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STUDY OF EXTRACTION, OPTIMIZATION AND PURIFICATION OF NATURAL FOOD COLORANT FROM THE SEEDS OF ANNATTO

Sangeeta A. Pingale*

Asst. Prof. Dept of Food Chemistry and Nutrition, K. K. Wagh College of Food Technology, Nashik, Maharashtra, India. Pin-422003

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***Corresponding Author**
Sangeeta A. Pingale.
Asst. Prof. Dept of Food Chemistry and Nutrition, K. K. Wagh College of Food Technology, Nashik, Maharashtra, India. Pin-422003

ABSTRACT

Annatto is the seed of the tropical bush *bixa ornellana* ,grows in India at coastal regions of Andhra Pradesh and Karnataka. Annatto products bixin and norbixin are carotenoids having colour shades of yellow, yellow orange to orange red.The present study was designed to study the extraction, purification and optimization of natural colorant from the seeds of Annatto. The seeds of annatto has been processed by following different methods for the extraction of natural colorant : Extraction with simple soaking and agitation method, Extraction with methanol by using sohxlet apparatus, Extraction with aqueous potassium or sodium hydroxide, Extraction with ethanol by using sohxlet apparatus. The colour yield from each method was 2.3%, 4.2%, 2.6% and 8.09 % respectively. Further the purification and optimization process was done for highest yield method i.e. extraction with ethanol by using sohxlet apparatus. For optimization various parameters such as Temperature, Time, No. of extractions, Varying ratio of seed/solvent were studied. Following results for each parameters were noted optimum for highest yield: Temperature: 35^0C, No.extractions: 13, Seed: Solvent ratio-1:20 and Time 1 hr. The investigator conclude that extraction with ethanol by using sohxlet apparatus is the best method for highest purified yield of 17.28 % bixin after applying the optimized set of parameters such Temp, No. extractions, Seed : Solvent ratio and Time.

KEYWORDS: bixin, norbixin, sohxlet extraction, food colorant.

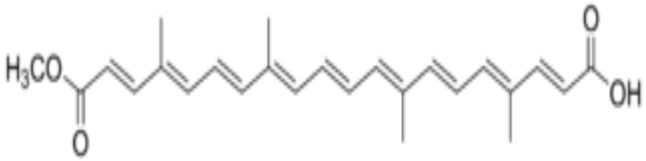
INTRODUCTION

Annatto is an orange-red condiment and food coloring derived from the seeds of the achiote tree (*Bixa orellana*). It is often used to impart a yellow or orange color to foods, but sometimes also for its flavor and aroma. The color of annatto comes from various carotenoid pigments, mainly bixin and norbixin, found in the reddish waxy coating of the seeds. The condiment is typically prepared by grinding the seeds to a powder or paste. Similar effects can be obtained by extracting some of the color and flavor principles from the seeds with hot water, oil which are then added to the food.[1] In the European Union, annatto has the E number E160 b. In the United States, annatto extract is listed as a color additive "exempt from certification" and is informally considered to be a natural coloring.[2, 3] Foods colored with annatto may declare the coloring in the statement of ingredients as "colored with annatto" or "annatto color."[4] The yellow to orange color is produced by the chemical compounds bixin and norbixin, which are classified as carotenoids.

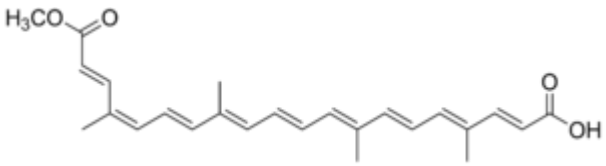
The fat-soluble color in the crude extract is called bixin. The seed contain 4.5–5.5% pigment, which consists of 70–80% bixin.[5] Unlike beta-carotene, another well-known carotenoid, annatto-based pigments are not vitamin A precursors.[6] A higher level of norbixin gives it a more yellow shade while the higher level of bixin gives it a more orange shade. Annatto condiments and colorants are safe for most people when used in food amounts, but they may cause allergic reactions in those who are sensitive.[7, 8] The Food and Drug Administration and experts at the Food Allergy Research and Resource Program (FARRP) at the University of Nebraska do not consider annatto to be one of the major food allergens.[9]

Bixin is an apocarotenoid found in annatto, a natural food coloring obtained from the seeds of the achiote tree (*Bixa orellana*). Annatto seeds contain about 5% pigments, which consist of 70-80% bixin.[1]Bixin is chemically unstable when isolated and converts via isomerization into *trans*-bixin (β-bixin), the double-bond isomer.[10] Bixin is soluble in fats but insoluble in water. Upon exposure to alkali, the methyl ester is hydrolyzed to produce the dicarboxylic acid norbixin, a water-soluble derivative.[10]

Bixin, the major apocarotenoid of annatto[5]



Chemical Structure of Trance Bixin



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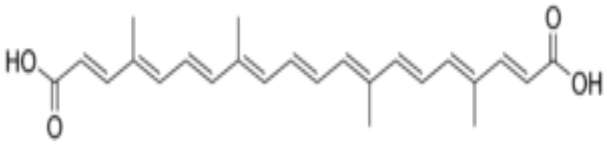

Chemical Structure of norbixin

Annatto is a rich source of tocotrienols, compounds similar in structure and function to the lipid-soluble antioxidant, vitamin E. The tocotrienols from annatto are the subject of current nutritional and medical research since these compounds are thought to have antiangiogenic effects.[11]

The annatto seed, unlike palm oil or rice bran, does not contain tocopherols, so it is a natural source of tocotrienol compounds with antioxidant activity *in vitro*.[12]

Norbixin isomers found in annatto extracts are responsible for the antimicrobial activity specific for Gram-positive bacteria.[13, 14] Bixin has also been found to protect the skin of mice against solar UV damage.[15]

AIMS AND OBJECTIVES

Present study was designed to study the extraction, purification and optimization of natural colorant from the seeds of Annatto. The seeds of annatto has been processed by following different methods for the extraction of natural colorant.

Extraction with simple soaking and agitation method, Extraction with aqueous potassium or sodium hydroxide, Extraction with methanol by using sohxlet apparatus, Extraction with ethanol by using sohxlet apparatus. The percentage of colour yield from each method was determined. Further the purification and optimization process was done for highest yield method i.e. extraction with ethanol by using sohxlet apparatus. For optimization various parameters such as Temperature,Time, No.of extractions, Varying ratio of seed/solvent were studied.

MATERIALS AND METHODS

Annato seeds were collected from the K.K. Wagh education society's babhaleshwar farm, Nashik, India. The seeds were shadow dried for 1 day and cleaned to remove unwanted stuff, dust etc. These seeds are grinded coarsely and used for extraction by various methods. All the solvents and chemicals were obtained from commercial sources (Sigma and Merck).

1) Extraction of water soluble norbixin by simple soaking and agitation method

In this method of extraction 50 gms seeds were kept for soaking in water by 1:10 proportion, the flask was kept on mechanical shaker for 4 successive days at 40^0C and filtered.After filtration, from the filtrate solvent was extracted by using vacuum rotary evaporator. The yield of crude norbixin; the water soluble coloring pigment was noted to be 2.03 %.

2) Extraction of water soluble norbixin by dilute sodium hydroxide

By this method, bixin was saponified into water-soluble norbixin. The 100 gms of seeds were kept for soaking in 100 ml water and dilute NaOH (0.1N) by 1:10 proportion, the flask was kept on mechanical shaker for 4 successive days at 40^0C and filtered. From the filtrate solvent was removed by using vacuum rotary evaporator. Finally the yield of crude norbixin was noted to be 2.60%.

3) Extraction of water insoluble bixin with Methanol by using Sohxlet apparatus

To the 50 gms granular seed powder, by 1:10 ratio methanol was added and kept for refluxing by using sohxlet apparatus for 1 hour at 50^0C.Total 10 extractions were conducted. The solvent was removed by rotary evaporator. The yield of water insoluble crude bixin; the coloring pigment was noted to be 4.6%.

4) Extraction of water insoluble bixin with Ethanol by using Sohxlet apparatus

To the 50 gms granular seed powder, the Ethanol was added by 1:10 ratio and kept for refluxing by using sohxlet apparatus for 1.5 hour at 50 ^{0}C. Total 10 extractions were conducted. The solvent was removed by rotary evaporator. The yield of water insoluble crude bixin; the coloring pigment was noted to be 8.09 %.

5) Optimization of bixin extraction method: The maximum yield of crude bixin was obtained by; extraction with ethanol by using Sohxlet apparatus method. Therefore this method was selected for further optimization of various parameters such as temperature,

seed/solvent ratio, time, no. of extractions to get highest yield of crude bixin. This was done by varying only one parameter at a time and remaining parameters were kept constant.

In the Set I: varying parameter was temp and stable parameters were: Time -1.5 hr, No. of Extractions-10, Seed - Solvent ratio -1:30.

In the SET-II: varying parameter was Time and stable parameters were**:** No. of Extractions-10, Seed-Solvent ratio - 1:30, Temp-35°C.

In the SET-III: varying parameter was No. of extractions and stable parameters were: Time -1 hr, Temp-35 °C, Seed-Solvent ratio -1:30.

In the SET-IV: Varying parameter was seed /solvent ratio and stable Parameters were: Time - 60 min, no. Extractions - 10, Temp- 35° C The optimized set of parameters for highest yield were noted to be: Temperature: 35^0C, No. extractions: 13, Seed: Solvent ratio – 1: 20 and Time 1 hr.

6) Purification process

i) Defatting of the crude bixin

The crude bixin was mixed with hexane by 1: 6 ratio .The mixture was stirred at room temp by magnetic stirrer for three hours. This yielded about 8 % fats from bixin, solvent was removed by using rotary evaporator. The variation in the temp and bixin ratio have yielded lower fat percentage. The primary purified bixin as such contained carotenoid as an active ingredient.

ii) Carotenoids extraction and total content

To determine the total amount of pure carotenoids approximately 15 g of the pure bixin extract, plus 3 g of celite 454 were weighed followed by successive additions of 25 mL of acetone were made ,which was transferred into a sintered funnel coupled to a 250 mL Buchner flask and filtered. This procedure was repeated three times or until the sample became colorless. The extract obtained was transferred to a 500 mL separatory funnel containing 40 mL of petroleum ether. The acetone was removed through the slow addition of ultrapure water, to prevent emulsion formation. The aqueous phase was discarded. This procedure was repeated four times until no residual solvent remained. Then, the extract was transferred through a funnel to a 50 mL volumetric flask containing 15 g of anhydrous

sodium sulfate. The volume was made up by petroleum ether, and the samples were read at 450 nm. The total carotenoid content was calculated using the following formula

$$\textbf{Carotenoid content (µg/g)} = \frac{A \times V\ (ml) \times 10^{4}}{A^{1\%}_{1\ cm} \times P\ (gms)}$$

Where

V= Total extract volume (ml)

A=Measured Absorbance.

P = Sample weight

$A^{1\%}_{1\ cm}$ = Specific absorption coefficient.

Specific absorption coefficient of Beta carotene in petroleum ether is 2592

RESULTS AND OBSERVATIONS

Table 1: Set I: Effect of varying temp on the yield of bixin by using ethanol.

Temp (°C)	Wt of dye (gm)	Bixin (%)
35	10.80	16.40
55	10.75	16.20
75	8.25	15.80
95	6.80	15.55

(**Stable Parameters:** Time -1.5 hr, No. of Extractions-10, Seed-Solvent ratio -1:30)

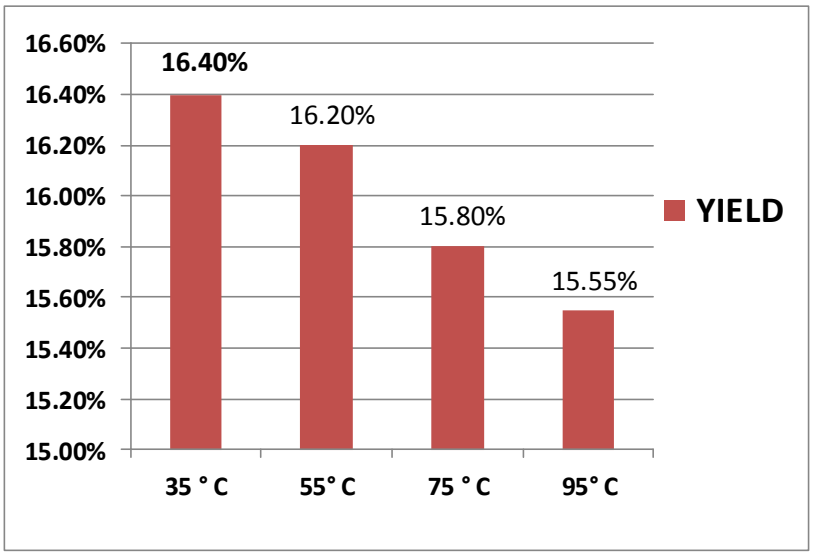

Figure -1: Set I: Temp Vs Yield%

Table 2: Set II: Effect of varying time on the yield of bixin by using ethanol.

Time (min)	Wt of dye (gm)	Bixin (%)
30	11.15	17.40
60	11.70	17.80
90	11.25	17.60
120	10.90	17.20

(**Stable Parameters:** Temp -35° C, No. of Extractions-10, Seed-Solvent ratio -1:30)

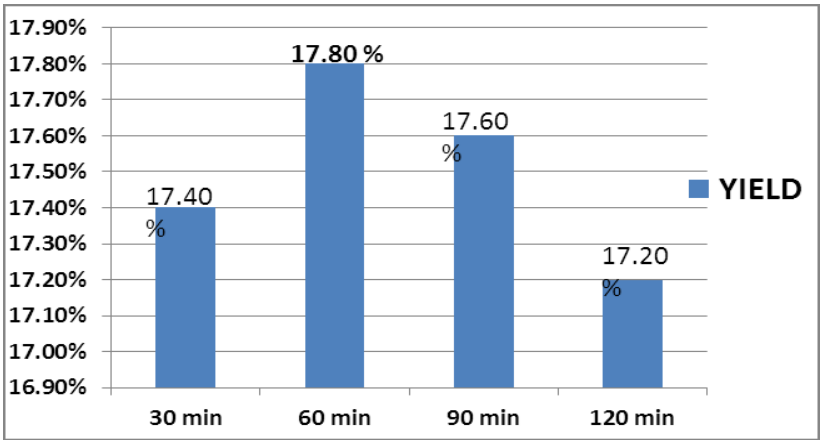

Figure-2: Set-II: Time Vs Yield%

Table 3: Set III: Effect of varying No. of Extraction on the yield of bixin by using ethanol.

No.of extractions (E)	Wt of dye (gm)	Bixin (%)
10	11.10	17.50
13	11.75	17.87
16	11.25	17.70
19	11.20	17.55

(**Stable Parameters:** Time -1.5 hr, Temp-35° C, Seed-Solvent ratio -1:30)

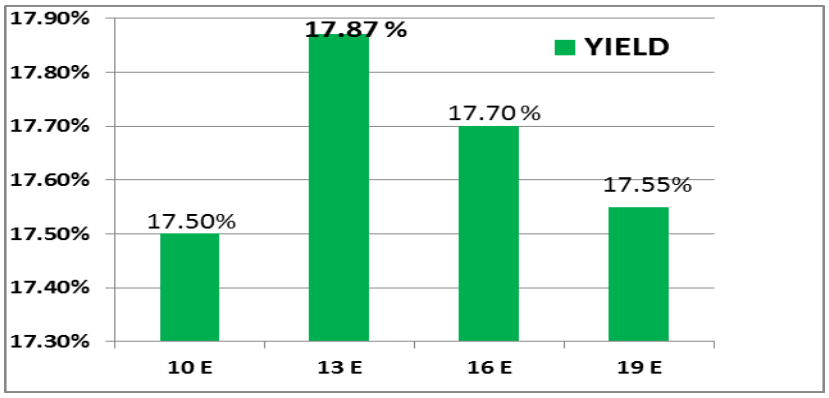

Figure-3: Set III : No. of extractions Vs Yield %

Table 4: Set IV: Effect of varying seed/solvent ratio on the yield of bixin by using ethanol.

Seed / Solvent ratio	Wt of dye (gm)	Bixin (%)
1:10	10.50	16.60
1:20	11.20	17.25
1:30	11.00	17.20
1:40	10.60	16.65

(Stable Parameters were: Time -1.5 hr, No. of Extractions-10, temp:35° C)

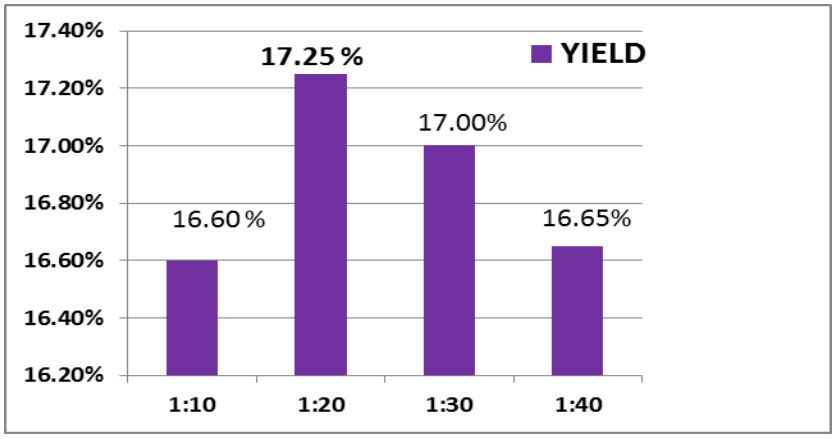

Figure -4: Set IV: Seed / Solvent ratio Vs Yield %

DISCUSSION

The parameters like temperature, seed/solvent ratio, number of extraction and time of each extraction has been optimized. The results of these parameters are shown in table no. 1-4.

The table no.1 shows that changes in the temperature from 35,55,75 and 95°C also changes the yield of bixin % by keeping the other parameters constant such as- Time -1.5 hr, No. of Extractions-10,Seed/Solvent ratio-1:30.The optimum yield was noted to be 16.40% bixin at 35°C.

The table no. 2 shows that changes in the time from 30, 60, 90, 120 minutes also changes the yield of bixin % by keeping the other parameters constant such as-Temp -35°C, No. of Extractions-10, Seed-Solvent ratio-1:30. The optimum yield was noted to be 17.80 % bixin for 60 minutes.

The table no.3 shows that, varying the No. of Extraction from 10,13,16,19 also changes the yield of bixin % by using ethanol at constant parameters such as- Time -1.5 hr, Temp-35° C ,

Seed-Solvent ratio -1:30 The optimum yield was found to be 17.87 % bixin for 13 extraction cycles.

The table no. 4 shows that, varying the No. of seed/solvent ratio from 1:10,1:20,1:30,1:40 also changes the yield of bixin % at constant parameters such as- Time -1.5 hr, No. of Extractions-10, Temp-35° C. The optimum yield was found to be 17.25 % bixin for 1:20 seed-Solvent ratio.Thus after setting the optimum parameter set such as : Temperature : 35^0 C, No. extractions : 13 , Seed : Solvent ratio-1:20 and Time: 60 mins (1 hr). The optimum yield was found to be 17.28 % bixin.

CONCLUSION

The investigator conclude that extraction with ethanol by using sohxlet apparatus is the best method for highest purified yield of 17.28 % bixin after applying the optimized set of parameters such as Temperature : 35^0 C, No. extractions :13, Seed :Solvent ratio-1:20 and Time : 60 mins.

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