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Ultrasound-assisted extraction of active compounds from *Beta vulgaris* using deep eutectic solvents

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Abstract

The aim of this study is to investigate the application of ultrasound in the extraction of betanin from beets (*Beta vulgaris*) using Deep eutectic solvents (DES). The extraction process was optimized using response surface methodology. The time variable was found to be correlated with the extraction performance. Three DES (choline chloride: urea (CC:U), choline chloride: glycerol (CC:G), and choline chloride: citric acid (CC:Ac)) were studied. The concentration of betanin extracted with CC:U, CC:G, and CC:Ac was 41.27-67.51, 82.46-104.45, and 50.06-111.93 mg/100 g. The maximum betanin extraction of 111.193 mg/100 g was achieved with CC:Ac in 38 min using 44% DES in water in an ultrasonic bath. These results demonstrate that DESs are excellent solvents for the extraction of betanin with superior efficiency to conventional extraction solvents. Ultrasound significantly improved the efficiency of betanin extraction from beets. However, a higher betanin content in the extracts did not translate into greater active antioxidant capacity, which may be related to the synergistic effects of other compounds present in the beet extracts. This study is the first attempt to optimize the parameters for ultrasound extraction of betanin from *B. vulgaris* using eutectic solvents.

Keywords: ultrasound; green solvents; red beet root; antioxidant.

Practical Application: Natural deep eutectic solvents (DES) are excellent sustainable solvents for the extraction of betanin. In combination with ultrasound with DES preserved and contribute to stabilize the betanin profile of the food due to its antioxidant capacity, preventing losses in nutritional quality and have high technological application as antioxidants and natural food additive in industry.

1 Introduction

Beta vulgaris (Chenopodiaceae), popularly known as beet, is a vegetable that has attracted the interest of the population due to its health benefits. These benefits are derived from the presence of bioactive compounds, including betalains, ascorbic acid, carotenoids, polyphenols, flavonoids, and saponins (Chhikara et al., 2019; Goldman & Janick, 2021).

Betalains are heterocyclic, nitrogenous, and water-soluble compounds that exhibit bioactive potential owing to their high free radical scavenging activity (Slimen et al., 2018). Betalains also have therapeutic properties for preventing diseases such as hypertension, dyslipidemia, cancer, neurological disorders, and vascular stenosis (Rahimi et al., 2019). Studies have reported that betalains are responsible for the pigmentation of beets, and red beetroot powder is an important ingredient in instant beverages for athletes, acts as a natural color enhancer for food products (Ng & Sulaiman, 2018), and is used as an alternative to nitrite or other colorants in meat products (Sucu & Turp, 2018). According to their chemical structure, these pigments can be subdivided into red-violet betacyananins and yellow betaxanthines. This study aims to investigate betanin (CI Natural Red 33, E-number E162, and betanidin 5-*O*-β-glucoside).

The extraction method is important for obtaining betanin a with the desired quality and quantity. Finding an extraction method that avoids the use of conventional volatile organics is a challenge. Generally, the procedures for extracting bioactive compounds from plant species use volatile, toxic organic solvents, often with carcinogenic properties, resulting in low extraction yields and harmful environmental impacts due to the generated residues (Cunha & Fernandes, 2018).

To solve these problems, several studies have been carried out on the organic chemistry of natural products, developing solvent formulations that are less harmful to the environment, and replacing conventional organic solvents in the extraction of bioactive substances from plant species (Belwal et al., 2018; Chanioti & Tzia, 2018; Choi & Verpoorte, 2019; Santoso et al., 2022).

Ultrasound is a key technique for achieving the goal of sustainable "green" chemistry, where its use can reduce the extraction time, energy consumption, solvent, and post-treatment compared to conventional procedures (Chemat et al., 2017; Rocha et al., 2017; Alarcon-Rojo et al., 2019; Chen et al., 2020; Monteiro et al., 2022; Wang et al., 2022).

Demirci et al. (2022), working with *Berneries crataegina* DC, demonstrated that ultrasonic waves can efficiently improve the extraction of bioactive compounds. This study suggested that anthocyanin content could be increased at 100% ultrasound power

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at 40 kHz, 258 W and 40 and 60 °C and Wang et al. (2022), observed that the use of ultrasound in the extraction of polysaccharides in fig leaves, the yield increased with the increasing ultrasonic power, and the highest yield was 5.05% at 250 W.

Deep eutectic solvents (DES) are a class of "green" solvents prepared from eutectic mixtures (Cunha & Fernandes, 2018; Lee et al., 2019). They are prepared by mixing two or more components, a hydrogen bonding receptor (HBR), and a hydrogen bond donor (HBD), in different proportions (Roy et al., 2021; Kucan & Rogosic, 2019). The most common DES are formed using choline chloride, a non-toxic quaternary ammonium salt, as the HBR, together with natural uncharged compounds such as alcohols, amines, carboxylic acids, sugars, and vitamins such as DLH (Ruesgas-Ramon et al., 2017). These compounds have good prospects for wider use in green extraction technologies (Belwal et al., 2018; Zainal-Abidin et al., 2017; Procentese et al., 2018).

There are no documented reports on the optimization of betanin extraction from *Beta vulgaris* using a DES, assisted by ultrasound. In this context, the originality of the present study is the development and optimization of the betanin extraction method using a DES and ultrasound by employing response surface methodology, aiming at a higher extraction yield.

2 Materials and methods

2.1 Instruments and chemicals

Choline chloride, citric acid, glycerol, urea, acetic acid, acetonitrile, phosphate buffer, potassium ferrocyanide, trichloroacetic acid, iron(III) chloride, 2,6-di-*tert*-butyl-4-methylphenol (BHT), 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH), ethanol, and betanin standards were acquired from Sigma-Aldrich.

2.2 Plant material

Fresh beets (*Beta vulgaris*) were purchased at an organic products fair in Viçosa, Minas Gerais, Brazil. The genetic patrimony/CTA of *Beta vulgaris* was registered in SisGen No. A61E5D4. Beets (100 g) were weighed, cleaned, crushed using a food processor, and subjected to lyophilization to obtain a fine powder that was packed in plastic bottles in a desiccator at room temperature.

2.3 DES preparation

Three deep eutectic solvents were prepared. Choline chloride as the HBR was combined with citric acid, glycerol, or urea as the HBD. Initially, choline chloride was weighed in a beaker and the HBD was added, according to the previously established molar ratios (see Table 1). The reagents were homogenized using a glass stick. The mixture was placed in a glycerin bath stabilized at 70 °C, and the bath rotation was fixed at 300 rpm. The eutectic mixture was kept in a bath under magnetic stirring until a clear and homogeneous liquid was formed. Finally, the DES was removed from the bath and stored in an amber bottle in a desiccator.

Table 1. Combinations of components of DES for extraction method.

Sample name	Component 1	Component 2	Molar ratio
CC-Ac	Choline chloride	Citric acid	1:2
CC-G	Choline chloride	Glycerol	1:2
CC-U	Choline chloride	Urea	1:2

2.4 Ultrasound-assisted extraction

Ultrasonic extraction experiments were performed using an ultrasonic probe (Elma Sonics P180H) with the temperature controlled at 35 °C, using a frequency of 37 kHz, and amplitude of 100% W. The material was filtered and an aliquot was analyzed using high-performance liquid chromatography (HPLC).

2.5 HPLC analysis

The extracts were analyzed using HPLC with ultraviolet (UV) detection for quantification of betanin, using the apparatus Shimadzu model QP5050, bomb LC10AD, a C18 column (25 cm x 2.5 cm). Gradient elution was carried out using two mobile phases: phase A comprised water with 0.5% v/v acetic acid and phase B comprised 40% v/v acetonitrile/water with 0.5% v/v acetic acid. The analysis was performed with a linear gradient of 5% to 95% B over 40 min, flow rate: 1 mL min^{-1} and temperature of approximately 23 °C. The UV-Vis absorption of the samples at 536 was monitored. To quantify betanin, an external standard curve was constructed by injecting the standard at different concentrations. A betanin standard (Sigma-Aldrich) was used to construct the analytical curve. The analytical curve was prepared from the absorbance of successive dilutions of a 1.0 mg mL^{-1} stock solution. The concentrations used to construct the analytical curve were 1.0, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, 0.0125, and 0.00625 mg mL^{-1}. For the quantification of betanin, the equation of the line was $y = 204868x + 236.46$, with a correlation coefficient (r^2) of 0.99.

2.6 Extraction and concentration of betalain pigment

In a test tube, lyophilized beet powder (0.1 g) was combined with the DES and water in the proportions specified in Table 2. Subsequently, the samples were subjected to extraction by ultrasound. The extracted solution was then filtered and immediately subjected to HPLC analysis.

2.7 Experimental design

The extraction process was optimized using response surface methodology. A central composite planning with two factors was used, consisting of four factorial tests obtained from the combination of levels +1 and −1, five repetitions at the central point denoted by the levels (0.0), and four tests on the axial points obtained from the combinations of the levels ($\pm \sqrt{2}$, 0) and (0, $\pm \sqrt{2}$), with the time (X1; min) and proportion of deep eutectic solvent in water (X2; %: v/v) as the independent variables.

2.8 Mathematical model

The response surface models were adjusted to generate second-order polynomial equations. The response function (Y)

Table 2. Range of design variables.

Test	Coded variable		Original variable	
	X1	X2	t (min)	Conc. DES in water (%)
1	-1	-1	16	10
2	+1	-1	60	10
3	-1	+1	16	30
4	+1	+1	60	30
5	$-\sqrt{2}$	0	7	20
6	$+\sqrt{2}$	0	69	20
7	0	$-\sqrt{2}$	38	6
8	0	$+\sqrt{2}$	38	44
9	0	0	38	20
10	0	0	38	20
11	0	0	38	20
12	0	0	38	20
13	0	0	38	20

X1 = time variable (min.); *X2* = solvent concentration variable (%).

was divided into linear, quadratic, and interaction components, as follows (Equation 1):

$$Y = b_0 + b_1x_1 + b_2x_2 + b_{11}x_{11} + b_{22}x_{22} + b_{12}x_1x_2 \quad (1)$$

where b_0 is the constant coefficient; b_1 and b_2 are the linear coefficients; b_{11} and b_{22} are the quadratic coefficients; b_{12} is the interaction coefficient; x represents the independent variables or factors; and *Y* represents the dependent variable or response. Regression coefficients were obtained by multiple linear regression (RLM) using the least squares method. These coefficients were statistically evaluated for their significance and interpreted according to their importance to the system.

2.9 Statistical analysis

Analysis of variance (ANOVA) was used to verify the validity of the adjusted models. Response surface plots were obtained from the values estimated using the fitted models. The F-test for lack of adjustment was performed when evaluating the adjusted models. The Student's t-test was used to test the significance of the regression coefficients. All calculations were performed using Statistica 7.0.

2.10 Antioxidant activity

An aliquot of each sample (1.0 mL) was transferred to a 25 mL test tube. The sample was combined with 2.5 mL of 0.2 mol L^{-1} phosphate buffer (pH 6.6) and 2.5 mL of 1% m/v potassium ferrocyanide ($K_3[Fe(CN)_6]$). The mixture was incubated at 45 °C for 20 min, and 2.5 mL of 10% w/v trichloroacetic acid was added to the solution in the test tube with subsequent stirring. A 2.5 mL aliquot of the mixture was transferred to another test tube, to which 2.5 mL of Milli-Q water and 0.5 mL of 0.1% w/v $FeCl_3$ were added with stirring. The absorbance was measured at 700 nm. The readings were performed in triplicate; the absorbance of the 2,6-di-*tert*-butyl-4-methylphenol (BHT) standard was used to indicate 100% activity. DPPH (1 mL of 0.5 mmol L^{-1}) was added to 4 mL of the sample, equally diluted in ethanol. The mixture was then packed in an amber test tube and stirred. After 30 min, the absorbance was measured at 517 nm.

3 Results and discussion

3.1 Effects of operational variables on extraction of betanin

The experimentally determined betanin contents for each set of combination of variables and DES used in this study are presented in Table 3.

The concentration of betanin extracted with CC:U, CC:G, and CC:Ac was 41.27-67.51 82.46-104.45, and 50.06-111.93 mg/100 g, respectively. The maximum betanin extraction of 111.93 mg/100 g was obtained with CC:Ac in 38 min. The lowest concentration of betanin (50.06 mg/100 g) was obtained with the highest percentage of solvent (44%), indicating no relation of the extraction efficiency to the percentage of solvent used. However, the time required for contact with the ultrasonic bath was only 69 min. In the extractions with CC:G, the highest concentrations of betanin were 104.45 and 102.26 mg/100 g, both with the longest extraction times. The extractions with CC:U also afforded similarly high concentrations with long extraction times. The extractions were reproducible, with good repetition at the central point and a lower error in the estimation of the coefficients.

3.2 Response surface analysis

To optimize the conditions for betanin extraction, the results were subjected to regression analysis, and a polynomial equation was derived using the significant values of the regression coefficients estimated in Table 4. The adequacy of the model was verified by comparing the experimental and predicted values using ANOVA. The values were statistically acceptable within the 95% confidence level. Three-dimensional plots were constructed to show the variation in the betanin extraction as a function of the time and extractor concentration.

Table 3. Effect of the experimental conditions on the yields of betanin (mg/100 g).

Test	Original variables		Deep eutectic solvents		
	Time (min)	Solvent concentration (%)	CC-Ac	CC-G	CC-U
1	16	10	80.01	96.99	41.84
2	60	10	51.68	90.72	52.49
3	16	30	72.72	94.09	55.87
4	60	30	88.36	104.45	67.51
5	7	20	105.70	90.43	59.89
6	69	20	50.06	102.26	62.14
7	38	6	61.58	82.93	51.11
8	38	44	111.93	82.46	60.75
9	38	20	86.66	92.18	50.58
10	38	20	89.06	92.90	43.11
11	38	20	87.75	92.85	41.27
12	38	20	90.95	91.62	42.44
13	38	20	88.43	93.66	42.39

Table 4. Regression coefficients.

Coefficient	Estimated values of the coefficients for the different extractors and their errors					
	CC-Ac		CC-G		CC-U	
	Value	Error	Value	Error	Value	Error
b_0 (average)	92.3	± 2.00	96.9	± 1.92	136	± 9.33
b_1 (time)	-0.417	± 0.109	-0.744	± 0.0602	-2.71	± 0.292
b_2 (conc.)	Not signif.	-	0.574	± 0.109	-3.62	± 0.529
b_{11} (time)^2	-0.0143	± 0.00127	0.00637	± 0.000621	0.0223	± 0.00301
b_{22} (conc.)^2	-0.0163	± 0.00229	-0.0262	± 0.00171	0.0406	± 0.00829
b_{12} (time)x(conc.)	0.0494	± 0.00287	0.0189	± 0.00176	0.0466	± 0.00854

Significance at $p < 0.05$.

By applying the regression equation for CC:Ac as the extractor (Table 4), the extraction time and extractor concentration, when interpreted in isolation, appeared to contribute negatively to the response when their levels were increased. In the joint interpretation of the variables, a synergistic interaction was observed, which caused an increase in the response. This fact can best be verified from the graph of the surface concentration versus time shown in Figure 1a, which provides the contours of the responses obtained through the predicted values of the adjusted models, where it is possible to observe the effect of the interaction between the variables. It can be concluded that when the levels of the variables are increased simultaneously, betanin extraction is maximized when the proportion of solvent is 45% and the time is 40–60 min.

When CC:G was considered as the extractor (Table 4), the behavior was similar to that obtained using CC:Ac with respect to the extraction time, because the isolated action of time does not promote an increase in the extraction. The high value of the coefficient b_2 (concentration) when analyzed in isolation contributes to an increase in the response; however, this increase in the response only occurs when the value of this variable is relatively low. For higher concentration values, the response tends to decrease because of the negative value of b_{22} (concentration)^2. The effects of the variables, when interpreted together, indicate synergistic interactions that promote an increase in the response. Figure 1 provides a visualization of these effects. When the levels of the time and concentration variables were increased simultaneously, the best extraction was achieved with: 20% < concentration 40% and 60 min; 60% and 69 min, affording betanin extractions of 100-110 mg/100 g.

However, the acidity/basicity of the medium compromises its stability. Water was deemed the best extractor solvent because of its high polarity. Betacyanins precipitate in acidic medium to form betaxanthins, where the extraction performed with acidic solvents was inefficient compared to that with neutral solvents. By adding other solvents to water (such as extractions with ethanol and ethanol/water 50%), the polarity was modified, thus increasing the concentration of extracted betanin. In the extracts obtained with the DES, CC:Ac afforded a higher betanin concentration due to the pH of the extractor solution, which stabilized the compound, while DES CC:G presented similar values. CC:U DES extracted a smaller amount of betanin than the other DES, owing to its basicity.

The use of CC:U as the extractor produced an unusual result (Table 4), where the linear coefficients b_1 (time) and

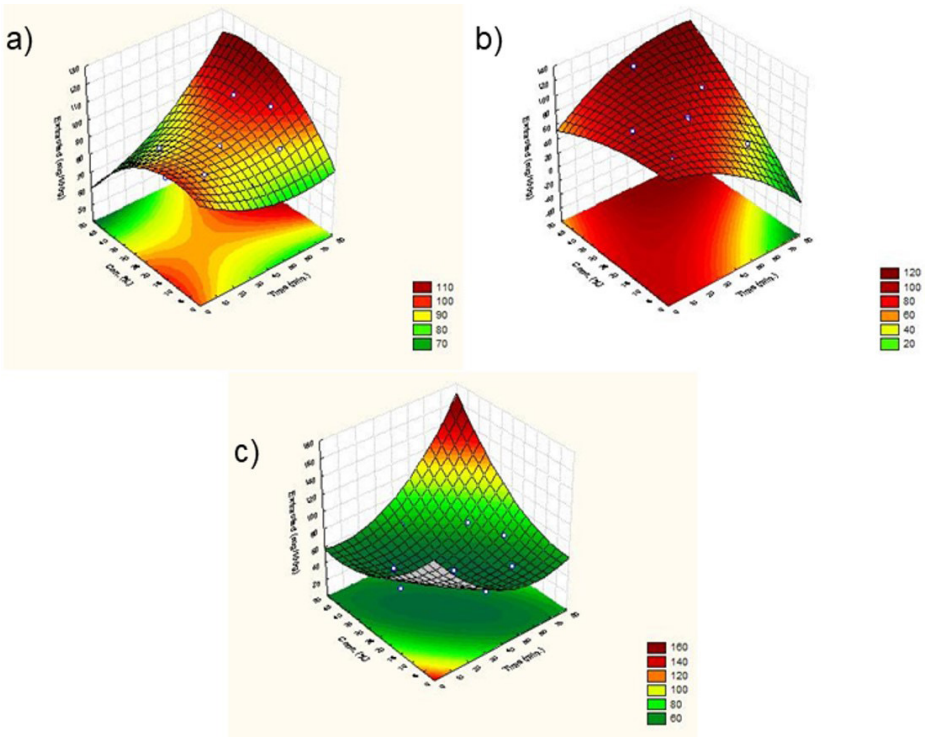

Figure 1. Response surfaces for the interactions of independent variables (C: concentration of DES in water; t: extraction time) on (a-c) betanin yield.

b_2 (concentration) had high and negative values, respectively, with low extraction values around the central point. The extraction increased on moving away from the central point, described by the quadratic coefficients and by the synergistic interaction between the variables. The behavior of the system can be better understood by the graphical analysis in Figure 1c. The minimum extraction was observed in the central region of the experimental space, followed by a small increase towards the limits of the investigated levels. Thus, the best results provided by the equation were found around the points defined by (16 min, 10%) and (16 min, 30%).

3.3 Antioxidant activity

Elimination of the stable free radical DPPH and analysis of the reducing power were used to investigate the potential antioxidant properties of the extracts obtained by ultrasound. The antioxidant activity of the extracts followed the order: CC:G > CC:U > CC:Ac. The highest antioxidant capacity was achieved with the lowest concentration of CC:G, both in the radical elimination test using DPPH (77.05 ± 0.08) and in the inhibition of lipid oxidation test (63.86 ± 0.12).

The extracts obtained with CC:U and CC:Ac exhibited similar antioxidant capacity, with values of 51.75 ± 0.05 and 51.00 ± 0.02, respectively, for elimination of DPPH radicals and values of 57.72 ± 0.13 and 60.17 ± 0.03 in the reducing power test. For the DPPH assay using betanin, Pearson's correlation coefficient (r) was 0.93, whereas for the reducing power test, $r = 0.81$. The highest correlation coefficient was obtained in the DPPH assay using betanin, showing a very strong correlation ($0.8 < r < 1$) based on Pearson's correlation. In the reducing power test using betanin, the Pearson's correlation coefficient of $r = 0.81$ indicates strong correlation. The results of both tests show the strong antioxidant activity betanin. However, it is possible that the antioxidant potential is not only due to betanin, but may also be related to the synergism of other compounds present in the beet extracts used in the present study, which are likely to contribute to the radical scavenging activity of beet extracts.

This paper presents the results of optimizing betanin extraction from red beetroot (*Beta vulgaris*) using natural Deep Eutectic Solvents (DES) and ultrasound. When considering the betanin content and the use this technique it is a sustainable process. This new procedure can be developed for this natural dye in the pharmaceutical and food industries.

4 Conclusion

The extraction time was determined to be an important factor for the ultrasound-assisted extraction of betanin using DES. Betanin extracts from beets (*Beta vulgaris*) have antioxidant potential. Suitable extraction conditions for ultrasound-assisted extraction were determined to maximize the antioxidant ability and obtain a reasonable betanin content. Response surface methodology is a reliable and efficient method for determining the optimal conditions for ultrasound-assisted extraction. This study provides an example of the use of natural DES solutions to replace volatile organic solvents and to enable efficient extraction. The results demonstrate that DES extracts have the potential to be applied safely in the cosmetic and pharmaceutical fields without additional steps of product isolation.

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