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Sustainable extraction of phenolics from beetroot leaves via hydrophobic DES

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Article

Keywords: Beta vulgaris L., Deep Eutectic Solvent, Phenolics, Extraction, Ultrasound, Ethanol.

Posted Date: October 30th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11287015/v1

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Additional Declarations: No competing interests reported.

Abstract

This study evaluates the efficiency of ultrasound-assisted extraction (UAE) for recovering total phenolic compounds (TPC) from dried beetroot leaves (Beta vulgaris subsp. cicla var. flavescens), an agro-industrial by-product with high bioactive potential. A hydrophobic deep eutectic solvent (DES) composed of menthol and lactic acid (1:1 molar ratio) was assessed as a sustainable alternative to absolute ethanol (99.8%). The DES was synthesized via sonication at 40 ± 5°C for 1 h and characterized using FTIR, TGA, and UV–Vis spectroscopy to confirm eutectic formation and thermal stability. A full 2^3 factorial design evaluated the effects of solvent type, liquid-to-solid ratio (12 and 16 mL/g), and extraction time (5 and 10 min) at a constant temperature of 50°C. TPC was determined using the Folin–Ciocalteu method at 765 nm. Factorial ANOVA revealed significant main effects and interactions ($p < 0.05$), with the DES significantly outperforming absolute ethanol across all conditions. The highest TPC yield achieved was 5912 ± 234 mg GAE/100 g dry leaves using DES at a 1:16 ratio for 5 min. These findings demonstrate that menthol–lactic acid DES combined with UAE provides an efficient extraction approach with potential for the sustainable valorization of beetroot leaf residues.

1. Introduction

Beetroot (*Beta vulgaris subsp. cicla var. Flavescens*) is a crop of global importance, cultivated primarily for human consumption owing to the nutritional properties of its root (Chhikara et al., 2019). However, the harvesting of this tuber generates a substantial volume of residual biomass; the leaves are estimated to account for between 25 and 75 % of the total fresh wight of the plant, with a reported mean of approximately 51 % (Nutter et al., 2021). Traditionally, thi by-product has been underutilized as compost, animal feed, or organic waste, disregarding its chemical profile and bioactive properties (Almeida et al., 2025; Shah et al., 2025). Nevertheless, within the current framework of the circular economy, agro-industrial residues are recognized as promising sources of high-value-added compounds (Reguengo et al., 2022).

Leaves of Beta vulgaris subsp. cicla var. flavescens contain a diverse phenolic profile that includes vitexin-type C-glycosyl flavones, isorhamnetin derivatives, and hydroxycinnamate-related compounds; 19 phenolics were identified in leaves, with several vitexin derivatives quantified in Swiss chard extracts (Almeida et al., 2025). Swiss chard leaf extracts have shown antioxidant, hypoglycaemic, and hepatoprotective-related activities, although these effects should be attributed to the extract matrix rather than to any single identified phenolic (Hashem et al., 2016; Mzoughi et al., 2019).

The efficient recovery of bioactive compounds from the beta vulgaris leaf matrices continues to pose a technological challenge. Ultrasound-assisted extraction (UAE) has gained prominence due to its ability to enhance mass transfer through acoustic cavitation. This process reduces processing times by between 32 and 36% compared to traditional methods while simultaneously protecting thermolabile compounds. The efficiency of UAE depends on the precise control of operating variables such as solvent type,

frequency, power, temperature, time, and liquid-to-solid ratio .(Mella et al., 2024)(Chemat et al., 2017; Kyriakoudi et al., 2024)(Shirsath et al., 2012; Wen et al., 2018)

Among the variables evaluated, solvent selection is a significant factor that influences extraction efficiency. Conventional liquid-solid extraction techniques typically employ volatile organic solvents such as ethanol, methanol, and hexane. Nevertheless, absolute ethanol (99.8%) is frequently adopted as the reference solvent in numerous investigations due to its superior extractive performance (Cunha & Fernandes, 2018). Ethanol is deemed safe for food and cosmetic applications. In contrast, the use of other solvents is restricted due to residual toxicity, substantial energy consumption, and the generation of hazardous environmental waste (Chemat et al., 2017). To mitigate these impacts, industry is seeking to intensify processes through emerging technologies. Green chemistry has driven the development of deep eutectic solvents (DES), systems formed by specific combinations of hydrogen-bond acceptors and donors (Cao & Su, 2021). DES are distinguished by their low toxicity, biodegradability, and tunable polarity, which position them as sustainable alternatives to conventional solvents for the dissolution of bioactive compounds (van Osch et al., 2020). Hydrophobic DES based on menthol and lactic acid exhibit properties that enable the extraction of weakly polar compounds, prevent aqueous dilution, and facilitate its subsequent separation (Sánchez San Segundo, 2019).

In Beta vulgaris, DES-UAE has been successfully demonstrated for the recovery of betalains from root tissues, where choline chloride-based systems significantly outperformed conventional solvents. However, published studies on beet studies remain centered on hydrophilic DES and root pigments, leaving leaf phenolic extraction largely unexplored (Demuner et al., 2023). Broader leaf-extraction studies show that DES-UAE effectively recovers flavonoids and phenolic acids from leaves of blueberry, broccoli, and Moringa oleifera, often surpassing ethanol controls (Y. Cao et al., 2023; Siddiqui et al., 2025; Wang et al., 2024). The extraction efficiency depends strongly on matching solvent polarity to the target compound, suggesting that systems optimized for hydrophilic beet pigments may not suit lipophilic leaf phenolics (C. Ferreira & Sarraguça, 2024; Siddiqui et al., 2025). Hydrophobic deep eutectic solvents, including menthol-based systems, are promising selective extraction media for phenolics, but their low water miscibility and viscosity require controlled dispersion or phase-management steps before aqueous Folin–Ciocalteu analysis; non-ionic surfactants can assist this process by improving micellar dispersion and extract handling (Jin et al., 2020; Rosa et al., 2026; Siddiqui et al., 2025).

Against this background, the present study evaluated a menthol–lactic acid eutectic solvent (1:1) as a hydrophobic alternative to absolute ethanol (99.8%) for ultrasound-assisted extraction from dried leaves of Beta vulgaris subsp. cicla var. flavescens. The solvent was prepared and spectroscopically characterized by FTIR, TGA and UV–Vis to support eutectic formation and assess its physicochemical behavior before extraction.

2. Methods

2.1. Plant material and sampling

Red beetroot leaves (*Beta vulgaris subsp. cicla var. Flavescens*) were obtained from local growers in the city of Machachi, Pichincha Province, Ecuador (2886 m a.s.l.; 0.70 atm; 12.7°C; 70%RH). The Fig. 1 sequentially depicts the stages of the process leading to the powdered leaves, prior to extraction.

Sampling was carried out within the first hour after harvest to preserve the integrity of the secondary metabolites. Only leaves in optimal physiological condition were selected, discarding those with mechanical damage, signs of disease, or visible deterioration.

The selected leaves underwent a thorough cleaning process. Initially, they were immersed in tap water for a duration of 30 minutes, effectively removing any external soil particles and impurities. Subsequently, the leaves were meticulously wiped with absorbent paper to eliminate any remaining surface moisture. The conditioned leaf biomass was then subjected to dehydration in a tunnel dryer at a constant temperature of 65°C. The drying process was maintained for a duration of 12 hours, with a consistent air velocity of 1 m/s. Upon completion of the drying cycle, the leaves achieved a precise residual relative humidity of 5.85%. The dried tissues were subsequently ground into a fine powder using a cutting mill (Retsch, model SM 300). The milling process was facilitated by a 1000 µm (1 mm) sieve operating at a constant speed of 1500 rpm, ensuring the attainment of particle size homogeneity. The powdered sample was then accurately weighed using an electronic balance and stored at room temperature (25 ± 2°C) within hermetically sealed containers. These containers were designed to safeguard the sample from light exposure until the extraction process commenced.

2.2. Reagent and DES preparation

Absolute ethanol (99.8% purity) of analytical grade served as the benchmark organic solvent for comparative extraction performance. Solid (-)-menthol ($C_{10}H_{19}OH$, 99.38% purity) was used as the hydrogen-bond acceptor (HBA), while L-lactic acid ($C_3H_6O_3$, 88% purity) was used as the hydrogen-bond donor (HBD).

The hydrophobic deep eutectic solvent (DES) was prepared by mixing these components in a 1:1 equimolar ratio. To prepare a standard batch, 157.24 g of crystalline (-)-menthol was combined with 90.032 g of L-lactic acid (equivalent to a volume of 84.83 mL, assuming a density of 1.206 g/mL) in an Erlenmeyer flask. Homogenization was conducted in an ultrasonic bath (Isolab, model 621.05.015, Germany) at 40 ± 5°C for 60 min (1 hour) until a stable, completely transparent liquid phase was formed. The synthesized DES was cooled to room temperature (25 ± 2°C) and stored. Visual evaluation was conducted over a 7-day storage period at 25 ± 2°C to assess the physical stability of the eutectic mixture, with no visible phase separation or recrystallization observed.

2.3. Physicochemical and thermal characterization of the DES

Prior to extraction, the physical properties, chemical coordination, and thermal window of the eutectic mixture were analyzed.

2.3.1. ATR-FTIR spectroscopy

Characterization of the DES was conducted using attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR, Perkin Elmer Spectrum Two spectrometer) to identify functional groups and examine intermolecular hydrogen bonds. Vibrational profiles were scanned across the 4000–450 cm^{-1} wavenumber range. To assess physical stability, the protocol outlined by Ojeda et al. (2023) was employed, which involved visually inspecting for signs of crystallization after seven days of storage at 25 ± 2°C.

2.3.2. Thermogravimetric analysis (TGA)

Thermal stability was evaluated using a Mettler Toledo TGA 1 STAR System under an inert, high-purity nitrogen atmosphere (99.999% purity) with a gas flow of 20 mL/min. The dynamic heating program began at 25°C, increasing at a rate of 10°C/min up to 100°C, where it was held isothermally for 10 min to assess localized moisture volatilization. Dynamic heating then continued from 100°C to 800°C, followed by a controlled cooling phase of 10 min.

2.3.3. UV-Vis spectrophotometric profile

The optical absorption of the DES was scanned in the wavelength range of 200 to 800 nm using an Agilent Cary 60 UV-Vis spectrophotometer. This measurement was essential to verify the absence of solvent absorption above 300 nm—particularly at the target analytical wavelength of 765 nm—ruling out spectral interference with the Folin–Ciocalteu colorimetric reagent.

2.4. Ultrasound-assisted extraction (UAE) protocol

A sequential extraction protocol combining static maceration and ultrasound-assisted extraction (UAE) was adapted from established methods (Chemat et al., 2017). For each experimental run, 1.0 g of dried, ground leaf powder was weighed and transferred into a 30 mL amber glass vial to prevent photodegradation of the target phenolics.

The appropriate volume of extracting solvent (either absolute ethanol or the synthesized menthol–lactic acid DES, both used neat) was added to achieve liquid-to-solid ratios of 12 mL/g (12 mL) and 16 mL/g (16 mL). The mixtures were first subjected to an undisturbed static maceration phase for 24 h at room temperature (25 ± 2°C) to promote tissue swelling, cellular hydration, and solute mobilization prior to sonication.

Following maceration, the vials were placed in a preheated ultrasonic bath (Isolab, model 621.05.015, operating at a fixed frequency of 40 kHz) at a controlled temperature of 50 ± 4°C for either 5 or 10 min according to the design matrix. Bath temperature was monitored continuously, and sample vials were confirmed to have reached the set point before the sonication timer was started. Immediately after sonication, the vials were cooled to room temperature. The samples were then filtered through Whatman No. 40 filter paper. The clear liquid extracts were collected in clean amber glass vials and stored at 4°C, protected from light, until colorimetric quantification. All extractions were performed in triplicate.

2.5. Experimental factorial design

A full 2^3 factorial design was employed to assess the influence of three independent extraction variables on process performance, as such designs facilitate estimation of main effects and interactions among multiple factors on one or more response variables. The variables examined—solvent type (menthol:lactic acid deep eutectic solvent at a 1:1 molar ratio versus absolute ethanol at 99.8%), liquid-to-solid ratio (12 and 16 mL/g), and ultrasound extraction time (5 and 10 min)—are recognized as critical parameters in ultrasound-assisted and deep eutectic solvent-based extraction protocols. The experimental matrix consisted of eight treatment combinations (E1–E8), consistent with a two-level, three-factor design. Each treatment was conducted in triplicate, yielding 24 total runs; the sequence was randomized to minimize systematic bias and enhance statistical validity. Fixed parameters—sample mass (1.0 g), particle size (1 mm), and extraction temperature (50°C)—were maintained across all trials, in accordance with standard practice in factorial extraction studies where selected variables are manipulated while other influential conditions remain constant.

2.6. Quantification of total phenolic content (TPC)

2.6.1. Analytical calibration and validation

Total phenolic content was determined by the Folin–Ciocalteu method using an Agilent Cary 60 UV–Vis spectrophotometer operated with Cary WinUV software (version 5.0; Agilent Technologies, Santa Clara, CA, USA). Absorbance was measured at 765 nm in a 10 mm quartz cuvette, and quantification was performed against a gallic acid calibration curve, since gallic acid is a widely used reference standard for this assay.

A gallic acid stock solution was prepared by dissolving 400 mg of gallic acid in 20 mL of distilled water. Five calibration standards were then prepared at nominal concentrations of 400, 800, 1200, 1600, and 2000 mg/L. Because the reference standard was corrected for its certified purity and moisture content, the corresponding corrected concentrations were 392.04, 784.08, 1176.12, 1568.16, and 1960.20 mg/L; these corrected values were used for calibration calculations.

Each calibration level was analyzed in triplicate. For the colorimetric reaction, 0.5 mL of standard or appropriately diluted sample solution was mixed with 0.25 mL of Folin–Ciocalteu reagent (1 N), 0.5 mL of sodium carbonate solution (10%, w/v), and 3.75 mL of distilled water, and a reagent blank was prepared in parallel under the same conditions.

The reaction mixtures were incubated in the dark for 60 min at room temperature before absorbance measurement. This incubation time and wavelength are consistent with validated Folin–Ciocalteu protocols, although the assay should be interpreted as an operational estimate of total reducing capacity expressed as gallic acid equivalents rather than as a measurement specific only to phenolic compounds.

Calibration data were fitted by linear regression, and model adequacy was assessed from the regression equation, residual distribution, and analysis of variance rather than from R^2 alone, because correlation-based statistics do not by themselves demonstrate linearity. The validated working range corresponded to the concentration interval over which proportional response was maintained, and sample solutions were diluted as necessary to fall within that range.

The limit of detection (LOD) and limit of quantification (LOQ) were estimated by the ICH regression approach as LOD = 3.3σ/m and LOQ = 10σ/m, where σ was the residual standard deviation of the calibration curve and m was its slope. Because LOD and LOQ values depend on the estimation approach and can vary under heteroscedastic conditions, these values should be interpreted as model-based estimates for the validated calibration range.

2.6.2. Extraction assay and phase stabilization

To overcome water immiscibility during the colorimetric assay, hydrophobic menthol–lactic acid DES extracts were pre-treated by combining 0.5 mL of extract with 0.5 mL of Tween 20 (1:1, v/v), providing a homogeneous single phase with a twofold dilution factor (DF = 2).

For the assay, an aliquot of 0.5 mL of the pre-treated DES mixture (or 0.5 mL of ethanolic extract, appropriately adjusted during final quantification) was mixed with 0.25 mL of Folin–Ciocalteu reagent (1 N), 0.5 mL of aqueous sodium carbonate (10% w/v), and 3.75 mL of distilled water to attain a final reaction volume of 5.0 mL.

The mixtures were incubated in complete darkness for 2 h at room temperature (25 ± 2°C) to complete color development. Absorbance was recorded at 765 nm against matrix-matched blanks containing corresponding proportions of solvent, surfactant, and water without sample. All extractions and analyses were performed in triplicate. Final total phenolic contents were calculated from a gallic acid standard curve and expressed as milligrams of gallic acid equivalents per 100 grams of dry leaf weight (mg GAE/100 g DW) after incorporating all specific dilution factors.

TPC was calculated according to Eq. (1), considering the concentration obtained from the gallic acid calibration curve, the total extraction volume, the corresponding dilution factor, and the dry sample mass.

$$TPC = \frac{C \times V \times DF}{m} \times 100$$

Eq. (1)

where:

C = concentration obtained from the gallic acid calibration curve;

V = total volume of the extract;

DF = total dilution factor applied; and

m = dry mass of the sample used.

2.7. Statistical analysis

Experimental data were compiled and expressed as the mean ± standard deviation (n = 3). To evaluate the individual and interactive effects of the three independent variables (solvent type, liquid-to-solid ratio, and extraction time) on total phenolic content (TPC) extraction yield, a three-way factorial analysis of variance (ANOVA) was executed. The statistical model was checked for normality of residuals, homoscedasticity, and independence of residuals to ensure model validity.

Post-hoc multiple comparisons of treatment means were conducted using Tukey's Honestly Significant Difference (HSD) test at a 95% confidence level (α = 0.05). Statistical computations, analysis of variance, and multiple range tests were processed using Statgraphics Centurion XIX (Statpoint Technologies, Inc., Warrenton, VA, USA), with parallel verification performed using the R statistical computing environment (R Core Team).

3. Results and discussion

3.1. Physicochemical and thermal characterization of the DES

3.1.1. ATR-FTIR spectroscopy

The FTIR spectrum (Fig. 2) showed characteristic bands consistent with the formation of a menthol:lactic acid deep eutectic solvent through intermolecular hydrogen bonding between the components (K. C. Ferreira et al., 2026; Olawuyi et al., 2026; Zheng et al., 2025).

The broad band at 3393.20 cm^{-1} is assigned to O–H stretching and is consistent with hydrogen-bonded hydroxyl groups, while the bands at 2923.65 and 2869.68 cm^{-1} correspond to aliphatic C–H stretching vibrations associated with menthol (Cherniakova et al., 2024; Martín et al., 2024; Zheng et al., 2025). The band at 1724.66 cm^{-1} is assigned to the carbonyl stretching vibration of the carboxylic acid moiety, whose position can shift upon DES formation because of changes in the hydrogen-bonding environment (Cherniakova et al., 2024; Indah Puspita Sari et al., 2024; Pires et al., 2022). The signals at 1372.37 cm^{-1} and in the 1128.29–919.65 cm^{-1} region are consistent with menthol-related aliphatic vibrations and C–O stretching modes, whereas the 1500–600 cm^{-1} interval corresponds to the fingerprint region of the mixture (Busato et al., 2022; Martín et al., 2024; Zheng et al., 2025). Overall, the FTIR data support DES formation by noncovalent interactions in menthol:lactic acid.

3.1.2. Thermogravimetric analysis (TGA)

The thermogravimetric analysis (TGA) profile presented in Fig. 3 demonstrates a multistep mass-loss pattern for the menthol–lactic acid deep eutectic solvent (DES).

The material maintains thermal stability up to approximately 100°C, after which a mass reduction of about 7% (93% residual mass) is observed. Under isothermal conditions at 100°C, an additional 10% mass loss (83% residual mass) occurs, presumably attributable to the evaporation of adsorbed moisture and other low-boiling constituents. The principal mass-loss event, occurring between approximately 120°C and 210°C, corresponds to the primary thermal degradation of the DES, likely involving concurrent volatilization and decomposition of the components. Above this range, minimal residual mass remains up to 400°C, indicating extensive conversion of the organic system into volatile products with negligible non-volatile residue or char (less than 1% residual mass) (Delgado-Mellado et al., 2018; González-Rivera et al., 2022; S. Liu et al., 2022).

3.1.3. UV-Vis spectrophotometric profile

The spectroscopic characterization of the menthol:lactic acid DES, illustrated in Fig. 4, supported its use as an extraction medium.

In comparable lactic acid–based DES systems, UV–Vis absorbance is mainly confined to the 240–400 nm region, with characteristic features reported at 248 nm, 254 ± 1 nm, and 277 ± 1 nm, plus a tail extending to about 380 nm (Paradiso et al., 2016). By contrast, the Folin–Ciocalteu assay is typically monitored at 761–765 nm, so the solvent is unlikely to cause direct spectral interference in TPC measurement under these conditions (Paradiso et al., 2016; Soares Macello Ramos et al., 2024). On that basis, the solvent itself is unlikely to cause direct spectral overlap at the TPC detection wavelength under the specified experimental conditions (Soares Macello Ramos et al., 2024).

3.2. Analytical calibration and validation

The gallic acid calibration curve demonstrated a robust linear relationship across the concentration range of 392.04–1960.20 mg/L, as illustrated in Fig. 5.

The regression model was statistically significant ($p < 0.001$), with a correlation coefficient (r) of 0.9995 and a coefficient of determination (R^2) of 0.9991. These results indicate that 99.91% of the variability in the analytical response was explained by the calibration model.

Based on the regression output, the calibration relationship was expressed as: $C = 985.8594A - 22.0018$, where C is the gallic acid concentration (mg/L) and A is the absorbance measured at 765 nm. Student's t-test indicated that the intercept was not significantly different from zero ($p > 0.05$), suggesting that no statistically significant constant bias was detected within the evaluated calibration range.

Residual analysis showed no evident systematic pattern, supporting the assumptions of linearity and homoscedasticity and the use of an unweighted linear regression model. The estimated limits of detection and quantification were 0.0803 and 0.2432 mg/L, respectively. Overall, the calibration

performance was considered suitable for the quantification of total phenolic content under the analytical conditions used in this study.

3.3. Ultrasound-assisted extraction results

The effect of extraction conditions—specifically solvent type (menthol–lactic acid DES vs. absolute ethanol), liquid-to-solid ratio (12 and 16 mL/g), and extraction time (5 and 10 min)—on the total phenolic content (TPC) was evaluated. As shown in Table 1, extraction efficiency varied significantly across treatments.

Table 1
Total phenolic content (TPC) under different extraction conditions.

Treatment	Solvent	Liquid-to-solid ratio [mL/g]	t [min]	mg GAE/mL	TPC mg GAE/100 g dry leaves
E1	Deep eutectic solvent (DES)	1:12	5	0,8950	2150 ± 155
E2		1:12	10	1,7370	4168 ± 210
E3		1:16	5	1,8475	5912 ± 234
E4		1:16	10	1,8193	5822 ± 102
E5	Absolute ethanol	1:12	5	0.2329	280 ± 23
E6		1:12	10	1.3492	1619 ± 247
E7		1:16	5	1.2645	2023 ± 319
E8		1:16	10	1.7603	2817 ± 52

Note: Values are expressed as the mean ± standard deviation (n = 3). Different superscript letters (a–c) within the same column indicate significant differences among treatments ($p < 0.05$) according to one-way ANOVA followed by Tukey's test.

Total phenolic content (TPC) in the deep eutectic solvent (DES) treatments varied from 2150 ± 155 to 5912 ± 234 mg gallic acid equivalents (GAE)/100 g dry leaves. The highest mean value was recorded at a 1:16 ratio for 5 minutes; no significant difference ($p > 0.05$) was observed at 10 minutes. This suggests that, at higher solvent proportions, extraction reached a plateau within the evaluated time frame rather than exhibiting continued measurable growth. Similar plateau behavior after an initial increase has been reported in DES-UAE systems when extraction time is extended beyond the most productive interval (Meng et al., 2025; Siddiqui et al., 2025; Wang et al., 2024). This interpretation is compatible with ultrasound-assisted extraction kinetics, in which cavitation accelerates early mass transfer and cell disruption, followed by a slower stage as easily accessible compounds are depleted, and the system moves toward saturation or diffusion control (Li et al., 2024; Siddiqui et al., 2025). Studies on leaf matrices further show that ultrasound can fragment plant tissue, disrupt cell-wall integrity, and improve

solvent penetration, which supports the faster recovery observed with DES under short treatment times (Chen et al., 2025; Li et al., 2024).

Absolute ethanol yielded lower TPC values than DES under all tested conditions, ranging from 279.5 ± 23 to 2816.5 ± 52 mg GAE/100 g dry leaves, and it showed a stronger dependence on extraction time, especially at the lower solvent proportion. That pattern is consistent with comparative studies showing that optimized DES-UAE systems often recover more phenolics than conventional solvents such as ethanol, methanol, or water, although the magnitude of improvement depends on solvent composition and matrix type. Similar DES-over-ethanol advantages have been reported for mulberry leaves, Moringa leaves, blueberry leaves, dogbane leaf-tea, and soursop leaves (Oton et al., 2025; Santos-Martín et al., 2023; Wang et al., 2024; Wu et al., 2020).

The three-way factorial ANOVA confirmed that the three factors evaluated (solvent type, liquid-to-solid ratio, and extraction time) exert statistically significant effects on TPC ($p < 0.0001$) (Table 2).

Table 2
Summary of the factorial ANOVA for total phenolic content (TPC).

Factors	**F statistic**	**p-value**
A: Solvent	105.95	< 0.0001
B: Liquid-to-solid ratio	645.83	< 0.0001
C: Time	194.12	< 0.0001
Interactions		
AB: Solvent × Feedstock-to-solvent ratio	1.10	0.3044
AC: Solvent × Time	27.64	< 0.0001
BC: Feedstock-to-solvent ratio × Time	51.83	< 0.0001
ABC: Solvent × Feedstock-to-solvent ratio × Time	5.21	0.0316
Note: Significance level: $p < 0.05$.		

The two-way interactions AC (solvent × time) and BC (ratio × time), as well as the three-way interaction ABC, were significant, indicating that the influence of each factor depends on the levels of the others. When menthol:lactic acid DES was compared with absolute ethanol for extracting phenolic compounds from beetroot leaves, TPC varied with both liquid-to-solid ratio and extraction time, which is consistent with broader DES-UAE literature identifying solvent composition, solvent-to-sample ratio, and extraction duration as key extraction variables. (Y. Liu et al., 2022; Siddiqui et al., 2025)

Tukey's multiple-comparison test ($\alpha = 0.05$) indicated that the deep eutectic solvent (DES) produced a significantly higher total phenolic content (TPC) than absolute ethanol. The 1:16 ratio demonstrated

superior performance relative to the 1:12 ratio, while the effect of extraction time depended on solvent type and liquid-to-solid ratio, as illustrated in Figs. 6a, 6b and 6c.

Based on the literature, the superior performance of DES is best explained by their tunable physicochemical properties, since polarity, viscosity, water content, and HBA/HBD composition jointly determine solvent penetration, mass transfer, and solute–solvent interactions (Li et al., 2024; Y. Liu et al., 2022; Meng et al., 2025). Acid-based DESs, including lactic-acid-containing systems, are often especially effective for phenolic extraction, but these effects remain solvent- and matrix-dependent (Oton et al., 2025; Santos-Martín et al., 2023; Sun et al., 2025).

Menthol–lactic acid likely enhances recovery of polar phenolics through a cooperative mechanism in which menthol provides a hydrophobic extraction phase, while lactic acid tunes the solvent's acidity and hydrogen-bonding capacity, improving solute–solvent interactions with hydroxylated phenolics. Under ultrasound-assisted extraction, acoustic cavitation disrupts plant cell structures and accelerates solvent penetration and mass transfer, which can further promote the release of matrix-associated phenolics. Accordingly, the higher recovery observed for this system is more precisely attributed to the combined effects of HDES composition and UAE intensification than to menthol or lactic acid alone (Jin et al., 2020; Le et al., 2026; Li et al., 2024).

3.4. Limitations of the study

One limitation of the present study is that phenolic content was assessed only as total phenolic content by the Folin–Ciocalteu method, which does not permit identification or quantification of individual compounds (Thovhogi et al., 2023). This is relevant because Swiss chard leaves contain a diverse phenolic profile that includes vitexin-type flavones and other flavonoid and phenolic-acid derivatives, and these compounds can vary across tissues and genotypes (Almeida et al., 2025; Sytar et al., 2018). In addition, comparison with published TPC values should be made cautiously because reported concentrations are influenced by extraction conditions, plant part, genotype, and environmental factors (Čeryová, 2025; Thovhogi et al., 2023). The study also did not address seasonal or harvest-stage variability, although evidence from leafy vegetables and Beta vulgaris indicates that phenolic accumulation can change with radiation, temperature, irrigation regime, and harvest time (Marin et al., 2015). Therefore, the high TPC observed here should be interpreted as a promising result under the tested extraction conditions rather than as a fixed phenolic benchmark for Swiss chard residues across seasons or cultivation environments.

4. Conclusions

The present study demonstrates the technical potential and environmental feasibility of using a hydrophobic deep eutectic solvent (DES) (menthol:lactic acid, 1:1) as a superior alternative to absolute ethanol for the recovery of total phenolic compounds (TPC) from Beta vulgaris L. leaves by ultrasound-assisted extraction (UAE). Physicochemical characterization (FTIR, UV–Vis, and TGA) confirmed the analytical compatibility of the DES and indicated that it remained thermally stable under the extraction

conditions applied at 50°C, indicating no relevant direct absorbance overlap at 765 nm under the evaluated conditions in quantification. The results demonstrated that the DES system significantly outperformed ethanol in extractive efficiency under all evaluated conditions ($p < 0.0001$), reaching a maximum yield of 5912 ± 234 mg GAE/100 g. This outstanding performance, which exceeds the ranges typically reported for conventional solvents and hydrophilic eutectic systems, is attributed to the capacity of the DES to tune its polarity and to establish specific hydrogen bonds with the target metabolites, potentiated by acoustic cavitation, which promotes cell disruption.

Declarations

Acknowledgements

The authors gratefully acknowledge Universidad Central del Ecuador and the Faculty of Chemical Engineering for the institutional, technical, and laboratory support provided during the development of this research.

Author contributions

M.D.A.R. contributed to the investigation, experimental work, methodology, and development of the study. C.A.A.P. and G.J.P.C contributed to the conceptualization, methodology, supervision, guidance of the research, and interpretation of the results. C.G.F.O. and W.G.C.G. contributed to the final review and critical revision of the manuscript. All authors contributed to writing, reviewing, and editing the manuscript and approved the final version for submission.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Additional information

The authors declare no competing interests.

Funding

The authors declare that no specific funding was received for this research.

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Figures

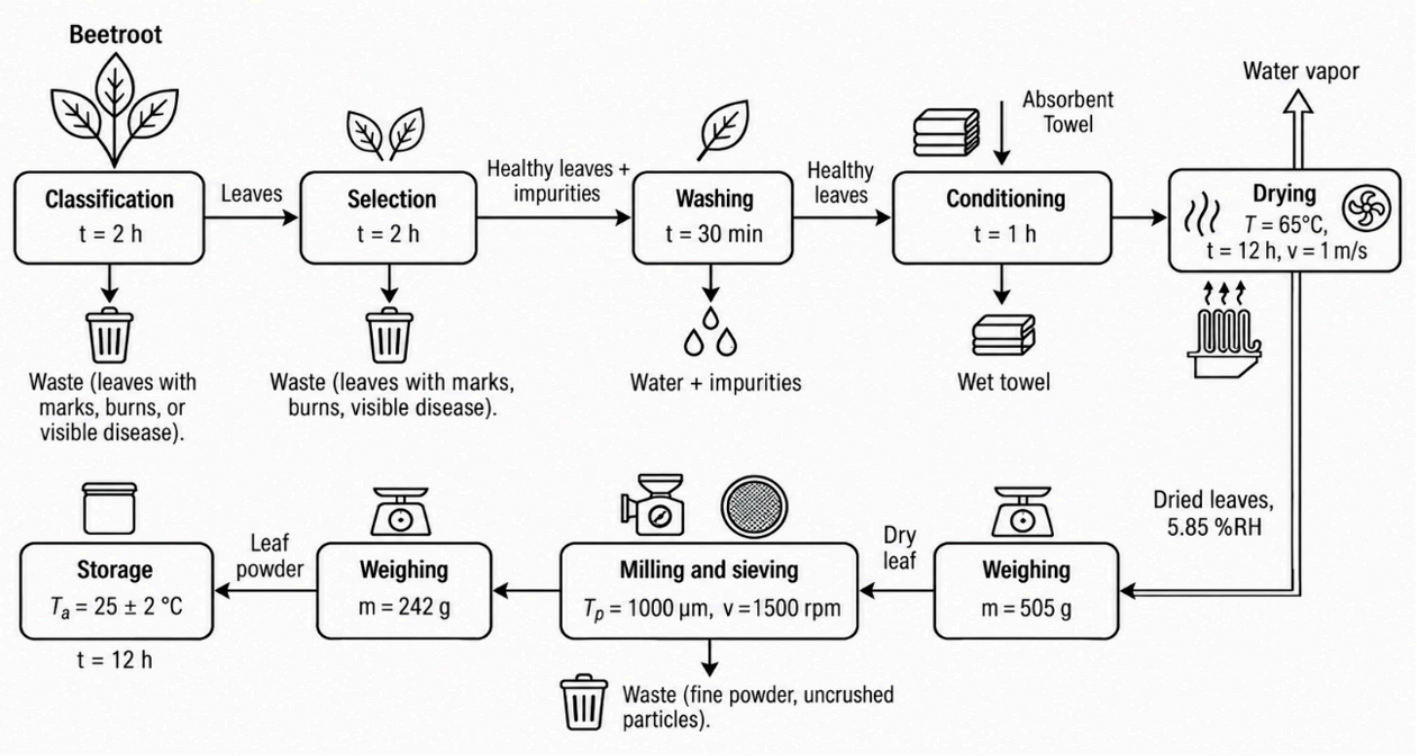


Figure 1

Sample preparation flow diagram.

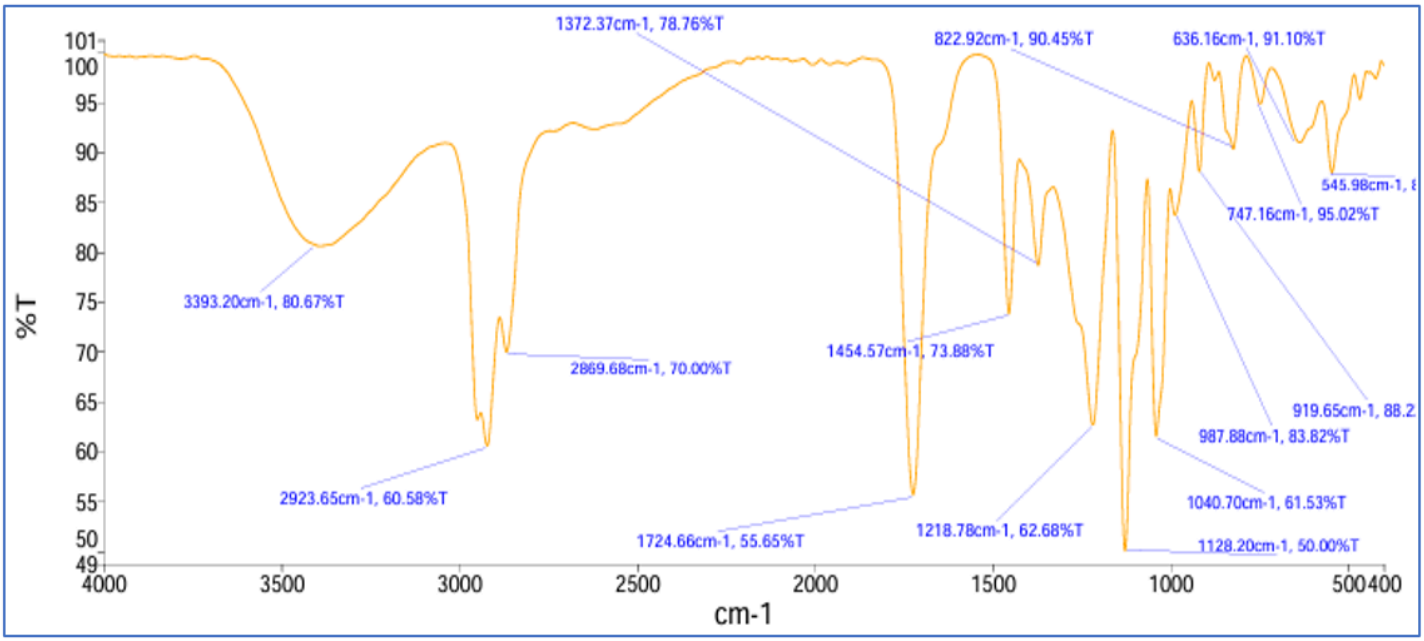


Figure 2

Fourier-transform infrared spectroscopy – FTIR spectrum of the DES.

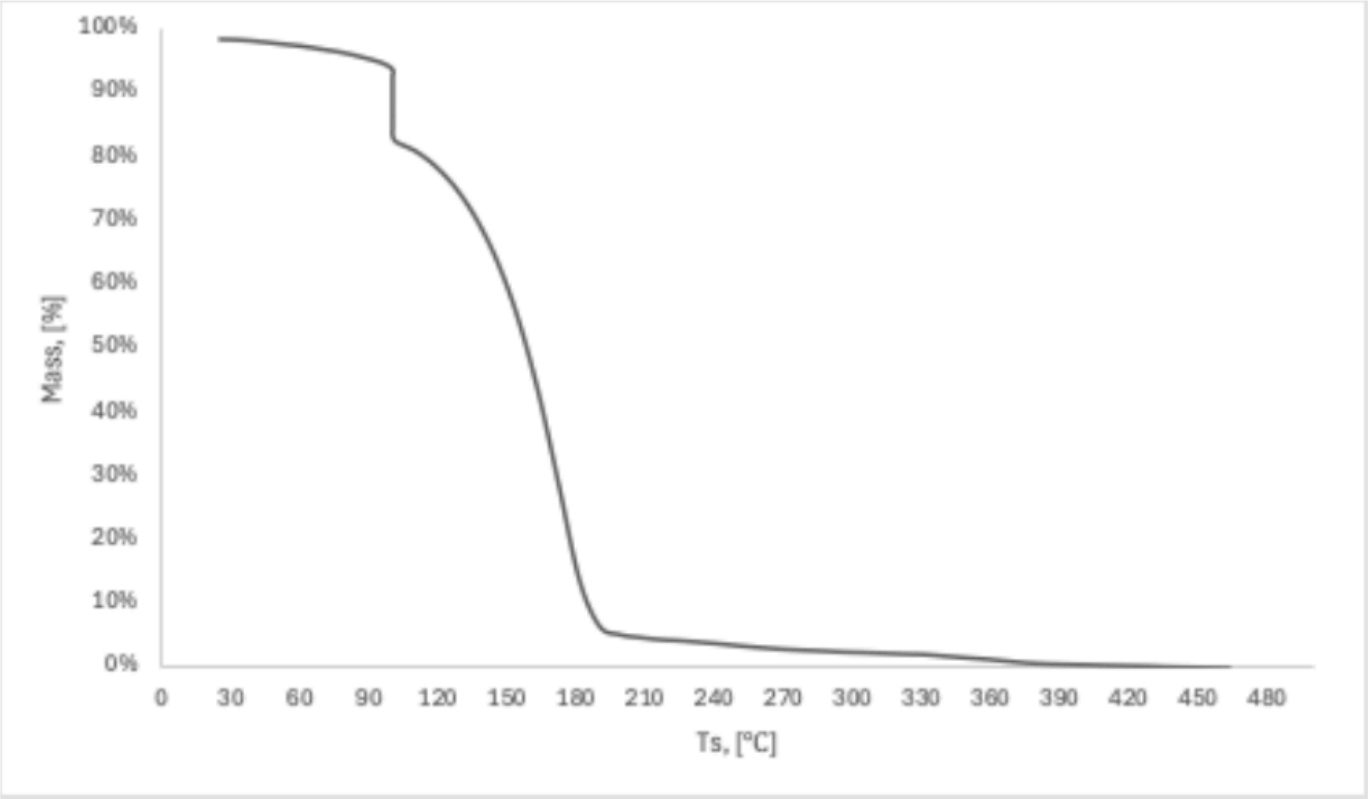


Figure 3

Thermogravimetric analysis of the DES.

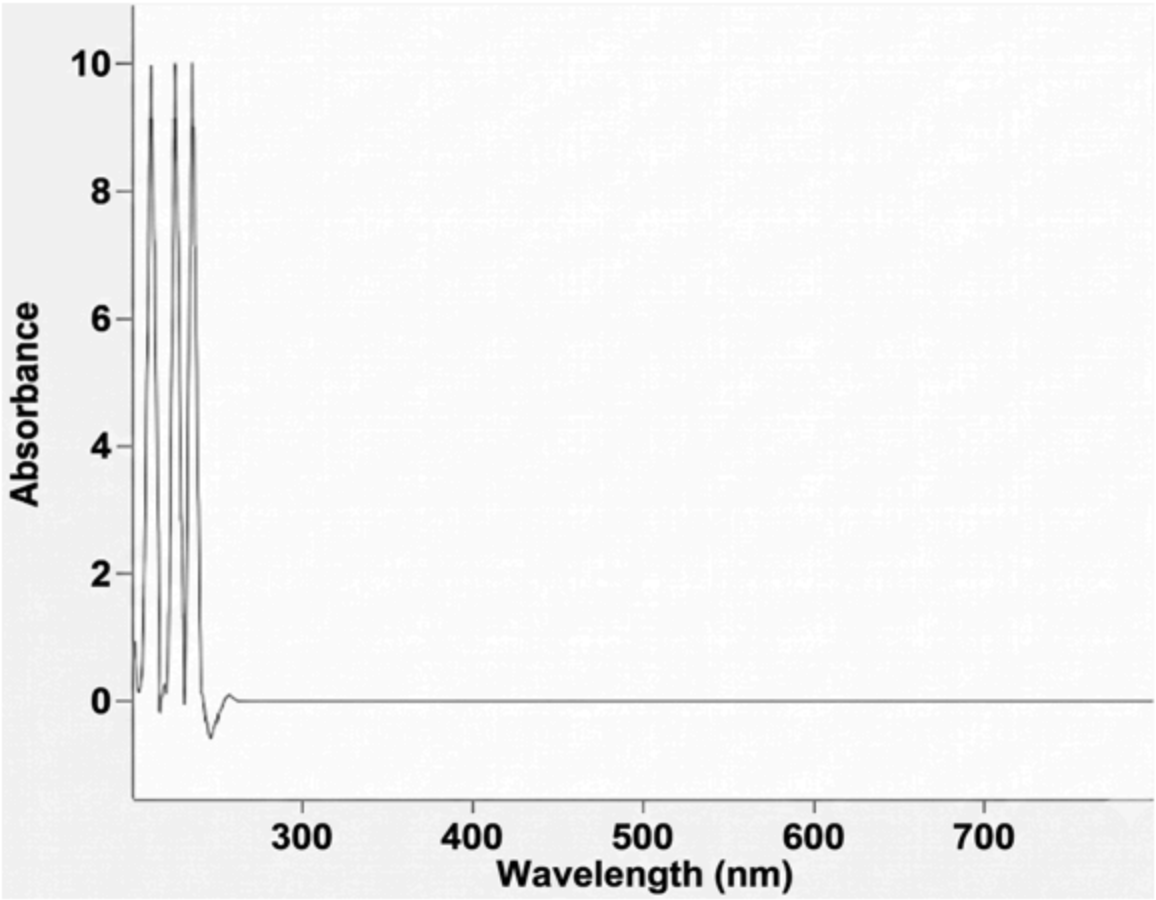


Figure 4

UV–Vis spectroscopic characterization of the DES.

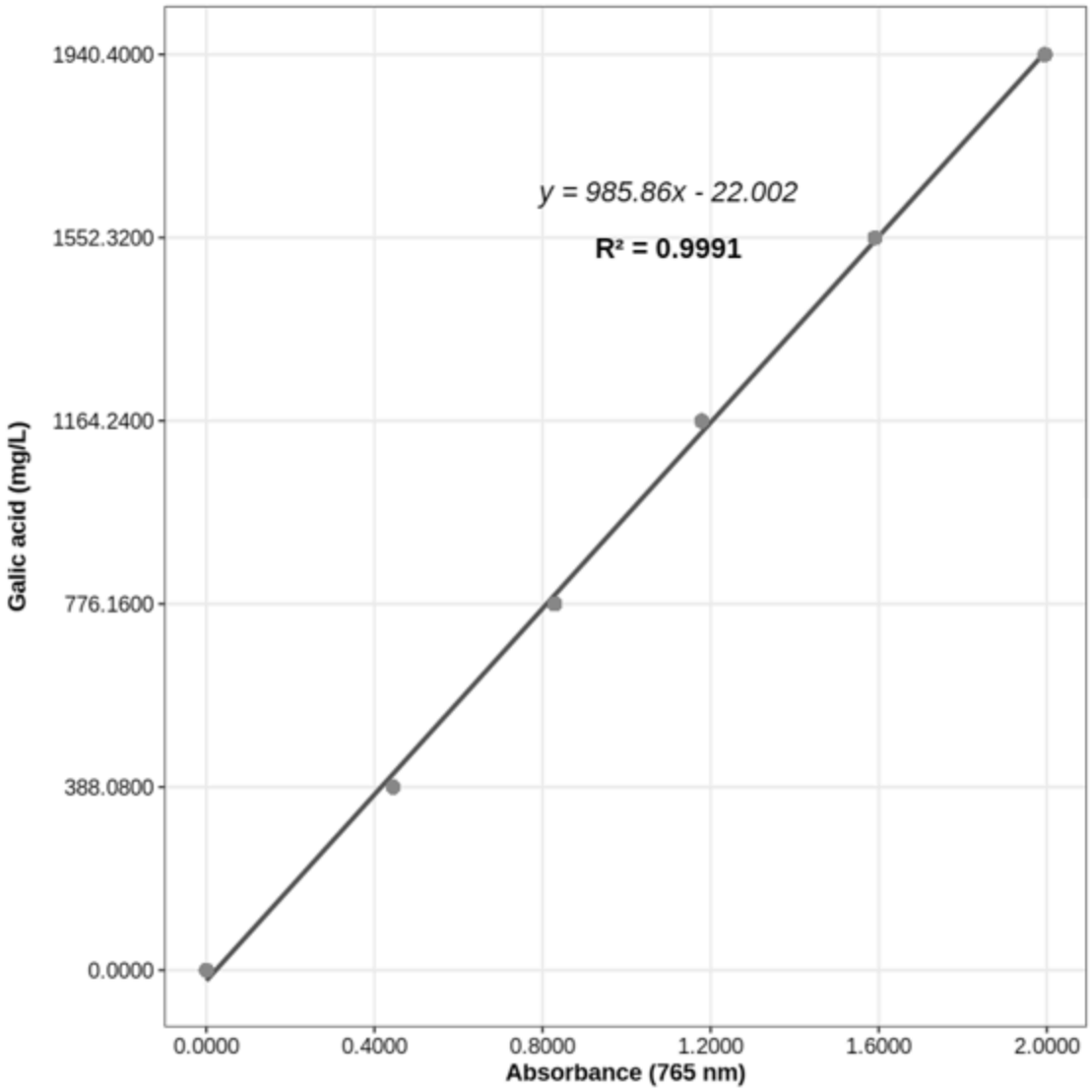


Figure 5

Gallic acid calibration curve for TPC assay at 765 nm.

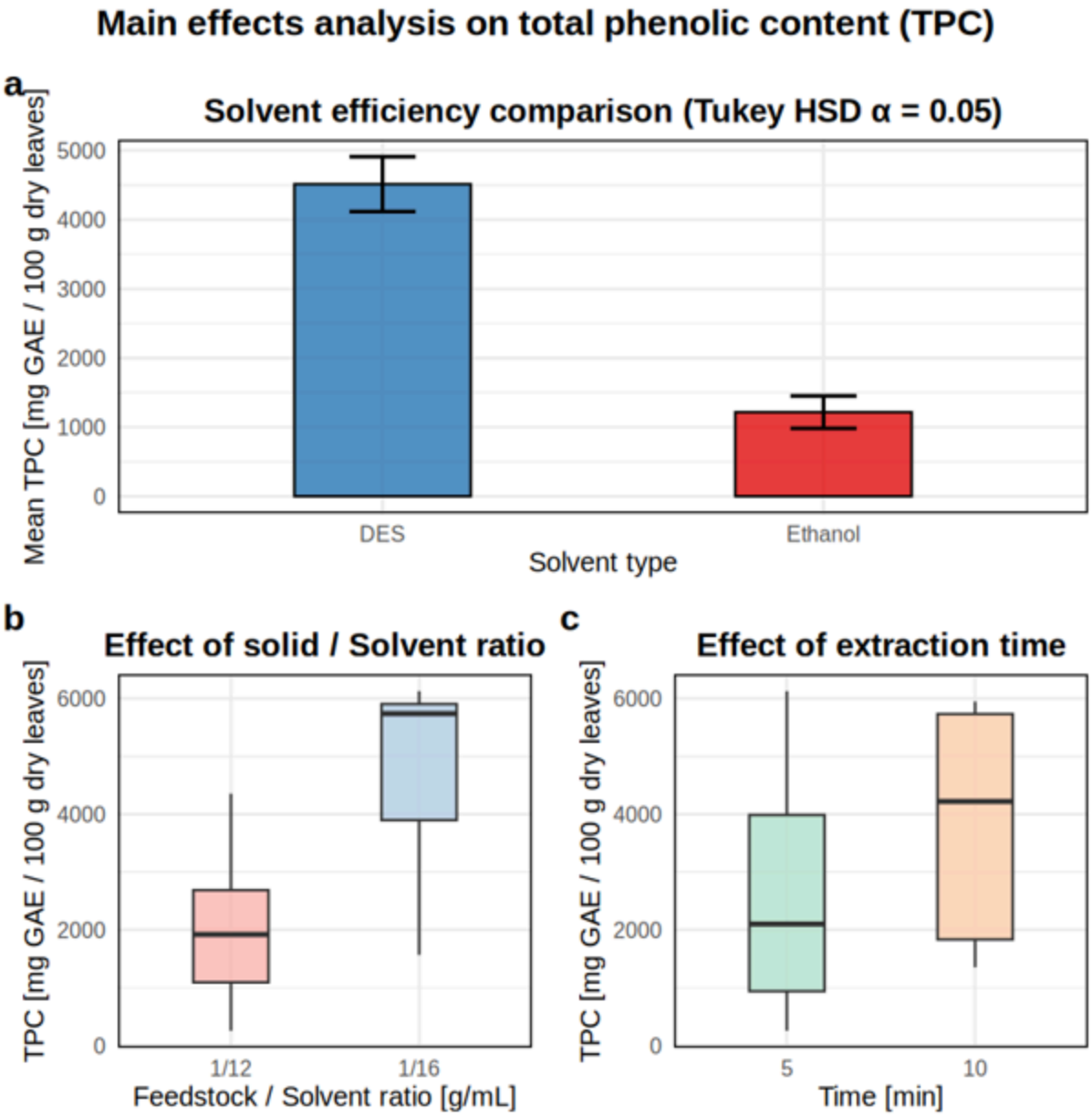


Figure 6

(a) Main effect analysis on total phenolic content; (b) Liquid-to-solid ratio interaction on TPC; (c) Influence of extraction time on TPC.