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Enhancing extraction of betalains from beetroot (*Beta vulgaris* L.) using deep eutectic solvents: optimization, bioaccessibility and stability

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Abstract

Deep eutectic solvents (DESs) are the next generation of green solvents that are considered for their stability and biocompatibility. This study used 10 different DESs synthesized from choline chloride, alcohols, organic acids and sugars. Red beet betalains were extracted using three conventional solvents and DESs. Characterization experiments of DESs suggested that the electrical conductivity, pH, viscosity, water activity, density and chemical structure were greatly affected by the composition of the hydrogen bond acceptors (HBA) and hydrogen bond donors (HBD). Betacyanin, betaxanthin and total betalain contents ranges were 23.68–702.17, 21.49–467.77, and 45.17–1169.94 mg kg^{-1}, respectively, with choline chloride (ChCl): glucose (Glu) (1:2) giving the highest values. ChCl:Glu was chosen for the optimization process considering the molar ratio (ChC = 1:Glu = 0.75–1.75), water content (15–35%) and temperature (30–60 °C) factors for the central composite design. The optimum conditions were recorded as 1:0.75 molar, 30.83% water content, and 30 °C, respectively. Under optimum conditions, the yields of betalain, betacyanin and betaxanthin were found to be 1192.17 ± 23.63, 738.83 ± 17.87, and 453.34 ± 5.93 mg kg^{-1}, respectively. Bioaccessibility analysis and stability tests were performed on the extracts obtained under optimum conditions. Stability tests revealed that the betalains of red beetroot are less stable in the light than in the dark. Bioaccessibility values for betacyanin, betaxanthin, and betalain were found to be 44.67 ± 1.40, 75.02 ± 1.20, and 56.21 ± 1.33%, respectively. Green extraction of betalains from red beetroot using DES, such as ChCl:Glu, is promising for a strong stabilization and high bioaccessibility of betalains.

Keywords Red beetroot, Betalain, Deep eutectic solvents, Storage stability, Bioaccessibility

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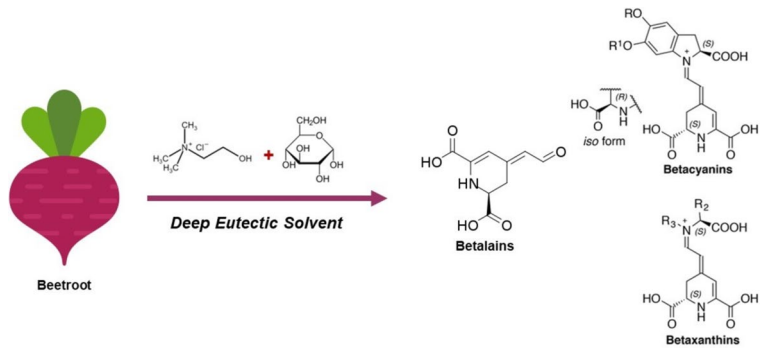
Graphical Abstract



Introduction

In the last decades, the preservation of the environment and human well-being have been the key focus of researchers. The increasing awareness of the harmful effects of human action on the environment led to the use of green methods as an alternative to the conventional methods applied in industries. One of the priorities of the EU agenda 2010–2050 for environmental policy and legislation is the low usage of petrochemical solvents and volatile organic compounds since most of these solvents are associated with high flammability, volatility and toxicity hazards (Radošević et al. 2016). Accordingly, various research groups have risen and focused on designing new, eco-friendly, and tuneable solvents.

Deep eutectic solvents (DESs) are one of the emerging green solvents promoted for green extraction of phytochemical compounds (Radošević et al. 2016). They consist of the combination of one or further hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA) giving a eutectic mixture (Moni Bottu et al. 2022). DESs are formed from renewable, inexpensive, and non-toxic natural components (Dai et al. 2013, 2014). In addition, they are environmentally friendly, easy to prepare, non-combustible, low volatile, miscible with water and their use does not imply further purification (Barbieri et al. 2020; Chanioti & Tzia 2018). Nonetheless, some of the DESs could present high viscosity which limits their application (Dai et al. 2013, 2014), but a controlled addition of water alleviates this drawback.

Betalains are water-soluble, nitrogen-containing plant colorants giving red-violet color for betacyanins and yellow color for betaxanthins (Kujala et al. 2002; Pavokovi & Krsnik-Rasol 2011). Betacyanins are also named betanin and contain phenolic group and cyclic amine group which strongly exhibit radical scavenging effects. Unlike betacyanins, the application of yellow betaxanthin as a pigment has not emerged due to its high sensitivity (Zin et al. 2020). Betalains are accumulated in the cell vacuoles of flowers, fruits, and leaves of plants. It has high contents of vitamins C, B_1, B_2, B_3, B_6, and B_{12}, and polyphenols (Ramírez-Melo et al. 2022). Beetroot contains carotenoids, nitrates, flavonoids and minerals (Chhikara et al. 2019). It exhibits numerous health properties including antioxidant, anti-inflammatory anti-aging, antimicrobial and anticarcinogenic properties (Chhikara et al. 2019; Nowacka et al. 2019; Pavokovi & Krsnik-Rasol 2011; Ravichandran et al. 2013). Beetroot is consumed raw and cooked, as salads, desserts, and fruit juices (Ramírez-Melo et al. 2022). It is widely used in milk and dairy products, soups, tomato paste, sauces, jelly, fruit juices, breakfast products and sausages. It also has various applications in foods such as sweets, confectionery and dry mixes (Azeredo 2009).

DESs have exhibted higher efficient for the recovery, detection and stability of phytochemical compounds as well as biological properties of many plants, byproducts and waste (Barbieri et al. 2020; Dai et al. 2014; Wei et al. 2015). In vitro digestion studies showed that the bioaccessibility of betalains was limited by food matrix and 26 to 60% of betalains, especially betacyanins or betanin, were significantly metabolized during simulated gastro-intestinal digestion (Tesoriere et al., 2004, 2008; Khan, 2016). Nonetheless, the extraction with DESs could enhance the bioaccessibility of betalains since the previous in vitro digestion studies performed on the DES-based extracts issued higher bioavailability of bioactive compounds (da Silva et al. 2021). Similarly, DES-based extracts have exhibited strong thermal, storage and light stabilties (Dai et al. 2014; Bi et al., 2020).

However, very few studies applied DESs for the recovery of betalains. A previous study successfully used DES

constituted of magnesium chloride hexahydrate and urea to extract and stabilize betalains from beetroot wastes (Hernández-Aguirre et al. 2021). Moreover, DESs of choline chloride: urea, choline chloride: glycerol and choline chloride: citric acid have been tentatively used to promote the recovery of betalains from beetroot (Demuner et al. 2023; Dias et al. 2022). However, more DESs should be tested to identify the most efficient DES capable to recover the maximum betalains from beetroot. Therefore, this study aimed to i) use ten DESs for the extraction of betalain from beetroot, ii) choose the most efficient DES and optimize the extraction conditions and iii) determine the bioaccessibility as well as heat and light stability of betalain-rich extract obtained at the optimum conditions.

Material and methods

Plant material

Detroit dark red variety of beetroots (*Beta vulgaris* L.) was used in the present study. The beetroots were harvested in December 2022 from Samsun, Turkey. Beetroots (3 kg) were washed thoroughly (3 L of water), peeled and pulped (using Aura Maxi Kitchen Robot). The pulp was preserved at -20 °C (in a freezer) in sealed bottles until analysis.

Preparation of DESs

A total of 10 DESs were prepared according to a method described previously (Zannou & Koca 2022). DESs used in the study contained two components an HBD and an HBA at 1:2 molar ratio and addition of 20% water (w/w). They were synthesised by combining HBD and HBA components at a 1:2 molar ratio and the mixture was stirred (100 rpm) at 70–80 °C until a transparent liquid formed. Choline chloride, ascorbic acid, lactic acid, and malic acid were used as the HBA and ethylene glycol, butanediol, acetic acid, glycerol, glucose, choline chloride, sorbitol, xylitol, and glucose were used as HBD (Table 1).

Characterization of DESs

Determination of density, water activity, pH, and conductivity

The density of DESs was determined by weighting 1 cm^3 of DES at room temperature (25 °C) using an analytical balance (± 0.0001) and the results were expressed as g cm^{-3} (Santana et al., 2019). Water activity was determined at 25 °C using a water activity device (4TE, AquaLab, USA). The pH was determined using a digital pH meter (Starter 3100, OHAUS, USA) in the range of 0–14. The conductivity was measured using a conductivity probe (Orion Star A215, ThermoScientific, USA) at 25 °C and the results were expressed as mS cm^{-1}. All measurements were done in triplicate.

Table 1 Composition of the prepared DESs

No	Hydrogen bond acceptor	Hydrogen bond donor
DES1	Choline chloride	Ethylene glycol
DES2	Choline chloride	Butanediol
DES3	Choline chloride	Acetic acid
DES4	Choline chloride	Glycerol
DES5	Choline chloride	Glucose
DES6	Ascorbic acid	Choline chloride
DES7	Lactic acid	Sorbitol
DES8	Malic acid	Xylitol
DES9	Ascorbic acid	Xylitol
DES10	Ascorbic acid	Glucose

The molar ratio selected for all solvents was 1:2

Viscosity determination

The viscosity of DESs was determined using a rheometer (CH-9230 Flawil 1, Buchi, Spain) according to Zannou and Koca (2022). All DESs were measured with a constant shear rate of 0.1 s^{-1} at 25 ℃ for 10 s, and the final viscosity was the average of three replicates.

Fourier transform infrared spectroscopy analysis

The functional groups of DESs and extracts were analyzed by measuring FT-IR (Spectrum-Two, PerkinElmer, USA). The extracts were embedded in KBr pellets and scans were taken with the wave number range of 4000–400 cm^{-1}.

Total betalain content

A portion (0.2 g) of beetroot pulp was mixed with 23.8 mL DESs or conventional solvents (water, methyl alcohol, and ethyl alcohol). The extraction was carried out in a shaking water bath (ST 30, Nüve, Turkey) for 30 min at 30 °C. Afterwards, the extracts were filtered through Whatman filter paper No. 1 three times and betalain content was determined using a spectrophotometer (LAMBDA 365, PerkinElmer, USA) at wavelengths of 535 and 480 nm (Mohamed et al., 2018; Chew et al., 2019). Betacyanin (BCY) and betaxanthin (BTX) content were calculated by the following equation:

$$\text{BCY or BTX}\left(\frac{\text{mg}}{\text{g}}\right) = \left[\frac{A \times D \times V \times Mw}{\varepsilon}\right] \quad (1)$$

where,

A; optical density of the extracts (535 nm for betacyanins and 480 nm for betaxanthins),

D; dilution factor,

V; final volume of extracts (mL),

Table 2 Actual and coded values of independent variables used in response surface methodology

Coded values	Actual values		
	X_1 molar ratio	X_2 water content, %	X_3 temperature, °C
-1.68	0.409	8.18	19.77
-1	0.75	15	30
0	1.25	25	45
+1	1.75	35	60
+1.68	2.09	41.81	70.22

Mw; molecular weight (BTX: 308 g mol^{-1} and BCY: 550 g mol^{-1}),

ε; Molar extinction coefficients (ε = 60,000 L mol^{-1} cm^{-1} for BCY; ε = 48,000 L mol^{-1} cm^{-1} for BTX).

The sum of BCY and BTX was considered as the total betalain content (BT).

Central composite design

Response surface methodology (RSM) was employed to ascertain the optimal extraction conditions for betalains from beetroot. The central composite design, utilizing Design-Expert software version 13.0, was executed involving three independent variables: molar ratio (X_1), water content (X_2), and temperature (X_3). The actual and coded values of these independent variables were detailed in Table 2. The responses (Y) under consideration encompassed BCY, BTX, and BT. All experimental runs were conducted in a randomized manner to mitigate the influence of systematic errors on the outcomes. Analysis of variance (ANOVA) was employed to validate the statistical significance of the regression model, and the *p* value was employed to determine the interaction effects of each variable. The second-order polynomial equation was determined for the response variables as follows:

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

where, Y is the response variable, β_0, β_i, β_{ii} and β_{ij} represent the regression coefficients of intercept, linear, quadric and interaction, respectively. X_i and X_j are the independent variables. k is a variable number.

Stability test

Stability tests

The storage stability was assessed by storing the extract obtained at the optimum conditions (1:0.75 molar, 30.835% water content, and 30 °C temperature) at 4 °C and 22 °C for 0, 3, 5, 7 and 9 days. At each set day, the samples were taken and analysed for BCY, BTX and BT content in triplicate to determine changes in concentration. For the storage in light, a modified method of Dai et al. (2014) was followed. Briefly, samples were placed in an incubator (ES 110 Incubator, Nüve, Turkey) at room temperature (25 °C) and light intensity of 3000 Lux for 0, 3, 5, 7, and 9 days. The extracts were placed at 30 cm to the source of light.

Analysis of degradation kinetics

The first-order kinetic model of the degradation of betalains was calculated with the following formula:

$$\ln\left(\frac{C}{C_0}\right) = -k.t \quad (3)$$

In the formula; C_0 is the initial betalain content (t = 0); C is the anthocyanin content at time t; t = time (seconds); k represents the degradation rate constant (s^{-1}).

The half-life ($t_{1/2}$) was calculated with the degradation rate constant using the following equation:

$$t_{(1/2)} = \frac{-\ln(0.5)}{k} \quad (4)$$

Betalain bioaccessibility

The in vitro bioaccessibility of BT was determined to be the fraction of BT that was solubilized within the mixed micelles and which became accessible for intestinal adsorption (Zannou et al. 2022). Briefly, a portion of the raw digesta was taken after simulated small intestine digestion and centrifuged with 5000 × g for 15 min at 4 °C. 3 mL of the supernatant was mixed 3 mL methanol and then centrifuged with 5000 × g for 15 min at 25 °C. Afterwards, the supernatant was collected for the determination of the total betalains, betaxanthin and betacyanin. The bioaccessibility was then determined using the following equation:

$$\text{Bioaccessibility } (\%) = (C_{\text{Micelle}}/C_{\text{Digesta}}) \times 100 \quad (5)$$

where C_{Micelle} and C_{Digesta} are the concentrations of the BT in the micelle phase and the overall digesta at the end of the in vitro digestion, respectively.

Statistical analyses

All experiments were executed in triplicate, and the outcomes were presented as the mean ± standard deviation (SD), with statistical significance considered at $p < 0.05$. The process of RSM analysis and evaluation of regression coefficients were carried out using Design Expert software version 10 (Stat-Ease Inc., USA). The adequacy of the acquired models was confirmed by assessing parameters including R^2, adjusted R^2, coefficient of variation (CV), and Fisher's test (*F*-value). Model and

Table 3 Physical properties of red beetroot samples

Parameters	Value
Color	
L^*	17.28 ± 2.10
a^*	14.51 ± 3.43
b^*	2.52 ± 0.90
Total solids, %	17.03 ± 0.22
Soluble solids, %	11.00 ± 0.00
pH	6.64 ± 0.10
Acidity, %	0.70 ± 0.00

Note: L^* represents perceptual lightness and a^* and b^* represent the four unique colors of human vision: red, green, blue and yellow

regression coefficient significance were evaluated at a threshold of $p < 0.05$. The connection between independent variables and responses was explored through 3D visualizations. Optimal conditions were determined utilizing the desirability function. Mean differences were subjected to ANOVA and the Duncan test for statistical testing.

Results and discussion

Physical and chemical properties of red beetroot

The physical and chemical characteristics of red beetroot are given in Table 3. Color values of the red beetroot were characterized as L* value 17.28 ± 2.10, + a* value 14.51 ± 3.43 and + b* value 2.52 ± 0.90. These values showed that the color features of red beetroot were dominated by brightness and redness. Similarly, a previous study reported an L* value of 17.40 for the red beetroot juice (Kayın et al. 2019). The dry matter and soluble solids of red beetroot used in the present study were found to be 17.03 ± 0.22% and 11.00 ± 0.00%, respectively. Accordingly, a study determined similar dry matter and total soluble solids as 8.80–11.0% and 8.35–9.90%, respectively (Sapronaitė et al. 2022). The pH value was determined as 6.64 ± 0.10 while the titration acidity was found as 0.70 ± 0.00% citric acid equivalent. These findings are in the same agreement with those a range pH of 6.01–6.14 and a titration acidity of 0.11–0.12% citric acid equivalent (Properties 2023).

Properties of DESs

Viscosity

The viscosities of the DESs used in the present study was displayed in Table 4. The viscosity of the detected DESs was in the range of 0.01–0.99 mPa. Malic acid-xylitol (DES 8) exhibited the highest viscosity, while choline chloride-ethylene glycol (DES 1), choline chloride-acetic acid (DES 3), and ascorbic acid-xylitol (DES 9) exhibited the lowest viscosity values. A previous study found that the viscosity decreased with increasing temperature differences (Chemat et al. 2015). In that study, the viscosities of the choline chloride-glycerol with 1:2, 1:3 and 1:4 ratios were reported to be 318.12, 338.69, and 353.52 mPa s, respectively. In the present study, choline chloride-glycerol (DES 4) exhibited a viscosity of 0.04 Pas which was lower than the value reported by previously (Chemat et al. 2015). This is potentially due to addition of 20% water during preparation. The strong hydrogen bonding network formed between the components of DESs is the main reason why DESs have high viscosity values, resulting in lower mobility of free species in the mixture (Ghaedi et al. 2017a, 2017b). Dai et al. (2015) reported that dilution with water leads to a large decrease in the viscosity of DES as a result of the gradual weakening of the hydrogen bond interactions between the components. The viscosities of eutectic blends are primarily influenced by the chemical composition of the DES constituents, which encompass salts and HBDs (Hayyan et al. 2015). Additionally, the elevated thickness of DES might result from the molar proportion of HBA and HBD. It is important to select the molar ratio properly to prevent the formation of excessively dense or saturated DES (Zannou & Koca 2022).

Table 4 Physical properties of the prepared DESs

	pH	Density (g/cm^3)	Viscosity (f in Pas)	Conductivity (µS/cm)	Water activity
DES 1	7.67 ± 0.05	1.10 ± 0.01	0.01 ± 0.00	19.59 ± 0.06	0.7351 ± 0.00
DES 2	7.41 ± 0.10	1.05 ± 0.01	0.03 ± 0.01	6.30 ± 0.00	0.8435 ± 0.01
DES 3	0.97 ± 0.03	1.09 ± 0.00	0.01 ± 0.00	17.15 ± 0.56	0.6167 ± 0.00
DES 4	4.95 ± 0.47	1.19 ± 0.02	0.04 ± 0.00	7.80 ± 0.47	0.4151 ± 0.02
DES 5	5.17 ± 0.36	1.28 ± 0.01	0.13 ± 0.01	2.71 ± 0.53	0.6250 ± 0.00
DES 6	1.21 ± 0.00	1.23 ± 0.00	0.07 ± 0.03	3.21 ± 0.40	0.4669 ± 0.00
DES 7	2.11 ± 0.04	1.29 ± 0.02	0.16 ± 0.00	12.82 ± 0.78	0.7777 ± 0.00
DES 8	1.18 ± 0.03	1.32 ± 0.02	0.99 ± 0.01	19.08 ± 0.00	0.5693 ± 0.00
DES 9	5.35 ± 0.50	1.14 ± 0.00	0.01 ± 0.00	10.06 ± 0.00	0.5749 ± 0.00
DES 10	2.87 ± 0.01	1.29 ± 0.01	0.26 ± 0.09	10.07 ± 0.00	0.5337 ± 0.00

pH

As seen in Table 3, the pH values of DESs with different components differed from each other. The highest pH value (7.67 ± 0.05) belongs to DES 1. Choline chloride-acetic acid (DES 3) was found to have the lowest pH value (0.97 ± 0.03). Alcohol-based DESs (DES 1 and DES 2) had the highest pH values. The pH values of DES (DES 6, DES 7, DES 8) in which xylitol and sorbitol were added to organic acids varied between 1.23 and 1.32 (Table 3). The presence of the organic acids lowered the pH values of DESs. A previous study determined the pH value to be 7.55 ± 0.37 in the ratio of choline chloride-ethylene glycol 1:2 (Zannou & Koca 2022). Glucose-based DESs are nearly neutral with pH values ranging around 7. As it can be concluded, the components that make up the structure of DESs directly affect the pH results of the prepared DESs.

FTIR analysis

Figure 1a, b, c, d, and e display the FTIR spectra of the investigated DESs categorized as choline chloride-alcohols, choline chloride-organic acids, choline chloride-sugars, organic acids-choline chloride, and organic acids-sugars, respectively. Evidently, the bands within the 3700–3100 cm^{-1} range are commonly associated with different hydroxyl (OH) stretching vibrations. The observed OH stretching vibrations are subject to significant influence from hydrogen bonding, thus their characteristics hinge on the potency of these hydrogen bonds. A broad hydrogen bonding, usually at wave numbers between 3550 and 3230 cm^{-1}, indicates the presence of an OH stretch band in the dense phase. Where water and alcohols are present, OH stretch bands can generally be observed at 3400 cm^{-1} (Alomar et al. 2016; del Amo-Mateos et al. 2023; Ghaedi et al. 2017a, 2017b). The peaks at 2880–3000 cm^{-1} belong to the C-H tensile bands. In Fig. 1b, d, and e, the peak at 1725 cm^{-1} wave number is related to the carboxylic acid (COO) group and confirms the presence of organic acid in the mixtures. The peaks observed at approximately 1645 cm^{-1} in Fig. 1a, c, and e correspond to stretching vibrations of C=C bonds (Zannou & Koca 2022). The peaks at 1450–1420, 1410–1400, and 1370–1320 cm^{-1} are related to CH_2 shear vibration, C–OH bending vibration, and $=CH_2$ bending, respectively (Ghaedi et al. 2017a, 2017b). In addition, moderately intense absorption patterns ranging from 800 to 1300 cm^{-1} were collectively identified as the fingerprint region of C–O–C stretching, OH bending, and CH_3 deformation (Jiang et al. 2012). Bands at 800–500 cm^{-1} were considered as O–C–O, C–O=O, O–C=O and CH deformation (del Amo-Mateos et al. 2023; Gan et al. 2010; Jiang et al. 2012).

Conductivity

Electrical conductivity is one of the fundamental physical properties that represents how well a material can conduct electric current. It is also an indicator of how resistant a material is to the movement (resistance) of electrons within its molecules (Bagh et al. 2013). The electrical conductivity of the studied DESs was given in Table 3. The conductivity varied between 2.71 and 19.59 mS cm^{-1}. DES 1 (19.59 ± 0.06 mS cm^{-1}) showed the highest conductivity, followed by DES 3 (17.15 ± 0.56 mS cm^{-1}) and DES 7 (12.82 ± 0.78 mS cm^{-1}), respectively. Those with the lowest conductivity were determined as DES 5 (2.71 ± 0.53 mS cm^{-1}) and DES 6 (3.21 ± 0.40 mS cm^{-1}). The values of the conductivity vary greatly depending on the components of DES, water content and viscosity as well as temperature. A previous study determined the conductivity value of choline chloride-ethylene glycol as 7160 mS cm^{-1} which differs from the value found for DES 1 (choline chloride-ethylene glycol) in the present study (Ibrahim et al. 2019). This difference might refer to the difference in water quantity added (Dai et al. 2015) measured lactic acid: choline chloride 1:1 ratio at room temperature as 6.76 mS cm^{-1}. (Adeyemi et al. 2018) and (Tang & Row 2013) found 7.73 mS cm^{-1}, and 7.61 mS cm^{-1} in the ratio of choline chloride-ethylene glycol 1:2, respectively. (Dai et al. 2015) found the conductivity of glycerol: choline chloride: water (2:1:1) to be 13.78 mS cm^{-1}. Regardingly, the DESs containing choline chloride had high conductivity while those containing organic acid or amino acid exhibited low values of conductivity. DES made from a polyalcohol is more conductive than sugar-based DES (Dai et al. 2015).

Water activity

Modification of HBA has no significant effect on interactions between DESs and water (Florindo et al. 2017). The a_w effect on betalain stability can be attributed to the reduced mobility of the reactants or the limited oxygen solubility (Delgado-Vargas et al. 2000). The law of the studied DESs were in Table 4. The range a_w values found in the present study was 0.4151 ± 0.02—0.8435 ± 0.01 where the lowest value was found with DES 6 (Ascorbic acid-Choline chloride) and the highest value with DES 2 (Choline chloride-Butanediol). According to Serris and Biliaderis (Serris & Biliaderis 2001), the a_w of 0.64 might induce the degradation of betanin since the a_w above 0.64 reduced the mobility of reactants. The DESs 7 (Lactic acid-Sorbitol) and 1 (Choline chloride-Ethylene glycol) gave also higher a_w values being 0.7777 ± 0.00 and 0.7351 ± 0.00, respectively. In contrast, DESs formed with choline chloride-glycerol and ascorbic acid-choline chloride provided the lowest viscosity, suggesting that the composition of DESs greatly influences their water

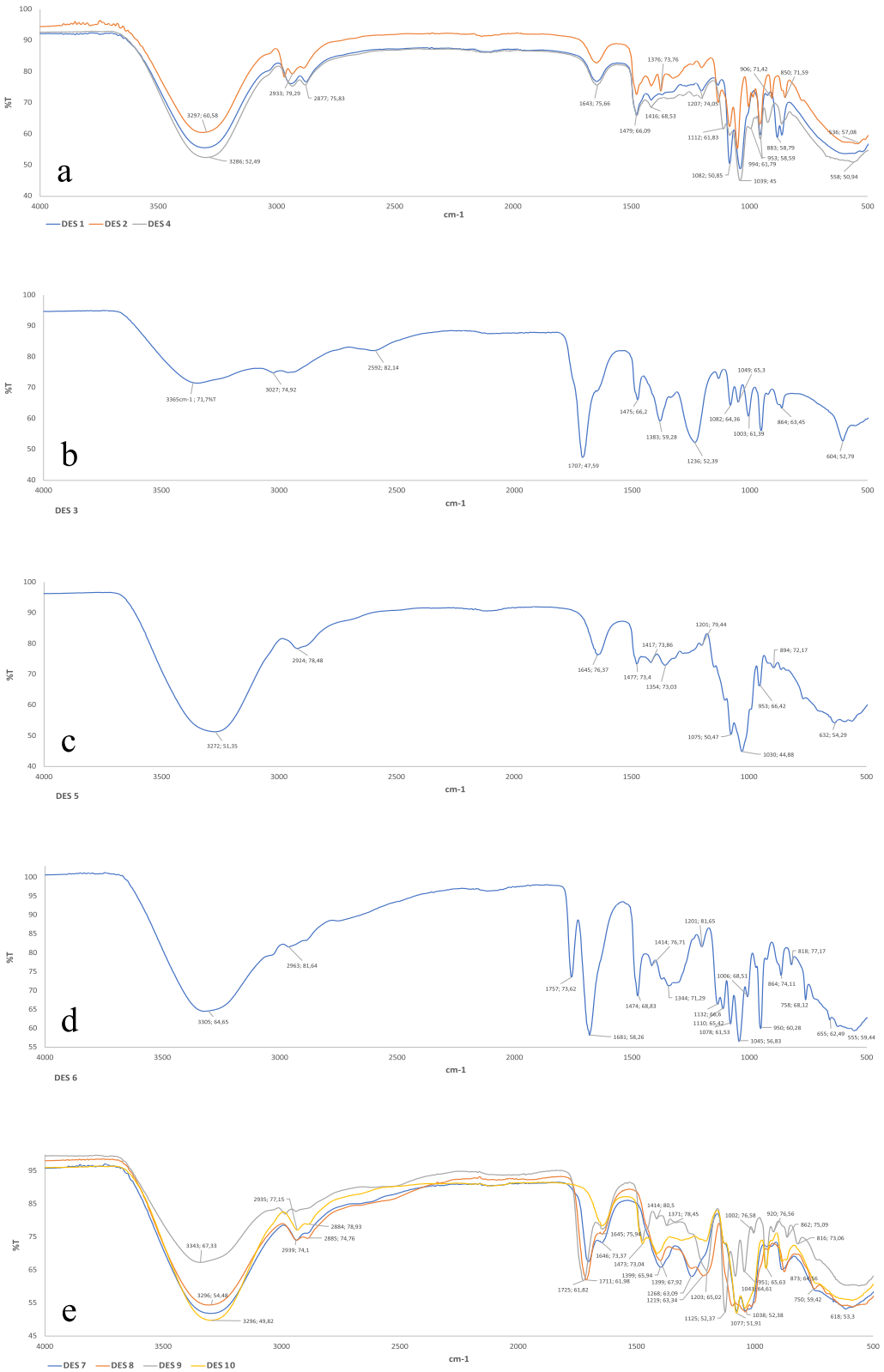


Fig. 1 FTIR spectra of DESs (wavenumber (cm^{-1}) vs. transmittance (%T)); **a**) choline chloride-alcohols, **b**) choline chloride-acids, **c**) choline chloride-sugars, **d**) acids-choline chloride, and **e**) acids-sugars

activity. A previous study found the water activity of glycerol: choline chloride: water (2:1:1), lactic acid: glucose: water (5:1:3), and xylitol: choline chloride: water (1:2:3) solutions as 0.126, 0.496, and 0.116, respectively (Dai et al. 2013). Compared with our findings, it can be assumed that the apart from the DES composition, the molar ratio affects the water activity of DES.

Density

Density measurement is essential for designing, working on, and optimizing chemical stages in mass transfer and fluid mechanics. The change in the densities of DESs is not linearly dependent on their molar ratio (A. Hayyan et al. 2013). In general, most DESs have higher densities than water (Dai et al. 2013; El Achkar et al. 2019; Haghbakhsh et al. 2019). The highest densities of DESs were determined to be 1.32 ± 0.02 g cm⁻3 (DES 8), 1.29 ± 0.02 g cm⁻3 (DES 7) and 1.29 ± 0.01 g cm⁻3 (DES 10), respectively (Table 4). Whereas, the lowest densities were found with DES 2 (1.05 ± 0.01 g cm⁻3) and DES 3 (1.09 ± 0.00 g cm⁻3). The most significant differences in the intensities of the DES variants may be due to the difference in the molecules of the components or their concentrations in the final DES mixture (Haghbakhsh et al. 2019). A previous study found the densities of zinc chloride-glucose (1:1), zinc chloride-fructose (1:1) and zinc chloride-lactic acid (1:1) as 1.002, 1.125, and 1.325 g cm⁻3, respectively (Sarjuna & Ilangeswaran 2020). Another study found the maximum density of choline chloride-ethylene glycol to be 1.12 g cm⁻3 at room temperature (Ibrahim et al. 2019). The density of choline chloride-ethylene glycol at a 1:2 ratio was determined at the range of 1.11 g cm^{-3}–1.12 g cm^{-3}, respectively (Adeyemi et al. 2018; Jafari et al. 2022; Tang & Row 2013). Due to their higher dispersion and faster mass transfer, DESs offering lower density and viscosity have higher extraction efficiency.

Betalain extraction using DESs

In this study, BT, BCY and BTX were extracted from the red beetroot using ten DESs and water, methanol and ethanol as conventional solvents. The amounts of BT, BCY and BTX were given in Table 5. As seen in Table 5, betacyanin content ranged from 23.68 to 702.17 mg kg^{-1}, betaxanthin content ranged from 21.49 to 467.77 mg kg^{-1} and betalain content ranged from 45.17 to 1169.94 mg kg^{-1}. The ethanolic extract displayed the lowest values while the DES 5 exhibited the highest values. The distilled water was more efficient than other conventional solvents, however, the DESs 1, 2 and 5 were found more efficient than all the solvents tested. Betalains are greatly sensitive the pH. The DESs 1, 2 and 5 had a pH range of 5.17–7.67 suitable for extraction and preservation of betalains. (Dias et al. 2022) reported that the betanin-containing extracts are generally relatively stable at a pH ranging from 3 to 7. For the betalain-rich extract, at a pH below 3.5, the absorption maximum shifts towards lower wavelengths, above pH 7 the change is towards upper wavelengths and outside the pH range of 3.5–7.0, the intensity of the visible spectra decreases (Azeredo 2009). A previous study determined the maximum betalain concentration (92 mg 100 g^{-1}) from *Opuntia joconostle* with methanol/water (20:80) at a pH value of 5 at 25 °C (Sanchez-Gonzalez et al. 2013). It was observed that the pH of DES 1 and DES 2 were slightly above 7, but they gave high values of betalain. This result might be associated with the strong hydrogen bonding network that the alcohol-based DESs such as choline chloride-ethylene glycol and choline chloride-butanediol generated (Zannou & Koca 2022). The findings in the present study are supported by (Wu et al. 2020) who reported the highest extraction efficiency of bioactive compounds with amide-based DESs, acid-based DESs, and alcohol-based. The lower yields of DESs 7, 8 and 10 could be related to the high the viscosity which hindered the mass transfer during the extraction. Similar to our findings, a previous study found that the DES composed of urea and magnesium chloride provided a higher yield of betalain at a 2:1 molar ratio (lower viscosity) than at 1:1 molar ratio (higher viscosity) (Hernández-Aguirre et al. 2021). Another study investigated ultrasound-assisted extraction with choline chloride-ethylene glycol from red dragon fruit peel and found that using DES in the extraction had a high kinematic viscosity (200–500 mm^2 s^{-1}) (Prajapati & Jadeja 2023). As a result, they reported that

Table 5 Betalain, betacyanin and betaxanthin contents (mg kg^{-1}) extracted using different solvents

Solvents	BCY	BTX	BT
DES 1	639.83 ± 34.46^{ab}	402.76 ± 12.07^{b}	1042.58 ± 46.38^{b}
DES 2	632.04 ± 7.33^{ab}	382.43 ± 4.81b^{c}	1014.47 ± 12.14^{bc}
DES 3	528.15 ± 24.62^{cd}	314.95 ± 11.97^{de}	843.10 ± 36.59^{e}
DES 4	374.69 ± 31.86^{e}	455.29 ± 16.55^{a}	829.97 ± 31.58^{e}
DES 5	702.17 ± 3.99^{a}	467.77 ± 21.24^{a}	1169.94 ± 20.84^{a}
DES 6	545.42 ± 87.87^{bcd}	363.61 ± 11.71^{bc}	909.03 ± 91.24^{cde}
DES 7	524.94 ± 78.42^{cd}	349.07 ± 35.69^{cd}	874.01 ± 70.97^{de}
DES 8	296.59 ± 40.86^{e}	223.76 ± 20.26^{f}	520.35 ± 60.07^{f}
DES 9	505.85 ± 12.31^{d}	301.58 ± 2.42^{e}	807.43 ± 10.74^{e}
DES 10	556.26 ± 131.82^{bcd}	356.34 ± 73.98^{bcd}	912.60 ± 205.69^{cde}
Water	606.37 ± 6.87^{abc}	390.61 ± 3.37^{bc}	996.99 ± 10.24^{bcd}
Methyl alcohol	488.12 ± 11.53^{d}	342.76 ± 6.75^{cde}	830.88 ± 17.71^{e}
Ethyl alcohol	23.68 ± 4.11^{f}	21.49 ± 1.95^{g}	45.17 ± 6.06^{g}

Note: There is no statistical difference ($p > 0.05$) between the averages shown with the same letter in the same column

high temperature and water addition reduced the viscosity for more efficient betalain production.

Optimization of operational parameters for betalain extraction

The DES 5 (choline chloride:glucose (1:2)) was considered for the optimiztion process since it was determined to be the most effective DES for the extraction of betalain from red beetroot. Three independent variables such as molar ratio, water content and temperature were used in the experimental design and the results were given in Table 6. The findings of the experimental design resulted in a total betalain content of 566.41–1262.75 mg 100 g^{-1}, betacyanin of 248.71–788.56 mg kg^{-1} and betaxanthin of 275.6–474.19 mg kg^{-1} (Table 6). The lowest total betalain content, betacyanin and betaxanthin were obtained at run 11 (1:1.75 molar, 35% water content, 60 °C temperature), run 14 (1:1.25 molar, 8.18% water content, 45 °C) and run 11 (1:1.75 molar, 35% water content, 60 °C), respectively. While the highest total betalain content, betacyanin and betaxanthin were obtained at run 8 (1:1.25 molar, 25% water content, 19.77 °C).

Table 7 displays the outcomes of the ANOVA conducted on the experimental data points. The quadratic models yielded responses that were notably well-fitting. These models exhibited significant outcomes ($p < 0.002$), and the lack of fit did not show significance ($p > 0.06$). With R^2 and adjusted-R^2 values exceeding 0.9665 and 0.9234, respectively, all these metrics collectively affirm the appropriateness of the quadratic model for effective navigation within the design space.

In general, the models for betacyanin, betaxanthin, and total betalain content exhibited a minimum of two significant linear factors. To summarize, temperature (X_3) was followed by the molar ratio (X_1) as the most influential linear factor. Among the interaction factors, the total betalain content revealed numerous significant interactions, while BCY displayed no noteworthy interaction terms. Based on the count of significant interaction terms, the interaction between molar ratio and water content (X_1X_2) emerged as the most pertinent interaction for all responses. The 3D visual representations (Fig. 2) confirmed the pronounced interactive effect of molar ratio and water content on the responses. Additionally, quadratic terms highlighted water content (X_2X_2) as the foremost contributor for all responses. The ultimate polynomial equations, expressed in terms of the coded factors, for all responses are presented as follows (Equations 6, 7, 8):

$$\text{BCY}, \frac{mg}{kg} = 514.87 - 32.72X_1 + 2653.00X_2 - 132.34X_3 - 37.90X_1X_2 - 0.43X_1X_3 - 3.91X_2X_3 + 12.26X_1^2 - 83.18X_2^2 + 2.95X_3^2 \quad (6)$$

Table 6 Independent factors and experimental results for the responses

Run	Factor 1 X_1 Molar ratio	Factor 2 X_2 Water content (%)	Factor 3 X_3 Temperature (°C)	Response 1 BCY (mg kg^{-1})	Response 2 BTX (mg kg^{-1})	Response 3 BT (mg kg^{-1})
1	1.25	25	45	501.42	365.11	866.53
2	0.75	35	30	735.63	463.77	1199.4
3	1.75	35	30	643.04	318.37	961.41
4	0.75	15	60	317.40	320.99	638.39
5	0.75	35	60	394.40	312.33	706.73
6	1.25	25	45	525.25	378.74	903.99
7	1.25	25	45	522.73	375.70	898.43
8	1.25	25	19.77	788.56	474.19	1262.75
9	1.25	41.818	45	282.50	295.49	577.99
10	1.75	15	30	558.02	365.43	923.45
11	1.75	35	60	290.81	275.60	566.41
12	0.75	15	30	508.29	433.29	941.58
13	1.25	25	70.227	348.71	331.26	679.97
14	1.25	8.18	45	248.71	331.26	579.97
15	0.409	25	45	641.90	429.92	1071.82
16	2.09	25	45	429.23	311.53	740.76
17	1.75	15	60	374.69	322.76	697.45

Table 7 ANOVA results of the optimization of betacyanins, betaxantins and total betalains extraction

	BCY			BTX			BT		
Source	**SS**	**F-value**	***p*-value**	**SS**	**F-value**	***p*-value**	**SS**	**F Value**	***p* value**
Model	403,400	22.44	0.0020	56,224.42	63.84	< 0.0001	711,900	36.85	< 0.0001
X_1	14,619.41	7.32	0.0304	14,652.11	149.74	< 0.0001	58,543.02	27.27	0.0012
X_2	9611.73	4.81	0.0644	1286.67	13.15	0.0084	3865.04	1.19	0.2216
X_3	23,920	119.73	< 0.0001	25,450.85	260.10	< 0.0001	420,700	195.97	< 0.0001
X_1X_2	11,491.28	5.75	0.0476	1683.16	17.20	0.0043	21,970.27	10.23	0.0151
X_1X_3	1.48	0.0007	0.9791	3973.86	40.61	0.0004	3822	1.78	0.2239
X_2X_3	12,739.27	6.38	0.0395	192.47	1.97	0.2035	16,063.49	7.48	0.0291
X_1^2	1694.81	0.8483	0.3877	108.29	1.11	0.3277	946.26	0.44	0.5280
X_2^2	78,007.20	39.05	0.0004	6160.02	62.95	< 0.0001	128,000	59.63	0.0001
X_3^2	6468.23	3.24	0.1150	760.68	7.77	0.0270	11,665.64	5.43	0.0525
Residual	13,985.14			684.95			15,027.18		
Lack of Fit	13,642.36	15.92	0.0602	582.56	2.28	0.3329	14,209.92	6.95	0.1305
Pure Error	342.78			102.39			817.26		
Corr Total	417,400			56,909.3			726,900		
R^2	0.9665			0.9880			0.9793		
Adj R^2	0.9234			0.9725			0.9527		
Pred R^2	0.7498			0.9132			0.8488		
Adeq precision	16.6336			25.9465			21.1313		
C.V. %	9.37			2.75			5.54		

$$\text{BTX}, \frac{\text{mg}}{\text{kg}} = 73.83 - 32.75X_1 - 9.71X_2 - 43.17X_3 - 14.50X_1X_2 + 22.29X_1X_3 - 4.90X_2X_3 - 3.10X_2^1 - 23.38X_2^2 + 8.21X_3^2 \quad (7)$$

$$\text{BT}, \frac{mg}{kg} = 888.70 - 65.47X_1 + 16.82X_2 - 175.51X_3 - 52.41X_1X_2 + 21.86X_1X_3 - 44.81X_2X_3 + 9.16X_1^2 - 106.56X_2^2 + 32.17X_3^2 \quad (8)$$

Multi-response of RSM

Optimum conditions were determined based on the desirability function. The optimum conditions were found to be 1:0.75 molar, 30.83% water content, and 30 °C temperature for molar ratio, water content, and temperature, respectively. Under these conditions, the predicted responses for total betalain, betacyanin, and betaxanthin were determined as 1223.13, 748.27, and 474.86 mg kg^{-1}, respectively. Experiments were carried out in triplicate under the same optimum conditions to verify the theoretical values. The experimental optimum results for betalain, betacyanin and betaxanthin were found to be 1192.17 ± 23.63, 738.83 ± 17.87, and 453.34 ± 5.93 mg kg^{-1}, respectively. As can be seen, the estimated and experimental values were found to be very close, indicating that the response surface methodology applied is reliable and reproducible. A previous study reported a total betalain content of 3.65–3.99 mg g^{-1} using a DES composed of urea and magnesium chloride (Hernández-Aguirre et al. 2021). These values are higher than our findings since different DESs were used. However, the results found in the optimum points are in the same line as those by another study which used choline chloride-urea (1:2), choline chloride-glycerol (1:2) and choline chloride-citric acid (1:2) being 41.27–67.51, 82.46–104.45 and 50.06–111.93 mg 100 g^{-1}, respectively (Dias et al. 2022).

According to their chemical structure, it is relatively simple to extract betalains. The present study revealed that the betalain counted for 62.81% betacyanins and 37.19% betaxanthins. These results are found close to the previous study which stated that the beetroots have

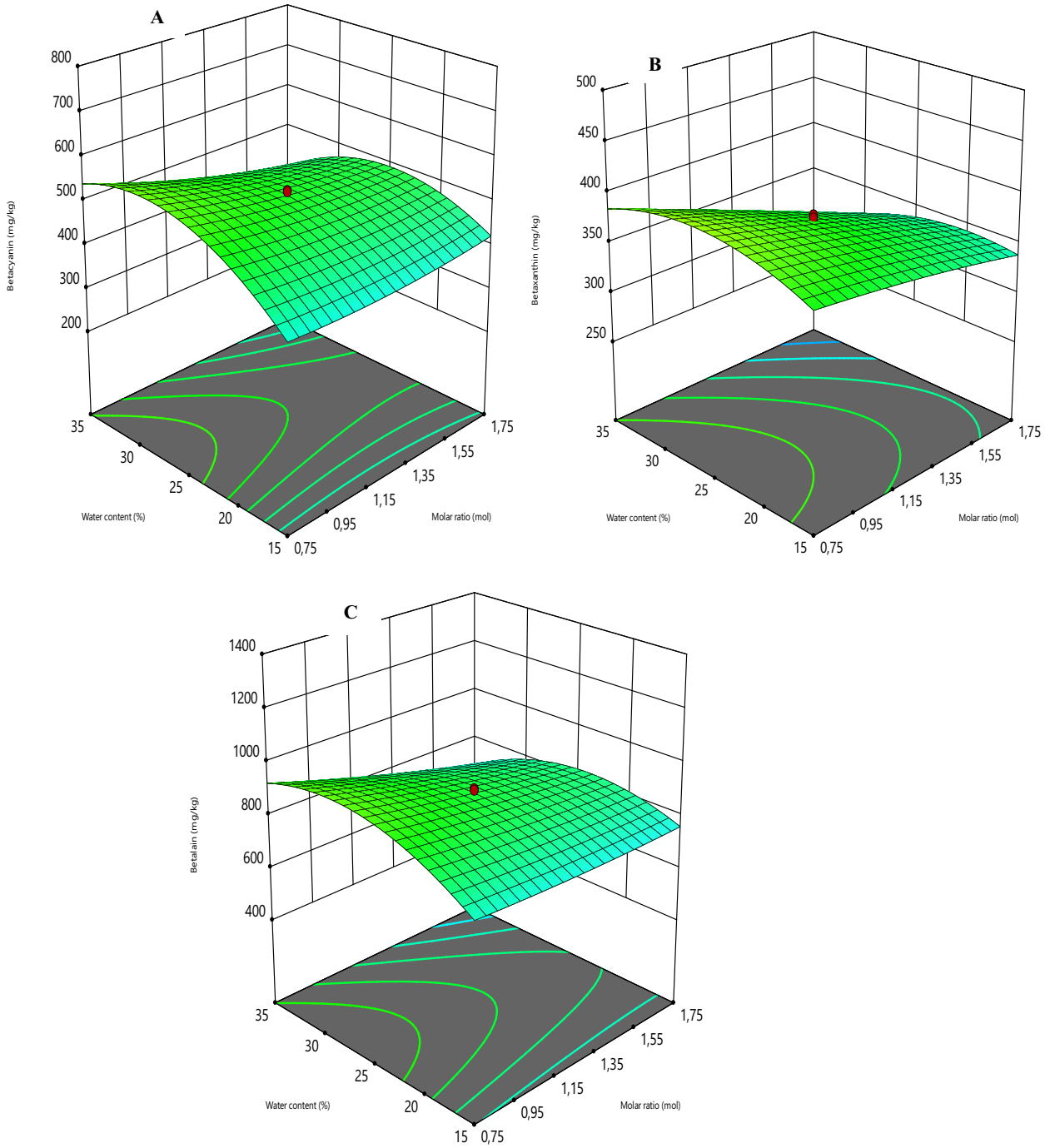


Fig. 2 3D Graphics of betacyanin **A**, betaxanthin **B** and betalain **C**

approximately 75–95% betacyanins and 5–25% betaxanthins (Delgado-Vargas et al. 2010). This suggests that DES exhibited compatibility in the recovery of betalains. Structurally, the major betacyanin compound (betanin) is betanidin 5-O-β glucoside with a cyclic amine group and a phenolic group, acting as extremely excellent electron donors (Fu et al. 2020). Thus, betalains might behave as the HBD in the extraction medium, allowing the DES to embrace them and facilitate their recovery during the extraction.

Table 8 Characteristics of DES obtained under optimum conditions

Properties	Choline chloride:glucose (1:0.75)
pH	7.39 ± 0.02
Density (g cm^{-3})	1.24 ± 0.00
Viscosity (f in Pas)	0.04 ± 0.00
Conductivity (μS cm^{-1})	6.52 ± 0.00
Water activity	0.65 ± 0.00

Characteristics of optimum DES

The DES obtained under optimum conditions was characterized for the pH, density, viscosity, electrical conductivity and water activity (Table 8). DES at the optimum conditions had 1:0.75 molar and 30.835% water content. The pH, viscosity, conductivity, density and water activity were recorded as 7.39 ± 0.02, 0.04 ± 0.00 mPa, 6.52 ± 0.00 μS cm^{-1}, 1.24 ± 0.00 g cm^{-3} and 0.65 ± 0.00 (Table 8), respectively. As seen in the results, the change in the molar ratio of HBD and the water content showed a significant effect on the characteristics of DES. The increase in the amount of water added to DES led to a decrease in the viscosity and an increase in the conductivity. The further addition of water to the DES provoked the movement of DES molecules and ions availability.

FTIR spectra of DES and extract obtained at the optimum conditions were shown in Fig. 3. In the FTIR spectrum of the extract, the band at 3356 cm^{-1} was associated with the stretching vibration of the –OH bond in betalain chemical structure while the band at 1643 cm^{-1} was for the stretching vibration of the C=N bond (Aztatzi-Rugerio et al. 2019; Kumar et al. 2017). The band at 1372 cm^{-1} is assigned to the elongation-strain vibration of the C–H bond while the band centered at 1241 cm^{-1} corresponds to the stretching vibration of the C–O bond of the carboxylic acid. Another band at 1073 cm^{-1} was attributed to the symmetrical stretching vibration of the C–O–C junction. FTIR spectra of both the optimum DES and optimum extract were found to be very similar, suggesting the sample was inlaid in the solvents. Nonetheless, the band at the peak of 3256 cm^{-1} presented higher intensity for the extract, while at the peak of 1643 cm^{-1}, the extract was weaker than the optimum DES.

Effect of storage on betalain extract obtained under optimum conditions

The storage stability of the extract obtained under the optimum condition was evaluated for 9 days at room temperature (20 °C) and in light (30 cm from the light source; from a 3000 Lux) at room temperature in an incubator. The results of the betalain losses during the storage were given in Table 9. As can be seen, the betalain losses in the dark at room temperature were determined as 29.17, 41.75, 50.69, and 59.47% for the 3rd, 5th, 7th, and 9th days, respectively. Whereas, losses in light were 70.57, 78.74, 82.34, and 84.90% on the 3rd, 5th, 7th, and 9th days compared to day 0, respectively. It can be evidenced that betalain extracts exposed to light deteriorated more quickly than those in the dark at room temperature. This suggested that betalains of red beetroot even in DES are less stable in the light system than in the dark. It has been previously mentioned that the exposure of betalain pigments to light leads to an excitation of the electrons of the betalain chromophore, affecting their stability (Dos Santos et al. 2018). Furthermore, many factors such as oxygen, temperature, pH, light, and food matrices were determined to affect the stability of betanins (dos Santos et al. 2018). A previous study observed that the betalain content of beet extract stored at 100 °C deteriorated even before the third day of storage (Mohammed et al. 2021). Upon 9 days of storage, it was observed that betacyanins were slightly less stable in both dark and light than betaxanthins. In contrast, another study reported that betacyanins are more stable than betaxanthins both at room temperature and when heated (Azeredo 2009). This fact might be related to the DES used in the present study.

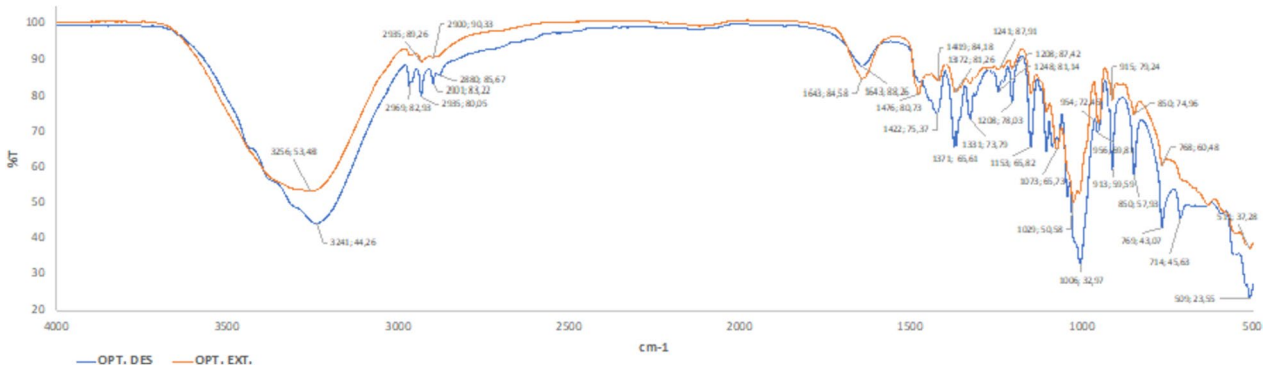


Fig. 3 The FTIR Spectrum of DES and extract at optimum conditions

Table 9 Percentage of loss (%) of BCY, BTX and BT in DES extract in different conditions throught the storage period

Compounds	Condition	Storage (days)			
		3	5	7	9
BCY	Dark	34.13	47.04	56.26	64.38
	Light	72.75	81.69	85.30	87.16
BTX	Dark	21.05	33.11	41.58	51.42
	Light	66.97	73.89	77.51	81.24
BT	Dark	29.17	41.75	50.69	59.47
	Light	70.57	78.74	82.34	84.90

It seems that DES preserved more betaxanthins than betacyanins.

The kinetic rate constant (k) and half-life ($t_{1/2}$) values of betalains degradation during the storage were shown in Table 10. As can be seen, the degradation of betalains increased with increasing lightness. The kinetic rate constant (k) values for the degradation of total betalain content, betacyanin and betaxanthin were higher in light than in the dark, confirming the adverse effect of light on betalains. The lowest values were obtained with betaxanthin suggesting it was more protected by DES. Similar to our results, previous studies demonstrated that the degradation of betalains from quinoa skins, purple pitaya and beetroot follows a first-order kinetic model (Herbach et al. 2004; Yang et al. 2021). The $t_{1/2}$ values of betacyanins varied from 5.58 ± 0.47 to 8.76 ± 0.18 days in the dark and from 2.29 ± 0.62 to 2.86 ± 0.81 days in the light. It was remarked that the storage in light reduced the half-life of betalains by 3–3.5 times compared to the storage in dark.

Bioaccessibility of DES red beetroot betalains

The in vitro bioaccessibility of BT was determined as 56.21 ± 1.33% while the highest value was found with BTX (75.02 ± 1.20%). The in vitro bioaccessibility of BCY was 44.67 ± 1.40%. These findings are supported by a previous study which reported that the in vitro recreational of betanin degradation in the small intestine, stomach, and mouth stages revealed approximately 50% loss of these pigments (Hussain et al. 2018). Moreover, it has been reported that betanin absorption occurs mainly in the gut (Khan 2016). The bioaccessibility and bioavailability of betalains, betanin, and indicaxanthin plays a key role in health by improving the redox state of the human body (Gandía-Herrero et al. 2016). Contrary to the findings of the present study, the study by (Hussain et al. 2018) and (Moreno et al. 2008) mentioned that the bioavailability of betanin and isobetanin was in the range of 0.28–0.90% after ingestion of beetroot juice. The high in vitro bioaccessibility of betalains found in the present study suggested that DES exerted a protective effect on the betalains. These results are found in the same agreement with the previous studies where DESs prevented bioactive compounds from degradation throughout the simulated digestive tract (da Silva et al. 2021).

Table 10 Effect of the light factor on the *k* and $t_{1/2}$ values of betalains degradation in DES extract

Compounds	k value (day^{-1})		$t_{1/2}$ (day)	
	Dark	Light	Dark	Light
BCY	0.12 ± 0.01	0.32 ± 0.09	5.58 ± 0.47	2.29 ± 0.62
BTX	0.08 ± 0.00	0.26 ± 0.08	8.76 ± 0.18	2.86 ± 0.81
BT	0.11 ± 0.00	0.29 ± 0.08	6.55 ± 0.41	2.51 ± 0.69

Conclusion

In this study, DES was used to investigate the betalains from red beetroot in comparison with conventional solvents such as methanol, ethanol and distilled water. The results showed that distilled water was more efficient than other conventional solvents, however, DES 1 (choline chloride: ethylene glycol), DES 2 (choline chloride: butanediol) and DES 5 (choline chloride: glucose) were found more efficient than all the solvents tested. DES 5 was found as the most prominent and was then chosen to undergo the optimization process. Optimum extraction conditions were determined as 1:0.75 molar, 30.835% water content, and 30 °C temperature. Experiments were performed in triplicate under optimum conditions and the results of betalain, betacyanin, and betaxanthin were 1192.17 ± 23.63, 738.83 ± 17.87, 453.34 ± 5.93 mg kg^{-1}, respectively. From the findings of the storage stability in dark and light, the exposure of the extracts to light provoked greater loss of betalain. The in vitro bioaccessibility of the total betalain content was determined as 56.21 ± 1.33%. These findings revealed that DESs are green and effective alternatives to obtain biocompatible extracts selectively enriched in bioactive compounds.

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Authors' contributions
Beyza Kaba: Conceptualization, Methodology, Investigation, Software, Data curation, Writing – original draft. Oscar Zannou: Conceptualization, Methodology, Investigation, Software, Data curation, Writing – original draft. Ali Ali Redha: Validation, Writing – review & editing. Ilkay Koca: Conceptualization, Methodology, Investigation, Data curation, Visualization, Supervision, Software, Validation, Writing – review & editing.

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Availability of data and materials
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Declarations

Ethics approval and consent to participate
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Consent for publication
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Competing interests
All authors declare no conflict of interest.

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