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Natural pigments and antioxidants properties of annatto extract at various pH of distilled water solvent and extraction times

*Handayani, I., Septiana, A.T. and Sustriawan, B.

Department of Food Technology, Faculty of Agriculture, Universitas Jenderal Soedirman. Jl. Dr.Soeparno, Karangwangkal Purwokerto, 53123, Central Java, Indonesia

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Abstract

Annatto is a natural colouring agent obtained from the outer coats of the seeds of the *Bixa orellana*. Carotenoid pigment in annatto consists of bixin and norbixin. Pigment extraction from annatto seeds is commonly done by indirect extraction using organic solvents and direct extraction using oil or aqueous alkali. This research aims to characterize the natural pigment and antioxidants of annatto extract. The annatto was extracted by maceration using distilled water at various pH and extraction times. The distilled water pH variations used in this study were 4, 7, and 9 while the extraction times were 5, 7.5 and 10 mins. Analyzing bixin and norbixin revealed the potential of annatto extract as a natural pigment, while phenol and flavonoids disclose the potential as a natural antioxidant. The results showed that varying pH of distilled water and time of extraction influences the bixin, norbixin, total phenol, and flavonoid contents of annatto extract. Annatto extraction using distilled water produces higher levels of bixin than norbixin. The highest levels of bixin (61.06%) were built in distilled water pH 7 for 7.5 mins of extraction, while the highest levels of norbixin (30%) were produced in distilled water pH 7 for 10 mins. Extraction using pH 4 creates a higher level of total phenol while increasing the extraction time up to 10 mins decreased the total phenol content. However, high levels of flavonoids were made in extraction using alkaline pH distilled water (pH 9) for 10 mins. The results showed that all extracts have the potential as natural pigments and antioxidants.

1. Introduction

Annatto is one of the world's oldest natural pigments obtained through the pericarp of *Bixa orellana* seeds. Annatto is a safe dye that has been recommended by the US Food and Drug Administration (US FDA) (Gallardo-Cabrera and Rojas-Barahona, 2015). The color of annatto is caused by carotenoid compounds found in the seed's outer layer. Annatto produces colors that vary from yellow to red depending on the concentration of the color-forming components (Taham *et al*., 2015) Bixin is the primary pigment in *Bixa orellana* seeds, and its concentration can be as high as 80% (de Paula *et al*., 2009; Husa *et al*., 2018). A red-orange color was produced from a double bond in the bixin's chemical structure (Suparmi and Leenawaty, 2009; Husa *et al*., 2018). Norbixin is a dark red-brown to red-purple orange -yellow carotenoid (Suparmi and Limantara, 2008). Bixin is soluble in non-polar solvents, while norbixin is soluble in polar solvents (Mala *et al*., 2013). Several solvents used for *Bixa orellana* seeds extraction include essential oil, hexane, chloroform, ethyl acetate, butanol, methanol, and vegetable oil 6.9 (Silva *et al*., 2008; Husa *et al*., 2018). Annatto is used to color candy, sausage, cheese and meat (Silva *et al*., 2008).

Extracts from plants can be extracted by Soxhlet and maceration (Diem *et al*., 2013). Stratified annatto extraction by washing has also been carried out by previous researchers using hexane and followed by a polar solvent, namely acetone (Pamidighantam *et al*., 2012). Other solvents used for annatto extraction include essential oil, hexane, chloroform, ethyl acetate, butanol, methanol and vegetable oil (Hossain and Rahman, 2011).

Factors that affect the extraction process include solvent pH, extraction time and the type of solvent (Hossain and Rahman, 2011; Paryanto *et al*., 2014). The pH the color, the antibacterial activity, and the toxicity level of the extract were thought to be influenced by the pH of the solvent and the extraction time (Hossain and Rahman, 2011; Diem *et al*., 2013). Solvent pH and heating time affect the total carotenoids of the palm fruit (Anggreini *et al*., 2018). Total carotenoids decreased

*Corresponding author.
Email: *isti.handayani@unsoed.ac.id*

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with increasing pH. Increasing the extraction time increased the total carotenoids of pumpkin extract (Wahyuni and Widjanarko, 2015).

Specific solvents such as benzene and chlorocarbon are toxic and harmful to the environment. They are inappropriate when used for food. Venugopalan and Giridhar (2012) performed annatto extraction using ethanol, while Yolmeh *et al*. (2014) performed annatto extraction using acetone. Annatto extraction using water (distilled water) is one of the profitable annatto extraction alternatives because water is a solvent that is easily obtained at a low price. Rosamah *et al*. (2009) performed annatto extraction using water.

In addition to functioning as a colorant, annatto extract is also known to be a source of antioxidants because it contains carotenoids (Kurniawati *et al*., 2010; Cuong and Chin, 2016). Substances at low concentrations can inhibit the oxidation of cell components such as proteins, carbohydrates, lipids, and deoxyribonucleic acid, known as antioxidants (Abayomi *et al*., 2014). Besides carotenoids, plant components that can act as antioxidants include phenols and flavonoids (Hossain and Rahman, 2011; Diem *et al*., 2013). According to Pratima *et al*. (2016), annatto seeds contain flavonoids, whereas Handayani *et al*. (2021), reported that annatto seeds contain phenol. Flavonoids and phenols are antioxidants. The study evaluated the characteristics of the pigment (bixin and norbixin) and antioxidant (total phenols and flavonoids) components of annatto extract produced from distilled water as a solvent at various pH and extraction times.

2. Materials and methods

2.1 Annatto extraction

The extraction of annatto was carried out by Handayani *et al*. (2021). A total of 25 g of *Bixa orellana* seeds were added to 90 mL of distilled water as the solvent. Maceration was performed through a magnetic stirrer at various pH of distilled water (4, 7 and 9) and duration of extraction (5, 7.5, 10 mins) of 80°C. The extract was then analyzed for bixin, norbixin, total phenol and flavonoid.

2.2 Bixin analysis

Bixin was measured utilizing the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (2012) method. A total of 0.1 g of annatto extract was transferred into a 100 mL volumetric flask added by 10 mL tetrahydrofuran and mixed to dissolve the pigment. An aliquot (1 mL) portion of the solution was transferred into another 100 mL volumetric flask and diluted to 100 mL with acetone. This solution measured the absorbance A at the peak wavelength (about 487 nm).

$$\%\text{Total Carotenoid (Expressed as bixin)} = \frac{\text{A} \times 100000}{3.090 \times \text{sample weight (mg)}} \times 100$$

2.3 Norbixin analysis

Norbixin was tested using the JECFA (2012) method. A total of 0.1 to 1 g of annatto extract was dissolved and diluted to 100 mL in 0.5% w/v potassium hydroxide solution. An aliquot (1 mL) portion of the solution was transferred into another 100 mL volumetric flask and diluted to 100 mL. The absorbance A of this solution was measured at the peak wavelength (about 482 nm):

$$\%\text{Total Carotenoid (Expressed as norbixin)} = \frac{\text{A} \times 100000}{2.870 \times \text{sample weight (mg)}} \times 100$$

2.4 Total phenol analysis

Total phenol was carried out using the Folin-Ciocalteu method (Orak, 2007). Gallic acid standards were made with varying concentrations of 5-125 ppm. After that, the absorbance was measured using UV-VIS spectrophotometry at a length of 765 nm. Samples were weighed as much as 100-150 mg, added 0.5 mL of methanol, 2.5 mL of distilled water and 2.5 mL of Folin – Ciocalteu 50% reagent. The mixture was allowed to stand for 5 mins and 3 mL of 7.5% Na_2CO_3 was added. After that, it was vortexed and then incubated for 15 mins at 45°C. The absorbance of the sample was measured at a wavelength of 765 nm using UV-VIS spectrophotometry. The content of phenolics in extracts was expressed in terms of gallic acid equivalent (mg GE/ mL)

2.5 Flavonoids analysis

The flavonoid content of annatto extracts was determined using the method by Mukhriani *et al*. (2015). The test sample was dissolved in methanol with a concentration of 2000 µg/mL. A total of 0.5 mL of the test sample was added with 1.5 mL of methanol, then added with 0.1 mL of 10% aluminum (III) chloride, 0.1 mL of 1 M sodium acetate, and 2.8 mL of distilled water. The solution was incubated for 30 mins. The solution's absorbance was measured with a spectrophotometer at 436 nm. Total flavonoids (mg/mL) were determined from the previously measured quercetin calibration curve using a linear regression equation.

2.6 Statistical analysis

Statistical analysis data were subjected to analysis of variance (ANOVA). Significant differences between means were determined by the Duncan Multiple Range Test (DMRT) at significant differences ($p < 0.05$). Values are presented as mean±SD.

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3. Results and discussion

3.1 Bixin and norbixin

Bixin is a pigment component in *Bixa orellana* seeds that is non-polar and soluble in oil or other organic solvents. Bixin is a carotenoid compound with a methyl ester structure (Gallardo-Cabrera and Rojas-Barahona, 2015). This pigment gives a yellow-orange color (Suparmi and Leenawaty, 2009). The result on the levels of bixin in the treatment of various pH and extraction times showed that the pH of distilled water and extraction time affected the levels of bixin (Figure 1)

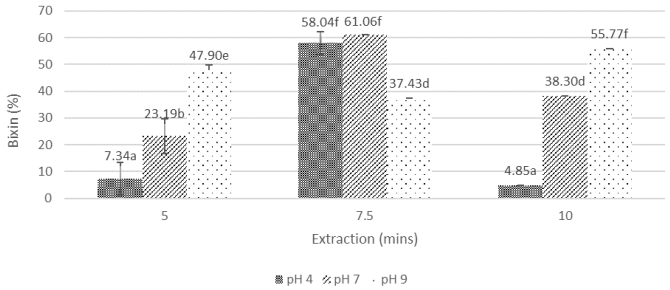

Figure 1. The bixin content of annatto extracts in the variations of pH distilled water and extraction time. The experiments were repeated in triplicate. Data was expressed as mean±SD of triplicates. Values with different superscripts are statistically significantly different by the Duncan Multiple Range Test at $p < 0.05$.

Norbixin is a pigment component of *Bixa orellana* seeds that is polar and soluble in water. Norbixin is a carotenoid with a carboxylic acid structure (Gallardo-Cabrera and Rojas-Barahona, 2015). The results showed that various pH and extraction times affected the norbixin levels (Figure 2).

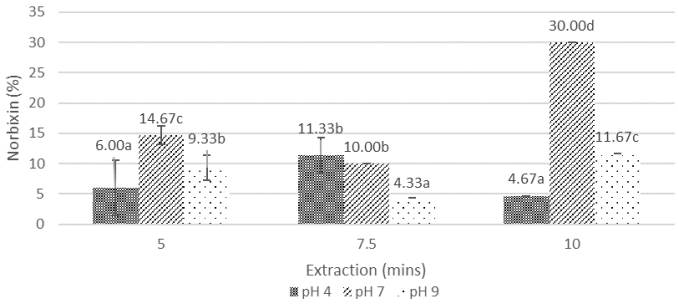

Figure 1. The bixin content of annatto extracts in the variations of pH distilled water and extraction time. The experiments were repeated in triplicate. Data was expressed as mean±SD of triplicates. Values with different superscripts are statistically significantly different by the Duncan Multiple Range Test at $p < 0.05$.

The highest norbixin content (30%) was produced at pH 7 of distilled water for 10 mins of extraction. Norbixin is a polar carotenoid. It has a high solubility in distilled water. The longer the extraction time, the more intense the contact between the solvent and the solute, and thus, the more components in the *Bixa orellana* seeds are dissolved. Norbixin solubility is affected by stirring during maceration (Aflaki, 2012). Extraction of *Bixa orellana* seeds using acetone produces 19.19±1.56% of norbixin (Suparmi and Limantara, 2008). Using acid and alkaline pH distilled water resulted in lower norbixin levels than the neutral pH of distilled water. It was suspected that at acidic and alkaline pH, norbixin was degraded. The results of this study are in line with Suparmi and Limantara (2008), who reported that the absorbance of norbixin was more negligible at pH 4 compared to pH 7. The double bonds in carotenoids caused these compounds to be degraded by pH (Gallardo-Cabrera and Rojas-Barahona, 2015). The double bonds in carotenoids can undergo isomerization which causes a structural change from trans to cis. Isomerization of carotenoids from trans to cis causes carotenoid compounds to be more unstable and more susceptible to oxidation which can lead to degradation. The results also showed that extraction for 10 mins at distilled water pH 4 and 9 resulted in lower norbixin.

3.2 Total phenols

Phenols include various compounds of plant origin that have an aromatic ring with one or more hydroxyl or methoxy groups (Sanchez-Maldonado *et al*., 2011) The formation of a green or blue-green color when the sample is exposed to $FeCl_3$ indicates the presence of phenolic compounds (Kamal and Ahmad, 2014). The result on the levels of total phenol in the treatment of various pH of distilled water and extraction time showed that the pH of distilled water and extraction time affected the levels of bixin (Figure 3)

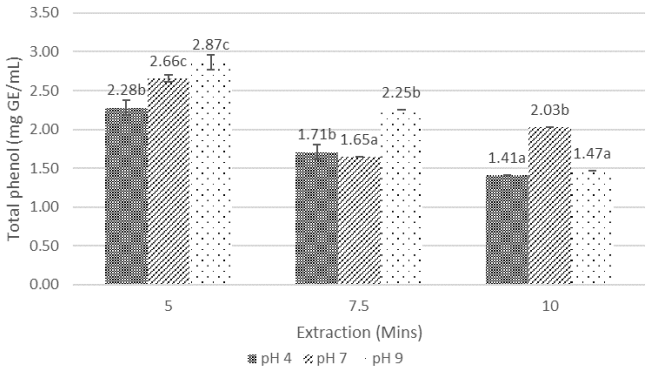

Figure 3. The total phenol content of annatto extracts in the variations of pH distilled water and extraction time. Data was expressed as mean±SD of triplicates. Values with different superscripts are statistically significantly different by the Duncan Multiple Range Test at $p < 0.05$.

The highest levels of total phenol (2.87 mg GE/mL) were produced by extraction using distilled water pH 4 for 5 mins. The acid of the solvent caused a change in ionic strength, which could increase the extraction. Phenol extraction from *Allium cepa* peel resulted in higher phenol levels in the pH 5.5 solvent than in the pH

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7 and 8 solvents (Machavarapu *et al*., 2013). Extraction using pH 3 and 4 solvents also resulted in higher total phenol than pH 7 and 8 but lower than pH 5.5. Harbourne *et al*. (2009), demonstrated the effect of solvent pH on phenol extraction from meadowsweet (*Filipendula ulmaria* L.). According to Harbourne *et al*. (2009), increasing the pH of distilled water from 3.9 to 6.4 helped increase the phenol content. The phenol content of pomegranate (*Punica granatum* L.) peel extracted in a solvent of pH 5.5 resulted in higher phenol levels than pH 3.5 and 4.5 (Wissam *et al*., 2012). The phenol content in the solvent pH 9 was not different from that at pH 4.5. This difference is thought to be due to the different compositions of the constituent compounds. Total phenol is composed of several compounds that have different characteristics. Polyphenols include more than 8,000 compounds with great structural diversity (although each has at least one aromatic ring with one or more hydroxyl groups) (Wissam *et al*., 2012). They can be divided into ten different classes depending on their chemical structure. The annatto extract is thought to be made up of various phenolic compounds that dissolve differently in different pH solvents. Diluting the pH of the aquadest solvent produced different polarities and thus different levels of total phenol in the annatto extract. Increasing the extraction time to 10 mins decreased the total phenol content significantly. The total phenol content was thought to be degrading as extraction time increased. Phenol compounds can be degraded due to external and internal factors. Oxygen is one of the critical external factors that can cause the degradation of phenol compounds. The annatto extraction was performed at 80°C with stirring using a magnetic stirrer, which is suspected of causing phenol oxidation and degradation at high temperatures. Increase in the strength of hydrogen bonds between polyphenols and proteins causes the low solubility of polyphenols in organic solvents (Wissam *et al*., 2012). In contrast, adding water to organic solvents weakened hydrogen bonds or increased polyphenol ionization in the solution, as a result, extending the extraction time reduced the total phenol content.

3.4 Flavonoids

Flavonoids are the largest group of polyphenolic compounds, commonly found in plants, and are bound to sugars as glycosides and flavonoid aglycones. Flavonoids are soluble in polar solvents (Sa'adah and Nurhasnawati, 2015). Figure 4 shows that pH and extraction time affect flavonoid levels in annatto extract. Extraction using pH 9 of distilled water for 10 mins produced the highest flavonoid content (3.70 mg/mL), while extraction using pH 9 of distilled water for 7.5 mins had the lowest concentration of 1.19 mg/mL. The results also showed that the variation of the distilled water pH and the extraction time gave varying flavonoid levels. It is suspected that the solvent pH temperature and extraction time affect the stability of the flavonoids in the extract. The study results by Settharaksa *et al*. (2012) on the flavonoid content of Thai hot curry paste extract showed that extraction using acid solvents (pH 4 and 5) resulted in lower flavonoid levels than extraction using pH 6. The low flavonoid level of annatto extract at pH 4 is thought to be caused by changes in the structure, thereby reducing flavonoid levels. Plant flavonoids are generally protein-bound. Acidic pH solvents are thought to damage the bond between flavonoids and proteins. Flavonoids that are not bound to protein are considered to be more easily degraded under acidic conditions.

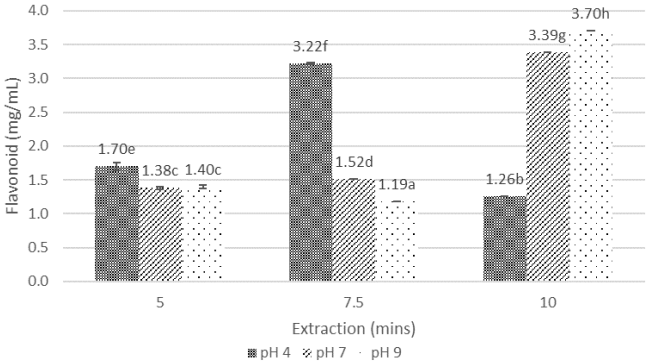

Figure 4. The flavonoid content of annatto extracts in the variations of pH distilled water and extraction time. Data was expressed as mean±SD of triplicates. Values with different superscripts are statistically significantly different by the Duncan Multiple Range Test at $p < 0.05$.

4. Conclusion

The distilled water can be used as an alternative solvent to extract the annatto pigment. Extraction using distilled water by maceration is considered profitable because it is easy to obtain, with a low price, and it can produce high levels of bixin (61.06%) and norbixin (30%) both of which have pigment potential. Extraction using distilled water can also extract phenols and flavonoids, which have the potential as antioxidants.

Conflict of interest

The authors declare no conflict of interest.

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References

Abayomi, M., Adebayo, A.S., Bennett, D., Porter, R. and Shelly-Campbell, J. (2014). *In vitro* antioxidant activity of *Bixa orellana* (annatto) seed extract. *Journal of Applied Pharmaceutical Science*, 4(2), 101-106. https://doi.org/10.7324/JAPS.2014.40216.

Aflaki, N. (2012). Optimization of carotenoid extraction in peel and flesh of cantaloupe (*Curcumis melo* L.) with ethanol solvent. France: Universite' Laval, Thesis.

Anggreini, R.A., Winarti, S. and Heryanto, T. (2018). Pengaruh suhu, lama waktu pemanasan, pH, garam dan gula terhadap kestabilan karotenoid licuala. *Jurnal Teknologi Pangan*, 12(2), 82-86. https://doi.org/10.33005/jtp.v12i2.1292 [In Bahasa Indonesia].

Cuong, T.V. and Chin, K.B. (2016). Effects of annatto (*Bixa orellana* L.) seeds powder on physicochemical properties, antioxidant and antimicrobial activities of pork patties during refrigerated storage. *Korean Journal for Food Science of Animal Resources*, 36 (4), 476-486. https://doi.org/10.5851/kosfa.2016.36.4.476

de Paula, H., Pedrosa, M.L., Júnior, J.V.R., Haraguchi, F.K., dos Santos, R.C. and Silva, M.E. (2009). Effect of an aqueous extract of annatto (*Bixa orellana*) seeds on lipid profile and biochemical markers of renal and hepatic function in hypercholesterolemic rats. *Brazilian Archives of Biology and Technology*, 52(6), 1373-1378. https://doi.org/10.1590/S1516-89132009000600008

Diem, Q., Elisa, A. and Tran-nguyen, P.L. (2013). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. *Journal of Food and Drug Analysis*, 22(3), 296-302. https://doi.org/10.1016/j.jfda.2013.11.001

Gallardo-Cabrera, C. and Rojas-Barahona, A. (2015). Stability study of an aqueous formulation of the annatto dye. *International Food Research Journal*, 22(5), 2149-2154.

Handayani, I., Haryanti, P. and Sulistyo, S.B. (2021). Color and antibacterial activity of annatto extracts at various pH of distilled water solvent and extraction temperature. *Food Research*, 5(6), 247-253. https://doi.org/10.26656/fr.2017.5(6).740

Harbourne, N., Jacquier, J.C. and O'Riordan, D. (2009). Optimization of the aqueous extraction conditions of phenols from meadowsweet (*Filipendula ulmaria* L.) for incorporation into beverages. *Food Chemistry*, 116(3), 722-727. https://doi.org/10.1016/j.foodchem.2009.03.017

Hossain, M.A. and Rahman, S.M.M. (2011). Total phenolics, flavonoids, and antioxidant activity of tropical fruit pineapple. *Food Research International*, 44(3), 672-676. https://doi.org/10.1016/j.foodres.2010.11.036

Husa, N.N., Hamzah, F. and Said, H.M. (2018). Characterization and storage stability study of bixin extracted from *Bixa orellana* using an organic solvent. *IOP Conference Series: Materials Science and Engineering*, 358(1), 012035. https://doi.org/10.1088/1757-899X/358/1/012035

Joint FAO/WHO Expert Committee on Food Additives (JECFA). (2012). Annatto extracts - 67th Joint FAO/WHO Expert Committee on Food Additives (JECFA) Meeting - Chemical and Technical Assessment (CTA) 2006, p. 1-21. Rome, Italy: Food and Agriculture Organization of the United Nations.

Kamal, A. and Ahmad, I.Z. (2014). Phytochemical studies of different phases of germination of *Nigella sativa* Linn - a medicinally important plant. *International Journal of Pharmacy and Pharmaceutical Sciences*, 6(4), 318-323.

Kim, S.H., Park, J.D., Lee, L.S. and Han, D.S. (1999). Effect of pH on the green tea extraction. *Korean Journal of Food Science and Technology*, 31(4), 1024-1028.

Kurniawati, P.T., Soetjipto, H. and Limantara, L. (2010). Antioxidant and antibacterial activities of bixin pigment from annatto (*Bixa orellana* L.) seeds. *Indonesian Journal of Chemistry*, 7(1), 88-92. https://doi.org/10.22146/ijc.21719.

Machavarapu, M., Sindiri, M.K. and Vangalapati, M. (2013). Optimization of physico-chemical parameters for the extraction of flavonoids and phenolic components from the skin of *Allium cepa*. *International Journal of Innovative Research in Science, Engineering and Technology*, 2(7), 3125-3129.

Mala, K.S, Rao, P.P, Prabhavathy, M.B. and Satyanarayana, A. (2013). Studies on application of annatto (*Bixa orellena* L.) dye formulations in dairy products. *Journal of Food Science and Technology*, 52(2), 912-919. https://doi.org/10.1007/s13197-013-1038-3

Mukhriani, Nonci, F.Y. and Munawarah, S. (2015). Analisis kadar flavonoid total pada ekstrak daun sirsak (*Annona muricata* L.) dengan metode spektrometri UV-Vis. *Jurnal Farmasi*, 3(2), 37-42. [In Bahasa Indonesia].

Orak, H.H. (2007). Total antioxidant activities, phenolics, anthocyanins, polyphenoloxidase activities of selected red grape cultivars and their correlations. *Scientia Horticulturae*, 111(3), 235-

241. https;//doi.org/10.1016/j.scienta.2006.10.019

Pamidighantam, P.R., Babu, P.M. and Akula, S. (2012). Selection and application of annatto (*Bixa orellana* L.) dye formulations in some traditional sweetmeats: Jilebi and jangri. *Journal of Scientific and Industrial Research*, 71(12), 788-793.

Paryanto, Hermiyanto, D.S. and Sanjaya. (2014). Pembuatan zat warna alami dari biji kesumba dalam bentuk konsentrat tinggi untuk pewarna makanan. *Metana,* 9(2), 55-58. https://doi.org/10.20961/ekuilibrium.v13i2.24844 [In Bahasa Indonesia].

Pranata, A., Raharjo, S., Santoso, U. and Haryadi. (2006). Degradasi Biksin selama pemanasan dan pencahayaan. *Agritech*, 26(4), 169-175. [In Bahasa Indonesia].

Prathima, D., Sujitha, A. and Usha, R. (2016). Phytochemical screening and antimicrobial activity of *Bixa orellana* Linn. *International Journal of Pharmacognosy and Phytochemical Research,* 8(7), 1078-1082.

Rios, A.O., Borsarelli, C.D. and Mercadante, A.Z. (2005). Thermal degradation kinetics of bixin in an aqueous model system. *Journal of Agricultural and Food Chemistry,* 53(6), 2307-2311. https://doi.org/10.1021/jf0481655

Rosamah, E., Ramadan, R. and Kusuma, I.W. (2009). Stabilitas warna biji tumbuhan annatto (*Bixa orellana* L.) sebagai bahan pewarna alami, presented at the Seminar Nasional XVI, Masyarakat Peneliti Kayu Indonesia (MAPEKI), p. 209-214. Balikpapan, Kalimantan Timur, Indonesia. [In Bahasa Indonesia].

Sa'adah, H. and Nurhasnawati, H. (2015). Perbandingan pelarut etanol dan air pada pembuatan ekstrak umbi bawang tiwai (*Eleutherine americana* Merr) menggunakan metode maserasi. *Jurnal Ilmiah Manuntung*, 1(2), 149-153. https://doi.org/10.51352/jim.v1i2.27. [In Bahasa Indonesia].

Sanchez-Maldonado, A.F., Schieber, A. and Ga¨nzle, M.G. (2011). Structure function relationships of the antibacterial activity of phenolic acids and their metabolism by lactic acid bacteria. *Journal of Applied Microbiology*, 111, 1176-1184. https://doi.org/10.1111/j.1365-2672.2011.05141.x.

Settharaksa, S., Jongjareonrak, A., Hmadhlu, P., Chansuwan, W. and Siripongvutikorn, S. (2012). Flavonoid, phenolic contents and antioxidant properties of Thai hot curry paste extract and its ingredients as affected of pH, solvent types and high temperature. *International Food Research Journal*, 19(4), 1581-1587.

Silva, G.F., Gamarra, F.M.C., Oliveira, A.L. and Cabral, F.A. (2008). Extraction of bixin from annatto seeds using supercritical carbon dioxide. *Brazilian Journal of Chemical Engineering*, 25(2), 419-426. https://doi.org/10.1590/S0104-66322008000200019.

Suparmi and Leenawaty, L. (2009). Pengaruh berbagai faktor eksternal terhadap stabilitas pigmen bixin dari selaput biji kesumba (*Bixa orellana* L.) potensi sebagai pewarna alami makanan. *Sains Medika*, 1(1), 8191. [In Bahasa Indonesia].

Suparmi, S. and Limantara, L. (2008). Stability characterization of norbixin pigment from kesumba keling (*Bixa orellana* L.) seeds, presented at the 8th National Student Conference Smart Food: Lifestyle and Health Reconciled. Semarang, Indonesia: Soegijapranata Catholic University.

Taham, T., Cabral, F.A. and Barrozo, M.A.S. (2015). Extraction of bixin from annatto seeds using combined technologies. *Journal of Supercritical Fluids*, 100, 175-183. https://doi.org/10.1016/j.supflu.2015.02.006.

Venugopalan, A. and Giridhar, P. (2012). Bacterial growth inhibition potential of annatto plant parts. *Asian Pacific Journal of Tropical Biomedicine*, 2(3 Suppl.), S1879-S1882. https://doi.org/10.1016/S2221-1691(12)60513-9.

Wahyuni, D. and Widjanarko, S.B. (2015). Pengaruh jenis pelarut dan lama ekstraksi terhadap ekstrak karatoneid labu kuning dengan metode gelombang ultrasonik. *Jurnal Pangan dan Agroindustri*, 3(2), 390-401. http://jpa.ub.ac.id/index.php/jpa/article/viewFile/155/164. [In Bahasa Indonesia].

Wissam, Z., Ghada, B., Wassim, A. and Warid, K. (2012). Effective extraction of polyphenols and proanthocyanidins from Pomegranate peel. *International Journal of Pharmacy and Pharmaceutical Sciences*, 4(3 suppl.), 675-682.

Yolmeh, M., Najafi, M.B.H. and Farhoosh, F.S. (2014). Modeling of antibacterial activity of annatto dye on *Escherichia coli* in mayonnaise. *Food Bioscience*, 8, 8-13. https://doi.org/10.1016/j.fbio.2014.09.001.

