Extraction of bixin from annatto seeds with microwave

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Abstract. This study aims to determine the effect of extraction time (5, 10, 15 minutes) and the ratio of the aquadest solvent: ethyl acetate (1: 6.5, 1: 7, 1: 8 v/v) to the yield of pigment. The optimum condition was found in the ratio of the solvent of distilled water: ethyl acetate (1: 7 v/v) extraction time of 15 minutes with low microwave power resulted in maximum pigment yield of 0.469%. The isolate compounds were analyzed using IR spectroscopy, mass spectroscopy, and High-Performance Liquid Chromatography (HPLC) showed that the isolates were boxing pigments having a molecular weight of 394 g/Mol with wavenumbers for OH groups, C = O carboxylate groups, CH bonds, duplicate C = C, and CO ester bonds. As well as showing the retention time of standard and standard pigments at minutes to 12.849 and 12.843.

1. Introduction
The color is a mutual attraction to food and drink. The addition of dyes in foods and beverages has a tremendous influence on consumer tastes and appeal. Dyes are widely used throughout the world, both synthetic dyes and natural dyes. Currently, more than 100,000 dyes are commercially available. Worldwide, nearly 1 million tons of synthetic dyes are produced annually [1]. However, the dangers posed by synthesis dyes to health cause their use in food and beverages to be closely watched by the Food and Drug Administration (BPOM) and the Food and Drug Administration.

Natural dyes are the most appropriate choice because natural dyes are safe to eat, non-toxic, non-allergenic, non-carcinogenic, and biodegradable [1]. One of the plants that have natural dyes is the seeds of annatto (Bixa Orellana L.) The primary pigment found in the seeds of anatto is carotenoid bixin and norbixin [2]. Genolans contain natural dyes that are widely used in the pharmaceutical industry, cosmetics, and food products such as cheeses, ice cream, butter, cereals, and sausages [3]. This is because the pigment bixin has a right color intensity, more excellent stability, and color range from yellow to red, based on its molecular structure, bixin is a non-polar compound and shows a high affinity for a polar solvent such as acetone, ethyl acetate, methanol, and ethanol. Based on Cardarelli et al., ethyl acetate has the highest solubility efficiency for bixin extraction from the seed of annatto [4].

Various methods have been developed to extract natural dyes from different plants, such as maceration, socket lets, liquid-liquid extraction, and hydro distillation. However, such purposes use large amounts of solvents and long extraction times. Therefore, a new technique is needed to improve the mechanism of extraction of natural dyes. One of the fastest-growing methods of mining is the Microwave-Assisted Extraction (MAE) method. The advantages of MAE include solvent usage, little energy, short extraction time, and yield higher yield [5]. The main factors affecting the extraction process with the MAE method are the properties of the solvent, the extraction time, and the strength of the radiation. The choice of solution for MAE is determined based on the solubility of the analyst by the
interaction between the solvent and the matrix, as well as the solvent nature, which can absorb microwaves [6]. Advances in extraction with the help of microwaves have led to the development in techniques such as microwave hydro distillation (MWHD), vacuum microwave hydro distillation (VMHD), microwave-assisted hydraulic disturbance (MHG), microwave-assisted extraction (MAE) and solvent-free microwave extraction (SFME) [7]. Microwave-Assisted Extraction (MAE) method to extract the bixin from Bixa Orellana [3]. The results showed that microwave heating at 210 W for 18 minutes yielded a yield of 16.281%, and conventional heating yielded a return of 8.231% for 90 minutes. This shows that with the MAE method, the extraction time is 72 minutes faster, and the yield is higher than the socket method. Sinha et al., used the MAE method for extracting natural dyes from seeds of annatto (Bixa Orellana) and using surface and artificial neural network (ANN) response methods [1]. To predict the optimum conditions of extraction parameters. The results showed that the ANN method had a better prediction of RSM. The results of carayuru plants (Arrabidaea chica) and achiote (Bixa Orellana) extracted by the MAE method yielded a yield of 19% and 11.4% respectively [6].

2. Methods

Annatto seeds were acquired in Yogyakarta, and hexane, acetone, ethyl acetate, dichloromethane, acetonitrile, acetic acid, and chloroform were obtained from Sigma-Aldrich. High-pressure Liquid Chromatography Dionex, Fourier Transform Infra-Red Spectrometer (FTIR) Shimadzu, TLC. Seeds of annatto put into a round flask as much as 10 grams. Added 75 ml of ethyl acetate and 10 ml distilled water, then inserted into the microwave. The seeds were extracted for 5, 10, and 15 minutes with aquades and ethyl acetate ration ratio 1: 7 v/v. After the extraction is complete, the extraction results are cooled and filtered. Water phase and the organic phase separated by separating funnel, then add sodium sulfate anhydrate into the organic phase, filter and filtrate are evaporated with a rotary evaporator at a temperature of 40°C. Put crudely on a cold-water bath until crystal form. The formed crystals are then filtered and dried. The dried crystals are recrystallized by adding 3 mL dichloromethane and methanol (1: 4 v/v), and 12 mL of methanol drop by drop. The solution is heated and then placed in the freezer. The formed crystals are filtered, rinsed with methanol, and dried. Calculate the percentage of bixin obtained by the equation.

\[
\text{Mass of bixin (g)} \times 100
\]

\[
\text{Mass of annatto seeds}
\]

Bixin purification is done by using TLC and melting point test. Bixin is dissolved with methanol, and as a mobile phase used acetone: n-hexane (1: 2 v / v), ethyl acetate: n-hexane (1: 2 v / v), and ethyl acetate: chloroform (1: 3 v/v).

The test bixin characterization includes a test with FTIR at wave number 4000 - 400 cm-1. Identification bixin using HPLC, using a reverse phase C18 Agilent ZORBAX Eclipse Plus (150x4.6 mm, particle size 5 & m; m). The motion used is a mixture of acetonitrile: 2% acetic acid: dichloromethane (65: 35: 2, v/v). The flow rate is set to 1.0 ml/min. Injecting the sample is done two times [8].

3. Results and discussion

Annatto seeds extracted using ethyl acetate and distilled water with the MAE method produced a brownish-red extract solution. Then the extract solution is separated by a separating funnel. The stored ethyl acetate phase is then added with anhydrous sodium sulfate, which functions to bind water. Then evaporated with a rotary evaporator at 40°C. The concentrated concentrate obtained is placed in a cold-water bath to form crystals in the bottom of the container. After that, the glasses are filtered and dried. Then it was recrystallized using dichloromethane and methanol solvents with a ratio (1: 4 v/v). This solution is heated to increase solubility, then cooled for some time until the crystal forms yield. The crystals are separated from the remaining solvents and then weighed the mass of crystals obtained. This recrystallization aims to remove impurities by dissolving in a suitable solvent and then re-crystallized so that the crystals obtained are crystals that have been free from contaminants.
3.1. Purity test bixin
Test purity bixin has done using thin-layer chromatography (TLC). The mobile phase used is acetone: n-hexane (1: 2 v/v), ethyl acetate: n-hexane (1: 2 v/v), and ethyl acetate: chloroform (1: 3 v/v). The resulting Rf values are 0.23, 0.36, and 0.5. These three results show that elution results still present a stain with a different solvent mixture, so it can be said that the bottled sample has been pure. Also, the melting point test of temperature at the first melting point to melt is wholly considered as a melting distance. The melting point of the crystal bixin is about 196-198°C. From these results, indicate that the crystal bixin quite pure because it has a narrow temperature range difference that is 2°C. Based on the TLC and melting point test, it is inevitable that the bixin obtained is pure.

3.2. Effect of time on bixin yield
Figure 1 shows that the return of the pigment bixin increases with the length of extraction time. This result is due to the influence of water. Water is a polar solvent that can absorb microwaves well, so that the longer the extraction time, the faster the hot water. The heat generated by the interaction of microwaves with polar solvents will increase solvent and analytical diffusion. The solution is capable of diffusing the matrix and extracting the analyst, then distributing out of the model carrying the soluble component. Therefore, rising temperatures will increase the efficiency of the extraction process because the desorption of the desired substance from the matrix will increase.

![Figure 1. Effect extract time on bixin yield (%).](image)

3.3. Molecular structure identification bixin

3.3.1. Infrared spectroscopy. The obtained molecular structure obtained is obtained using FTIR.

![Figure 2. FTIR of bixin.](image)

Figure 2 shows the bixin spectrum measured at the wavenumber of 4000-400 cm⁻¹. From Figure 2 we can see there are 13 peaks at the wavenumbers as follows: 3186.54 cm⁻¹, 3020.66 cm⁻¹, 2929.03 cm⁻¹,
2859.59 cm\(^{-1}\), 1707.16 cm\(^{-1}\), 1381.09 cm\(^{-1}\), 1298.15 cm\(^{-1}\), 1275 cm\(^{-1}\), 1185.30 cm\(^{-1}\), 967.34 cm\(^{-1}\) and 839.07 cm\(^{-1}\) respectively. The peak at 3186.54 cm\(^{-1}\) indicates the strain of the hydrogen-free OH group. The 3020.66 cm\(^{-1}\) wave number indicates the strain of CH sp2 (= CH), at wavenumbers 2929.03 cm\(^{-1}\) and 2859.59 cm\(^{-1}\) shows the presence of a CH strain, in the 1708.04 cm\(^{-1}\) wave number indicating a strain of C = O carboxylate, at a wavenumber 1605.81 cm\(^{-1}\) indicating a C = C strain, at 1437.03 cm\(^{-1}\) and 1381.09 cm\(^{-1}\) wavenumbers indicating a CH, at wavenumbers 1298.15 cm\(^{-1}\), 1275 cm\(^{-1}\) and 1158.30 cm\(^{-1}\) indicate the presence of CO strain of esters, and at 967.34 cm\(^{-1}\) and 839.07 cm\(^{-1}\) wavenumbers indicates a CH bend of = CH. Based on the results of spectrum analysis Infra-Red, it is known that bixin has an OH-group, OH, C = O carboxylate group, CH bond, C = C double bond, and CO bond of esters. The result of the FTIR spectrum has a wave number almost equal to the FTIR spectrum of the literature.

3.3.2. Mass spectroscopy. To determine the molecular weight of bixin tested using GC MS. The GC MS spectrum from bixin can be seen in Figure 3. The test results with GC MS, indicating a peak with a molecular ion mass [M + H]\(^+\) of 395 m/z and a molecular ion mass [M-H]\(^-\) of 393 m/z. In the positive mode to find out the molecular mass of the compound, the peak m/z is reduced by its ionizing H\(^+\). As for knowing the molecular mass in negative mode, the peak m/z is coupled with the ionizer. The molecular mass identified by the second the detector is a molecular mass of a pigment boxing of 394 g/Mol. In the mass spectrum with positive ion detectors, there is a fragmentation pattern at the peak of m /z 335, indicating that there is a missing fragment with a molecular weight of 60 to the peak of m /z 395. The disappeared piece is thought to be the loss of CH\(_3\)OH and CO. While on the mass spectrum with negative ion detectors found no pattern of fragmentation. Based on the results obtained from the measurement of mass spectroscopy, it can be seen that boxing has a molecular weight of 394 g/Mol. But on the mass spectrum shows many peaks that appear.

![Figure 3. Mass spectrum of bixin.](image)

3.3.3. Identification with HPLC. For test with HPLC, standard bixin and bixin extraction were dissolved with acetonitrile, using C18 column Agilent ZORBAX Eclipse Plus with 1.0 mL/min flow rate, 450 nm
wavelength, injection volume 20 & μ, its mobile phase is acetonitrile: 2% acetic acid: dichloromethane (65: 35: 2 v/v). The standard bromine chromatogram and the extracted bromine chromatogram are shown in figure 4. From the chromatogram, we can see the standard binding retention rate of 12.843 with the area of 10098841, while the retention time of the extraction result is 12.849 with the field of 9559317. Results show that the acquired bixin has a retention time of equal to the standard retention time from literature retention time of 12.7 [9].

**Figure 4.** a) Chromatogram standard of bixin & b) Chromatogram sample of bixin.

Based on Figure 4. Observation on temperature controller, when extraction time 5 minutes temperature in round flask reached 33°C, extraction time 10 minutes temperature in round flask reach 48°C, while extraction time 15 minutes round pump temperature reach 69°C. These temperatures include safe temperatures for extracting extraction because the bixin begins to degrade if the heating temperature is more than 80°C [9]. The results show that the maximum pigment yield is obtained at 0.446% with an extraction time for 15 minutes. So it can be known that the optimum extraction time with the MAE method in this study is 15 minutes.

4. **Conclusion**

Extracting results from Annatto seeds (Bixa Orellana L.) by Microwave-Assisted Extraction (MAE) method obtained optimum conditions on the ratio of aquades and ethyl acetate (1: 7 v / v) solvent extraction time for 15 minutes with the acquisition of bixin of 0.469 %. Based on the identification of the structure of the bixin pigment molecule with infrared spectrophotometer obtained wave numbers for O-H groups, C = O carboxylic groups, C-H bonds, C = C double bonds, and C-O bonds of esters.

The results obtained from mass spectroscopic measurements show that the bixin pigment has a molecular weight of 394 g / mol with a fragmentation pattern of m / z 335 [M + H-CH3OH + CO] +. Based on the identification of bixin components with KCKT, the standard Bixin retention time (TR) and bixin samples were obtained at 12.843 and 12.849 minutes, respectively.

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