Indian Journal of Natural Products and Resources
Vol. 9(4), December 2018, pp. 290-295

Extraction and rapid quantification of pure bixin from *Bixa orellena* L.

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Received 11 July 2016; Revised 29 December 2018

Bixin (9Z-6, 6'-diapocarotene-6, 6'-dioate), one of the carotenoid-based colour extracted from the seeds of the tropical tree *Bixa orellana* L., used primarily as a natural colouring agent in the food industry. In the present study, different solvents were used in both hot and cold conditions to extract bixin from *B. orellana* seeds, collected from two different geographical locations (Chhattisgarh and Uttaranchal) of India. The crude annatto extract was characterized by Thin Layer Chromatography (TLC). The major component, bixin was identified by TLC and further isolated from the crude extract by Preparative TLC (PTLC) method with a purity of 80%. Analysis of purified bixin contents isolated from samples was quantified by both High-Performance Liquid Chromatography (HPLC) and spectrophotometer. The results obtained from both the analytical techniques were comparable and lead to the conclusion that the highest amount of bixin was obtained from Chhattisgarh variety of annatto seed (21.13%), extracted by hot extraction method using acetone as a solvent. As compared to HPLC, the spectrophotometric analysis is an easy, rapid and cost-effective alternate route for the quantitative determination of isolated and purified bixin.

Keywords: Bixin, HPLC, Preparative TLC, Solvent extraction, Spectrophotometer.

IPC code; Int. cl. (2015.01)- C09B 61/00, C09B 67/00

Introduction

The demands for natural colours in food, textiles, pharmaceutical, and other chemical industries have increased due to its low or non-toxicity, appealing colour and health benefits. Among all the sectors food and food products play a major role in day to day life. The addition of harmful synthetic colours and other additives into the food products raises the concern of food safety. Hence, the demand for the utilization of natural colours in food industries is increasing gradually. Among the existing natural food colours, annatto is ranked as the second most economically important natural colourant in the world, and it is the most frequently used natural colourant in the food industry of the United Kingdom[1]. This natural colourant imparts colour ranging from yellow to red and is primarily being used in cheese and butter industry[1]. Recently, annatto colour is also used in foods such as meat and fish products, soft drinks, sugar confectionery, margarine, ice cream, and soups[1]. Annatto is not only limited to food sectors but also used in the drug industry as a possible substitute for Tartrazine (prohibited in many countries), which is a synthetic colourant used in various drugs[2,3].

Annatto colour is a carotenoid pigment basically obtained from the seed coat of the tropical shrub *Bixa orellana* L.[2,4]. This tree is native to tropical America but widely cultivated in Africa and other tropical countries[5-8]. Annatto tree has become naturalized in the hotter parts of India and is cultivated to some extent in the states of Odisha, Andhra Pradesh and Maharashtra for its seeds[9,10]. The major carotenoid pigment, which comprises 70-80% of the seed coat is bixin. Other than that annatto also contains a mixture of nor-bixin, β-carotene, cryptoxanthin, lutein, zeaxanthin, and methylbixin[11]. Various techniques have been applied for extraction of annatto pigments from the seeds, such as immersion of seeds in hot vegetable oil, dilute alkaline aqueous solutions and solvents. Recently, many other technologies like ultra-sound assisted extraction, microwave-assisted extraction, supercritical fluid extraction have also been developed for efficient extraction of major carotenoid pigments such as bixin and nor-bixin from seeds[1,12-14]. Several analytical methods for separation and detection of bixin has also been established. For the qualitative detection of bixin, two-dimensional

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thin layer chromatography has been suggested[15]. For quantitative detection of bixin, several analytical techniques like UV-visible spectroscopy, derivative spectroscopy[16], HPLC[16,17], Liquid Chromatography Mass Spectrometry (LC-MS)[18] and Gas Chromotography Mass Spectrometry (GC-MS)[19] have been used. But these detection methods have several limitations, like HPLC and other sophisticated methods consume more time for separation and it also needs halogenated solvents, which makes the process more costlier[2,4,16]. On the other hand, UV-visible spectroscopy or derivative spectroscopy provides the cost-effective and quick method for detection of bixin but affected by interfering compounds like β-carotene or other non-bixin compounds[16]. However, a purified sample can make the detection more accurate, cost-effective and less time-consuming.

In this study, a method for isolation of pure bixin from the crude sample was developed for easy and cost-effective quantification of the compound. The isolated bixin was quantified by the HPLC followed by spectrophotometry and both methods were compared for their efficiency for detection of bixin.

Materials & Methods

Plant materials

In this study, annatto seeds were collected from two distinct geographical locations of India, Kondagaon, Chhattisgarh (19.6 °N, 81.67 °E) and Pantnagar, Uttaranchal (28.97 °N,79.41 °E). The specimens were identified by Dr. Darshan Kaur Cheema, Department of Botany, S.G.T.B Khalsa College, University of Delhi. The seeds were dried at 80 °C in a hot air oven and stored in a desiccator for further analysis.

Chemicals

All solvents used for extraction and isolation were of analytical reagent grade obtained from Merck, India. Standard used for identification of bixin was purchased from Sigma-Aldrich, India. Isolated compound was identified by TLC on pre-coated silica gel G60 F_{254} (Merck, India). HPLC grade acetonitrile and acetic acid were purchased from Merck India. Distilled water and solvents for HPLC were filtered through 0.45 µm filter (Millipore, USA) and degassed in an ultrasonic bath (Remi instruments, Mumbai, India).

Extraction methods

Two types of solvent extraction methods namely hot extraction and cold extraction were performed for extraction of pigments from annatto seeds. Solvents of different polarity were also used to identify the suitable solvent responsible to give maximum recovery of bixin from annatto seed. The solvents used for the extraction are ethanol, acetone, and hexane, which are non-halogenated and ethanol having a higher polarity followed by acetone and hexane.

In hot extraction method, nearly 30 g of dried, powdered seed samples were taken in three different thimbles and extraction process was carried out in a Soxhlet apparatus for 12 hours with different solvents like ethanol, acetone and hexane at 70, 50 and 60 °C, respectively. Whereas, in the cold extraction method about 30 g of samples were taken in three different conical flasks and dissolved with 200 mL of solvents (ethanol, acetone, and hexane). The extractions were carried out for 24 h at room temperature (28 °C). The flask was being shaken at 0.25 h interval for 8 h for proper mixing of solvents with the seed sample and then kept fixed for the rest of the time. The solids were separated by filtration (Whatman filter paper 1). The solvents were evaporated using rotary evaporator and dried extract was kept at 4º C for further analysis. The yield of the extract obtained by both hot and cold extraction was calculated using the following equation (Eq.1).

$$\%\, Yield = \frac{Weight\ o\ the\ extract(g)}{Weight\ of\ sample\ taken\ (g)} X\ 100 \qquad \text{(Eq.1)}$$

Analysis of extracts

Qualitative analysis by thin layer chromatography

Qualitative analysis of both hot and cold extracted samples was carried out by TLC. TLC plates of 5 x 20 cm size were prepared using silica gel and activated in a hot air oven. Further, samples were applied on the plate. The plates were dried and developed using a mixture of n-butanol, methyl ethyl ketone and 10% aqueous ammonia (3:2:2 by v/v) until the solvent front has ascended about 16 cm. The saturation of the chamber was performed with the mobile phase for 10 min before the plate development followed by drying of the plate. Bixin and norbixin appear as yellow spots with R_f values of about 0.50 to 0.45, respectively.

Isolation of pure bixin by preparative TLC

Isolation of bixin from crude annatto extract was done by PTLC method. Samples were loaded on TLC plate made up of silica gel 60, (20 x 20 cm, and thickness of 2mm). The mobile phase and procedure

are same as in analytical TLC except that the solvent front was allowed to ascend till 18 cm height and the plate was dried. Bixin appeared as a dark yellow band with R_f values of about 0.50. The respective band was scraped off and dissolved in acetone. The sample extracted in acetone was separated from the silica by centrifuging at 5000 rpm for 5 min. Two times washing to the sediment of silica was given to ensure complete recovery of bixin from the silica and the acetone was evaporated in a rotary evaporator. The concentrated bixin was dissolved in 10 mL of acetone and analyzed in both HPLC and UV-visible spectrophotometer.

Quantitative analysis

HPLC analysis

The crude annatto extract and the isolated bixin compound obtained from PTLC method were analyzed by HPLC (Waters 600) equipped with a reverse phase C_{18} column (Waters Spherisorb, 5µm, 250 x 4mm), Waters 2998 photodiode array detector, Waters 600 HPLC quaternary pump, Waters inline degasser and Empower software. The column temperature was controlled at 40°C and the flow rate was 1.0 mL min^{-1} and the mobile phase consisted of solvent A (acetonitrile) and solvent B (0.4% v/v aqueous acetic acid), which was delivered at 65% of solvent A: 35% of solvent B at 1 mL/min. Detection was carried out at fixed wavelengths of 460 nm. All samples were filtered through a 0.45μm membrane filter prior to analysis. A calibration curve was prepared by using 1,2,3,4 and 5 mg/L bixin standard. Total colouring matter as a percentage of bixin content was calculated from the total bixin peak area by interpolation from the calibration graph.

Spectrophotometric method

Bixin samples isolated by PTLC method was also analyzed using UV-visible spectrophotometer (Perkin Elmer Lambda 25) following McKeown and Mark method[20]. Approximately 0.1 g of sample was dissolved in 100 mL of chloroform. The absorbance of the solution was scanned over the wavelength range 400-600 nm in a quartz cuvette of 1 cm path length against a chloroform reference. The wavelength of maximum absorbance (A_{max}) was found to be 510 nm. The total pigment content expressed as bixin content was calculated using the following equation (Eq. 2):

$$\text{Total pigment in \% (as Bixin)} = \frac{[(Amax + A404) - (0.256Amax)]}{282.6} X \frac{100}{sample\ weight\ (g)} X \frac{dilution\ volume\ (mL)}{1000} \quad \text{(Eq. 2)}$$

Where Amax is the maximum absorbance in the scanned range i.e. at 510 nm, A404 is the measured absorbance at 404 nm, the value 0.256 is the factor relating the absorbance at 404 and 501 nm for bixin in chloroform, and the value 282.6 is the absorptivity of bixin at 501 nm in chloroform.

Results and Discussion

Crude bixin extract (CBE) analysis

Extraction of bixin by using different solvents are more economical and efficient as compared to other developed techniques like microwave, ultrasonic, and supercritical carbon dioxide[21]. The bixin yields obtained by both hot and cold extraction methods using different solvents(ethanol, acetone, and hexane) are described in Table 1. The results also compare the bixin content of annatto seeds obtained from two different sources. Among all solvents, acetone and hexane under hot extraction method showed improved results. The total CBEs obtained by using acetone as a solvent and hot extraction method for Uttaranchal and Chhattisgarh annatto seeds are nearly the same i.e 9.21 and 9.20 %, respectively. Again the yield of CBE by hexane using hot extraction in Uttaranchal and Chhattisgarh annatto seeds are quite similar i.e.9.01and 8.80 %, respectively. For the solvents acetone and hexane, the cold extraction method has also produced a good yield i.e nearly 70-90 % of the amount obtained through hot extraction (Table 1). The performance of ethanol in both hot and cold extraction methods is not so impressive. The lowest yield of 3.01% was obtained in cold ethanol extract of Uttaranchal annatto seeds. The solvents like acetone and hexane resulted in more bixin yield as compared to ethanol. It could be due to the higher solubility of bixin in medium polar and non-polar solvents as compared to polar solvents[21,22]. In spite of distinct geographical locations, there is not much difference observed in bixin content between Chattisgarh and Uttaranchal annatto seeds.

Purification and analysis of bixin from CBE

In order to identify the presence of bixin in the CBE samples, analytical TLC was performed at the first stage. In TLC analysis, three distinct spots were detected i.e. one dark yellow pigment and two light

Table 1 — Crude bixin content extracted by different solvents and extraction method

Sample source	Sample code	Solvent used	Extraction temperature	Extraction duration (Hours)	% Yield of crude bixin extract	% Yield of crude bixin in cold extraction as compared to hot extraction
Uttaranchal, India	UHH	Hexane	60	12	9.01	
	UHA	Acetone	50	12	9.21	
	UHE	Ethanol	70	12	4.17	
	UCH	Hexane	28	24	7.79	84.58
	UCA	Acetone	28	24	8.03	89.12
	UCE	Ethanol	28	24	3.01	72.18
Chhattisgarh, India	CHH	Hexane	60	12	8.80	
	CHA	Acetone	50	12	9.20	
	CHE	Ethanol	70	12	5.97	
	CCH	Hexane	28	24	6.53	77.17
	CCA	Acetone	28	24	7.51	74.20
	CCE	Ethanol	28	24	5.37	89.95

Three letter abbreviation
1st letter U/C: Uttaranchal/Chhattisgarh
2nd letter H/C: Hot/cold
3rd letter stands for H/A/E: Hexane/Acetone/Ethanol

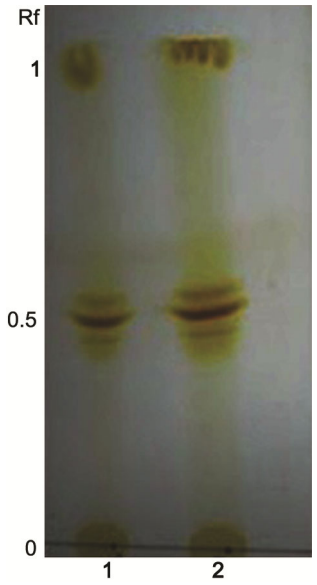

Fig. 1 — TLC analysis of crude annatto extract

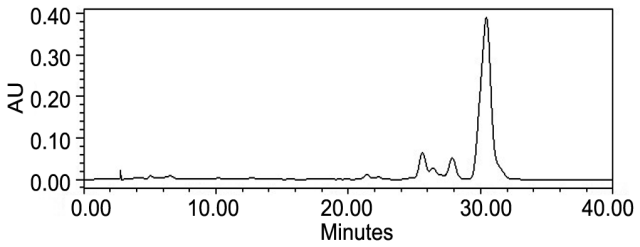

Fig. 2 — HPLC chromatogram of bixin obtained by Preparative TLC

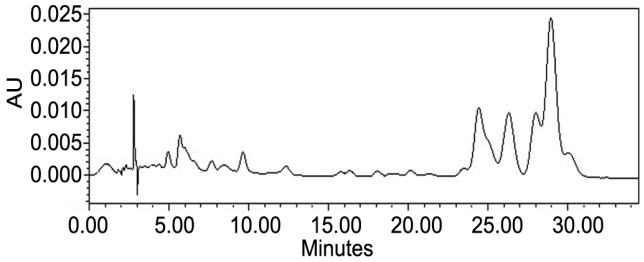

Fig. 3 — HPLC chromatogram of crude extract

yellow pigments (Fig.1). The dark yellow spot and the second light yellow spot (detected below the dark yellow spot) were appeared at R_f 0.5 and 0.45 and identified as bixin and norbixin, respectively, which is in agreement with the literature[23]. For isolation/purification of bixin from the mixture, CBE samples were loaded on preparative TLC plates. Bixinappeared at R_f 0.5 was isolated by extracting in acetone and the final extracted solution was analyzed both by spectrophotometer and HPLC. HPLC chromatograms of purified bixin by preparative TLC and CBE are shown in Fig. 2 and Fig. 3, respectively. A distinct peak of isolated bixin appeared at a retention time (RT) of 31 minutes. Whereas, multiple peaks appeared in the RT range of 20-30 minutes and overlapped with the bixin peak for CBE samples. This signifies the presence of impurities and interferes with accurate quantification of bixin. The purity of the isolated bixin was verified by the bixin standard (Sigma), which resulted in 80% purity. Further, bixin from other solvent extracted samples were quantified, following the

Table 2 — Comparison of purified bixin content in HPLC and spectrophotometry

Sample code	HPLC % Bixin	Spectro% Bixin	Mean	SD
CHH	11.0	10.59	10.80	0.29
CHA	21.13	21.62	21.38	0.35
CHE	4.41	3.73	4.07	0.48
CCH	12.35	12.54	12.45	0.13
CCA	10.25	9.63	9.94	0.44
CCE	5.44	5.22	5.33	0.16
UHH	11.05	11.62	11.34	0.40
UHA	19.5	18.7	19.10	0.57
UHE	9.42	9.32	9.37	0.07
UCH	9.04	8.79	8.92	0.18
UCA	10.25	10.32	10.29	0.05
UCE	4.91	5.02	4.97	0.08

Three letter abbreviation
1st letter U/C: Uttaranchal/Chhattisgarh
2nd letter H/C: Hot/cold
3rd letter stands for H/A/E: Hexane/Acetone/Ethanol

same procedure of isolation and quantification. A comparative analysis of total pure bixin content of all the extracted samples were done (Table 2). Although, the crude yield or CBE obtained in both Uttaranchal and Chhattisgarh seeds were same but the highest purified bixin content was observed in Chhattisgarh seeds i.e. 21.13 % (0.21 g/g) followed by Uttaranchal seeds i.e. 19.5 % (0.19 g/g) by hot extraction method using acetone as a solvent. On the contrary, the hexane extracted sample, which yields a similar crude content as acetone contained very less bixin ranging from10 to11 % in both hot and cold extraction method. However, the ethanol-extracted sample contained the lowest bixin (2-4 %).

The combination of medium polar solvent and higher temperature resulted into higher bixin yield (21.13 %) and reduced extraction time to almost half (i.e12 h) as compared to cold extraction with polar solvent, which gave a lower yield of 4.91 % at 24 hours[5]. Other researchers also reported maximum extraction of bixin using medium polar solvents like acetone and ethyl acetate[24,25]. But, as compared to other medium polar solvents, acetone as a food grade solvent is preferably used for extraction of annatto seed as it is used as a colouring agent for food and pharmaceutical industry. Although a remarkable difference in bixin yield was observed among different solvents and solvent extraction techniques, the bixin content was found to be nearly the same between the annatto seeds of two different geographical locations of India.

Comparison of estimation methods for bixin

The isolated bixin content was analyzed by HPLC method and further verified by spectrophotometer following the McKeown and Mark (1962) method[20]. The objective of the further spectrophotometric analysis is to establish a cost-effective analytical method as compared to HPLC, having the same accuracy of estimation (Table 2). The advantages of the spectrophotometric method over HPLC is that the method does not require extensive treatment and procedures, which is generally used in HPLC. As compared to HPLC, the spectrophotometric method is simple, fast and cost-effective, thus recommended for quantification of bixin[4]. Previously, bixin contents have been quantified by both spectrophotometric and HPLC methods, but the values obtained by the former method always remained on the higher end as compared to the later due to the presence of nor-bixin components like β-carotene or other impruties[16,21]. But, in this study, the results obtained by both analytical methods for estimation of bixin came nearly same and can be comparable (Table.2). The standard deviation (SD) value varied between 0.05 to 0.57 and the variation from the mean (% pure bixin), ranges between 0.8-11.8 %. Due to introducing PTLC in isolation of pure bixin, the drawbacks of other interfering compounds have been overcome and the quantification of pure bixin has been easily done by spectrophotometric method.

Conclusion

The medium polar solvent like acetone helps in increasing the extraction of bixin from annatto seeds as compared to highly non-polar or polar solvents like hexane and ethanol, respectively. Again hot extraction enhances the process resulting in less time and more yield of bixin. Sample isolation/purification by preparative TLC leads to more accurate quantification of bixin compared to crude annatto extract. Therefore, UV-visible spectrophotometry could be a cost-effective and less time-consuming method as compared to HPLC for quantification of isolated bixin.

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