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**Scientific Research**

# Optimization of annatto dye extraction in semi-industrial scale and evaluation of its stability in laboratory scale and food model system

**Hosseini, F. [1*], Saberian, H. [2], Bolourian, Sh. [3], Afshari, M. [4]**

1. Assisitant professor, Department of Food Additives, Food Science and Technology Research Institute, ACECR, Khorasan Razavi, Iran.
2. Assisitant professor, Technical center for agriculture, ACECR, Isfahan University of technology branch, Isfahan, Iran.
3. Associate professor, Department of Food Additives, Food Science and Technology Research Institute, ACECR, Khorasan Razavi, Iran.
4. Chief Executive Officer, Golchin-e Toos Natural Additive knowledge-based company (Afzooneh).

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*Corresponding Author E-Mail:
f.hoseini@acecr.ac.ir

**ABSTRACT**

In this research, the effects of solvent type, solvent to seed ratio, temperature and time of extraction on the yield, the color purity and the yield of bixin/ norbixin of annatto were studied. Central Composite Design with 4 parameters including of solvent type (acetone, NaOH and acetone-NaOH), solvent to seed ratio (1:1 to 5:1 ml/g), time (2-6 h) and temperature of extraction (25-65 ºC) in three levels (-1, 0 and +1) was employed. The results indicated that the quadratic model was significant for the yield of annatto and R2 = 0.915, indicating that 91.5 % of the change in the responses could be predicted by the fitted model. In addition, the temperature and time of extraction had no any significant effect on the extraction yield. The maximum yield was predicted by NaOH under the 46.41 ºC, 2.05 h and solvent to seed ratio of 5:1, which was 13.31 %. The quadratic model was significant for the color purity of annatto and R2 = 0.848. By changing the solvent from acetone to NaOH and increasing the solvent to seed ratio, the color purity increased. Furthermore, bixin/ norbixin yield, which is the weight ratio of bixin or norbixin to annatto seed, was determined and calculates as a comprehensive response. The most effective factor in the color change of annatto powder was determined as light and if it removed, using suitable packaging, it would be possible to maintain the best quality of the powder .Similar results were obtained regarding the stability of annatto dye in the whey powder .

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# 1. Introduction

Today, color additives are widely used in food products to create a desirable and desirable appearance for consumers. In the researches of various researchers, the negative effects of synthetic colors on human health have been proven and their role in the occurrence of disorders such as apathy, hyperactivity in children, restlessness, etc. It has been emphasized [1 and 2].

Annatto color ($E_{160b}$) is one of the oldest natural carotenoids known from the pericarp of tropical tree seeds.*Bixa Orlana*[1] it will be obtained. Although various carotenoids have been identified in annatto seeds, the main pigments are annatto bixin and norbixin, which constitute more than 80% of the total content of annatto carotenoids. These pigments are extracted in different ways to obtain oil-soluble extracts, oil suspensions or water-soluble extracts, each of which has its own special applications [3 and 4].

The color of annatto ranges from orange to red. Today, annatto color as a natural and healthy color is widely used in various food products such as margarine, butter, confectionery products, bakery, cheese, ice cream, pharmaceutical meat products, production of cosmetics, etc. [5]. From an economic point of view, annatto color is the second natural color additive used in the industry in the world, and the demand for it is expected to increase day by day [6].

Due to the large use of annatto color in the food industry, many studies have been conducted to ensure the safety of annatto color, and the results of these studies did not show any negative effects from the use of annatto color [7, 4]. Also, its antioxidant, anticancer and antimicrobial properties have been mentioned in various articles [8 and 9]. Among other features of annatto (besides coloring), we can mention its use in the treatment of various diseases, antimicrobial properties and antioxidant properties [5].

Annatto seeds are available at a low cost through countries such as India and can be used to extract dyes. In this research, due to the lack of sufficient information and documentation regarding the process of extracting color from annatto seeds in semi-industrial conditions, in the first stage, the effect of different treatments including the variables of type of solvent, ratio of seeds to solvent, extraction temperature and extraction time on the amount of extraction The color of annatto seeds was investigated. Also, the response level method was used to determine the optimal values of the studied factors, because in this method, in order to achieve the maximum efficiency and the maximum reliability of the test, fewer treatments and also a shorter time are needed. It should be noted that the goal of optimization was to maximize the amount of extraction efficiency both quantitatively and qualitatively (purity level of extracted color). In the next step, the stability of the color in different storage conditions was investigated and finally its efficiency for the food model system (cheese water powder) was evaluated. Improving the general health level of society, especially children and teenagers, and helping to produce healthy foods and preventing the spread of cardiovascular diseases and cancer have been among the goals of the research.

# 2- Materials and methods

## 2-1- Materials

Annatto seeds were purchased from India. Solvents including ethanol, ethyl acetate, acetone were prepared with Merck brand for experiments and Kian Chemi brand for semi-industrial production stage treatments. Profits were obtained from Arax Chemical Company.

## 2-2- Optimizing the conditions of annatto color extraction using the RSM method

In this study, in order to investigate the effect of annatto extraction conditions and optimization of the said process, response surface design with four variables is used to investigate the relationship between the obtained responses and process variables and optimization of these responses. The effect of independent variables included$_1$x halal type,$_2$x solvent to solid ratio,$_3$x time and$_4$x extraction temperature is shown in three levels in table (1).

Three repetitions of the central point (to calculate the repeatability of the process) were used to estimate the experimental error. The

1 *Bixa earellana*.L

variables were coded according to the following table:

**Table 1.** Independent variables and their levels used in the CCD design

| Coded Level | | | Symbol | Independent Variables |
|---|---|---|---|---|
| -1 | 0 | +1 | | |
| **NaOH** | **50% Acetone + 50% NaOH** | **Acetone** | $X_1$ | Solvent type |
| **1** | **3** | **5** | $X_2$ | Solvent/Solid ratio (ml/g) |
| **6** | **4** | **2** | $X_3$ | hour (min) |
| **65** | **45** | **25** | $X_4$ | Temperature (°C) |

The range of changes in the ratio of solvent to solid is from 1 to 1 to 5 to 1, for a time of 2-6 hours and for a temperature of 25 to 65 degrees Celsius.

Due to the fact that the most important issue in this research is the investigation of the main and mutual effects of factors such as the ratio of solvent to solid, type of solvents, extraction time and temperature on the percentage of extraction efficiency, therefore RSM statistical scheme was chosen.

In order to extract the color, first annatto seeds were filtered using a sieve system and their waste materials were removed. Then the seeds were washed with water and dried in the air. Five times the weight of the seed, hexane was added to it and it was subjected to gentle stirring overnight to degrease. Then the seeds were centrifuged and the solvent was separated from the seeds.

Then, the extracted fat seeds were transferred to the percolator tank and according to the determined treatments, solvent was added to it with a specific ratio. After the required time at the specified temperatures, the resulting colored extract was filtered using bag filters and transferred to the evaporator. According to the volume of the extract, concentration was done until reaching one fifth of the initial volume of the extract. Then the concentrated extract was dried in the air for 2 days. The obtained powder was milled and rolled to make the particle size uniform.

## 2-3- Determining the weight efficiency of color extraction

The yield of annatto color extraction was calculated by dividing the weight of the powder pigment obtained by the weight of the primary annatto seed and as a percentage.

## 2-4- determination of purity

The purity of the color extracted according to FDA standard method based on Beer-Lambert law and using UV-Vis spectrophotometer CAMSPEC M model$_{550}$ It was determined to be made in England. For this purpose, 80 mg of the extracted dye was weighed with an accuracy of 0.001 and placed inside a 100 ml volumetric flask ($V_1$) was poured and made up to volume with acetone. Then 5 ml ($v_1$) of this solution in a 100 ml volumetric flask ($V_2$) another one was poured and made up to volume again with acetone. 5 ml of this solution ($v_2$) was removed and transferred to another 100 ml volumetric flask (V3) and made up to volume with acetone. Acetone was chosen as the blank of the device and the absorbance of the samples was measured at 487 nm wavelength.

The color purity was calculated from the following equation [10]:

$$\% \text{ color purity} = \frac{Ab \times V1 \times V2 \times v3}{v1 \times v2 \times W \times 3090}$$

in this regard:

Ab absorption rate of colored solution, 3090 $E^{1\%}_{1cm}$ It is equal to the maximum absorption intensity of a pure sample of one percent in a one centimeter cell at a wavelength of 487 nm.

## 5-2-bixin/norbixin efficiency

In the extraction process, both the weight yield of the dye is important and the purity of the obtained dye, and the sample that has the highest weight yield does not necessarily have the highest purity. Therefore, in order to reach the best sample, an index called bixin/norbixin efficiency was defined according to the following relationship:

bixin/norbixin efficiency= Purity × color efficiency

efficiency of bixin/norbixin = 100× ( Color weight / (Weight of Bixin/ Norbixin) × (weight of annatto seeds/weight of color)

## 6-2-Tests to identify and confirm the extracted color structure





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*Nuclear Magnetic Resonance Spectroscopy (NMR)

Determining the structure and purity of the sample based on bixin was done using a Bruker brand NMR device (made in America) located in the chemistry department of Kashan University. For this purpose, a few milligrams of the sample were dissolved in chloroform solvent and placed in the special tube of the NMR machine.

*Infrared Spectroscopy (IR)

The extracted color sample was placed in a vacuum oven for 24 hours to make it completely free of any moisture. Then, a tablet was prepared from the mixture of color sample with potassium bromide (KBr) and placed inside the infrared spectrometer Double beam-Shimadzu-4300 (made in Japan). In order to ensure the purity of the extracted compound, the spectrum obtained from the sample was compared with the standard spectrum of Bixin.

## 2-7-Invitro study of annatto color stability under storage conditions

For this purpose, the obtained annatto color powder was packed in polyethylene bags. The air in the package is evacuated as much as possible and kept under the following conditions: temperature 4°C (refrigerator) and darkness, temperature 25°C (ambient) and darkness, temperature 25°C (ambient) and light and during a certain period of time, the color of the samples using It was measured by Huntlab colorflex device made in America and as CIELAB values including L (light), a (red-green) and b (yellow-blue).

For a better correlation between colorimetric and visual differences, the total colorimetric difference (ΔE) for each sample compared to the control sample was calculated from the following formula (the zero day sample was selected as a control):

$$\Delta E = \sqrt{\left(\Delta L\right)^2 + \left(\Delta a\right)^2 + \left(\Delta b\right)^2}$$

The parameter C or chroma, which determines the degree of color purity and saturation, was obtained by converting Cartesian coordinates (a, b) to polar coordinates based on the following formula:

$$C = \sqrt{\left(a\right)^2 + \left(b\right)^2}$$

## 2-8-Evaluating the stability of annatto color in food model system

In order to evaluate the stability of extracted pigment, cheese water powder was studied as a food model system. Colorless cheese water powder was obtained from Golshad Food Industries Co., Mashhad. Annatto color was added to cheese juice powder at three levels of 0.14, 0.28 and 0.42%. Whey powder prepared in the dragee formulation was used in the amount of 60% of the total dragee of vegetable oil and 40% of powder materials, and the final product was prepared as a snack consisting of 50% of the dragee and 50% of the base (extruded corn). The samples were packed in plastic bags and kept in two environments of light and darkness and at two temperatures of 37 degrees and ambient temperature for a specified period of time. The quality of the color stability of the samples during this period was evaluated by measuring the changes in color indices (L, a, b).

## 9-2- Experiment design and statistical analysis

Response surface method (RSM) was used to optimize color extraction. With the help of this statistical plan, the number of tests is reduced and all the coefficients of the quadratic regression model and the mutual effect of the factors can be estimated [11]. The central compound design (CCD) was used to evaluate the coefficients of the quadratic mathematical model and the analysis of variance (ANOVA) was performed using Design Expert 7 software. In the evaluation stage of color stability, the changes of indices during the storage time were measured by comparing the averages in Duncan's multi-range test.

# 3. Results and Discussion

## 3-1- Examining the effect of variables in the extraction of

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## annatto color by maceration method based on the amount of color yield

The response values (color efficiency, color purity, and bixin/norbixin efficiency of annatto seeds) under different test conditions can be seen in Table 2.

**Table 2** Experimental conditions from the CCD and the experimental results for color extraction from Annatto seeds and their responses (Yield, Color purity and yield of bixin/norbixin)

| No | solvent | Solvent/Solid ratio | Time (h) | Temperature (°C) | Yield (%) | Purity (%) | Provide/provide Yeild |
|---|---|---|---|---|---|---|---|
| 1 | -1 | 1 | 2 | 25 | 0.78 | 7.45 | 0.06 |
| 2 | 1 | 1 | 2 | 25 | 3.54 | 2.37 | 0.08 |
| 3 | -1 | 5 | 2 | 25 | 1.93 | 18.66 | 0.36 |
| 4 | 1 | 5 | 2 | 25 | 9.49 | 2.36 | 0.19 |
| 5 | -1 | 1 | 6 | 25 | 0.76 | 7.61 | 0.06 |
| 6 | 1 | 1 | 6 | 25 | 1.56 | 1.57 | 0.02 |
| 7 | -1 | 5 | 6 | 25 | 1.79 | 17.57 | 0.31 |
| 8 | 1 | 5 | 6 | 25 | 12.12 | 2.88 | 0.34 |
| 9 | -1 | 1 | 2 | 65 | 0.99 | 12.23 | 0.12 |
| 10 | 1 | 1 | 2 | 65 | 1.33 | 2.33 | 0.03 |
| 11 | -1 | 5 | 2 | 65 | 1.92 | 12.22 | 0.23 |
| 12 | 1 | 5 | 2 | 65 | 17.88 | 2.04 | 0.36 |
| 13 | -1 | 1 | 6 | 65 | 0.68 | 11.31 | 0.08 |
| 14 | 1 | 1 | 6 | 65 | 3.18 | 0.03 | 0.00 |
| 15 | -1 | 5 | 6 | 65 | 2.34 | 26.5 | 0.62 |
| 16 | 1 | 5 | 6 | 65 | 11.84 | 0.28 | 0.03 |
| 17 | -1 | 3 | 4 | 45 | 0.8 | 7.04 | 0.06 |
| 18 | 1 | 3 | 4 | 45 | 6.97 | 3.36 | 0.23 |
| 19 | 0 | 1 | 4 | 45 | 1.12 | 5.73 | 0.06 |
| 20 | 0 | 5 | 4 | 45 | 11.93 | 4.98 | 0.59 |
| 21 | 0 | 3 | 2 | 45 | 4.91 | 9.03 | 0.44 |
| 22 | 0 | 3 | 6 | 45 | 1.55 | 6.13 | 0.09 |
| 23 | 0 | 3 | 4 | 25 | 5.18 | 9.07 | 0.47 |
| 24 | 0 | 3 | 4 | 65 | 5.71 | 2.9 | 0.17 |
| 25 | 0 | 3 | 4 | 45 | 6.105 | 5.69 | 0.54 |
| 26 | 0 | 3 | 4 | 45 | 4.745 | 7.54 | 0.47 |
| 27 | 0 | 3 | 4 | 45 | 6.135 | 7.79 | 0.58 |

* In solvent type, -1: Acetone, +1: NaOH and 0: 50% Acetone and 50% NaOH

The results of the analysis of variance (ANOVA) of several regression models for the color yield of annatto seeds are shown in Table No. 3. The results indicated that the quadratic model was significant for the total color efficiency and the coefficient of explanation ($R^2$) calculated for it was 0.915, which indicates that 91.5% of the variation in responses can be explained by the fitted model. In other words, only 8.5% of the total changes cannot be predicted and explained by the model. Corrective explanatory coefficient ($R2_{adj.}$ =0.82) is close to the coefficient of explanation, which indicates the existence of a high correlation between the test values and the predicted values. The lack-of-fit test indicates the failure of the model to show the data in points that are not in the regression model range [12]. According to Table 4, the lack of fit was not significant. Therefore, all the results indicated that the quadratic regression model under different conditions of temperature, time and ratio of

solvent to solid material predicts the color yield well.

**Table 3.** Analysis of variance of different models for yield of Annatto

| Source | Std. Dev | R-Squared | Adjusted R-Squared | Predicted R-Squared | press | |
|---|---|---|---|---|---|---|
| Linear | 2.66 | 0.7001 | 0.6456 | 0.4905 | 264.17 | |
| 2FI | 1.93 | 0.8852 | 0.8135 | 0.5134 | | |
| Quadratic | 1.91 | 0.9154 | 0.8166 | 0.4190 | | Suggested |
| Cubic | 1.84 | 0.9739 | 0.8302 | -4.7358 | | |

Figure 1 shows a sample of concentrated extract and extracted powdered dye.

**Fig 1** Left to right: annatto seeds, annatto extract, annatto natural color

**Table 4** Analysis of variance and significance of regression coefficient for yield of Annatto (Quadratic model)

| Source | Sum of Squares | DF | Mean Square | F Value | Prob >F |
|---|---|---|---|---|---|
| **Model** | 474.63 | 14 | 9.27 | 33.90 | 0.0002 |
| $X_1$-solvent | 173.72 | 1 | 173.72 | 47.51 | < 0.0001 |
| $X_2$-solvent/solid ratio | 182.40 | 1 | 182.40 | 49.88 | < 0.0001 |
| $X_3$-Time | 2.68 | 1 | 2.68 | 0.73 | 0.4084 |
| X4-Temperature | 4.22 | 1 | 4.22 | 1.16 | 0.3036 |
| $X_1^2$ | 2.91 | 1 | 2.91 | 0.79 | 0.3902 |
| $X_2^2$ | 6.39 | 1 | 6.39 | 1.75 | 0.2107 |
| $X_3^2$ | 7.59 | 1 | 7.59 | 2.08 | 0.1752 |
| $X_4^2$ | 0.63 | 1 | 0.63 | 0.17 | 0.6843 |
| $X_1X^2$ | 85.33 | 1 | 85.33 | 23.33 | 0.0004 |
| $X_1X^3$ | 0.76 | 1 | 0.76 | 0.21 | 0.6564 |
| $X_1X^4$ | 2.93 | 1 | 2.93 | 0.80 | 0.3881 |
| $X_2X_3$ | 0.45 | 1 | 0.45 | 0.12 | 0.7331 |
| $X_2X_4$ | 5.19 | 1 | 5.19 | 1.42 | 0.2567 |
| $X_3X_4$ | 1.31 | 1 | 1.31 | 0.36 | 0.5613 |
| Residual | 43.88 | 12 | 3.66 | - | - |
| Lack of Fit | 42.62 | 10 | 4.26 | 6.76 | 0.1356 |
| Pure Error | 1.26 | 2 | 0.63 | - | - |
| Cor Total | 518.52 | 26 | - | - | - |

Equation (1) for the predicted responses of the weight yield of annatto color extraction was obtained in coded form:

$$\text{Yield (\%)} = 5.19 + 3.11\,X_1 + 3.18\,X_2 - 0.39\,X_3 + 0.48\,X_4 - 1.06\,X_1^2 + 1.58\,X_2^2 - 1.72\,X_3^2 + 0.50\,X_4^2 + 2.31\,X_1X_2 - 0.22\,X_1X_3 + 0.43\,X_1X_4 - 0.17\,X_2X_3 + 0.57\,X_2X_4 - 0.29\,X_3X_4 \quad (1)$$

In this regard, $X_1$ ، $X_2$ ، $X_3$ and $X_4$ According to the codes of solvent type, solvent/solid ratio, extraction time and temperature.

The significance of each parameter was determined by the P index. As can be seen in Table 4. Linear effect of X variables$_1$ ، $X_2$ and X interaction$_1X_2$ It significantly affected the weight yield of annatto color extraction ($p < 0.05$). So that by changing the type of solvent from acetone to soda and increasing the ratio of solvent to solid, the efficiency has increased,




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which can also be seen in Figure 2.

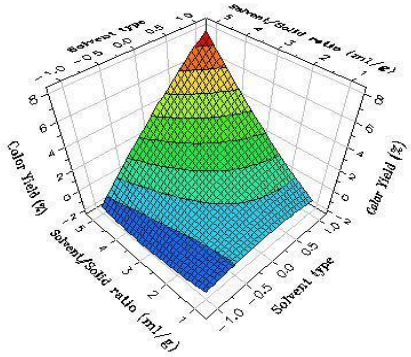


**Fig 2** Response surface plot of the interactional effect of solvent type and S/S on the yield during Annatto extraction

As seen in equation (1), the largest linear coefficient is related to the ratio of solvent to solid material (3.18) and as a result of increasing the ratio of solvent to solid material, it has had the greatest direct effect on extraction efficiency. In this research, temperature and extraction time did not show significant effect on extraction efficiency. Researches on the extraction of other natural pigments have also reported similar conditions, including Wasmond et al. (2006) observed that the extraction time has no effect on the extraction efficiency of the natural chlorophyll pigment [13]. Also, Sabrian et al. (2015) stated that the ratio of solvent to solid was the most significant and effective factor on the yield of chlorophyll extracted by the conventional method; Although the extraction time did not have a significant effect on the yield [14].

## 2-3- Examining the effect of variables in extracting annatto color by maceration method based on color purity

The response values (color purity) under different test conditions can be seen in Table 2. The results of the analysis of variance (ANOVA) of several regression models for color purity are shown in Table No. 5.

**Table 5** Analysis of variance of different models for color purity of Annatto

| Source | Std. Dev | R-Squared | Adjusted R-Squared | Predicted R-Squared | press | |
|---|---|---|---|---|---|---|
| Linear | 3.73 | 0.6867 | 0.6297 | 0.4854 | 502.58 | |
| 2FI | 3.27 | 0.8253 | 0.7161 | 0.2714 | 711.57 | |
| Quadratic | 3.51 | 0.8484 | 0.6716 | 0.0563 | 921.64 | Suggested |
| Cubic | 2.54 | 0.9735 | 0.8279 | -4.5428 | 5413.26 | |

The results indicated that the quadratic model (quadratic) was significant for the purity of annatto color and the coefficient of explanation ($R^2$) calculated for it was 0.848, which indicates that 84.8% of the change in responses can be explained by the fitted model. The lack-of-fit test indicates the failure of the model to show the data in points that are not in the regression model range [12].




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**Table 6** Analysis of variance and significance of regression coefficient for color purity of Annatto (Quadratic model)

| Source | Sum of Squares | DF | Mean Square | F Value | Prob >F |
|---|---|---|---|---|---|
| **Model** | 828.61 | 14 | 59.19 | 4.80 | 0.0049 |
| $X_1$-solvent | 593.63 | 1 | 593.63 | 48.12 | < 0.0001 |
| $X_2$-solvent/solid ratio | 75.48 | 1 | 75.48 | 6.12 | 0.0293 |
| $X_3$-Time | 1.50 | 1 | 1.50 | 0.12 | 0.7336 |
| X4-Temperature | 5.000E-003 | 1 | 5.000E-003 | 4.053E-004 | 0.9843 |
| $X_1^2$ | 0.34 | 1 | 0.34 | 0.028 | 0.8702 |
| $X_2^2$ | 0.11 | 1 | 0.11 | 9.258E-003 | 0.9249 |
| $X_3^2$ | 10.43 | 1 | 10.43 | 0.85 | 0.3759 |
| $X_4^2$ | 0.45 | 1 | 0.45 | 0.037 | 0.8514 |
| $X_1X^2$ | 76.96 | 1 | 76.96 | 6.24 | 0.0280 |
| $X_1X^3$ | 17.58 | 1 | 17.58 | 1.42 | 0.2557 |
| $X_1X^4$ | 14.96 | 1 | 14.96 | 1.21 | 0.2924 |
| $X_2X_3$ | 15.62 | 1 | 15.62 | 1.27 | 0.2824 |
| $X_2X_4$ | 3.36 | 1 | 3.36 | 0.27 | 0.6113 |
| $X_3X_4$ | 6.90 | 1 | 6.90 | 0.27 | 0.4688 |
| Residual | 148.02 | 12 | 12.34 | 0.56 | - |
| Lack of Fit | 145.39 | 10 | 14.54 | - | 0.0858 |
| Pure Error | 2.63 | 2 | 1.32 | 11.05 | - |
| Cor Total | 976.63 | 26 | - | - | - |

According to Table 6, the lack of fit was not significant. Therefore, all the results indicated that the quadratic regression model predicts the purity of annatto color well under different conditions of solvent and ratio of solvent to solid.

Equation (2) for the predicted responses of annatto color purity was obtained in coded form:

$$\text{Purity (\%)} = 6.05 - 5.74\,X_1 + 2.05\,X_2 - 0.29\,X_3 + 0.017\,X_4 - 0.37\,X_1^2 - 0.21\,X_2^2 + 2.01\,X_3^2 + 0.42\,X_4^2 - 2.19\,X_1X_2 - 1.05\,X_1X_3 - 0.97\,X_1X_4 + 0.99\,X_2X_3 - 0.46\,X_2X_4 + 0.66\,X_3X_4 \quad (2)$$

In this regard, $X_1$ ، $X_2$ ، $X_3$ and $X_4$ According to the codes of solvent type, solvent/solid ratio, extraction time and temperature.

The significance of each parameter was determined by the P index. As can be seen in Table 6. Linear effect of X variables$_1$ ، $X_2$ and X interaction$_1X_2$ It has significantly affected the purity of annatto color ($p<0.05$). So that by changing the type of solvent from acetone to soda and increasing the ratio of solvent to solid, the purity has increased, which can also be seen in Figure 3.

As can be seen in equation (2), the largest linear coefficient is related to the type of solvent (5.74) and as a result, the type of solvent has the most direct effect on the extraction efficiency. In this research, temperature and extraction time did not show any significant effect on purity.

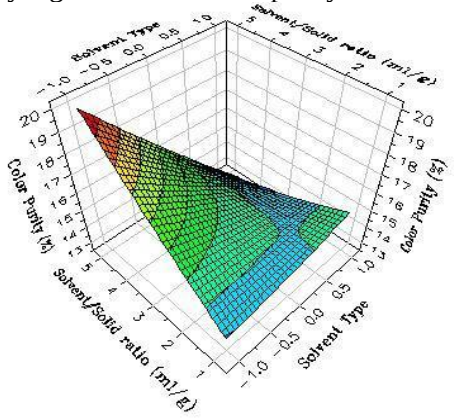


**Fig 3** Response surface plot of the interactional effect of solvent type and S/S on the color purity during Annatto extraction

Chaudhry et al. (2010) used various solvents to extract annatto color, including 95% ethanol (by cold extraction method), 95% ethanol (by hot extraction method), 1% acetic acid, 10% acetic acid and 100% acetic acid and solution They used 0.5% and 0.25% profit. The results of this research showed that among the solvents used, 0.25% soda solution showed the best color purity efficiency, which is consistent with the results of our research [15].

Chovin et al. (2012) extracted annatto seeds

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using acetone solvent with ratios of 1:1 and 1:4 solid to solvent under light and dark conditions. These researchers reported that the extraction efficiency decreases significantly with a decrease in the grain-to-solvent ratio. Also, removing light has little effect on increasing extraction efficiency, but it significantly reduces the amount of volatile compounds in annatto acetone extract [16].

In the research of Bulurian et al. (2013) on color extraction

natural curcumin, ratio of solvent to solid substance, ratio of solvents to each other and time were reported as the most effective factors on extracting the natural color of curcumin [17].

## 3-3- Examining the effect of variables in the extraction of annatto color by maceration method based on bixin/norbixin efficiency.

The results of analysis of variance (ANOVA) of bixin/norbixin yield index quadratic model showed that this model is not significant and the quality of the model for prediction is low ($R^2$= 63.8%) (Table 7).

**Table 7** Analysis of variance of different models for the yield of bixin/norbixin ratio

| Source | Std. Dev | R-Squared | Adjusted R-Squared | Predicted R-Squared | press |
|---|---|---|---|---|---|
| Linear | 0.18 | 0.3565 | 0.2395 | 0.1074 | 0.97 |
| 2FI | 0.20 | 0.4032 | 0.0302 | -0.8690 | 2.02 |
| Quadratic | 0.18 | 0.6376 | 0.2148 | -0.9027 | 2.06 |
| Cubic | 0.20 | 0.8456 | -0.0033 | -28.5141 | 31.95 |

Since the bixin/norbixin efficiency index is the result of the multiplication of the previous two indices (weight efficiency and color purity) and on the other hand, the change process of each of these indices is completely different from each other, so the product is also a different number that follows a specific trend. does not According to Table 2, the highest purity (26.5%) and the highest yield of bixin/norbixin (0.62% or 0.62 grams per 100 grams of annatto seeds) is related to sample 15 (that is, acetone solvent and the highest ratio of solvent to solid substance) , temperature and time) but the weight efficiency of this sample is very low. In fact, this index was proposed with the aim of solving the defects of the weight efficiency and purity index, because in the weight efficiency index, not all extracted compounds are bixin and norbixin, but other compounds are also isolated during the extraction process. In the purity index, the weight of bixin/norbixin is determined relative to the extracted color and not relative to the annatto seed. Therefore, since the bixin/norbixin efficiency index shows the weight of bixin/norbixin relative to annatto seeds, this index is more comprehensive.

## 3-4-Optimum extraction conditions to maximize the weight yield of annatto color

Based on the mentioned results, the optimal conditions of the test variables for color extraction from annatto seeds were predicted using Design Expert 7 software. The maximum efficiency was predicted under the conditions of sodium hydroxide solvent, temperature 41.46 $C^o$, time 2.05 hours and solvent to solid ratio 5, equivalent to 13.31.

## 5-3- The results of the tests to confirm the extracted color structure

Figure 4 shows the HNMR spectrum drawn for the sample extracted from annatto seeds. HNMR

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includes the application of nuclear magnetic resonance by considering the hydrogen atom in the molecule to determine the structure of the molecule, which corresponds to the structure of bixin as seen in the figure. Comparing the IR spectrum obtained from the extracted sample with the standard IR spectrum confirmed the presence of bixin in the structure of the sample (Figure 5).

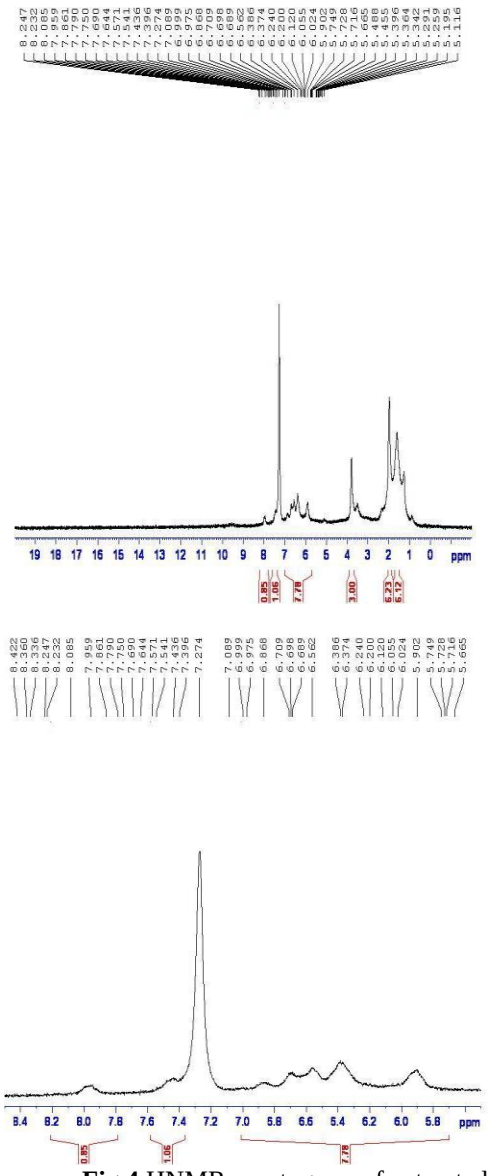
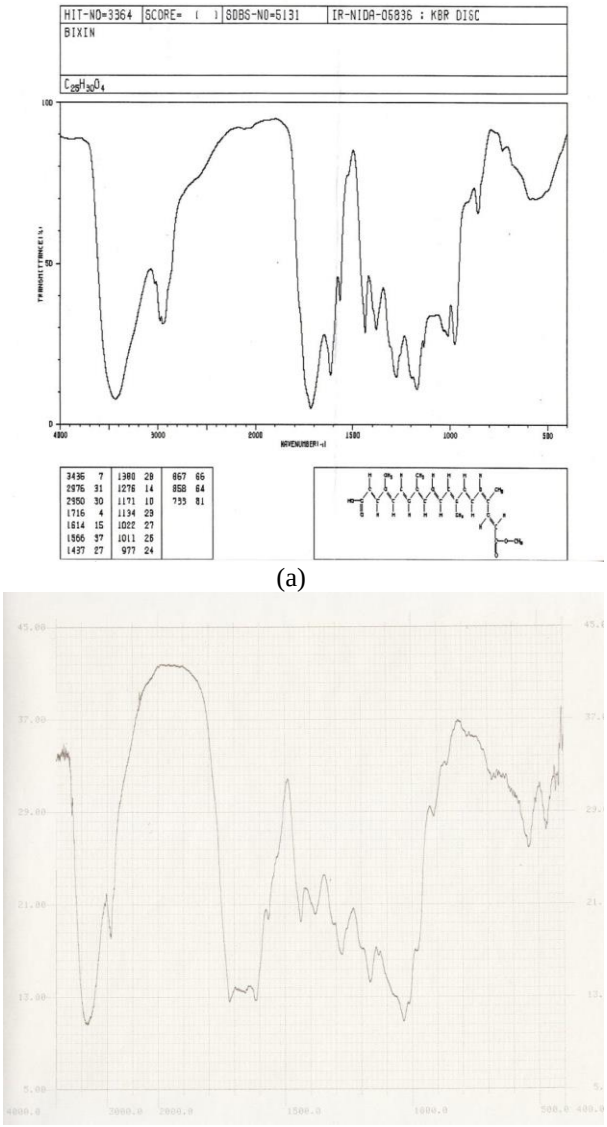


**Fig 4** HNMR spectogram of extracted color




(a)

(b)

**Fig 5** IR of standard sample (a), IR of extracted sample (b)

## 3-6- The results of evaluating the stability of annatto color in vitro

The results of measuring the color changes of annatto powder during storage in 3 treatments of light-ambient temperature, darkness-ambient temperature and darkness-refrigerator temperature are shown in the table below.

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**Table 8** Effects of preservation conditions on color changes of annatto

| Time Week)) | Storage condition | Color charachteristics | | |
|---|---|---|---|---|
| | | L | a | b |
| 0 | **Light- 25°C** | 15.96±0.035 | 9.91±0.05 | 4±0.05 |
| | **Dark- 25°C** | 15.96±0.035 | 9.91±0.05 | 4±0.05 |
| | **Dark- 4°C** | 15.96±0.035 | 9.91±0.05 | 4±0.05 |
| 2 | **Light- 25°C** | 16.12±0.23 | 11.6±0.3 | 5.99±0.11 |
| | **Dark- 25°C** | 15.65±0.1 | 11.31±0.08 | 5.94±0.06 |
| | **Dark- 4°C** | 14.85±0.3 | 11.84±0.31 | 6.139±0.3 |
| 4 | **Light- 25°C** | 16.53±0.41 | 12.18±0.37 | 6.86±0.22 |
| | **Dark- 25°C** | 16.14±0.37 | 11.59±0.29 | 6.32±0.16 |
| | **Dark- 4°C** | 14.65±0.19 | 11.43±0.13 | 5.85±0.08 |

According to the analysis of variance, only the effect of ambient light-temperature on the color changes of the samples was significant, and the two treatments of darkness-ambient temperature and darkness-refrigerator temperature did not have a significant effect on the changes in color characteristics. Therefore, it can be concluded that the most effective factor in changing the color of annatto powder is the light factor. The effect of light on the decomposition of bixin has been shown in various studies, such as Najjar et al. (1988) showed that light is the most important destructive factor among various factors such as air, antioxidants and peroxides on the stability of bixin in chloroform solution during the storage period. be [18]. Also, Balas Y et al. (2006) reported that a significant decrease in the amount of bixin was observed in annatto oleoresin, annatto powder and annatto seeds stored in room light conditions compared to treatments stored in the dark [19].

## 7-3- The results of annatto color stability evaluation in the food model system

The results of evaluating the stability of annatto color in the whey food model system are shown in Table 9.

**Table 9** Mean of CIE parameter changes in different condition during 2 months.

| 37°C | | | 25°C | | | Storage condition | Time (week) |
|---|---|---|---|---|---|---|---|
| b | a | L | b | a | L | | |
| 29.25±0.05 | 16.85±0.11 | 52.11±0.24 | 29.25±0.05 | 16.85±0.11 | 52.11±0.24 | **light** | **0** |
| 29.25±0.05 | 16.85±0.11 | 52.11±0.24 | 29.25±0.05 | 16.85±0.11 | 52.11±0.24 | **Dark** | |
| 30.28±0.08 | 19.04±0.04 | 49.37±0.41 | 29.86±0.09 | 18.74±0.47 | 48.30±0.08 | **light** | **4** |
| 29.69±0.16 | 16.75±0.17 | 51.16±0.24 | 28.48±0.08 | 19.04±0.24 | 47.76±0.24 | **Dark** | |
| 29.06±0.17 | 18.80±0.14 | 49.31±0.53 | 30.57±0.22 | 15.15±0.38 | 52.32±0.45 | **light** | **8** |
| 28.80±0.138 | 14.92±0.255 | 48.38±0.3 | 28.93±0.20 | 19.15±0.26 | 47.46±0.58 | **Dark** | |

According to Table 13-4, the independent and mutual effects of all environmental variables, including light and dark conditions, storage temperature and storage time on changes in brightness (parameter L) and changes in redness (parameter a) of the samples were significant ($p<0/05$). The only independent effect of storage temperature on factor b was not significant ($p<0.05$) and the samples stored at ambient temperature and at 37 degrees had similar yellowness.

It was also observed that the presence and absence of light in the storage environment causes a significant difference in the brightness, redness and yellowness of the samples. Light reduces the amount of color by accelerating the oxidation of conjugated double bonds of carotenoids (Ghafoor and Choi, 2009 and Zhang et al., 2010) [20, 21].

With the passage of time, the brightness of the

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samples decreased (the color of the samples became darker), the amount of redness increased

and the amount of yellowness decreased.

In general, it can be concluded that in the samples kept in the dark, the color of the samples is brighter (lighter and yellower), which shows the necessity of keeping the extracted color in light-impermeable packaging conditions. Also, more studies are necessary to determine the shelf life of these colors.

Annatto color stability has been studied in other researches. Among them, the color stability of commercial annatto solutions by exposing them to temperatures of 90, 100, 120 and 140 degrees Celsius for 450 minutes has been investigated by Fryer et al. (1999). In this study, brightness, redness and concentration of norbixin were determined. According to the report of these researchers, during heating, the amount of yellowness increases and the amount of redness decreases, which is caused by the destruction of bixin. Investigation of the kinetics of the reaction has shown that the degradation of color characteristics follows the first-order reaction and the degradation of norbixin fits with the second-order reactions [22].

Rao et al. (2004) also investigated the color stability of annatto in a food model system under different cooking conditions. They also reported that bixin has good stability at low temperatures, but high temperatures cause its structure to be destroyed.

## 4- General conclusion

In the near future, the use of natural colors in food products will increase with more consumer awareness and increasing legal restrictions for the use of synthetic colors. Therefore, it is necessary to produce, determine the application conditions and check the stability of natural colors in different food model systems. Considering the health importance of natural colors and the existence of many potential natural resources for the extraction of these types of colors, the extraction and application of natural colors in various food products will be a valuable step in improving the general health of the society. Based on the findings of this research, annatto color can be extracted under the optimal conditions of sodium hydroxide solvent, temperature 41.46 $C^o$, time 2.05 hours and solvent to solid ratio 5. Increasing the ratio of solvent to solid has the most direct effect on the extraction efficiency, and the extraction temperature and time did not show a significant effect on the extraction efficiency. The quadratic regression model predicts the purity of annatto color well under different conditions of solvent and ratio of solvent to solid material during extraction. Also, the most effective factor in the color changes of annatto powder was found to be the light factor, if it is removed by using suitable packaging, the quality of the extracted color powder can be maintained to a large extent. Similar results were observed regarding the stability of annatto color powder in the whey powder food model system.

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**مقاله علمی_پژوهشی**

# بهینه‌سازی شرایط استخراج رنگ آناتو در مقیاس نیمه صنعتی و ارزیابی پایداری رنگی آن در شرایط آزمایشگاهی و سیستم مدل غذایی

**فرشته حسینی[۱*]، حامد صابریان[۲]، شادی بلوریان[۳]، مجید افشاری[۴]**

۱-استادیارگروه پژوهشی افزودنی های غذایی، پژوهشکده علوم و فناوری مواد غذایی، سازمان جهاد دانشگاهی خراسان رضوی.

۲-استادیار مرکز خدمات تخصصی کشاورزی، جهاد دانشگاهی واحد صنعتی اصفهان، اصفهان، ایران.

۳-دانشیار گروه پژوهشی افزودنی های غذایی، پژوهشکده علوم و فناوری مواد غذایی، سازمان جهاد دانشگاهی خراسان رضوی.

۴-مدیر عامل شرکت دانش بنیان افزودنی های طبیعی گلچین توس (افزونه).

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$L^*$

**چکیده**

**در این پژوهش، تاثیر متغیرهای نوع حلال، نسبت دانه به حلال، دما و زمان استخراج بر بازده رنگی، خلوص رنگ و بازده بیکسین/ نوربیکسین دانه آناتو بود. طرح مرکب مرکزی با چهار فاکتور نوع حلال (استون، سود، سود-استون)، نسبت حلال به ماده جامد (۱:۱ تا ۱:۵ میلی لیتر بر گرم) و زمان (۲-۶ ساعت) و دمای استخراج (۲۵-۶۵ درجه سانتیگراد) در سه سطح (۱- ، ۰ و ۱) بررسی شد. نتایج حاکی از آن بود که مدل درجه دوم برای بازده رنگ آناتو معنی دار می باشد و ضریب تبیین ($R_2$) محاسبه شده برای آن ۰/۹۱۵ بود که بیانگر آن است که ۹۱/۵٪ تغییر در پاسخ ها توسط مدل برازش شده قابل تبیین است. همچنین دما و زمان استخراج تاثیر معنی داری بر بازده استخراج نشان نداد. بازده بیشینه تحت شرایط حلال هیدروکسید سدیم، دمای $C^o$ ۴۶/۴۱، زمان ۲/۰۵ ساعت و نسبت حلال به ماده جامد ۵ به ۱، معادل ۱۳/۳۱٪ پیش بینی گردید. مدل درجه دوم برای خلوص رنگ آناتو معنی دار بود و ضریب تبیین ($R_2$) محاسبه شده برای آن ۰/۸۴۸ بود. با تغییر نوع حلال از استون به سود و افزایش نسبت حلال به ماده جامد، خلوص افزایش یافت. شاخص بازده بیکسین/ نوربیکسین نیز که در واقع وزن بیکسین/ نوربیکسین نسبت به دانه آناتو می باشد، به عنوان یک شاخص جامع تر تعیین و محاسبه شد. همچنین موثرترین عامل در تغییرات رنگ پودر آناتو، عامل نور تشخیص داده شد که درصورت حذف آن با استفاده از بسته بندی های مناسب می توان کیفیت پودر رنگ های استخراجی را تا حدود زیادی حفظ نمود. نتایج مشابهی در خصوص پایداری پودر رنگ آناتو در سیستم مدل غذایی پودر آب پنیر مشاهده شد.**

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* مسئول مکاتبات:

f.hoseini@acecr.ac.ir