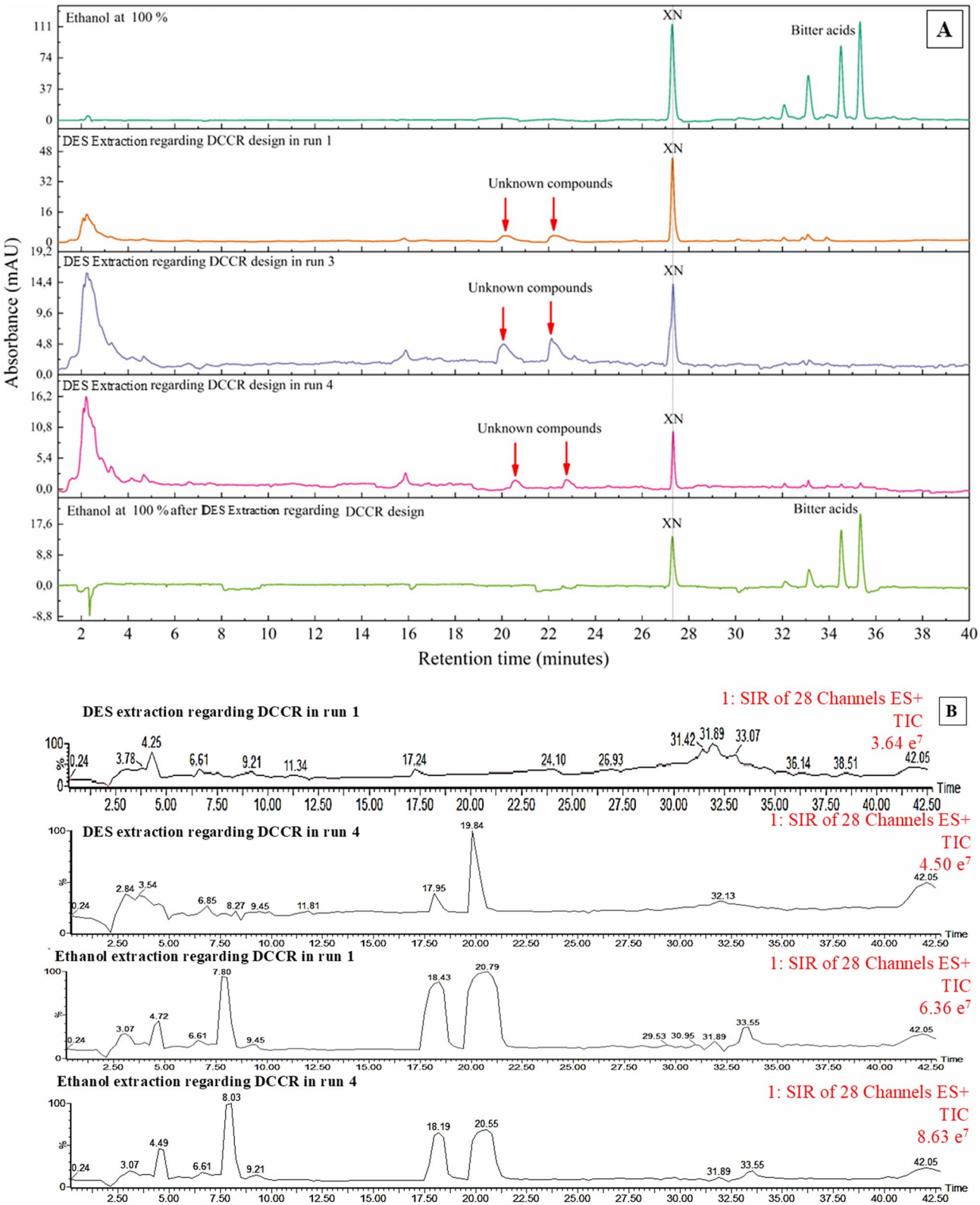

Fig. 3 (A) Chromatograms obtained at a wavelength of 370 nm *via* a photo-diode array detector concerning XN extraction processes. (B) Total ion chromatograms (TIC) of HT extracts provided by the mass detector with electrospray ionization.

DES extracts obtained in this work also comprised phytochemicals with molecular masses corresponding to caffeic acid, formononetin, biochanin A/glycitein, apigenin/genistein, naringenin, myricetin, isocohumulone, and desmethylxanthohumol, along with XN and bitter acid traces that had already been identified. Moreover, traces of daidzein,

kaempferol/luteolin, epicatechin/catechin, hesperetin, postlupulone, and naringin were also detected in the DES extracts, as shown in Fig. S1, S2, and S3. The subsequent ethanolic extract presented daidzein, apigenin/genistein, naringenin, biochanin A/glycitein, epicatechin/catechin, quercetin/morin, hesperetin, gallocatechin, myricetin, isocohumolune, desmethylxanthohumol, and chlorogenic acid. These compounds exhibited poor solubility in DES and remained in the raw material after the first extraction stage. Notably, gallocatechin was the only phytochemical found exclusively in ethanol extracts. Traces of postlupulone, naringin, epicatechin/catechin, formononetin, and kaempferol/luteolin were detected in ethanolic extracts. Based on the reported phytochemical profiles, DES extracts contained higher levels of phenolic acids than ethanolic treatments. Moreover, unlike studies that employ single organic solvents (*e.g.*, methanol, ethanol, or chloroform), DESs were able to extract compounds not previously reported for HT, such as desmethylxanthohumol and chlorogenic acid.[3,31] This compositional difference conferred superior antioxidant capacity to the DES extracts, as discussed in the following sections.

Furthermore, desmethylxanthohumol was identified as a previously unreported compound in DES extracts, as shown in Fig. 3, probably due to its higher solubility in aqueous media. The enhanced extraction performance of DES is related to its physicochemical properties, particularly its capacity to form hydrogen-bonding networks that promote interactions with more hydrophilic phenolic compounds.[19,32] Grudniewska and Popłoński[18] also reported the presence of an unknown compound in DES extracts of spent hops, later identified as desmethylxanthohumol. In addition, it showed that temperature affected its concentration, resulting in an approximately 30% increase, as indicated by the total ion current chromatograms in Fig. S1 and S2.

3.2. Experimental design of polyphenol extraction *via* DES

The DES extractions were selective for XN, as the chromatograms of these extracts did not show the bitter acid peaks observed in the ethanolic extract (Fig. 3A and B). The results of the experimental design of the DES extraction step are shown in Table 2. The XN range varied from 5.27 (run 07) to 36.23 (run 04) μg g^{-1} of dried HT (Y_1), and the antioxidant capacity from 14.42 (run 05) to 18.91 (run 04) mmol Trolox eq. kg^{-1} of dried HT (Y_2). Both models (Y_1 and Y_2) indicated that curvature was not statistically significant ($p \geq 0.05$). Therefore, first-order models without axial points are enough to describe the experimental data. The models constructed by a multiple regression analysis of the experimental data are shown below (eqn (3) and (4)):

$$Y_1 = 20.57 + 4.21X_1 + 10.79X_2 \quad (3)$$

$$Y_2 = 15.88 + 1.34X_1 + 0.90X_1X_2 \quad (4)$$

The results of ANOVA and the *F*-test with a significance of 0.05 are shown in Table S2. The good fit of the models to the experimental data is confirmed by the low average relative deviations of 7.20% and 2.87%, and the high R^2 values of 0.97 and 0.84 for Y_1 and Y_2, respectively. Models were statistically significant, $MQ_{Reg}/MQ_{Re} > F_{table}$, and did not exhibit a lack of fit because $MQ_{LF}/MQ_{PE} < F_{table}$, and the *p*-value for the lack of fit was greater than 0.05. The response surfaces and contour curves were constructed from these first-order polynomial models, as shown in Fig. 4.

Fig. 4 shows a direct correlation between temperature (X_1) and both responses, XN content (Y_1) and antioxidant capacity (Y_2), *i.e.*, increasing temperature increases both responses. The positive signs of the linear coefficients for variable X_1 in both models (eqn

Table 2 Real and coded (in parentheses) independent variables of experimental design and the corresponding responses (XN and FRAP) for DES extraction. It is important to highlight that HT mass remains constant for all the trials in 4 g

	Independent variables				Responses		Relative deviation[c] (%)	
					XN (Y_1)	FRAP (Y_2)		
Trials	Temperature (°C)		DES mass ratio[b](g)		μg XN g^{-1} of dried HT[a]	mmol Trolox eq. kg^{-1} of dried HT[a]	XN	FRAP
01	30.0	(−1)	4.00	(−1)	6.2	15.2	9.7	1.9
02	30.0	(−1)	8.00	(+1)	25.9	14.4	4.9	5.5
03	50.0	(+1)	4.00	(−1)	12.7	16.0	10.0	1.8
04	50.0	(+1)	8.00	(+1)	36.2	18.9	1.8	4.2
05	25.9	(−1.41)	6.00	(0)	23.8	14.4	—	—
06	54.1	(+1.41)	6.00	(0)	22.6	16.5	—	—
07	40.0	(0)	3.18	(−1.41)	5.3	15.2	—	—
08	40.0	(0)	8.82	(+1.41)	34.8	14.8	—	—
09	40.0	(0)	6.00	(0)	18.9	15.2	8.9	4.9
10	40.0	(0)	6.00	(0)	20.4	15.9	1.0	0.1
11	40.0	(0)	6.00	(0)	23.7	15.6	13.2	1.8
						Mean	7.2	2.9

[a] Mass of HT in dried weight. [b] HT mass is equal for all trials. [c] Relative deviation $= \left|\frac{(\text{experimental value} - \text{predicted value})}{\text{experimental value}}\right| \times 100$

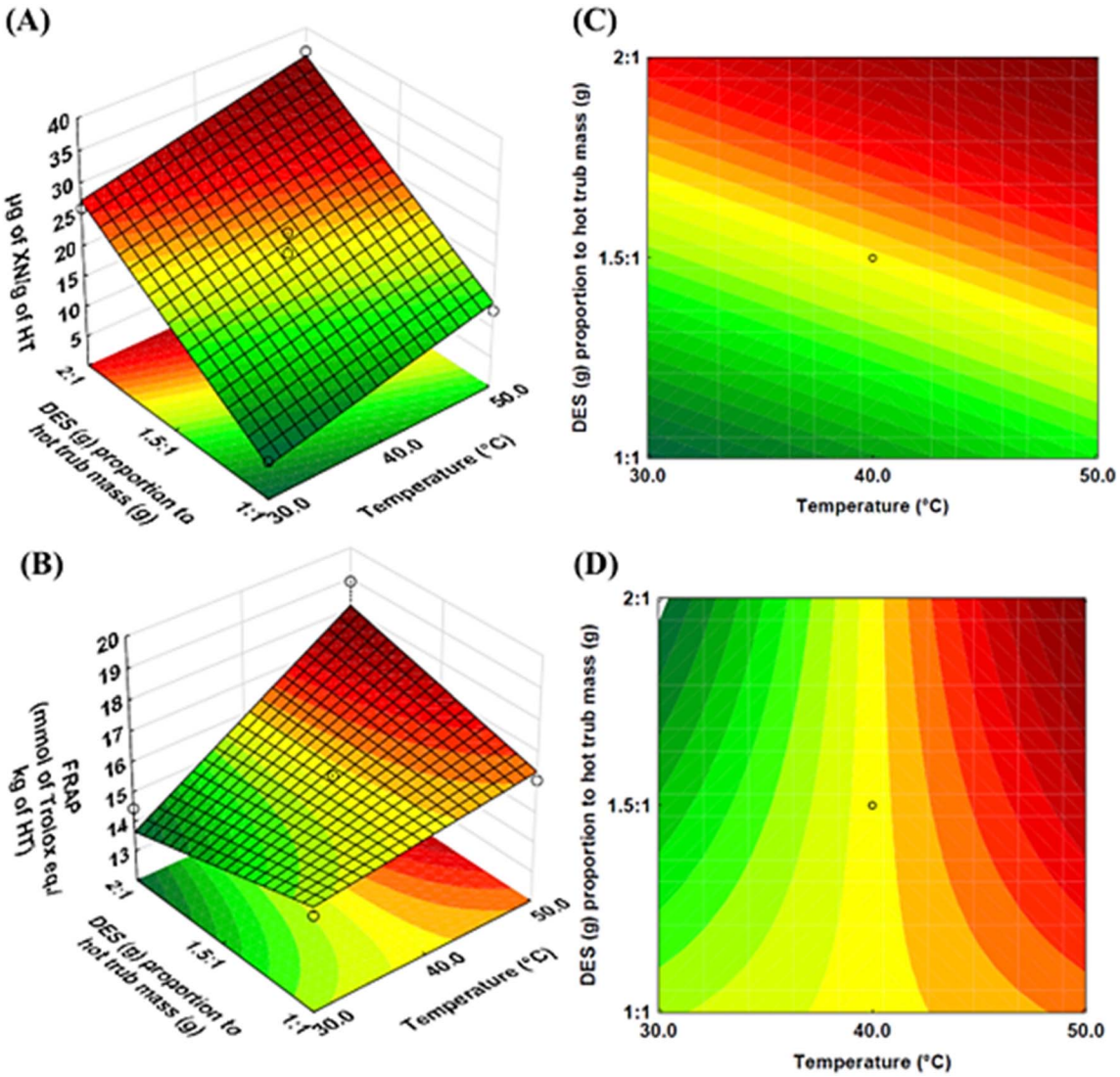

Fig. 4 (A and B) Response surfaces and (C and D) contour curves obtained from experimental design to evaluate the effects of temperature (X_1) and DES to HT ratio mass (X_2) in the responses of XN content (μg of XN g^{-1} of dried HT) and antioxidant activity by the FRAP method (mmol Trolox eq. kg^{-1} of dried HT).

(3) and (4)) indicate a positive effect of this variable on both responses. The increase in temperature promotes a decrease in solvent viscosity and solvent surface tension during the extraction of phytochemicals, allowing the improvement of solvent permeation in the sample matrices and resulting in a higher transfer rate.[33] Additionally, chemical interactions, such as hydrogen bonds, van der Waals forces, and electrostatic interactions, are the main factors that retain phytochemicals within the biomass matrix. As the temperature increases, these chemical interactions weaken, which enhances the extraction of the desired compounds from the biomass matrix into the solvents.[32] In the literature, the same pattern was observed by Grudniewska and Popłoński[18] working with XN extraction from spent hops using four different DES mixtures. The authors reported that temperatures between 40 and 60 °C increased the XN content. However, above 60 °C, the XN content started to decrease, indicating that using higher temperatures can lead to its degradation.[18]

Regarding the DES-to-HT mass ratio (X_2), it also yielded a positive linear coefficient for XN content (Y_2), as shown in Fig. 4 and eqn (3). The findings suggest that XN extraction is enhanced with increasing DES proportion. Conversely, the water content adversely affects the XN extraction, which in this work ranged from 30 to 44% (w/w) (data not shown), from the minimum (−1) to the maximum (+1) point of the experimental design. It is worth mentioning that the modulation of water content occurred indirectly; for instance, it was increased or even decreased by the growth of the DES ratio. This approach was chosen because the DES mixture naturally absorbs water from its environment, making the method more practical by eliminating the need for additional pre-treatments of raw materials, such as oven drying or freeze-drying, thereby reducing overall processing time and costs.[34]

The water ratio in the DES mixture plays a key role as it significantly influences the solvent's physicochemical

Table 3 The phytochemical composition of hot trub extracts from the CCRD, produced *via* ethanol extraction[a]

Trials	Cohumulone ($\mu g\ g^{-1}$ of dried HT[b])	*N*-+Adhumulone ($\mu g\ g^{-1}$ of dried HT[b])	Colupulone ($\mu g\ g^{-1}$ of dried HT[b])	*N*-+Adlupulone ($\mu g\ g^{-1}$ of dried HT[b])	Xanthohumol ($\mu g\ g^{-1}$ of dried HT[b])	FRAP (mmol Trolox eq. kg^{-1} of dried HT[b])
01	82.3 ± 1.0	329.9 ± 4.3	723.8 ± 14.6	1012.5 ± 12.7	107.0 ± 0.7^{b}	10.3 ± 0.2^{bcde}
02	63.9 ± 2.4	327.9 ± 3.8	556.2 ± 14.0	822.4 ± 19.2	84.4 ± 0.6^{d}	9.4 ± 0.2^{e}
03	—	—	—	—	113.9 ± 1.1^{a}	12.1 ± 0.1^{a}
04	—	—	—	—	70.1 ± 0.5^{e}	9.7 ± 0.2^{de}
05	48.2 ± 1.5	219.4 ± 2.7	534.5 ± 6.4	802.1 ± 11.1	92.4 ± 1.5^{c}	10.8 ± 0.1^{b}
06	—	—	—	—	92.9 ± 0.8^{c}	10.6 ± 0.5^{bc}
07	—	—	—	—	107.2 ± 0.4^{b}	10.7 ± 0.3^{b}
08	—	—	—	—	69.1 ± 2.8^{e}	9.7 ± 0.1^{cde}
09	—	—	—	—	84.7 ± 2.5^{d}	10.3 ± 0.2^{bcde}
10	—	—	—	—	84.1 ± 1.9^{d}	10.4 ± 0.6^{bcd}
11	—	—	—	—	84.3 ± 3.0^{d}	10.3 ± 0.5^{bcde}

[a] CCRD – central composite rotatable design; HT – hot trub; FRAP – ferric reducing antioxidant power. [b] Mass of HT in dried weight. Different letters in the same column indicate statistical distinctions (p-value ≤0.05). Mean ($n = 3$) ± standard deviation of three independent experiments.

properties, such as viscosity, conductivity, and polarity, which in turn affect its extraction capacity.[35,36] In our approach, the water content used is low compared to other studies in the literature, where it usually ranges from 50 to 80%.[37,38] This means that XN did not dissolve well in a high-polarity environment. A similar pattern was observed in our previous study on XN extraction from HT using hydroalcoholic mixtures as solvents, where high-polarity treatments (with water content above 45% in ethanol) were less effective, or even ineffective.[4] In this sense, Metaj *et al.*[39] explored several organic solvents and DES mixtures in order to understand the relationship between the hydrophobicity of bitter acids and XN from hop cones and the characteristic properties of these solvents. Their findings indicate that XN dissolves more effectively in low-polarity, low-viscosity environments, such as diethyl ether and ChCl: phenol. Therefore, to maximize XN extraction from HT using the DES approach, it may be necessary to adjust polarity and other physicochemical properties, such as viscosity.

On the other hand, the antioxidant capacity values did not show a significant effect of increasing DES proportion alone. However, when both DES proportion and temperature were increased simultaneously, antioxidant capacity increased significantly, as shown in Fig. 3B. This effect occurred because temperature alters chemical interactions and intensifies the interface between the solvent and the solute, thereby enhancing mass transfer from biomass to DES.[39] Additionally, increased temperature reduces DES viscosity, which, combined with a higher solvent proportion, can improve solute diffusion and enhance the mobility of a wide range of phenolic compounds and other phytochemicals within the solvent 25. Consequently, this leads to a higher rate of polyphenol transfer. Similar results were reported by Athanasiadis *et al.*,[40] who observed that temperature and DES proportion modulated the extraction of total phenolic compounds from peppermint. Additionally, as seen in RSM (Fig. 4), the increase in antioxidant capacity did not correlate with the pattern of increased XN content.

Based on the results, we can infer that according to the CCRD, the optimized conditions were a temperature of 50 °C (X_1; +1) and 2 parts of DES to 1 part of HT ratio mass (X_2; +1). Under these conditions, the highest yield of XN and significant antioxidant capacity were achieved. Furthermore, the predicted model demonstrated a strong ability to accurately describe the experimental data, with low relative deviations (XN at 1.8 and antioxidant capacity at 4.2) under the optimized conditions (+1; +1).

3.3. Ethanol-based subsequent extraction from experimental design and ethanol concentration scan

The subsequent ethanol extraction recovered the remaining polyphenols and bitter acids that DES had not extracted. Table 3 displays the composition of XN and bitter acids, as well as the antioxidant capacity evaluated by the FRAP method. The ethanol step yielded a higher XN content than that obtained *via* DES extraction, ranging from 69.1 ± 2.8 to 113.9 ± 1.1 $\mu g\ g^{-1}$ of dried HT. The highest XN content achieved in this step was correlated with the previous DES extractions at lower DES mass levels (−1), particularly in runs 1, 3, and 7. This result was

expected, as the first extraction step using DES yielded a low extraction efficiency under the extraction conditions of runs 1, 3, and 7 (Fig. 4 and Table 2). This result indicated that minimizing XN transfer to the solvent during the DES step was essential for the compound to remain in the solid residue, which can then be effectively extracted during the ethanol step. A low XN solubility in DES mixtures (1 ChCl: 2 glycerol; 1 ChCl: 2 propylene glycol; 1 ChCl: 2 ethylene glycol; 1 ChCl: 2 Lac) was also noted by Grudniewska and Popłoński.[18] They reported that organic solvents extracted almost 6 times more XN than DES mixtures, attributing this result to their poor solubility in polar systems.

Regarding antioxidant capacity, the ethanol extracts showed a 60% reduction compared to DES extraction, suggesting that this solvent has greater extraction efficiency for a wide range of phytochemicals. Furthermore, these findings suggest that residual phytochemicals in the biomass matrix were minimal across all conditions tested in the DES step. In the studies conducted by Chagnoleau *et al.*[41] (2 betaine: 3 citric acid, 1 ChCl: 1 malonic acid, 1 betaine hydrochloride: 10 ethylene glycol) and Lee *et al.*[42] (2 glycerol: 1 betaine; 3 glycerol: 1 betaine; 2 glycerol: 1 D-sorbitol; 3 glycerol: 1 D-sorbitol; 3 glycerol: 1 xylose; 3 glycerol: 1 glucose; 3 glycerol: 1 fructose; 1 glycerol: 1 urea), it was reported that DES mixtures produced extracts with significantly higher antioxidant capacity than traditional solvents, like ethanol and methanol.

Despite the strong antioxidant capacity of DESs, this study focused on identifying the method that maximizes XN extraction. Therefore, based on the high XN content extracted in step 2, run 1 (−1; −1) was selected as the best result. Additionally, the extracts obtained with ethanol presented a high content of bitter acids, which are the majority. The concentrations varied from 48.2 ± 1.5 to 1012.5 ± 12.7 μg g^{-1} of dried HT, corresponding to cohumulone and *n*+adlupulone. The bitter acids were detected only in ethanol extracts obtained from biomass treated with DES at 30 °C or lower. The presence of bitter acids in DES extracts was observed by Macchioni *et al.*,[19] who applied Lac-based mixtures at room temperature to dried hops cones. On the other hand, Grudniewska and Popłoński[18] did not report the presence of bitter acids in their study, which used a 60 °C extraction temperature. Therefore, higher temperatures (>30 °C) used in the DES step may have promoted the thermal degradation of bitter acids. Thus, DESs associated with temperature can be used to modulate the phytochemical composition of the extract.

Once the condition for DES extraction was determined for the point minimum of XN extraction (−1; −1), the approach of reducing ethanol concentration was applied, seeking to evaluate how the phytochemicals are affected by the increase in polarity in step 2. The ethanol concentrations ranged from 40 to 100% (v/v), with *n*-+adlupulone and cohumulone exhibiting the highest and the lowest concentrations, 1225.4 ± 2 μg g^{-1} of dried HT and 23.5 ± 2.2 μg g^{-1} of dried HT, respectively, as shown in Table 3. The maximum concentration of XN was achieved at 70% (v/v) ethanol, with a value of 108 ± 1 μg g^{-1} of dried HT, indicating no negative effect on increasing polarity by around 30%. However, this increase in polarity significantly affects antioxidant capacity,

Table 4 The composition of hot trub extracts concerning the ethanol concentration scan and the comparison between mixing strategies[a]

EtOH%[b]	Cohumulone (μg g^{-1} of dried HT)	*N*-+Adhumulone (μg g^{-1} of dried HT)	Colupulone (μg g^{-1} of dried HT)	*N*-+Adlupulone (μg g^{-1} of dried HT)	Xanthohumol (μg g^{-1} of dried HT)	FRAP (mmol Trolox eq. kg^{-1} of dried HT)
40	23.5 ± 2.2^{c}	56.8 ± 5.1^{d}	39.7 ± 4.1^{d}	28.1 ± 3.6^{d}	34.4 ± 2.6^{d}	2.8 ± 0.3^{d}
55	91.9 ± 6.9^{b}	279.6 ± 7.1^{c}	557.8 ± 10^{c}	682.3 ± 16.9^{c}	88 ± 3^{c}	3.9 ± 0.3^{c}
70	125.5 ± 4.2^{a}	371.7 ± 9.1^{a}	893 ± 12.7^{a}	1225.4 ± 23^{a}	108 ± 1^{a}	4.3 ± 0.1^{b}
100	96.3 ± 10.7^{b}	309.6 ± 5.2^{b}	671 ± 17.7^{b}	956.5 ± 24.3^{b}	103.8 ± 2.4^{b}	10.3 ± 0.2^{a}
Thermo shaking (extraction 1)	—	—	—	—	11.3 ± 0.4^{a}	9.8 ± 0.2^{a}
Magnetic mixer (extraction 1)	—	—	—	—	9.6 ± 0.9^{b}	9.8 ± 0.7^{a}
Thermo shaking (extraction 2)	125.5 ± 4.2^{a}	371.7 ± 9.1^{a}	893 ± 12.7^{a}	1225.4 ± 23^{a}	108 ± 1.2^{a}	4.3 ± 0.1^{b}
Magnetic mixer (extraction 2)	86.8 ± 10.1^{b}	343.7 ± 30.3^{b}	624.7 ± 66.3^{b}	851.1 ± 93.4^{b}	91.2 ± 7.8^{b}	4.7 ± 0.3^{a}

[a] Mass of HT in dried weight; HT – hot trub; FRAP – ferric reducing antioxidant power. [b] EtOH% – ethanol concentration% (v/v). Different letters in the same column indicate statistical distinctions (p-value ≤0.05). Mean (n = 3) ± standard deviation of three independent experiments.

suggesting that the major phytochemicals responsible for it were not soluble in a 70% ethanolic solution.

3.4. Effect of the mixing strategy on extraction yield and solid residue

The mixing strategy was investigated to assess the reproducibility and extraction efficiency of two different mixing methods (magnetic mixing and thermo-shaking). For this purpose, the minimum conditions for the DES (−1; −1) and the ethanolic extraction at 70% (v/v) were utilized. The extract produced by thermo-shaking was significantly higher (p-value ≤0.05) in terms of XN content and bitter acid composition when compared to that obtained *via* magnetic mixers involving both extraction steps, as displayed in Table 4. Regarding antioxidant capacity, no significant differences were observed between the two mixing strategies from the DES step. However, an 8.5% decline in antioxidant capacity was observed for the magnetic mixer compared with ethanolic treatments.

The impact of two mixer types on a solid raw material was also evaluated using SEM and FTIR, as presented in Fig. 5. The dried HT used as a control sample (Fig. 5A) displayed a particulate microstructure and a rough surface, with only small pieces of spent hops. After the extraction processes, the solid residues appeared more like pellets than particles, with an internal microstructure resembling a heap of cylindrical holes. In addition, the DES extractions (Fig. 5C and E) resulted in a smoother surface than the ethanolic treatments, indicating that the solvents remained in the raw material.

No differences in the shape of the microstructure of the solid residue were observed between the two mixing processes, indicating that neither promoted maceration of the raw material during extraction. It is worth noting that the solid residue exhibited distinct characteristics when compared between the DES and ethanolic extractions. Ethanolic treatments produced a more porous, fragmented structure than DES-treated samples, suggesting better dissolution of phytochemicals by the solvent. Ethanol extraction is not typically recognized for promoting expressive fragmentation during the process. However, DES solvents can induce biomass fragmentation. Huang *et al.*[43] highlighted the efficacy of DES in drawing out phytochemicals (crocins) by plant cell rupture, leading to fragmentation. They did not observe this damage when they performed the extraction in 50% ethanol. De Almeida Pontes *et al.*[26] also reported the expressive damage caused by DESs on olive leaves, and the same pattern was not noted for the extraction samples with ethanol. Thus, the fragmentation observed in the ethanol treatments can be attributed to the previous extraction with DESs, which was carried over to the subsequent steps.

Regarding the FTIR findings (Fig. 5B), the solid residue was compared to the spectra of HT and ChCl to detect any modifications. The HT spectrum exhibited the presence of vibration bands at around 1584, 1490, 1182, 1133 (deep valley), and 940 cm^{-1}, corresponding to C–N stretching, N_1H bending, and N_2H bending; C–O–C stretching; C–O stretching, and CO_2H bending on the plane, respectively,[44,45] while the ChCl spectrum displayed a band near 1480 cm^{-1}, referring to a specific vibrational mode of the CH_2 group bending in an angular deformation as a scissor.[26] After the extraction steps, significant modifications were observed in the solid residues, particularly in shifts and the disappearance of bands associated with characteristic functional

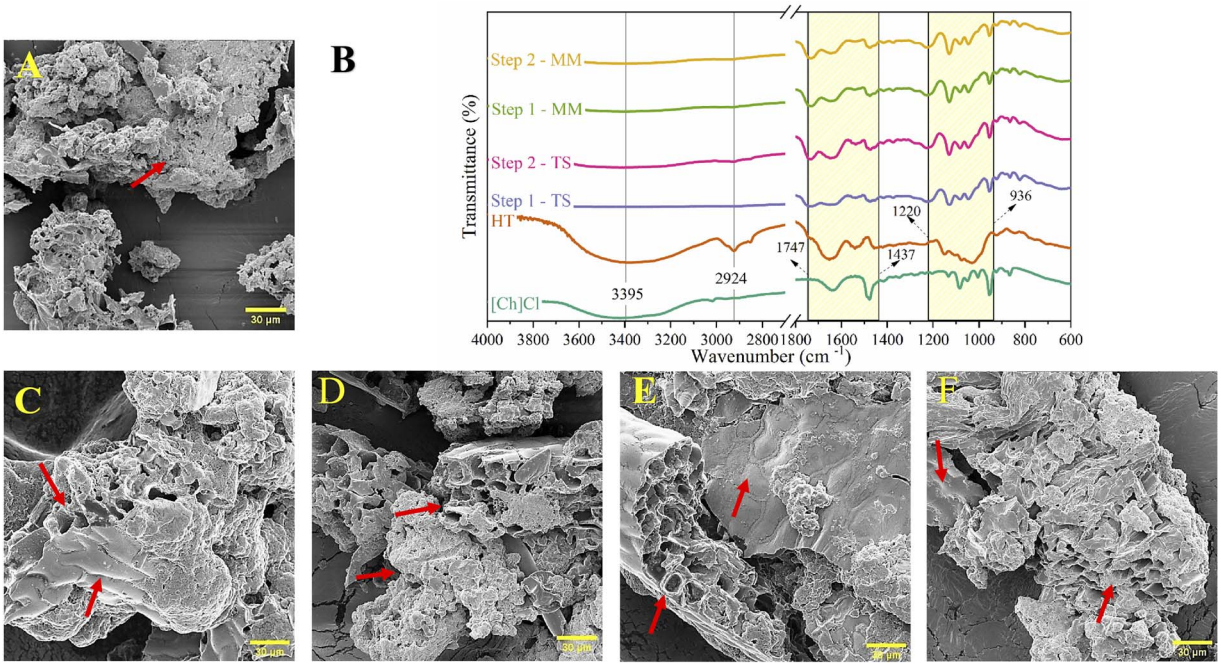

Fig. 5 The characterization of the solid residue after examining the mixing equipment by morphological analysis *via* SEM with a scale set at 30 μm and compositional analysis *via* FTIR spectra (B). (A) – HT without processing; (C) – DES extraction by thermo-shaking; (D) – ethanolic solution extraction by thermo-shaking; (E) – DES extraction in a magnetic mixer; (F) – ethanolic solution extraction in a magnetic mixer; step 1 – DES extraction; step 2 – ethanolic solution extraction; TS – thermo-shaking; MM – magnetic mixer.

groups of the raw material. Additionally, no differences were observed in the FTIR spectral profiles among the samples.

The most expressive alterations in solid residues were related to the alcohol and hydroxyl (–OH) functional groups, initially located at 1130 (C–O stretching) and 3395 cm^{-1} (OH– stretching).[46] In both situations, the extraction process resulted in a decrease in intensity and the disappearance of bands, leading to the partial or complete removal of compounds containing these functional groups. Furthermore, the DES was the primary cause of biomass modification, as the subsequent ethanol extraction did not show significant changes in the FTIR spectra. The disappearance of the band at 3395 cm^{-1}, which corresponds to intermolecular hydrogen bonds, clearly indicates that DES has caused some structural changes.

Another indication of these structural changes in solid residues was the appearance of a new band at 1700 cm^{-1}, revealing the presence of the amide I functional group. Moreover, a decrease in intensity was observed in peaks associated with nitrogenous compounds in the spectral range from 1437 to 1747 cm^{-1}. The appearance of the amide I signal suggests that the bonds connecting proteins to phytochemicals were cleaved during DES extraction. It is worth noting that the extraction process involving DESs was conducted at a controlled temperature not exceeding 30 °C. Then, any observed effects can be attributed exclusively to the properties inherent to the DES used in the experiment.

Therefore, the study of the mixing strategy showed that the two approaches did not exhibit significant differences in the microstructure and functional group profiles of the solid residues. The XN content and antioxidant capacity were affected by less than 10%, indicating good reproducibility. Moreover, the solid residue fragmentation and disappearance of functional groups corresponding to alcohol demonstrate that phytochemicals were effectively extracted from biomass. This protein-rich solid residue can be repurposed as a food and pharmaceutical ingredient.

3.5. Sustainability evaluation by *Path2Green*

The evaluation of XN, bitter acids, and other phenolic compounds extracted from hot trub using the sequential extraction process proposed in this study, assessed through the *Path2Green* metric, provides a comprehensive perspective on its sustainability (Table S3). In summary, the process received a positive score of +0.592 (from −1 to 1), as indicated by the pictogram in Fig. 6, indicating alignment with green chemistry and circular bioeconomy principles.

The use of HT as a raw material strongly contributes to sustainability (Principle 1: biomass choice) by valorizing an agro-industrial by-product and enabling the production of new high-added-value products. This valorization aligns well with circular economy strategies and received the highest score in the *Path2Green* assessment. Moreover, the use of non-treatment for subsequent extraction reduces the energy required for the process, earning a +1 in Principle 3. The process achieved exhaustive recovery of phytochemicals using solvents of renewable origin, highlighting a positive contribution to overall

Fig. 6 *Path2Green* pictogram of the proposed extraction process.

sustainability. Although the DES is inherently a higher-cost solvent, it enabled enhanced extraction selectivity, which may be advantageous for specific applications of XN. In addition, the DES based on ChCl and La combined with XN can be directly applied in several formulations and applications, such as active packaging materials.[47] In contrast, the use of ethanol as an extraction solvent is more complex, as it is volatile and must be removed from the environment before the phytochemicals are applied. However, ethanol recovery systems, such as vacuum rotary evaporation, can be employed to minimize industrial waste and reduce expenses associated with solvent consumption, which typically represents one of the highest cost contributors in economic assessments of extractive processes.[48] The score of each principle also reveals areas that merit improvement to enhance its overall sustainability. The process was carried out in batch mode, which makes it challenging to scale up. Additionally, it generated approximately 13% biomass residue. These points could be improved in the process to further enhance its sustainability, including adopting a process for converting residual biomass into a protein ingredient.

4. Conclusion

The DES based on a mixture of ChCl and Lac as a single solvent proved highly efficient for the recovery of very hydrophilic compounds in HT, such as chlorogenic acid and desmethylxanthohumol, although it was not the most effective solvent for the target compound (XN). Despite this limitation, the DES should not be considered unsuitable, as it plays a significant role in irreversibly modifying interactions and bonds between macromolecules and micromolecules. This pattern thereby enabled the extraction of a wide range of phytochemicals. Then, using DES as a pretreatment to increase selectivity during subsequent extraction is very useful, and different solvent-to-raw-material ratios and temperatures prompt distinct responses in phytochemical composition. It is important to emphasize that the DES chosen achieved a goal in avoiding the coextraction of bitter acids and XN. From a sustainability perspective, the proposed two-step extraction approach aligns with green chemistry principles and circular bioeconomy concepts by reducing the use of conventional organic solvents.

Our work contributes to SDG 12 (Responsible Consumption and Production) and SDG 15 (Life on Land), by promoting efficient methods to reduce waste and minimize environmental impact by recycling brewery by-products into food ingredients. The favorable *Path2Green* assessment further confirms the environmental advantages of integrating DESs into brewery by-product processing chains. The solid residue obtained after DES pretreatment is an underexplored resource with preserved protein content, highlighting HT as a promising source of plant-based proteins. Further research is required to investigate protein extraction strategies and their technological functionality, and evaluate the economic feasibility of industrial implementation for HT valorization in hop-derived product manufacturing.

Author contributions

Klycia Fidélis Cerqueira e Silva: writing – original draft, conceptualization, methodology, and investigation. Paula Virginia de Almeida Pontes: methodology and writing – original draft. Patrícia Tonon de Souza: writing – review & editing and investigation. Monique Martins Strieder: formal analysis and writing – review & editing. Eduardo Augusto Caldas Batista: writing – review & editing and supervision. Miriam Hubinger: writing – review & editing, supervision, and funding acquisition.

Conflicts of interest

The authors declare no competing interests.

Data availability

All data supporting the findings of this study are included in the article and supplementary information (SI). No additional data are available. Raw data can be obtained from the corresponding author upon request. The final, accepted version of the manuscript will be uploaded to the REDU platform (https://redu.unicamp.br/), an institutional repository of the University of Campinas (UNICAMP).

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