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Liquid-liquid System in One-step to Purify Betanin from its Natural Source

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Betalains are natural pigments, soluble in water, found in vacuoles of some plant species. This natural dye with excellent antioxidant, antitumor and anti-inflammatory properties. Concentration of natural color extracts by conventional method ch as evaporation results in low quality product due to loss of hue and chroma. In this sense, aqueous biphasic system (ABS) are relevant for the development of environmentally friendly and "biocompatible" separation processes. This research present in one-step a process for the purification of betanin from *Opuntia ficus-indica*. THF solvent was evaluated during the process and then added different salts for the formation of the ABS. The extraction using THF was optimized and the results show that the betanin was purified ≈ 13 fold, and the antioxidant capacity was increased ≈ 65% when ATPS at 43 wt% of THF and 6 wt% of sodium citrate buffer (pH 5.5) was used.

1. Introduction

Betalains are natural pigments, soluble in water, found in vacuoles of some plant species. They are divided into two classes: red-violet betacyanins and yellow betaxanthins. Among its functional properties, betalains are known to present antioxidant properties, anti-inflammatory, anticancer and antimicrobial (Brewer, 2011; Harlev, et al., 2013). Furthermore, betanin (CI Natural Red 33, E-number E162, betanidin 5-Ob-glucoside) are the only betalains approved for use in food. However, purification or concentration of natural dye extracts by conventional method such as evaporation results in low quality product due instability.

The liquid-liquid application method using aqueous biphasic system (ABS) can be an alternative for efficient purification of these biocompounds of industrial interest, such as DNA, proteins, enzymes, alkaloids, antibiotics, drugs, among others (Asenjo and Andrews, 2012; Matos, et al., 2014). ABS are composed of two immiscible aqueous phases that coexist in balance by the addition of soluble compounds in water (Albertsson, 1986). ATPS formed by hydrophilic organic solvents and inorganic salts have been proposed for having advantages over ABS based on polymers due to their low viscosity and fast separation of the phases. Additionally, they can easily be reused when the objective is to amplify the scale. These systems have been studied regarding the partition of different biomolecules, such as proteins, amino acids, and other natural compounds (Tan, et al., 2013).

This work focuses in integrate process of extraction and purification of betanin from fruit of *Opuntia ficus-indica*. The extraction process was performed using the organic solvent tetrahydrofuran (THF), which is a low cost hydrophilic solvent, low rate of toxicity, and that has already been used for the purification of enzymes (Fowles, et al., 2013; Souza, et al., 2015). Moreover, the purification process was integrated to the extraction process using ATPS though the addition of inorganic salts (sodium carbonate, sodium citrate, and sodium citrate buffer). ATPS equilibrium curves was defined with their respective tie-line (TL), tie-line length (TLL) and critical point, as well as the model parameters of curve adjustments. The purification conditions using ATPS such as salt nature, THF and salt composition were found.

2. Material and Methods

2.1 Materials

The materials used to perform the process were tetrahydrofuran (THF, ≥ 99%), inorganic salts such as sodium carbonate (Na_2CO_3, ≥ 99%), sodium citrate ($Na_3C_6H_5O_7$, ≥ 99%), citric acid ($C_6H_8O_7$, ≥ 99%), Folin & Ciocalteu's phenol reagent, 2,2-difenil-1-picrilhidrazil (DPPH), and gallic acid ($(HO)_3C_6H_2CO_2H$, ≥ 99%). All of them were purchased from Sigma-Aldrich. Deionized water was prepared using a Milli-Q Water Purification system (Millipore Corporation). The fruits from *Opuntia ficus-indica* were acquired at a local market in Propriá, semi-arid region of Sergipe, Brazil. The samples were collected at a maturation stage, conditioned, and transported in thermic isolated recipients to the Food Research Laboratory of Instituto de Tecnologia e Pesquisa - ITP at University Tiradentes, Aracaju, Sergipe, Brazil.

2.2 Bioactive Extraction

The optimal condition to extract betanin from fruit of *Opuntia ficus-indica* was determined by a Central Composite Design (CCD) of (2^3) where there are six axial points and three repetitions in center point, at a total of 17 trials.

The independent variables of the design experiment were: the ratio of the mass of the fruit to the volume of the solvent (THF, temperature (°C), and time (min). The response variable of the experiments was the ratio of the content of betanin to the concentration of the total content of phenols in the samples. The software STATISTICA® 8.0 was used to generate the data. During the extraction process in samples were kept closed and in thermostatic bath (MA 127 MARCONI), then filtered for further analysis. The humidity content of the samples was determined using the Karl-Fisher method (Karl Fischer Titrator - 870 KF Titrino plus).

2.3 Betanin Analysis

The quantification of betalains (betanin) was performed using the method described by Cardoso-Ugarte *et al.* (2014). The absorbance of the extracts was performed using a UV/Vis at wavelengths of 537 nm, 476 nm, and 600 nm. The percentage of betanin using the following equation:

$$x = 1.095\ (A_{537} - A_{600}) \quad (1)$$

where: "*x*" is the absorption of betanin. Subsequently, the concentration of betalains was calculated in mg/L applying Beer-Lambert law, using $E^{1\,cm}_{1\%}$ = 1120 for betanin.

2.4 Phenolic compounds analysis

The determination of the total content of phenols was performed applying Folin–Ciocalteu reagent (Aguirre at al. 2013), using gallic acid with stander.

2.5 Phase Diagrams

Aqueous solutions of THF at 60 wt% + inorganic salts at 40 wt%, were used to determine the phase diagrams. The inorganic salts used were sodium carbonate, sodium citrate and citrate buffer (citric acid/sodium citrate at a ratio of 3.2705 wt%, pH 5.5). The phase diagrams were determine by the cloud-point titration method at 25 ± 1ºC at atmospheric pressure (Souza et al., 2015). The mass of each point generated was quantified with a precision of 10^{-5} g using the scale Shimadzu, model AUW220D. Merchuk model (Merchuk et al., 1998) was used in order to correlate the experimented points that describe the systems. The experimental tie-lines (TLs), were measured with the procedure outlined in our previous work (Souza et al., 2015) and their respective length (tie line length – TLL) were determined through the application of Eq. (2), based on the concentrations of THF and salt) in the two phases.

$$TLL = \sqrt{(X_T - X_B)^2 - (Y_T - Y_B)^2} \quad (2)$$

where the indexes T and B are of top and bottom phases, respectively.

2.6 Optimization ATPS

The mixture points for the formation of ATPS were chosen through analysis of the phase diagrams. For these systems, the purification process was integrated to the extraction process, that is, the components were dissolved in the extract, rich in THF. The systems were prepared in graduated centrifuge tubes (15 mL) by weighing the appropriate amounts of THF extract (35-50 wt%) and salts (6-10 wt%). Different sodium salts

(carbonate, citrate, and citrate buffer) were used in order to verify the pH (pH 3.5 – 8.0) influence on the purification step and stability of betanin.
The systems were prepared gravimetrically within $\pm 10^{-5}$ g, vigorously stirred and then centrifuged 3,000 *g* for 20 minutes at 25°C. Subsequently, the phases were carefully separated, the volume and weights were determined in graduated test tubes (the total mass of the extraction systems prepared is 12.0g). All of the systems had a triplicate.
Partition coefficients were evaluated for betaine concentration (K_B) and total phenolic compounds (K_P), in the top and bottom phases of system, and were determined using Eq. (3). The recovery of betanin in the bottom phase (R_B) and total phenolic compound in the top phase (R_P) were determined using Eq. (4) and (5), respectively. The purification factor (PF) was determined by Eq. (6).

$$K_{B\text{ or }P} = \frac{[C_T]}{[C_F]} \quad (3)$$

$$R_B = \frac{100}{1 + R_V K} \quad (4)$$

$$R_P = \frac{100}{1 + \left(\frac{1}{R_V K}\right)} \quad (5)$$

$$FP = \frac{[BS]}{[BS]_i} \quad (6)$$

where: R_V is volumetric ratio between the phases (V_T is the volume of the top phase – mL and V_F is the volume of bottom phase – mL); C_T and C_F are the concentration of a determined bioactive at the bottom and top phases (mg/L); The ratio of the concentration of betaine to the concentration of total phenols after [BS] and before $[BS]_i$ the purification procedure corresponding to the purification factor.

3. Results and Discussion

3.1 Extraction Process

The extraction process of betanin from fruit of *Opuntia ficus-indica* was optimized using an experiment design of type DCCR 2^3, including 6 axis points and 3 repetitions in the central point, at 17 trials. The codified and real values of the parameters that were used are shown in Table 2.

Table 2: Codified values of the parameters and optimization conditions for the applied model used for the extraction of betanin from the Opuntia ficus-indica fruit .

Variables	Code	Level					Optimization conditions	
		-1.68	-1	0	1	1.68	**Experimental condition**	**Codified value**
Ratio (g/mL)	x_1	0.66	0.8	1	1.5	1.84	0.99 (wt%)	- 0.02623
Temperature (°C)	x_2	21.55	27	35	45	51.82	30 ºC	- 0.64630
Time (min)	x_3	1.32	2	3	5	6.36	2.93 h	- 0.06476

The response variable was related to the ratio between the content of betanin and the content of phenolic compounds extracted at each trial of experimental design. In order to develop more effective processes and less costly processes for the purification of betanin, the extraction was performed using the THF solvent. Although this organic solvent has a toxicity rate, recent studies have shown that THF is a polar solvent with low to moderate toxicity. In addition, *in vitro* and *in vivo* studies have shown that THF is not mutagenic, as well as having low bioaccumulation potential (Fowles et al., 2013). Thus, the solvation power of THF for organic substances has been shown in the extraction of luteins, beta-carotenes, neoxathins, and for enzyme purification (Khachik, 2013). Chen et al. (2016), compared the solvents THF and ethanol in the extraction of luteins from seaweed, and the best results were using THF with 99.5% of efficiency.
The studied variables (ratio fruit mass/solvent volume (X_1)Q, temperature (X_2)QL, and the extraction time (X_3)QL revealed to be significant for the control of the analyzed parameter. The temperature was the variable with biggest statistic relevance on the extraction process of betanin from its natural source, followed by the combination of the variables ratio (w/v) (L) and time (L). The bar lengths in the chart are proportional to their

estimated effect absolute value, and the vertical line represents the confidence interval of 90%. The confidence levels for this work (using DCCR 2^3) are considered satisfactory for levels above 85% (Rodrigues and Iemma, 2005). Therefore, the factors that have a significant effect on the answer are the ones beyond the line.

The response surface for the dependent variable is shown in Fig. 2. The three surface parts were generated shows the relationship between the response and the independent variables (Rodrigues and Iemma, 2005). The second order model obtained for the betanin extraction process is shown in Eq.(7). The statistic significant parameters were not eliminated from the model and added to the residuals. The values found for each parameter, such as the ratio (fruit mass/solvent volume) of 0.99, temperature of 30ºC, and extraction time of 2.93h. In this condition, 20.0 ± 0.2 mg/L of betanin and 224.1 ± 2.7 mg/L of total phenolic compounds were obtained. Our model is consistent, considering that the predicted values and the experimental values of the dependent variable (betanin concentration/total phenolic compounds concentration) are close (Table 2). The good correlation between the experimental and predicted values confirmed that the model obtained by CCRD could accurately predict in others works, as the antioxidants extraction yield of *Limonium sinuatum* flowers using ultrasound-assisted extraction and in the phenolic compounds extraction from cactus pear (Aguirre et al., 20013 and Xu et al., 2017).

$$Y = 0.0609\text{-}0.0214X_2\text{-}0.0118X_3\text{-}0.0159X_1^2\text{-}0.0165X_2^2\text{-}0.0117X_3^2 + 0.0129X_1X_3 \quad (7)$$

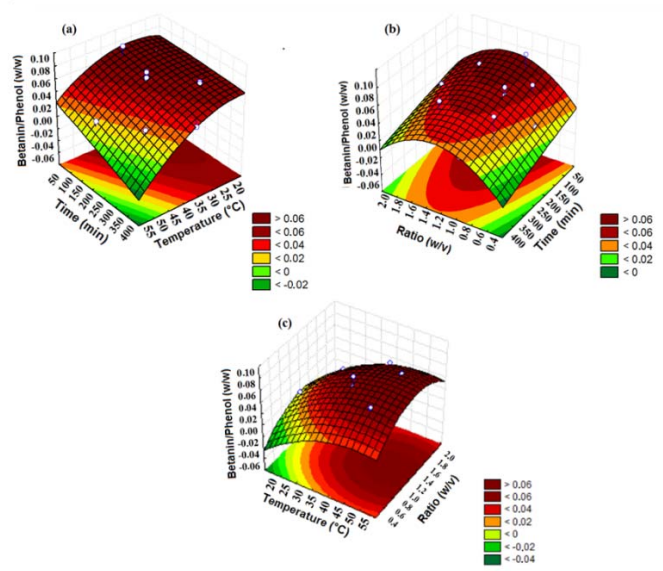

Figure 2: Surface response of the experiments performed for the extraction of betanin. a) betanin/phenol in relation to time and temperature; b) betanin/phenol in relation to the molar ratio and temperature; c) betanin/phenol in relation to the temperature and molar ratio (Santos et al., 2018).

3.2 Purification Process

The ATPS formed by different salts (sodium carbonate, sodium citrate, or sodium citrate buffer) were used in order to verify the pH influence in the purification and stability of betanin. For this purpose, citrate and carbonate salts associated with the cation $[Na]^+$ were used in this investigation. Analyzing the effect of pH in the phase that is rich in salt is especially important due to migration preference of betanin to the lower phase of the system (phase rich in salt, K_B << 1). Different pH values were investigated (pH 3.5-8.0) which revealed betanin instability (degradation) in more acid medium (pH 3.5) and more alkaline medium (pH >5.5). Betanin concentration was reduced from 23.2 ± 1.3 mg/L to 20.0 ± 1.5 mg/L in systems with a pH of 3.5 and significantly reduced to 0.24 ± 0.06 mg/L in systems at pH 8.0 in comparison to systems at pH 5.5. Betanin is a compound that is strongly dependent on pH; our results show that with values farther away from pH 5.5 its stability is strongly affected. Thus, the ATPS were conducted and optimized using sodium citrate buffer at pH 5.5.

In order to evaluate the effect of the concentration of THF in ATPS for the purification of betanin, the sodium citrate buffer concentration was set at 8 wt% and THF varied between 35 – 50 wt%, as shown in Fig. 2(a). For extraction and purification purposes, comparisons regarding the composition of the systems were established from the equilibrium curve shown in Figure 3. In the studied systems, K_B was always < 0.025 ± 0.002 with over

99% performance in the bottom phase. Ebrahimi et al. (2016), using PEG + sodium sulfate ATPS was able to recover commercial betanin by up to 76%. Therefore, the purification factor was determined by the migration of total phenolic compounds to the top phase, considered contaminants in the system. The value of K_P increased from 0.955 ± 0.02 to 1.33 ± 0.07 indicating that THF concentration increase, and consequently the hydrophobic character of the top phase in the ATPS (phase rich in THF, log K_{ow}= 0.94) induced a greater selectivity in the separation of betanin. In this case, it is clearly observed an inversion in the migration of total phenolic compounds (from the bottom to the top phase), which conducts better purification of betanin in the opposite phase, in other words, its isolation in the bottom phase of the system.

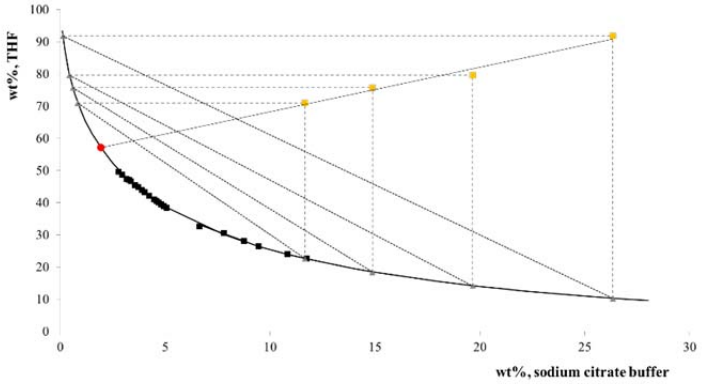

Figure 4: Phase diagram for ternary systems composed of THF + sodium citrate buffer + water, at 25 ± 1 ºC and at atmospheric pressure. ▬ calculated binodal from Eq. 1; ■, data of experimental solubility; ▬, tie-lines; ■, auxiliary data curve; ● , critical point.

The effect of the concentration of sodium citrate buffer on ATPS was also evaluated (set at 43 wt% of THF and sodium citrate buffer varying between 5 – 18 wt%) as shown in Fig. 4(b). The increase of salt concentration in the system (above of 6 wt%) reduces the capacity of the system to purify betanin. This can be due to the increase of the hydrophilic character, cause by the capacity of salt to attract the molecule of water to itself, and consequently reduce the migration of polar compounds to the top phase. Thus, betanin purification factor increased 3.80 ± 0.32 to 13.7 ± 0.53 fold, compared to initial extraction step. Chethana et al. (2007) using multiple partitioning of betalains from beetroot (*Beta vulgaris*) with PEG and ammonium wherein 70– 75% of betalains partitions to the top phase (PEG rich phase) with purification factor of 3.4 fold. The increase of the purification factor is related to the selectivity of the phases that constitute the system, which results, mainly, in the removal of contaminants. The results show a selectivity of total phenolic compounds to the top phase in comparison to betanin. Therefore, the capacity to isolate betanin in one of the phases is directly associated with the sensitive capacity of the components in controlling the hydrophilicity between the phases.

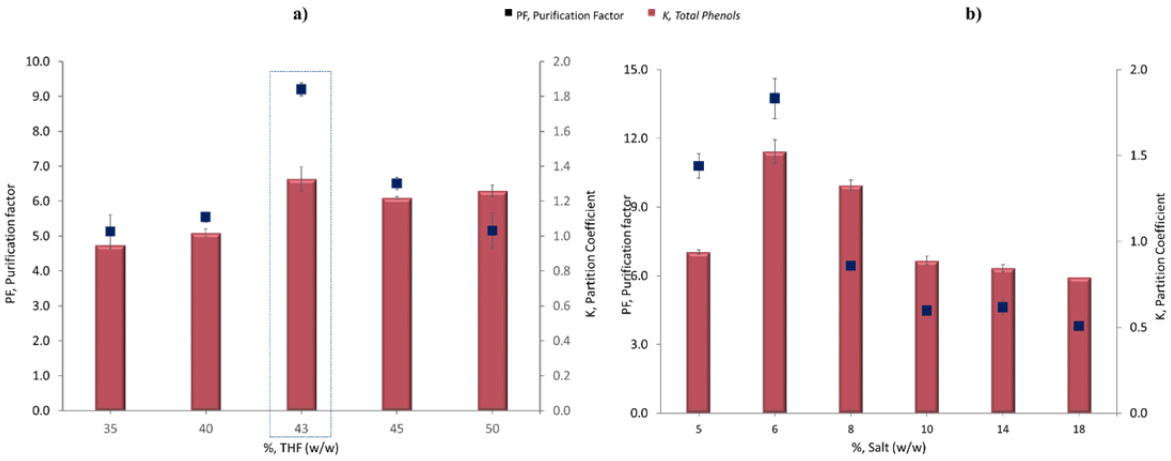

Figure 5: Effect of the concentration of sodium citrate buffer on the purification factor (PF) of betanin and on the partition coefficient (K) of total phenolic compounds, respectively, for systems based on THF extract + sodium citrate buffer (pH 5.5), at 25 °C and at atmospheric pressure.

4. Conclusions

The betanin was more extracted using THF solvent and the extraction process was optimized through a design experiment in the condition of 0.99 (w/v) of the ratio (fruit mass/volume of solvent used), at 30°C for 2.93h. In this extraction condition, 32.1 ± 0.3 mg/L of betanin and 246.03 ± 2.7 mg/L of phenolic compounds were obtained. The best purification system was, mainly, the result of the manipulation of contaminant phenolic compounds for the top phase of the system. Therefore, a concentration of 38.65 ± 1.37 mg/L with a PF of 13.74 ± 0.53 was obtained for betanin, using ATPS at 43 wt% of THF and 6 wt% of sodium citrate buffer (pH 5.5) at 25°C. In addition, the capacity inhibition of radical DPPH of the purify betanin was preserved and increased with a performance of 65.79 ± 1.55 %.

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