Characterization and Storage Stability Study of Bixin Extracted from *Bixa orellana* Using Organic Solvent

N N Husa¹, F Hamzah²* and H M Said³

¹,²Biocatalysis and Biobased Material Research Group, CoRe of Green Technology & Sustainable Development, Faculty of Chemical Engineering, Universiti Teknologi MARA, MALAYSIA
³Kelantan Biotech Corporation Sdn Bhd, Lot 3174, Batu 23, Jalan Kota Bharu-Machang, Kampung Wek, 18500 Machang, Kelantan, MALAYSIA

fazlena@salam.uitm.edu.my

Abstract. Colorant is one of the additives to give a better appearance or improve the colour of the product. Synthetic colorant has been widely used in industrial due to its readiness in the market and its colour stability. However the arising issues related to the safety, nutrition and also the therapeutic effect has encouraged users to become more concern about the colouring component. Thus, the present research was conducted in order to produce natural colorant called bixin from *Bixa orellana* or also known as annatto seeds. The study was focusing on the effect of the solvent on the characteristic and concentration of the extracted bixin. While stability of the bixin during different storage condition was determined to further validate the effectiveness of the solvent. The result indicated that methanol and acetone gave darker of the bixin colour as compared to the water. However, the deterioration rate of the bixin in acetone and methanol were faster as compared to the water. The extracted bixin was analysed using Fourier-transform infrared spectroscopy (FTIR) and spectrophotometer analysis. The strong band for the bixin was observed at absorbance range of 1704 – 1740 cm⁻¹. The study indicates that the concentration of the extracted bixin was the highest in methanol which gave 817.7 ppm of bixin. Meanwhile, bixin concentration in acetone and water was 602.9 ppm and 477.19 ppm respectively.

1. Introduction

Colorants or colour additive are the terms for all soluble or solubilized colouring agents (dyes or pigments) as well as insoluble pigments, employed to impart colour to a material. Colour has been widely used in pharmaceutical, food and cosmetic products because it work as sensorial characteristic that influences the expectations of the customer and also influences quality-related decisions during visual inspection[1]. Colouring agents can be classified by their origin as natural, synthetic or inorganic colorants. Synthetic organic dyes has been recognized for many years as the most reliable and economical colouring agents because they are superior to natural pigments in tinctorial power, consistence of strength, range and brilliance of shade, hue, stability, ease of application and cost effectiveness. However, the use of synthetic dyes is gradually receding due to an increased environmental awareness and to potential harmful effects of either toxic degraded products or their non-biodegradable nature [2]. Furthermore, the safety concern regarding to the synthetic colouring has been addressed due to the present of a carcinogenic component, high level toxicity and allergic reaction to the end user in some of the synthetic colorant [3]. Therefore, there is an increased interest
in substituting the usage of synthetic natural to natural colouring agent as a consequence of perceived consumer preferences as well as legislative actions. There are many sources that can be utilize to produce the colorant or colour additives, such as herbs, flowers, roots and even beans [4]. Flavonoids, carotenoids and chlorophyll are the major contributors to the natural colours of the most plant. One of the less exploring plant and have higher colouring potential in Malaysia is Bixa orellana (B. orellano) or annatto. B. orellano is a native plant in Brazil and also grown in some tropical countries such as Peru, Mexico, Kenya, Asia and East Africa [5]. Bixin which is extracted from the outer layer seed of B.orellana (Figure 1a) is group of carotenoid pigments which give higher high range of colour-comprising of red, orange and yellow hues [6]. It is lack of toxicity and has high tinctorial value. Bixin consists of 25 carbons with the chemical formula C_{25}H_{30}O_{4} with molecular weight of 394.51. Carboxylic acid and methyl ester are attached at end of the chain and the chemical structure is as shown in Figure 1(b). According to Santos et al. [7], several carotenoids, including bixin and norbixin, can be obtained from annatto seeds, depending on the extraction method used. Extraction of the bixin can be conducted by chemical using indirect extraction with solvents, direct extraction using aqueous alkali solutions and direct extraction using oil [8] or mechanical using ball milling or spouted bed [9] method. However, natural pigments generally are unstable and participate in different reactions. It is reported that the produced colour is strongly depend on the processing and also it storage condition. The chemical, toxicological, and antioxidant properties, degradations and extraction of bixin and norbixin have been extensively studied but for stability of the bixin in the powder form only a few studies can be found in the literatures. Thus in the presence study, storage capability of the powder bixin extracted using different solvent was studied. The study aims to determine the effect of different storage condition towards colour degradation of the bixin.

![Figure 1. a) Annatto seeds in the pod b) Chemical structure of Bixin](image)

2. Materials and Methods

2.1 Chemicals and Reagents

The main raw material which is B.orellana or annato was taken from Jeram Linang, Pasir Puteh, Kelantan with coordinate of 5.798517, 102.342852. Methanol and acetone are analytical grade reagents with > 99% purity purchased from Merck. The sodium hydroxide was purchased from R&M Chemicals, while the bixin standard was purchased from Spectrum Chemical.

2.2 Solvent Extraction of B.orellana

The raw material which is annatto seeds was removed from the pod. 2 g of the annatto seeds was weighed and mixed with 20 ml of solvent. The mixture of annatto seeds and the solvent was stirred using digital overhead stirrer (OS-20 Tuff) at 500 rpm until the colour was completely extracted from the seeds. The time taken for the stirring process was recorded and the same procedures were repeated for another type of solvent. The extracted colorant was filtered using Whatman paper grade 595 with the pore size ranging from 4 - 7 μm.
2.3 Characterization of Bixin

Colour analysis of the bixin was conducted using UV-Vis spectrophotometer (Perkin Lambda 750 UV/VIS Spectrophotometer) and the value was compared with the absorbance from bixin standard. Bixin standard solution was prepared by dilution of 0.05 g of standard bixin in 50 ml of solvent to produce a 1000 ppm stock solution. Then, further dilution was conducted to produce 750 ppm, 500 ppm and 250 ppm of bixin solution. The absorbance of the solution was analysed using UV-Vis spectrophotometer (Perkin Lambda 750 UV/VIS Spectrophotometer) at 540 nm to generate the standard curve. The bixin extracted from *B.orellana* was analysed using similar procedure for absorbance value. While, Fourier Transform Infrared Spectrophotometer (FTIR) - Jasco FT/IR-460 Plus in the range of 4000 to 400 cm⁻¹ analysis was used to analyse the chemical functioning group presence in the extracted bixin.

2.4 Colour Stability Test

Colour stability test for bixin was conducted at two conditions which are the storage condition influencer and sunlight influencer test. 0.1 g of annatto seeds was mixed with 10 ml solvent and extracted using digital overhead stirrer at 500 rpm for 5 minutes. 5 ml of the sample was used for 4 different tests for storage condition A, B, C and sunlight influencer as tabulated in Table 1. The absorbance reading for these samples were recorded in every 3 days for 25 days. Meanwhile for the sunlight influencer condition, the sample was exposed to the sunlight for everyday and the absorbance was recorded. All the set of test was repeated triplicate.

| Condition | Type of Vial | Storage temperature |
|-----------|--------------|---------------------|
| A         | Transparent  | Room temperature    |
| B         | Amber        | Room temperature    |
| C         | Transparent  | 4°C                 |

3. Results and Discussion

3.1 Effect of the solvent on the colour and concentration of the bixin

The color comparison between the extractions of the bixin using different types of solvents is shown in Figure 2. The results indicated that extraction of bixin using methanol as the solvent produce the brightest colorant while the distilled water produces the least bright colorant for the same mass of *B.orellana* used. Both of the methanol and acetone used producing a clear orange colorant while extraction using distilled water producing cloudy-orange colorant. This result show that methanol and acetone were extracted higher bixin amount as compared to the water. This might be due to the dissolution properties of the methanol and acetone that encouraged dilution of colouring material. According to Boeing *et al.*[10] methanol act as a better extraction solvent compared to acetone because it can simultaneously dissolving the cell membranes and the carotenoids. While Silva *et al.*[11] also claim that organic solvent such as acetone and methanol will extract higher pigment concentrations from 3.5 to 5.2% bixin. Extracted bixin was transformed into powder form using freeze and spray drying method and the output is illustrated in Figure 3. The freeze drying process producing a brighter powdered colorant compared to spray drying which produces a pale orange colour. The different temperature used in the powder formation significantly affects the colour of bixin. Fades or deteriorates of the bixin colour was occurred in spray dried process that result in pale of orange bixin powder. This might occur due to the high temperature up to 130°C used during spray dried method. According to the Wang *et al.*[12], temperatures over 80 °C negatively affect anthocyanin stability and results in degradation of the absorbance value. Similar finding was reported by Patras *et al.* [13] who demonstrated that the anthocyanins in blackberry and strawberry puree are significantly affected by thermal treatments at 70 °C for 2 min. Thus the results show that lower operating temperature able to preserve the colour from deteriorates.
Figure 2. Extract bixin using methanol, acetone and water

Figure 3. Powder form of bixin using freeze and spray dried process

The concentration of bixin in methanol, acetone and water is shown in Table 2. The results show that the highest concentration of bixin was obtained by using methanol with a value of 817.71 ppm followed by acetone at 602.90 ppm of bixin and the least was by using distilled water with 477.19 ppm of bixin. The extraction of the bixin in the solvent is influenced by solubility properties of the bixin. As reported by Pimentel and Stringheta,[14], bixin is an oil-soluble pigment and the solubility of bixin is significantly enhanced by the action of the oil present in the seeds. Thus, low concentration of the bixin in the water represents the characteristic of the oil soluble pigment. The result obtained is satisfying with the study by Boeing et al.[10], that demonstrate methanol is the better solvent for the extraction of natural colorant compared to the acetone and water. This is because methanol has the ability to dissolve the cell membrane and pigment as compared to acetone and water. Thus, the presence studies indicate that low polarity of methanol and acetone capable to extract higher concentration of the bixin as compared to water.

Table 2. Concentration prediction result of the extracted colorant

| Extraction solvent   | Absorbance reading | Concentration of Bixin in sample (ppm) | Polarity index |
|----------------------|--------------------|----------------------------------------|----------------|
| Distilled water      | 0.0602             | 477.19                                 | 10.2           |
| Acetone              | 0.0167             | 602.90                                 | 5.1            |
| Methanol             | 0.0671             | 817.71                                 | 5.1            |

3.2 Characterization of Bixin

Figure 4 shows the result for the FTIR analysis of the standard bixin which consist a strong band at 1704 cm\(^{-1}\) and a medium band on 970 cm\(^{-1}\). Yusá-Marco et al.[15] state that the carotenoids compound specifically bixin band can be determined at 1721 cm\(^{-1}\), 1603 cm\(^{-1}\), 1149 cm\(^{-1}\) and 962 cm\(^{-1}\). The effect of solvent on the extraction of bixin based on the functional group present in the solution is reveal in Figure 5. For the extraction with water, there are a very sharp band at 3274 cm\(^{-1}\) and 1634 cm\(^{-1}\) and the small band of bixin at 1737 cm\(^{-1}\) was detected. Besides, the presence of small bands which representing the functional group of C-O-C stretch was appeared at 1365 cm\(^{-1}\) and 1217 cm\(^{-1}\). On the other hand, functioning group of the bixin extracted using methanol shown in Figure 6 and the presence of the bixin spectrums were detected at 1737 cm\(^{-1}\) and 1020 cm\(^{-1}\). While, the alcohol group (O-H) representing methanol was observed at 3322 cm\(^{-1}\). Based on the spectrum, alkyl C-H functional group was detected at small band of 2943 cm\(^{-1}\) and 2832 cm\(^{-1}\). While, the two small bands of C-O-C were appeared at 1217 cm\(^{-1}\) and 1114 cm\(^{-1}\). As for the bixin extracted using acetone, the presence of
strong bixin band was observed at 1708 cm\(^{-1}\) and a weak band was detected at 903 cm\(^{-1}\). FTIR spectrum of the bixin extract using acetone is illustrated in Figure 7. Other functioning group present in bixin extracted using acetone was identified at 1220 cm\(^{-1}\) that representing C-O-C group. While, a small band at 3512 cm\(^{-1}\) and 3003 cm\(^{-1}\) was frequently diagnosed as unsaturation.

3.3 Storage Capability of the Extract

Colour stability test for different storage condition of the bixin extract is shown in Figure 8. The deteriorate rate was calculated based on the slope obtained from the results. Figure 8 (a), shows that acetone able to retain the highest bixin colour after certain exposure time as compared to methanol and distilled water. However, deteriorate rate of the bixin in acetone is faster as compared to water and methanol with the value of 0.1697 abs/day. While extract in distilled water shows a more stable colour deteriorate rate based on the better linearity of graph and slope of the graph (0.0574 abs/day). While for condition B, it was observed that deterioration of the bixin show a same deteriorate pattern as storage condition A. However, deterioration rate for each of the sample was lower than condition A. This is due to the characteristic of amber vials which capable to prevent photodegradation from occurring. The deterioration rate obtained was higher for bixin in acetone followed by methanol and distilled water with the values of 0.1375, 0.1132 and 0.0667 abs/day respectively. Similar trend was recorded for bixin deterioration in condition C. However, cooling condition was able to prevent the colour deterioration process of the bixin. Deterioration slope for bixin in each solvent was lower as compared to the condition A. The value obtained was 0.1192 abs/day for bixin in acetone, 0.0713 abs/day for bixin in methanol and 0.06 abs/day for bixin in water. Then for the exposure of the bixin to sunlight studies, a similar deterioration trend was also observed. Water was the most stable solvent to prevent fast deterioration
of the bixin colour. The deterioration rate for acetone, methanol and water were 0.2727, 0.1697 and 0.1645 abs/day respectively.

![Figure 8. Colour stability in different storage condition](image)

The deterioration rate might be influence by the characteristic of the solvent itself. Acetone have evaporation rate of >3.0 where it molecules can evaporated faster event at room temperature. This is because it molecule structure do not have pure hydrogen bonding that require less energy for it molecule to evaporate to the surrounding. This condition is explaining the highest deterioration rate of bixin. Similar characteristic is also reported for methanol. Methanol also have a boiling point near to room temperature that encourage the methanol molecule evaporate faster to the environment. While water is made up of many hydrogen bonds and higher temperature are required to break up this bond to make water evaporate. Thus at room temperature, slow evaporation will occurred and this phenomena answering the slowest degradation rate of bixin in distilled water.

### 4. Conclusion

Methanol, acetone and distilled water were a polar solvent that can be used to extract bixin from *B.orellana*. The present of the bixin in all of the solvent was determined at 1704 – 1740 cm\(^{-1}\). The study indicated that methanol is the optimum solvent in bixin extraction which gave up to 817.7 ppm of bixin. On the other hand in storage stability study, bixin in the water retained its colour after 25 days of each of the storage condition. The deterioration rate shows that acetone gave the highest reduction of bixin colour as compared to the methanol and water in every storage conditions. Thus, proper storage and extraction are required in order to retain colour of the extracted bixin.

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