Optimization of Variables Influencing The Stability of Betaxanthin Pigments From Yellow Prickly Pears Juice By Experimental Design

Fatima Dehbi¹, Aziz Hasib², Hicham El Batal³, Aziz Ouatmane⁴, Abderrahim Jaouad⁵

Sultan Moulay Slimane University, Faculty of Sciences and Technologies, Laboratory of Environment and Valorization of Agro-resources, B.P. 523, Beni-Mellal, MOROCCO.

Abstract— The crude pigments obtained from yellow prickly pears juice, were evaluated for their stability at series of pH: from 3 to 6.5 in the presence of different ascorbic acid or citric acid concentrations as stabilizers. The effect of exposure to light and dark between temperatures values: 70 and 100°C and varied heating time from 5 to 60 min were also estimated. The stability of betaxanthins pigments was measured on the basis of their UV-visible absorption spectrum. The six variables: pH, ascorbic acid, citric acid, light, heating time and temperature were selected and then, a screening of them was carried out with a design optimal experimental to determine the influential factors. Optimization was conducted with three factors: temperature, length of heating time and pH. They were modelled with a central composite experimental design. Then, the model was validated by statistical test and the optimum has been found and checked by three experiments. The optimal conditions that maximized the retention of betaxanthins pigments were: temperature of 76°C, heating time of 16.20 min and pH of 5.76. At these conditions, the predicted rate of retention of pigment was 79%.

Keywords— Prickly pears, pigments, Optimization, Response surface methodology (RSM).

I. INTRODUCTION

Cactus prickly pears “Opuntia ficus indica L.” can be considered as a plant of the future. It is a promising plant, widely distributed in Morocco and grows in many other countries such as Mexico, United States, South Africa, Australia, and Mediterranean basin countries. It is one of the most suitable plants to cultivate in arid and semiarid regions around the world [1,2]. The plant is grown as fruit, vegetable, forage, and fodder production [3,4]. It supplies two edible parts, the fleshy stem (cladode) and highly attractive and nutritious fruit.

The pulp exhibits a high pH value (5.3 to 7.1), low acidity (0.01% to 0.18% as citric acid equivalents), and total soluble solids content ranging from 10.7 to 17°Brix, mainly due to reducing sugars [3, 5-8]. These characteristics make the pulp a very good medium for microbial spoilage [9] but, on the other hand, very suitable to be added to low-acidic foods. Sugar, protein, dietary fibres, and ash contents are similar to those of other fruits [10]. In general, pulp is rich in nutraceuticals and functional properties [2,6,10,11]. Moreover, the fruit contents of pectin and mucilaginous components influence the pleasant flavour of pulp and could serve as thickening agents by forming viscous colloids [7,10].

This pulp has an attractive colour that varies from a soft green, greenish-white, canary-yellow, orange-yellow, lemon-yellow, red, and cherry-red to purple hues [5,12]. These colorants maintain their appearance over a wide pH range (from 4 to 7), which makes them ideal pigments for colouring many foods [9,13].

These attractive colours are due to the betalains comprising the red-violet betacyanins and the yellow-orange betaxanthins [14,15].

The use of prickly pears as a source of betalains may be interesting since the plants of the Opuntia genus need minimal requirements from soil and water. In this way, they may be a great alternative to agricultural economy in arid and semiarid regions [16].

Betalains are natural pigments that are found in natural form in most families belonging to the Centrospermae order that includes the cactaceae family in which the Opuntia genus is found. The chemical structure of these pigments is derived from the betalamic acid and, depending on the united components in this structure, the yellow betaxanthins and the red-violet betacyanins will be present. The most-studied betalains are found in red beets (Beta vulgaris) which main betacyanins are betanin and isobetanin. Betalains stability is affected by temperature, pH, oxygen, light, and aqueous activity [17].

During food processing and storage, several studies noted that temperature is the most important factor on betalain stability.

Concerning the pH factor, betalains is relatively stable over the broad pH range from 3 to 7 [18], which allow their application to low acidity foods. Below pH 3.5, the absorption maximum shifts toward lower wavelengths, and above pH 7 the change is toward upper ones; out of the pH range 3.5–7.0 the intensity of the visible spectra decreases. Optimal pH range for maximum betanin stability is 5–6 [16,19].

Betalain stability was also reported to be impaired by light exposure [20,21]. Authors showed an inverse relationship between betalain stability and light intensity [21].
Some food antioxidants, especially ascorbic and isoascorbic acids have been described to enhance betalain stability [22, 23].

In methodology, we opted for response surface methodology (RSM) as it is an affective statistical technique for optimizing complex processes. The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple parameters and their interactions. Therefore, it is less laborious and time-consuming than other approaches required to optimize a process [24]. It is widely used in optimizing the extraction process variables, such as polysaccharides, anthocyanins, vitamin E, phenolic compounds and protein from varied materials [25-27]. Box–Behnken design (BBD), one of RSM, only has three levels, and needs fewer experiments. It’s more efficient and easier to arrange and interpret experiments in comparison with others and widely used by many researchers [28,29].

In this study, we propose a screening design that can be used for the estimation of stability of pigment from Moroccan Opuntia fruits juice.

Figure 1: Chemical structures of proline-betaxanthin/isoproline-betaxanthin (a), γ-aminobutyric acid-betaxanthin (b)

II. MATERIALS AND METHODS

A. Sample measurements

Opuntia ficus indica fruits grown in Morocco were harvested and stored at -20°C. The fruits were thawed prior to manually squeezing. The obtained juice of pH 5.8 was centrifuged (4000 x g, 30min at 4°C) and the supernatant juice was stored at -20°C before being used.

The juice obtained was subjected to various treatments including thermal treatment, pH, stabilizing additives and light or dark exposure.

pH treatment: samples were subjected to pH adjustment using 1M HCl or 2% NaOH. Each set of samples was adjusted to 3 and 6.5; respectively. These samples were added with different percentages of stabilizing.

Ascorbic acid (Asc Ac) or citric acid (Cit Ac) was added into the juice with concentration of (0%; 0.1%; and 1%) (V/V). The juice incorporated with Ascorbic or citric acids were further treated at different temperatures.

Heat treatment: samples obtained from section 2 were treated at two different temperatures; respectively 70 and 100°C during 5 and 60min; each set of the sample treatment was treated in dark or with light exposure.

Pigment was extracted from the homogenate with methanol (1:5) and the supernatant passed through a 0.45µm nylon filter.

B. Spectrophotometer analysis:

All determinations of total betaxanthins contents were performed on an UV/Vis spectrometer (UV-1650PC Shimadzu, JAPON) equipped with quartz cuvette of 1cm. The pigments were extracted using methanol. The betalain content was expressed as mg/l and calculated according to literature equation (1) [48].

\[
\text{Bx (mg/L)} = \frac{(A(DF)(MW)V_d/\varepsilon L)}{(1)}
\]

Where A is the absorption value at 482 nm betaxanthins (figure 2), DF is the dilution factor, Vd is the juice solution volume (mL), and L is the path-length (1cm) of the quartz cuvette.

The molecular weight (MW) and molar extinction coefficient (ε) of betanin [MW = 550 g/mol; ε = 48,000 L/(mol cm)] in H2O were applied in order to quantify the betaxanthins (Bx).
C. Statistical analysis:

NeMRODW software (Mathieu D.Nony J; and Phan Tanluu R.; New Efficient Methodology for research using optimal Design; Windows version; LPRAT; Marseille France) has been used in this works.

III. RESULTS AND DISCUSSION

A. Screening design

This step is done after the exploratory research. We are quickly picks the factors potentially influential in the chosen experimental field. Because of the belief that an increasing number of factors increase the number of experiments exponentially, a high number of factors are often reduced to a number that seldom exceed three or four. This reduction is artificial and relies only on laboratory practices.

**TABLE 1**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Numbers Levels</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>U1</td>
<td>2</td>
<td>70, 100</td>
</tr>
<tr>
<td>U2</td>
<td>2</td>
<td>5, 60</td>
</tr>
<tr>
<td>U3</td>
<td>2</td>
<td>0, 1</td>
</tr>
<tr>
<td>U4</td>
<td>2</td>
<td>3, 6.5</td>
</tr>
<tr>
<td>U5</td>
<td>2</td>
<td>0.1, 1</td>
</tr>
<tr>
<td>U6</td>
<td>2</td>
<td>0.1, 1</td>
</tr>
</tbody>
</table>

This design was used to screen six factors supposed to have an influence on the stability of pigments obtained from *Opuntia ficus indica* juice (Table 1). The response studied is the rate of retention of pigment. The factors are giving the datasheet of the experiments to run (Table 2).

In a screening study, the effects are supposed to be additive; this implies that the relationship between the experimental responses and the studied variables is a first-order polynomial model with values of \( X_i = \pm 1 \) (equation 2):

\[
Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_6X_6
\]

(2)

From the experimental data, the estimations of the bi effects can be calculated (Table 3). These values can then be represented with various diagrams such as a bar chart (Figure 3).

These study clearly four influent key factors: The temperature, heating time and pH. The light, citric and ascorbic acid stabilizing are without any significant effect on the response in the chosen experimental field.

**TABLE 3**

<table>
<thead>
<tr>
<th>Name</th>
<th>Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>( b_0 )</td>
<td>56.665</td>
</tr>
<tr>
<td>( b_1 )</td>
<td>-18.217</td>
</tr>
<tr>
<td>( b_2 )</td>
<td>-19.655</td>
</tr>
<tr>
<td>( b_3 )</td>
<td>2.578</td>
</tr>
<tr>
<td>( b_4 )</td>
<td>12.027</td>
</tr>
<tr>
<td>( b_5 )</td>
<td>-0.113</td>
</tr>
<tr>
<td>( b_6 )</td>
<td>-4.025</td>
</tr>
</tbody>
</table>

**TABLE 2**

<table>
<thead>
<tr>
<th>N° Experience</th>
<th>Rand</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Light</th>
<th>pH</th>
<th>Asc ac %</th>
<th>Cit ac %</th>
<th>Response Y1 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>100</td>
<td>5</td>
<td>0</td>
<td>6.5</td>
<td>0.1</td>
<td>1</td>
<td>62.71</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>100</td>
<td>60</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>0.1</td>
<td>7.17</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>100</td>
<td>60</td>
<td>1</td>
<td>3</td>
<td>0.1</td>
<td>1</td>
<td>6.36</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>70</td>
<td>60</td>
<td>1</td>
<td>6.5</td>
<td>0.1</td>
<td>0.1</td>
<td>73.04</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>100</td>
<td>5</td>
<td>1</td>
<td>6.5</td>
<td>1</td>
<td>0.1</td>
<td>77.55</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>70</td>
<td>60</td>
<td>0</td>
<td>6.5</td>
<td>1</td>
<td>1</td>
<td>61.47</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>70</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>80.02</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>70</td>
<td>5</td>
<td>0</td>
<td>3</td>
<td>0.1</td>
<td>0.1</td>
<td>85.00</td>
</tr>
</tbody>
</table>
**B. Optimization**

1) Predicted model and statistical analysis

Table 3 shows the process variables and experimental data. The results of the analysis of variance, goodness-of-fit and the adequacy of the models are summarized. The percentage of rate of retention ranged from 26.48 % to 79.00 %. The maximum value was found at the temperature 70°C, length of heating time 32.5 min and pH 4.75. The application of RSM offers, based on parameter estimates, an empirical relationship between the response variable (rate of retention of betalain pigment) and the test variables under consideration. By applying multiple regression analysis on the experimental data, the response variable and the test variables are related by the following second-order polynomial equation (3):

\[
Y = 54.41 - 11.35X_1 - 8.82X_2 + 6.54X_3 - 1.11X_1X_2 + 2.30X_1X_3 + 1.45X_2X_3 + 1.38X_1X_1 + 0.99X_2X_2 + 0.52X_3X_3
\] (3)

Where X1, X2 and X3 were the coded values of the test variables: The temperature (°C), heating time (min) and pH, respectively.

The statistical significance of regression equation was checked by F-test, and the analysis of variance (ANOVA) for response surface quadratic polynomial model was done by software Nemrodw. The ANOVA of quadratic regression model demonstrated that the model was highly significant. And the Fisher’s F-test had a very high model F-value (201.90) and a very low P-value (P < 0.0001). The value of R2Adj (0.9896) for Eq. (3) is reasonably close to 1, and indicates a high degree of correlation between the observed and predicted values.

A very low value of coefficient of the variation (C.V.) (2.47%) clearly indicated a very high degree of precision and a good deal of reliability of the experimental values. The lack-of-fit measures the failure of the model to represent the data in the experimental domain at points which are not included in the regression.

The F-value (3.99) and P-value (0.0774) of lack-of-fit implied the lack-of-fit was not significant relative to the pure error. It indicates that the model equation is adequate for predicting the rate of retention of the pigment under any combination of values of the variables. The lack-of-fit measures the failure of the model to represent the data in the experimental domain at points which are not included in the regression. The coefficient estimates of model equation, along with the corresponding P-values, were presented in Table 3. The P-values are used as a tool to check the significance of each coefficient, which also indicate the interaction strength between each independent variable. Smaller the P-value is, more significant the corresponding coefficient is [30]. When value of “probability > F” is less than 0.05. It can be seen from this table that the linear coefficients (X1, X2, X3), a quadratic term coefficient (X12, X22) and cross product coefficients (X1X2, X1X3, X2X3) were significant, with very small P values (P < 0.01). The other term (X32) is not significant (P > 0.05).

2) Response surface plot

The 3D response surfaces are the graphical representations of regression equation. They provide a method to visualize the relationship between responses and experimental levels of each variable and the type of interactions between two test variables.
In the present study, the effects of the three factors as well as their interactive effects on the rate of retention are shown in Figures.

3) Effect of temperature and the pH:

Figure 4 denotes the three dimensional surfaces plots of effect of temperature (X1) and the pH (X3) on response. As can be seen, enhancing the temperature values (X1) from 70 to 100°C could decrease the Rate of retention of the pigment according to the results previously observed by [31, 38]. Also, this decrease is more significant on the Rate of retention of the pigment when the pH value (X3) is minimal. In the same way, the increase in the pH from 3 to 6.5 increases the Rate of retention of the pigment significantly when temperature (X1) is minimal.

![Figure 4: Response surface plots and showing the effect of temperature T (X1) and the pH (X3) on the Rate of retention of the pigment.](image)

According to [32] anaerobic conditions favor betanin stability at lower pH (4.0-5.0). The same conclusion is drawn by [33]. who says that on the basis of an absorptivity of 1.00 at the respective λmax-values, cactus pear juice showed betaxanthin contents of 5.6 mg/L (pH 3), 5.8 mg/L (pH 5), and 5.7 mg/L (pH 7), but betacyanin contents in purple pitaya juice amounted to 7.5 mg/L (pH 3), 8.0 mg/L (pH 5), and 7.5 mg/L (pH 7). For the purified fractions from yellow–orange cactus pear, betaxanthin contents of 5.4 mg/L (pH 3), 5.8 mg/L (pH 5), and 5.4 mg/L (pH 7) respectively. This shows that betalain contents only slightly differ over the pH range from 3 to 7 both in juices and in pigment solutions, successively.

In some studies the effect of the pH also studied but with the presence of one stabilizing such as ascorbic, isoascorbic, and citric acid [34]. This study shows that samples heated at pH 6 exhibited considerably higher betaxanthin retentions directly after heating contrary to juice at pH 4.

This result is concordant with previous findings [16,19] providing evidence of maximum betalain stability at pH values close to the natural pH values of the respective betalain-containing plant tissue.

According to the same study and interestingly, betaxanthin regeneration was found in cactus pear juices heated at two different pH 4 and 6; whereas, recondensation was about four times higher at pH 6. The outcomes for betaxanthins agree with earlier observations for betacyanins stating enhanced regeneration after thermal treatment at pH 6 as compared to pH 4 [35]. Moreover [36] observed a stronger betalain loss upon heating at lower pH-values of 4.0 and 3.2.

Temperature is the most important factor on betalain stability during food processing. Some studies reported increasing betalain degradation rates resulting from increasing temperatures because during heat processing, betaxanthin may be degraded by isomerisation, decarboxylation or cleavage resulting in a gradual reduction of yellow color. Thermal betaxanthin degradation in indicaxanthin solutions as well as in red beet and prickly pear juices was reported to follow first-order reaction kinetics [37, 38].

Like the Moroccan juice of Opuntia ficus-indica, Montefiori D. [39] reported that by combination of the effect of the pH and the temperature, the stability of the Italian pigments of cultivars of Opuntia ficus-indica was better with the pH low for treatments of 60 and 70°C.

Generally the degradation rates were found to depend on both temperature and pigment concentration [40]. As confirmed more recently by [36,41, 42] color degradation was higher at elevated temperatures and slower for higher concentrations, respectively.

4) Effect of time and the pH:

Figure 5 shows the effect of length of heating time (X2) and the pH (X3) on the Rate of retention of the pigment. It was observed that Rate of retention of the pigment in yellow-colored juices from Opuntia ficus-indica increased with the decrease in the length of heating time (X2) from 5 to 60min. This increase is also more significant on the Rate of retention of the pigment when the pH (X3) is minimal. Similarly, the increase in the pH from 3 to 6.5 increases the Rate of retention of the pigment significantly when length of heating time (X2) is minimal.

The same remarks are observed by Hasib A. et al. [37] in another study on cactus-pear juice which noted that in addition to the temperature of treatment and the duration of incubation, the pH largely influences the stability of the betaxanthins. Really, according to the value of the pH, various rates of retention were obtained with an optimal stability with pH 5. The results obtained are comparable with those obtained for betaxanthins extracted from fruit juice of various origins.
Coskuner Y. et al. [43] reported that the optimal retention was obtained with pH 5, whereas [44] quoted that the optimal pH is 5.5.

**Figure 5**: Response surface plots and showing the effect of length of heating time (X2) and the pH (X3) on the Rate of retention of the pigment.

5) **Effect of temperature and time of incubation:**

Figure 6 shows the interaction between temperature and time. From the plot it can be seen that when temperature (°C) was increased from 70°C to 100°C the Rate of retention decreased in a linear manner. In addition, the length of heating time also demonstrated a linear decrease for stability of the betaxanthin pigment. This effect, according to [37,45] who also admits that the pace of the absorption spectra was not deteriorated by the heat treatment.

In addition, they note a fall of the intensity of absorption to 482 nm as the temperature of treatment increases. This is observed for the various durations of incubation: 5, 10, 20 and 30min. They also conclude that the reduction in the intensity of this peak of absorption corresponds to a reduction in the concentration in betaxanthins following a thermal degradation.

Studies on heat stability of indicaxanthin for orange-colored juices from *Opuntia ficus-indica* were carried out by several researchers using absorption spectra to monitor pigment loss. As reported for betacyanins [45], absorbance of indicaxanthin decreased steadily with increasing temperature and heating time. The same relation was observed by [38] upon heating of fermented yellow-orange cactus pear juice.

**Figure 6**: Response surface plots and showing the effect of temperature (X1) and the length of heating time (X2) on the Rate of retention of the pigment.

6) **Verification of predictive model**

To ensure the predicted result was not biased toward the practical value, experimental rechecking was performed using this deduced optimal condition. A mean value of 79 ± 2.13 (N = 3), obtained from real experiments, demonstrated the validation of the RSM model.

The good correlation between these results confirmed that the response model was adequate for reflecting the expected optimization.

**IV. CONCLUSION**

The performance of the Rate of retention of the pigment in yellow prickly pear juice was studied with a statistical method based on the response surface methodology in order to identify and quantify the variables which may maximize the Rate of retention.

The three variables chosen, namely temperature, length of heating time, and pH all have an influence on the Rate of retention of the pigment using the conditions of treatment.

The optimal conditions obtained by RSM for Rate of retention of the pigment include the following parameters: temperature 76°C length of heating time 16.20 min, and pH 5.76. Under these conditions, the experimental Rate of retention of betaxanthin pigment was 79%.
Acknowledgements

We thank Professor A. Bacaouli, Faculty of Sciences of Marrakech, for the helpful discussions and the generous access to the NeMRODW software.

REFERENCES


[28] Box G. and Behnken D. Technometrics 1960, 2, 455.


