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**ORIGINAL RESEARCH PAPER**

# OPTIMIZATION OF *HIBISCUS SABDARIFFA* L. (ROSELLE) ANTHOCYANIN AQUEOUS-ETHANOL EXTRACTION PARAMETERS USING RESPONSE SURFACE METHODOLOGY

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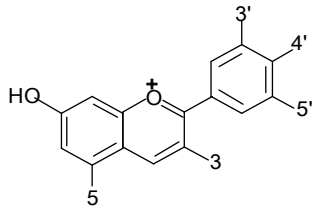
**Abstract:** Anthocyanins along with protocatechuic acid and quercetin have been recognized as bioactive compounds in *Hibiscus sabdariffa* L. aqueous extracts. Characteristic anthocyanin absorption in the visible region makes their quantification possible without the interference of the other two compounds, and also can favor its potential application as an alternative to organic-based dye sensitized solar cell, in various forms. In order to optimize measurable factors linked to the extraction of these flavonoids, an optimization was performed using a Box-Behnken experimental design and response surface methodology (RSM). Three levels of ethanol concentration, temperature and solid-solvent ratio (SSR) were investigated. The optimization model showed that with 96 % EtOH, 65 °C, and 1:50 SSR, the highest anthocyanin concentration of 150 mg/100 g was obtained.

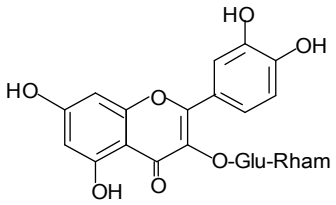
**Keywords:** *anthocyanin extraction, bioactive compounds, dye sensitized solar cells, modelling, roselle pigments*

## INTRODUCTION

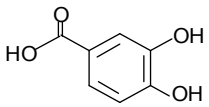
The chemical compounds present in aqueous extracts of *Hibiscus sabdariffa* L. calyces, common name jamaica (*Malvaceae* family) and which have been recognized as bioactive, are anthocyanins, protocatechuic acid and quercetin (Figure 1). This bioactivity, as seen in preclinical and some clinical tests, has been linked with lipid metabolism, anti-hypertensive activity and apoptosis [1 – 4]. On the one hand, *H. sabdariffa* calyx refreshing water based drinks have traditionally been consumed in different countries and regions, particularly in tropical and sub-tropical ones; however, the therapeutic use that this aqueous or other polar solvent-based extracts can be put to, must adhere to a standardization which guarantees the efficiency, security and tolerability of this extract.




| Anthocyanin | 3' | 4' | 5' | 3 | 5 |
|---|---|---|---|---|---|
| Cyanidin-3-sambubioside | OH | OH | H | 2-O-β-D-xylosyl-D-glucose | OH |
| Cyanidin-3-glucoside | OH | OH | H | glucosyl | OH |
| Delphinidin-3-sambubioside | OH | OH | OH | 2-O-β-D-xylosyl-D-glucose | OH |
| Delphinidin-3-glucoside | OH | OH | OH | glucosyl | OH |
| Cyanin | OH | OH | H | glucosyl | glucosyl |
| Malvin | $OCH_3$ | OH | $OCH_3$ | glucosyl | glucosyl |




Quercetin-3-O-rutinoside




Protocatechuic acid

***Figure 1.*** *Bioactive compounds in Hibiscus sabdariffa L. calyx aqueous extracts*

Anthocyanins present in *H. sabdariffa* extracts, apart from affording them (along with aqueous and polar extracts in general) their characteristic red color, have been bioactive compounds showing beneficial effects for health, a fact that may attract the attention of both the pharmaceutical and food industry [5]. As for their property as a pigment, anthocyanins in general isolated from *H. sabdariffa* L., or other natural sources, have become a point of interest to the cosmetic industry as well as the food industry as potential substitutes for certain artificial dyes.

The food industry needs to replace these artificial food dyes, as, although up to now it can be stated that they "are not a major cause of ADHD (attention-deficit/hyperactivity disorder) *per se*, they seem to affect children regardless of whether or not they have ADHD" [6]. Finally another possible use for anthocyanins in general has been proven, considering their capacity to absorb radiation in the visible light spectrum and it is

related to their application in photovoltaic technology specifically in dye-sensitized solar cells (DSSC) [7 – 11].
In short, for whatever application of anthocyanins in general or anthocyanins of *H. sabdariffa* L. calyces in particular (health, cosmetics, food, and energy), their availability by means of a solid-liquid extraction is necessary. Mention has been made [12] that even with the knowledge of the potential of these pigments in a variety of applications, their use has been limited by "their relative instability and low percentages of extraction" and that consequently the emphasis of research studies on these flavonoids is to overcome these problems.
Some of the previous studies on anthocyanin extraction processes from *H. sabdariffa* L. calyces with polar solvents [13 – 21] have been carried out using water as the solvent. As far as can be ascertained, no studies on extraction condition optimization using aqueous ethanol have been found. Consequently, the aim of this study has been to optimize aqueous ethanol extraction of anthocyanins from *H. sabdariffa* L. calyces.

## MATERIALS AND METHODS

### Raw materials

*Hibiscus sabdariffa* L. dried calyces were used, provided by farmers of Paso del Macho district in the state of Veracruz, Mexico. After removing inert material, clean calyces were ground in a ball mill (Pulvex 100, Mexico) and sieved through a vibratory sieve shaker (Retsch 3D, Germany). The different particle diameter ground samples were stored in polyethylene vacuum bags at room temperature until extraction was carried out.

### Extraction assembly and procedure

Solid-liquid extractions were performed in triplicate on ground calyces, using round-bottomed, three necked 250 mL vertical flasks with 24/40 joints as extractors, placed on disposable molded fiber cup carriers and then on a hot plate agitator (Gallenkamp, SWT550-0300, United Kingdom). A West type condenser, a thermometer and a glass stopper were connected to the flask through the three necks. The condenser was connected to a refrigerated bath circulator (Haake K F3, Germany). Agitation was constant (800 rpm) provided by the plate magnetic control and a PTFE-coated ovoid magnetic stirring bar (Bel-Art, Spinbar, USA) was used inside each flask.

### Experimental design and analysis

A Box-Behnken experimental design was applied to study the effect of three variables (solvent concentration, temperature and solid-solvent ratio) on anthocyanin extraction yield and a subsequent Response Surface Methodology (RSM) was applied to optimize the results. The highest point on the RSM contour plot indicates where the greatest yields are produced and the best values for each factor can be read on the axes. A desirability value nearer to one shows the best suitability of combine factor values. In this case, the values of the variables investigated (Table 1) were ethanol concentration

(44, 70, 96 % v/v), temperature (25, 45, 65 °C) and solid-solvent ratio SSR (1:50, 1:75, 1:100). The Design Expert 10 program (Stat-Ease, Minneapolis, USA) was used for data analysis: an ANOVA was performed with a post hoc Tukey range test to pinpoint the source of any significant differences between the factors involved.

***Table 1.*** *Variable codes and levels*

| Independent variable | Code | -1 | 0 | 1 |
|---|---|---|---|---|
| Ethanol concentration [% v/v] | $X_1$ | 44 | 70 | 96 |
| Temperature [°C] | $X_2$ | 25 | 45 | 65 |
| Solid-solvent ratio [g·mL$^{-1}$] | $X_3$ | 1:50 | 1:75 | 1:100 |

## Analytical methods

A spectroscopic method was used to measure total anthocyanins, specifically a *p*H differential absorption spectrophotometric method based on anthocyanin color change due to a variation in the chemical structure caused by *p*H conditions [22, 23]. At *p*H 1, anthocyanins are mainly in the form of a cation (oxonium or flavylium ion) that is colored, which turns into hemiketal or carbinol, colorless at *p*H 4.5.
Difference in absorbance at 510 nm of both forms is proportional to anthocyanin content, reported in terms of cyanidin-3-glucoside (ε = 26 900 M$^{-1}$·cm$^{-1}$, MW = 449.2 g·mol$^{-1}$). Absorbance correction due to turbidity was carried out through readings at 700 nm. A diode array UV-VIS spectrophotometer (Hewlett-Packard 8452 A, Germany) was used.

## RESULTS AND DISCUSSION

Samples were run in a randomized manner in triplicate and the results were analyzed using the Box-Behnken method (Tables 2 and 3).

***Table 2.*** *Box-Behnken analysis with results*

| Run | Ethanol [% v/v] | Temp. [°C] | Sol/solv. ratio | Observed value | Predicted value | Residual |
|---|---|---|---|---|---|---|
| 1 | -1 | 1 | 0 | 0.31 | 1.01 | -0.7 |
| 2 | -1 | 0 | -1 | 1.35 | 1.12 | 0.23 |
| 3 | -1 | 0 | 1 | 0.47 | 0.48 | -0.017 |
| 4 | -1 | -1 | 0 | 0.42 | 0.58 | -0.17 |
| 5 | 0 | 0 | 0 | 0.95 | 0.88 | 0.076 |
| 6 | 0 | 0 | 0 | 0.95 | 0.88 | 0.073 |
| 7 | 0 | 0 | 0 | 0.98 | 0.88 | 0.1 |
| 8 | 0 | -1 | -1 | 1.18 | 0.98 | 0.21 |
| 9 | 0 | 1 | -1 | 1.57 | 1.41 | 0.17 |
| 10 | 0 | 1 | 1 | 1.2 | 0.77 | 0.43 |
| 11 | 0 | -1 | 1 | 0.59 | 0.34 | 0.25 |
| 12 | 1 | 1 | 0 | 1.32 | 1.17 | 0.15 |
| 13 | 1 | 0 | -1 | 1.01 | 1.27 | -0.25 |
| 14 | 1 | -1 | 0 | 0.49 | 0.74 | -0.24 |
| 15 | 1 | 0 | 1 | 0.33 | 0.63 | -0.31 |

***Table 3.*** *Statistical analysis of the results*

| Source | Type III sum of squares | df | Mean square | F | Sig. |
|---|---|---|---|---|---|
| EtOH | 0.139 | 1 | 0.139 | 4.809 | 0.036 |
| Temp | 1.113 | 1 | 1.113 | 38.384 | 0.000 |
| Ratio | 2.418 | 1 | 2.418 | 83.394 | 0.000 |
| EtOH * Temp | 0.642 | 1 | 0.642 | 22.154 | 0.000 |
| EtOH * Ratio | 0.030 | 1 | 0.030 | 1.030 | 0.318 |
| Temp * Ratio | 0.037 | 1 | 0.037 | 1.264 | 0.269 |
| EtOH * Temp * Ratio | 0.000 | 0 | | | |
| Error | 0.928 | 32 | 0.029 | | |
| Total | 42.500 | 45 | | | |
| Corrected Total | 8.058 | 44 | | | |

This analysis indicated that all factors showed significant differences, ethanol concentration at the $p \leq 0.05$ level and both temperature and SSR at a $\leq 0.01$ level. Additionally, there was a highly significant difference ($p \leq 0.01$) in the interaction between ethanol concentration and temperature; this occurs when one of the independent variables exerts an effect on another of the independent factors of the experimental design. Post hoc Tukey tests indicated that the ethanol concentration level that gave the highest results was 70 % v/v but this was influenced by the temperature factor, as seen in the RSM graph in Figure 2. Regarding the temperature factor, although the 45 °C level gave significantly different results, the 65 °C level rendered the highest. For the SSR factor, the -1 or 1:50 level gave the highest results.

The experimental values were analyzed by multiple regressions in order to adjust to the second order polynomial eqn. (1):

$$y = 0.87486 + (0.76231x10^{-1})\,(SSR) + (0.21536)(T) - (0.31744)(EtOH) + (0.23139)(SSR)(T) + (0.49890)(SSR)(EtOH) + (0.55269)(T)(EtOH) \quad (1)$$

The predictive model calculation (second order polynomial equation model) leads to the optimal theoretical calculation of extraction in order to achieve the maximum value of 1.575 mg anthocyanins·$g^{-1}$ raw material, using 96 % EtOH, 65 °C, and 1:50 SSR.

A subsequent RSM graph showing greatest anthocyanin concentration achieved at 65 °C and 96 % ethanol (SSR factor held at 1:50) was carried out to observe the effect of simultaneous variations in the variables analyzed (Figure 2).

The residual errors in Table 2 (observed – predicted values) show a certain level of difference, both positively and negatively. A normal plot of residuals was generated (Figure 3) and it can be appreciated that there is one outlier which could cause noise in the optimization process, with the rest of the results grouped reasonably near to, and on either side of, the straight 45 ° line.

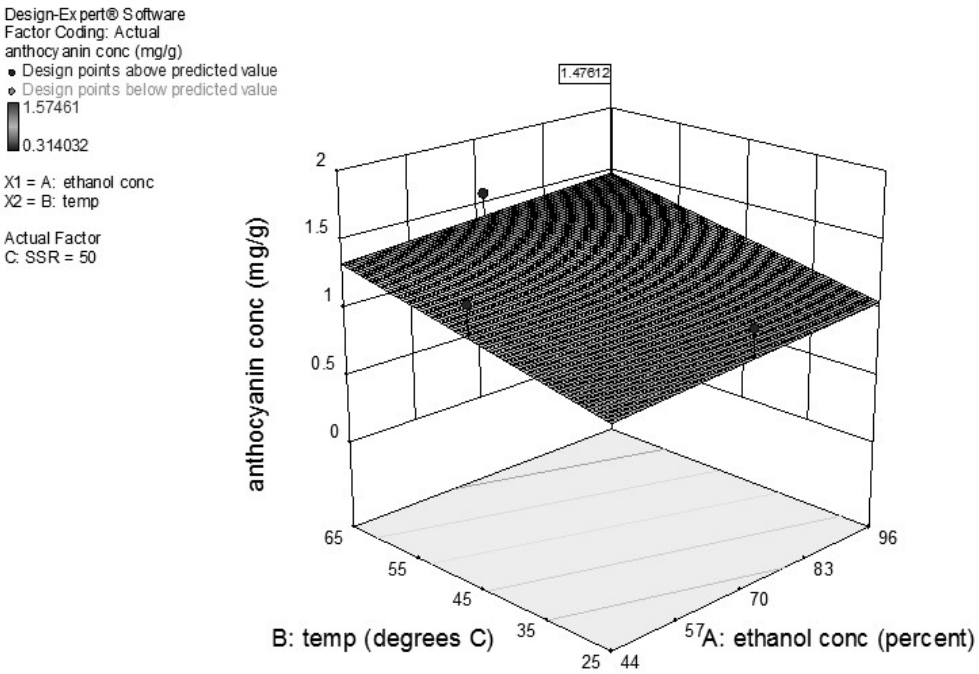



***Figure 2.*** *RSM graph for anthocyanin concentration*

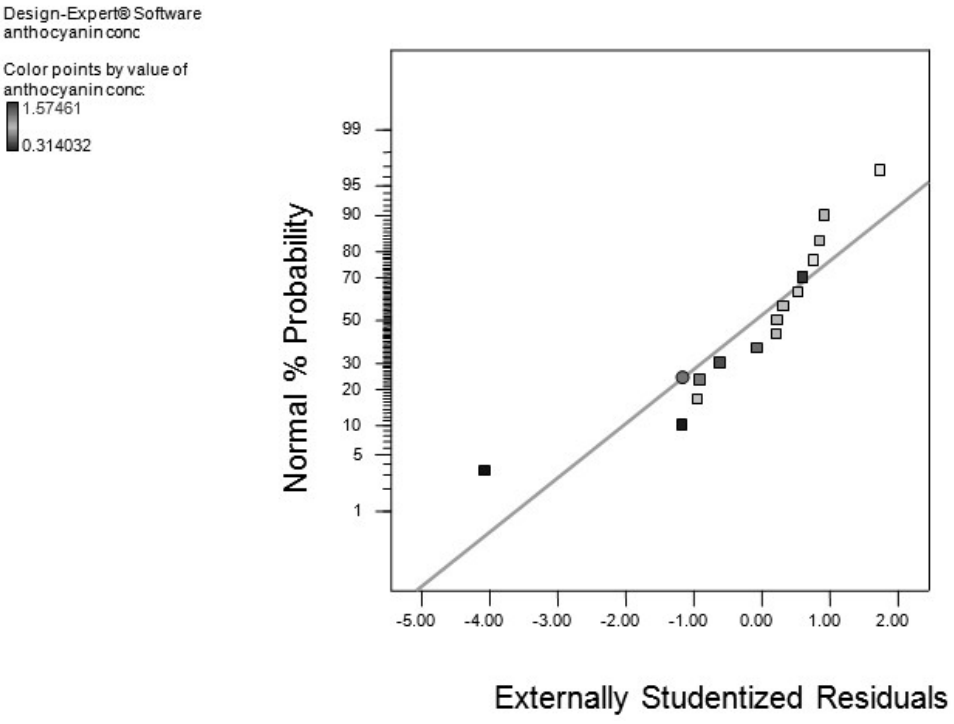



***Figure 3.*** *Normal plot of residuals*

A subsequent Cook's distance test (Figure 4) shows the outlier to be within the accepted range of divergence, therefore not causing unacceptable leverage on results, and the outlier deletion from the results was rejected.

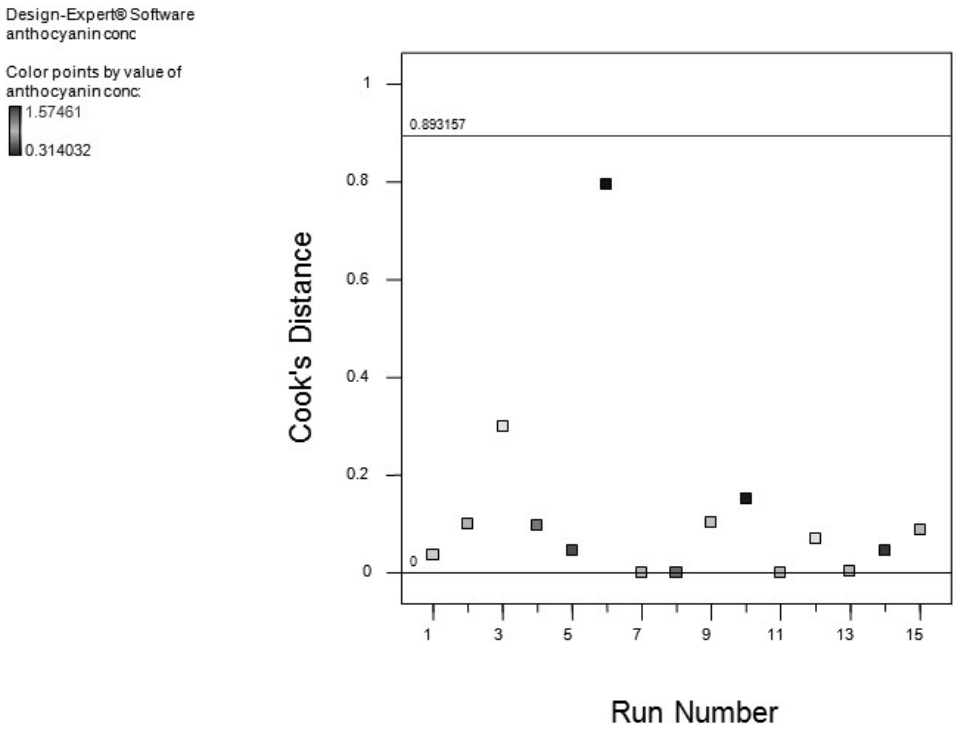



***Figure 4.*** *Cook's distance plot*

When the numerical optimization step was taken, the plots for optimum levels for each factor and the combined desirability function (values from 0 to 1) were obtained (Figure 5). These were 96 % ethanol concentration and 65 °C at an SSR of 1:50.

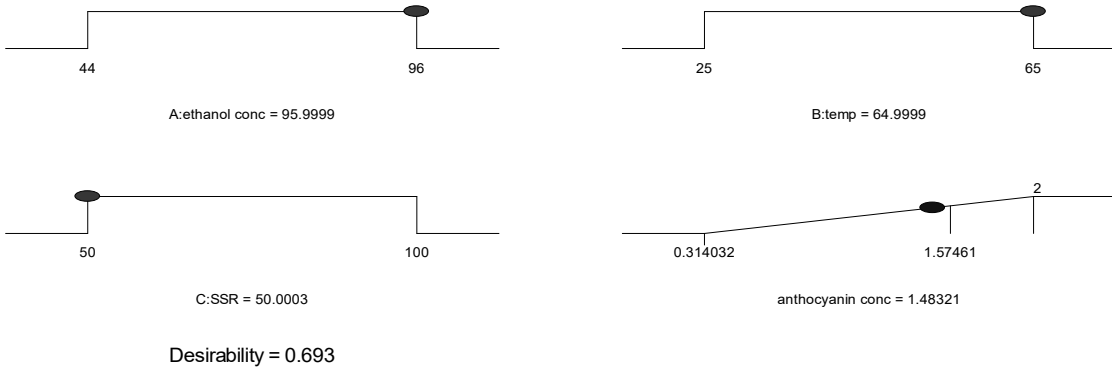



***Figure 5.*** *Numerical solutions for optimization*

A contour plot also shows the optimum anthocyanin production conditions (Figure 6). A desirability function measurement reveals how acceptable a set of conditions are, in this case good at 0.693 (Figure 6). A contour plot (Figure 7), where SSR was held at 1:50, was carried out where the desirability function was demonstrated graphically.

Validation of eqn. 1 was carried out by further experiments in triplicate, using the optimal conditions resulting from the original experiments; the accuracy and suitability of the developed model were confirmed (data not shown).

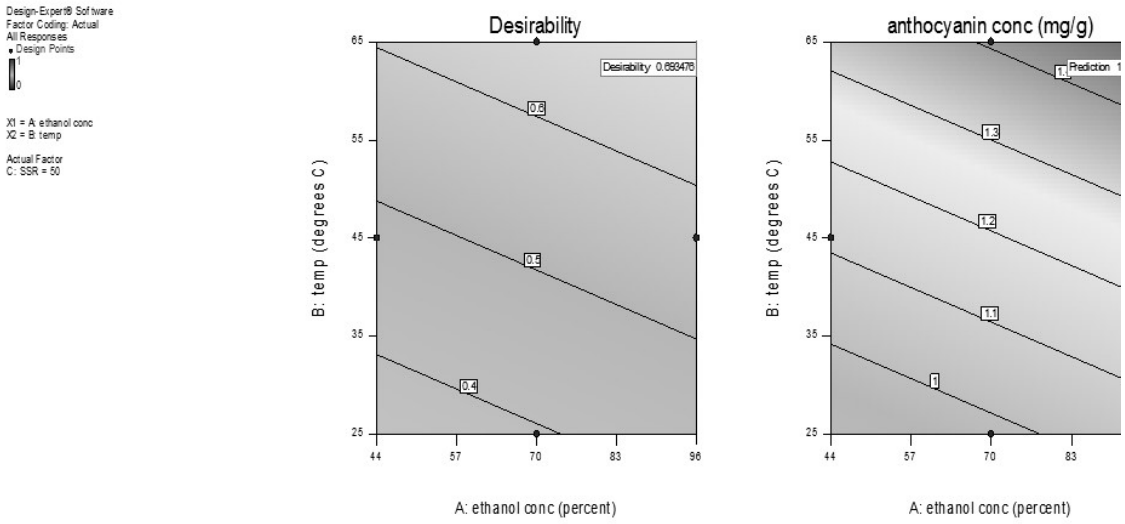



***Figure 6.*** *Contour plots for desirability and anthocyanin production*

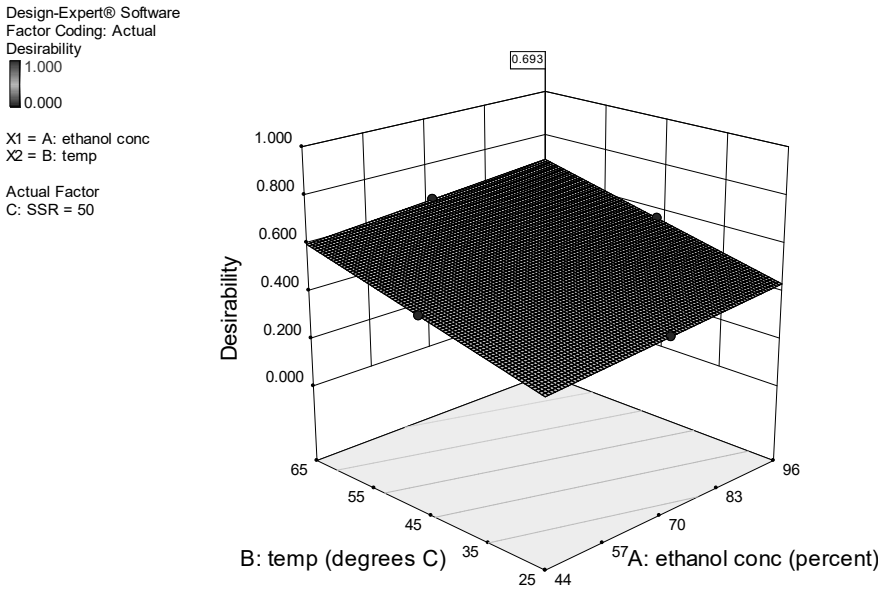



***Figure 7.*** *RSM graph indicating the 0.69 desirability value of ethanol concentration vs temperature, holding SSR at 1:50*

As in any solid-liquid extraction process, there are main parameters that affect the desired solute diffusion into the solvent, such as particle size, solvent polarity, solvent concentration, solid-solvent ratio, extraction time and temperature. In terms of solvent polarity, and considering the polar behavior of *H. sabdariffa* calyx anthocyanins, former studies mentioned above have performed aqueous extractions. In such studies, as well as in the present one, the aim has been to find conditions that result in the highest anthocyanin concentration with the particular solvent chosen. The idea of using aqueous ethanol instead of water as the extraction solvent confers possible advantages in

subsequent process steps, such as concentration of the extract, i.e. a fast and less energy demanding solvent evaporation, a condition that better preserves anthocyanin chemistry. The use of *H. sabdariffa* is well known as a traditional drink (aqueous based), but the potential of these pigments for other applications (health, energy-DSSC, cosmetics) have to overcome what has been stated: "their relative instability and low percentages of extraction" [11]. Therefore this study has contributed with knowledge about the behavior of *H. sabdariffa* calyx anthocyanins in aqueous-ethanol extraction, particularly extraction optimization.

## CONCLUSIONS

*Hibiscus sabdariffa* L. calyx anthocyanin extraction optimization, based on aqueous ethanol solvent, revealed that the best combination of factors rendering the highest anthocyanin yield was as follows: 96 % ethanol concentration, 65 °C temperature and 1:50 SSR. In order to generate a straight comparison of solvent polarity and its influence on extraction performance it is recommended to carry out a study using these optimized extraction conditions with aqueous ethanol *versus* water optimized extraction conditions already published, applied at the same time to a specific *H. sabdariffa L.* calyx batch.

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