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ScienceDirect

Procedia Chemistry 14 (2015) 455 – 464

Procedia Chemistry

2nd Humboldt Kolleg in conjunction with International Conference on Natural Sciences, HK-ICONS 2014

Effects of Cyclohexane/Acetone Ratio on Bixin Extraction Yield by Accelerated Solvent Extraction Method

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Abstract

In this study, accelerated solvent extraction (ASE) was applied to the quantitative extraction of bixin. The effects of cyclohexane/acetone ratios on bixin extraction yield were evaluated. Acetone was used in the process of pigment extraction and also played a major role in its analysis by UV-Vis spectrophotometry. Pure *cis*-bixin isolated by flash chromatography and characterized by Fourier Transform Infra Red spectrometry was used as a bixin standard for qualitative and quantitative analysis of annatto extracts which were obtained by accelerated solvents extraction. UV-Vis spectrophotometry analysis shows that the extraction using 100 % cyclohexane gives the lowest bixin yield. This is different from the UV-Vis spectra generated with acetone extraction. The difference in percentage of acetone shows significant effects on bixin extraction yields. The use of cyclohexane:acetone (60 % : 40 %) solution at 50 °C for 5 min heating time results the highest total bixin extraction yield (48.00 %) as compared to the other solvent ratios, and to the usual extraction methods using the same solvent (29.14 %). High pressure liquid chromatography analysis shows that bixin extracted by this method has purity degree of 68.16 %.

Peer-review under responsibility of the Scientific Committee of HK-ICONS 2014

Keywords: Accelerated solvent extraction; cis-bixin; flash chromatography; high performance liquid chromatography; UV-Vis spectrophotometry

Nomenclature

ASE	accelerated solvent extraction
FAO	Food and Agriculture Organization
WHO	World Health Organization

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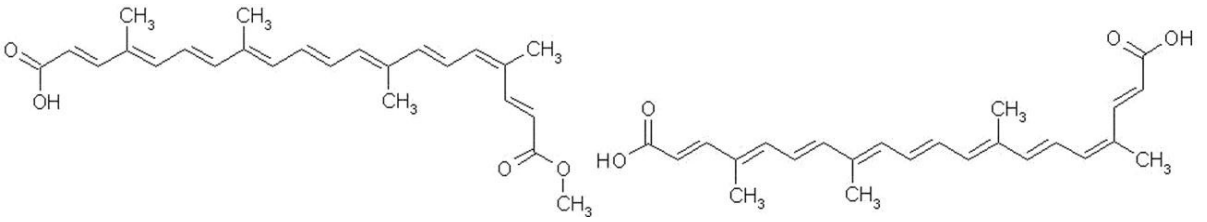
Peer-review under responsibility of the Scientific Committee of HK-ICONS 2014
doi:10.1016/j.proche.2015.03.061

1. Introduction

Annatto tree (*Bixa orellana* L.) is native to tropical South America. However, it is also cultivated in many countries in Central America, Africa and Asia. The main carotenoid found in its seeds is bixin (methyl hydrogen 9'-*cis*-6,6'-diapocarotene-6,6'-dioate, $C_{25}H_{30}O_4$; Fig. 1a). Small amounts of norbixin (9'-cis-6,6'-diapocarotene-6,6'dioic acid, $C_{24}H_{28}O_4$; Fig. 1b) are also found. Bixin becomes the major carotenoid (80 %) of the annatto extracts. It is responsible for the reddish-orange color of the annatto seeds and their extract.

Commercial annatto preparations have been added to numerous foods to impart yellow to red colors[1–3]. In addition, this pigment is used for textiles, varnishes, cosmetics, tattoos, and medicinal purpose[4–7]. Recently, natural bixin has been explored and used as a photosensitizer in organic solar cells[8–10].



(a) (b)

Fig. 1. Structure of *cis*-bixin (a) and cis-norbixin (b)

Annatto pigment can be separated from annatto seeds by many ways, including immersion of seeds in hot vegetable oil, dilute alkaline aqueous solutions and solvents. Recently, besides the usual methods with variation of solvents at room temperature, some extraction techniques using soxhlet, microwaves[11,12], ultrasounds and supercritical carbon dioxide fluid[13–15] have also been investigated. However, the results of these studies seemed to be unsatisfactory both in terms of economics and efficiency. As a carotenoid, bixin is very susceptible to processing and storage conditions, which might cause significant color loss of annatto pigment[16,17].

For these reasons, it is necessary to develop extraction methods to separate analyte from its matrix effectively, quantitatively and rapidly with minimal solvent usage. Experiments were conducted to extract pigment from annatto seeds using accelerated solvent extraction under various conditions in order to obtain the optimum bixin extraction yield. This method is advantageous over the traditional maceration or soxhlet method in that it uses only about 10 mL to 20 mL of solvent per g samples and an extraction cycle can be completed within a very short period 10 min to 15 min. Generally, three to four cycles are required to completely extract the pigment contained in the sample[18].

2. Materials and methods

2.1. Materials

Annatto seeds were acquired in Pontianak city, West Kalimantan, Indonesia. Bixin was obtained commercially from *molecula.com*. The following solvents, all analytical grades: acetone (> 99.5 %), acetonitrile (99.8 %), acetic-acid glacial (99 % to 100 %), cyclohexane (> 99.5 %) and hexane (97 %) were obtained from Sigma-Adrich, Germany.

2.2. Instrumentation

UV-Vis Spectrophotometer U-1800 and Fourier Transform Infra Red Spectrometer (FTIR) SHIMADZU, Flash Chromatography CombiFlash COMPANION automated purification methods, Accelerated Solvent Extraction Dionex-ASE 350, High Pressure Liquid Chromatography DIONEX HPLC summit ASI-100.

2.3. Procedure

2.3.1. Determination of total bixin content of annatto seed

The bixin concentration of the seeds was determined using an adaptation of the methodology described by FAO/WHO[19]. Around 1g of the seeds weighed precisely and the pigment thoroughly extracted with acetone until the seeds are colorless. Aliquots (0.1 mL) of the extracts were evaporated under N_2 flow and re-suspended in 10 mL with acetone. Absorbance was measured with an UV-Vis spectrophotometer at 487 nm and the bixin concentration was calculated according to the Lambert–Beer law, using $E^{1\%}_{1cm} = 3\ 090$.

2.3.2. Preparation of purified bixin

The crude residue obtained from method 2.3.1. was purified by flash chromatography over silica gel particles (250 mesh to 400 mesh) using n-hexane (solvent A) and acetone (solvent B). Pressurized gas (10 psi to 15 psi [1 psi equal 6 894.76 Pascals]) is used to drive the solvent through the column of stationary phase. Several thin layer chromatographies (TLC) using mixtures of hexane : acetone were done to determine ideal parameters for flash chromatography. The best separation obtained with the use of hexane:acetone (2 : 1 v/v) and the target compound are presented at R_f 0.33 The purification conditions of annatto extract in this experiment are shown in Fig. 2.

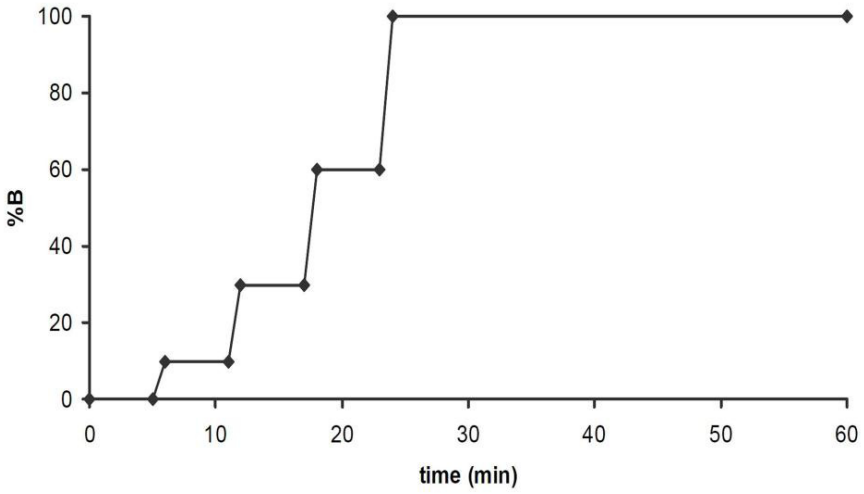


Fig. 2. Condition of bixin purification by flash chromatography

Eluates were collected and analysed by UV-Vis spectrophotometry, FTIR spectroscopy and HPLC. UV–Vis spectra were obtained in acetone. HPLC methods are explained in the method 2.3.5.

2.3.3. Accelerated solvent extraction

Annatto seeds were dried for 24 h at 50 °C in oven. A 22 mL stainless steel extraction cell was prepared by placing a cellulose filter in the capped end. The cell was packed with a mixture of ± 1 g samples and ± 3 g sands. Finally a second cellulose filter was placed before capping the cell. The ASE cell was placed into ASE carousel for extraction process.

Solvent extraction of pigment from annatto seeds was carried out using the Dionex-ASE 350. Extraction was performed with different percentages of cyclohexane/acetone (100 % : 0, 80 % : 20 %, 60 % : 40 %, 40 % : 60 %,

20 % : 80 %, 0 : 100 %). Extraction conditions involved a five min sample heating time at 50 °C followed by 100 min of total extraction time at 1 500 psi. Each 15 mL extract was transferred in glass vials. Extraction process on the same samples was repeated three cycles. The extracts were stored at -8 °C until analysis. All experiments were performed in triplicates.

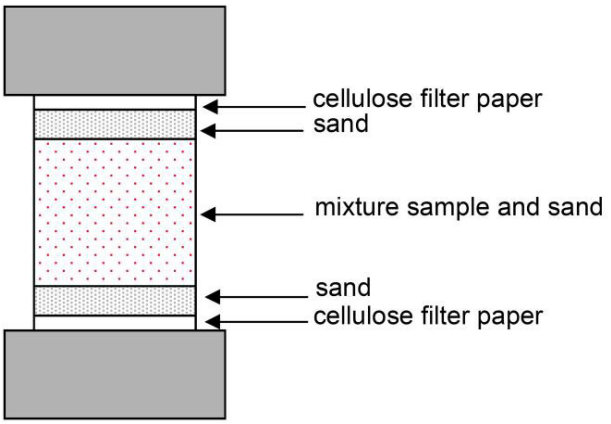


Fig. 3. Packing of ASE extraction cell

2.3.4. Usual extraction method

Amount of ± 1 g dried annatto seeds were added to 15 mL of the optimum solvent ratio obtained from method 2.3.3 and stirred magnetically for 6 h. The obtained solution was filtered with cellulose filter paper. Extraction process on the same samples was repeated three cycles. The extracts were stored at -8 °C until analysis. All experiments were performed in triplicates.

2.3.5. Quantitative analysis of bixin

Quantitative analysis of bixin was performed by UV-Vis spectrophotometer and HPLC equipped with ASI-100 automated sample injector and a photodiode array detector (PDA). The UV-Vis spectrophotometry method of the extract was described in the method 2.3.1.

HPLC analysis of the annatto extracts from ASE was performed on C-18 Spherisorb ODS-2 column, 150 mm × 4.6 mm i.d., 3 µm particle size, with acetonitrile / 0.5 % acetic acid (70 : 30 v / v) under isocratic conditions at a flow rate of 1.0 mL · min^{-1}. Samples were detected at 455 nm on a PDA detector equipped with the Chromeleon software. All samples were filtered through a 0.22 mm filter prior to injection. A stock solution of bixin (25 mg · L^{-1} in acetonitrile) purified as indicated in methods 2.3.2. was used to generate the calibration. Four dilutions were prepared to give concentrations of 20 mg · L^{-1}, 15 mg · L^{-1}, 10 mg · L^{-1}, and 5 mg · L^{-1}, respectively. The calibration curve for bixin was generated by Chromeleon manager software.

3. Result and discussion

3.1. Characterization of pure bixin extracted from annatto seeds

In this study, pure bixin isolated from annatto seeds was used as a standard bixin for qualitative and quantitative analysis of annatto extracts which are obtained by accelerated solvent extraction. UV-Vis spectrophotometry analysis shows that the annatto seeds prepared by repeated extraction in this study presented 27.90 mg bixin · g^{-1} ± 0.02 mg bixin · g^{-1} (2.79 % ± 0.02 % weight). The average concentration of bixin was reported to vary from 12 mg · g^{-1} to 23 mg · g^{-1} seeds, depending on edaphic–climatic (such as temperature, illumination, rainfall and soil) and genetic (cultivar) factors[20].

In Fig. 4, the UV–Vis absorbance spectrum is shown for the purified bixin in acetone. The absorption maxima for bixin expected at 487 nm, 457 nm and 429 nm[19] are shown a good agreement with the experimental results. In this study, acetone was used in the process of extracting pigment and also played a major role in the pigment analysis by UV-Vis spectrophotometry. The acetone solvent tends to produce pigments with the highest absorption peak[20]. Hence, the spectrophotometric respon of pigments facilitate its qualitative and quantitative analysis. The contribution of this solvent to the extraction in various species was comparatively studied[19].

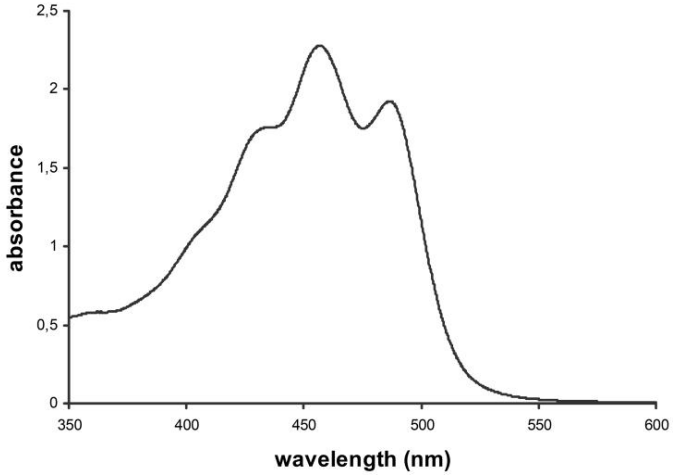


Fig. 4. UV-Vis spectrum for pure bixin in acetone

The FTIR spectrum of annatto extract (Fig. 5a) shows the following bands: at 3 417 cm^{-1} the -O-H stretching vibration is observed, at 2 924 cm^{-1} and 2 853 cm^{-1} the H-C-H bending vibration, at 1 717 cm^{-1} the carboxylic C=O group, at 1 617 cm^{-1} the O-H bending vibration, at 1 562 cm^{-1} and 1 426 cm^{-1} the alkene C=C streching, at 1 378 cm^{-1} the C–H bending of the methyl groups, at 1 256 cm^{-1} the C=O streching, at 1 160 cm^{-1} symmetric and asymmetric vibrations of the C–O–C ester group, at 1 008 cm^{-1} asymmetric C-H bending, 963 cm^{-1} the methylene rocking vibration of *trans*-carotenoid, at 846 cm^{-1} the coupling of the C-O stretching vibrations, at 826 cm^{-1} the terminal methylene and at 722 cm^{-1} the methylene rocking vibration of *cis*-carotenoid[21].

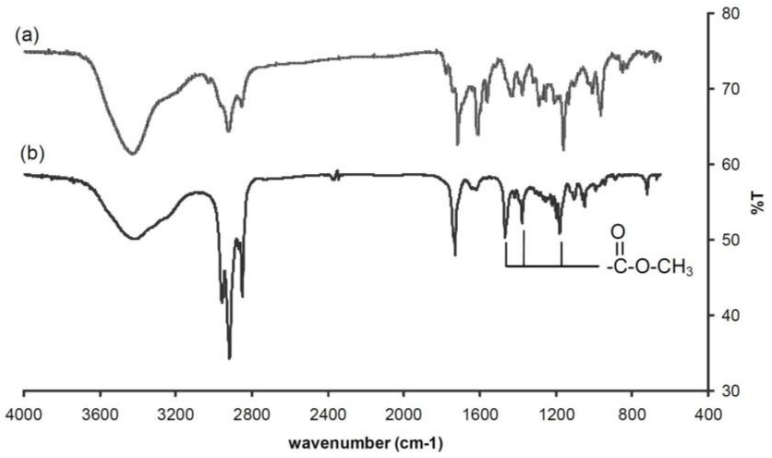


Fig. 5. FTIR spectra for annatto extract (a) and pure bixin (b)

The FTIR spectra show the difference between annatto extract and pure bixin spectrum. This suggests that there are other components contained in the annatto extract. Traces of bixin diesters may be found in the annatto extract. Tirimanna[22] postulated the presence of other minor carotenoids in annatto, which included β-carotene, cryptoxanthin, lutein, zeaxanthin and methyl bixin. The presence of a range of lycopenoate analogues and other minor carotenoids in annatto was also reported by Mercadante et al.[23].

However, the major colouring component of annatto is confirmed as bixin because the difference between annatto extract and pure bixin spectra are not significant. Spectra of bixin obtained from flash chromatography in Fig. 5b are confirmed as pure *cis*-bixin. The following bands were observed in the bixin spectrum: at 3 420 cm^{-1} the -O-H stretching vibration is observed, at 2 957 cm^{-1}, 2 917 cm^{-1} and 2 850 cm^{-1} the H-C-H bending vibration, at 1 731 cm^{-1} the C=O ester group, at 1 620 cm^{-1} the O-H bending vibration, at 1 469 cm^{-1} the alkene C=C streching, at 1 378 cm^{-1} the C–H bending of the methyl groups, at 1 220 cm^{-1} the C=O streching, at 1 180 cm^{-1} symmetric and asymmetric vibrations of the C–O–C ester group, and at 720 cm^{-1} the methylene rocking vibration of *cis*-carotenoid[21].

HPLC analysis was also performed to compare the retention time between bixin obtained commercially and bixin purified by flash chromatography. The results from both bixin samples indicated the same retention time. HPLC analysis of the bixin purified by flash chromatography indicates a purity degree of 88.11 % which the other contain are 11.75 % di-cis bixin and 0.14 % unknown compound. This purity degree was calculated as the percentage of the bixin peak area relative to total area.

3.2. Bixin extraction yield by accelerated solvent extraction

Extraction is a function of the solvation energy in the aqueous phase as well as the solvation energy in the organic phase. The important factors are physical properties of the solvent, such as dielectric constant, dipole moment and solubility parameters. The solubility parameters can describe the energetic cost for cavity formation, while measured values for polarity and hydrogen bonding can describe the gain in energy for solvation. Table 1[24] shows the physical properties of cyclohexane and acetone.

Table 1. Physical properties of cyclohexane and acetone

Physical properties	Cyclohexane	Acetone
Boiling point (°C)	80.80	56.20
Dielectric constant	2.00	20.70
Density ($g \cdot mL^{-1}$)	0.78	0.79
Dipole moment (D)	0.00	2.70
pKa	52.00	19.20

Based on Table 1, cyclohexane is very non-polar compared to acetone. Achondo et al.[10] calculated the dipole moment of *cis*-bixin by computational methods, which is 1.88 D. For these reasons, mixtures of cyclohexane and acetone were made to obtain a polarity range of solvent in order to obtain the optimum solvent ratio for bixin extraction.

UV-Vis spectrophotometry analysis of bixin extraction obtained by ASE based on the methodology described by FAO/WHO is presented in Table 2. In this study, bixin extraction yields were calculated by the formula:

$$\text{Bixin extraction yield (\%)} = \frac{\text{Bixin content in annatto extract obtained by ASE (\%)}}{\text{Total bixin content of annatto seeds (\%)}} \text{ x } 100\% \quad (1)$$

Based on the methodology described by FAO/WHO, the use of 100 % cyclohexane gives the lowest bixin yield. Its UV-Vis spectra do not show the typical spectra of bixin. This is different from the UV-Vis spectra generated by the extraction using 100 % acetone and also the other solvents with addition of acetone in the solvent (Fig. 6). Acetone is a good extraction solvent for plant pigments as it breaks down cell walls and is miscible with the pigments[25]. Acetone which has pKa of 19.20 is a weaker acid of Brønsted-Lowry than bixin (pKa 4.90[10]). Stronger acids will react with the conjugate bases of weaker acids. Hence, acetone is a good solvent for bixin.

The difference in volume of acetone shows significant effects on bixin extraction yields. A significant increase in bixin extraction yields was observed with the use of cyclohexane:acetone (60 % : 40 %) solution at 50 °C for 5 min heating time (48.00 % total of three cycles extraction). This result is higher than the other solvent ratios, even higher than bixin extracted by the usual methods using cyclohexane:acetone (60 % : 40 %) solution (29.14 %). This improvement was possibly due to a polarity increase of the solvent.

The effect of solvent polarity in the bixin extraction was evaluated by Cardarrelli et al.[26], who used methanol/water (1 : 1 v / v), ethanol/water (1 : 1 v / v), methanol, ethanol, ethyl acetate and hexane. They indicated that the best solvent for bixin extraction was ethyl acetate (4.9 mg bixin · g^{-1} seeds) in ultrasonic bath with mass/volume ratio of 1 : 2, at room temperature. This suggests that bixin is soluble in the most polar organic solvents. The structure of bixin with 25 carbons and an acid and methyl ester end-groups is more polar than the carotenoids usually found in foods (chain with 40 carbons) and shows more affinity for medium polar solvents.

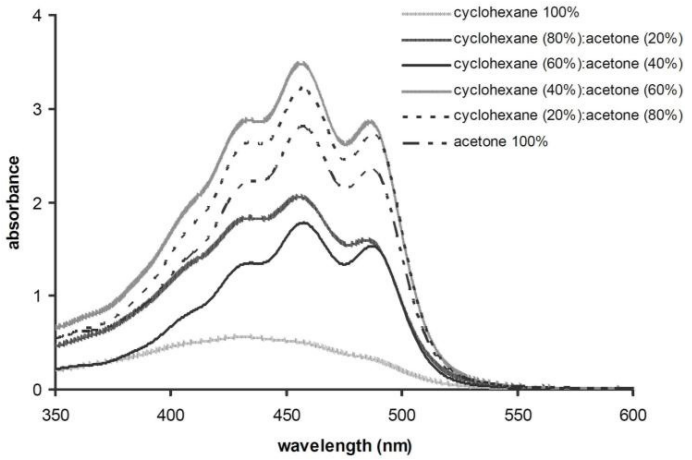


Fig. 6. UV-Vis spectrum of annatto extracts obtained by accelerated solvent extraction after the first extraction cycles excepted cyclohexane:acetone (60 % : 40 %) (the third extraction cycles)

Table 2. Yields of bixin extracted by ASE with the various cyclohexane and acetone ratios based on the methodology described by FAO/WHO

Percent of solvent (%)		λmax (nm)	Percentage of bixin extracted by ASE (%)			
Cyclohexane	Acetone		I	II	III	T
ASE						
100	0	431	1.87 ± 0.03	0.38 ± 0.04	0.17 ± 0.04	2.42 ± 0.05
80	20	434; 455.5; 485	9.14 ± 0.04	4.98 ± 0.03	3.57 ± 0.04	17.69 ± 0.09
60	40	434; 456; 487	26.93 ± 0.03	12.16 ± 0.02	8.92 ± 0.03	48.00 ± 0.05
40	60	433.5; 456; 486.5	16.43 ± 0.13	11.00 ±0.13	9.07 ± 0.10	36.50 ± 0.21
20	80	433.5; 456.5; 487.5	15.76 ± 0.02	13.08 ± 0.03	7.33 ± 0.04	36.17 ± 0.03
0	100	435; 456; 486.5	13.55 ± 0.03	10.03 ± 0.03	6.44 ± 0.03	30.02 ± 0.03
USUAL METHOD						
60	40	434; 456; 487	16.23 ± 0.05	8.04 ± 0.05	2.87 ± 0.06	29.14 ± 0.05

I: the first extraction, II: the second extraction, III: the third extraction, T: total percentage of bixin extracted after three cycles extraction

However, an increase in the ratio of acetone in the solvents ratio (cyclohexane:acetone) 40 % : 60 %, 20 % : 80 % and 0 : 100 %, decrease bixin extraction yields. Some studies reported that the least polar and the most polar solvents tested showed very low bixin extraction efficiency[25,26].

3.3. High performance liquid chromatography study

HPLC method proposed provided good linearity, sensitivity, procedure accuracy, system precision and suggested its suitability for analysis of carotenoids in annatto seeds[27]. The retention time of purified bixin used in this study is 4.11 min (Fig. 7). For this reason, the effect of solvents to bixin purity degree in annatto extracts from ASE was

confirmed by comparing its retention times. For the target compounds, linear regression analysis was performed by using external calibration curves.

The results from the HPLC analysis of bixin extraction yields showed reasonable agreement with both spectrophotometry methods. The results in Table 3 show a significant increase in bixin extraction yields with the use of cyclohexane:acetone (60 % : 40 %). The results from spectrophotometry methods were slightly higher than the result found by HPLC analysis, indicating the presence of non-bixin absorbing species.

Based on HPLC analysis, the use of 100 % cyclohexane does not allow a good extraction, while the use of cyclohexane:acetone (80 % : 20 %) gives a purity degree of 5.49 %. The use of cyclohexane:acetone (60 % : 40 %) gives bixin extracted which has purity degree of 68.16 %. An increase of acetone ratio did not cause a significant increase in bixin purity degree. The use of cyclohexane:acetone (40 % : 60 %, 20 % : 80 %, 0 : 100 %) give 72.58 %, 64.17 %, and 70.02 % purity degree respectively.

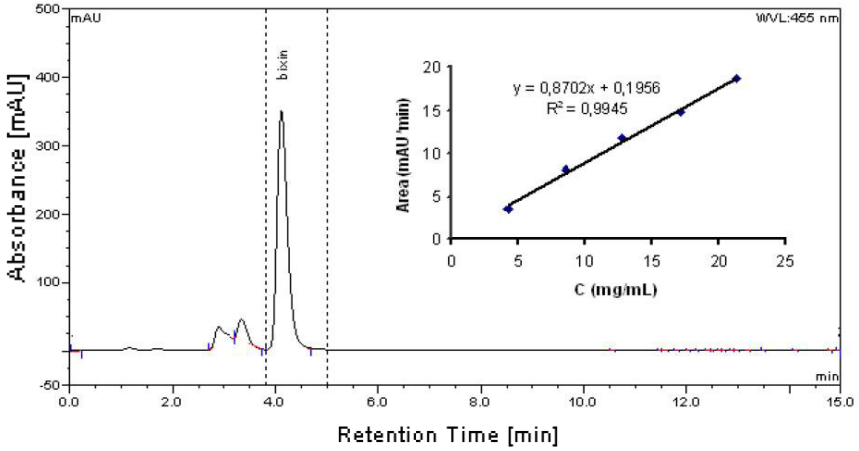


Fig. 7. HPLC chromatogram and calibration curve of purified bixin obtained by flash chromatography

Table 3. Yields of bixin extracted by ASE with the various cyclohexane and acetone ratios based on the HPLC methods

Percent of solvent (%)		Percentage of bixin extracted by ASE (total of three cycles extraction) (%)
Cyclohexane	Acetone	
ASE		
100	0	0.96
80	20	19.87
60	40	42.76
40	60	28.05
20	80	28.92
0	100	26.59
USUAL METHOD		
60	40	29.09

Crystalline bixin products of 80 % to 97 % purity may be obtained by extraction of annatto seeds with certain organic solvents and subsequently produced as a solvent-free product[17]. Low purity bixin produced in this experiment may be caused by the heating temperature of 50 ^{o}C. This is evidenced by the purity of bixin generated through the usual method with a purity degree of 81.30 %.

The formation of several degradation products was confirmed by HPLC (Fig. 8) and possible identification in Table 3. The area of the peak 5 with a retention time of 3.14 min in Fig. 8a is greater than peaks with similar retention times (2.86 min and 3.29 min). The retention times of these products were close to that of bixin, indicating that these products could be geometrical isomers of bixin. Rios et al.[28] indicated that the degradation product which has close retention time with bixin may be di-*cis*-bixin. The di-*cis*-bixin isomers can be considered as reaction intermediates that produce all-*trans*-C-17 irreversibly (peak 1) or return reversibly to bixin (peak 6)[28]. Peaks 3 and 4 showed a smaller retention time than those of bixin and peaks 5 and 6. These features are coincident with those reported for all-*trans* norbixin in a similar solvent mixture.

Table 4. Possibly identification of degradation products caused by temperature

No. Peak Fig. 8a	Retention time (min)		Possibly identification
	Fig. 8a	Fig. 8b as comparison	
6	4.05	4.05	bixin
5	3.14	3.20 2.86	di-*cis*-bixin
4	2.47	2.33	all-*trans*-norbixin
3	2.28		
2	1.62	1.14	not identified
1	1.26		all-*trans*-C-17

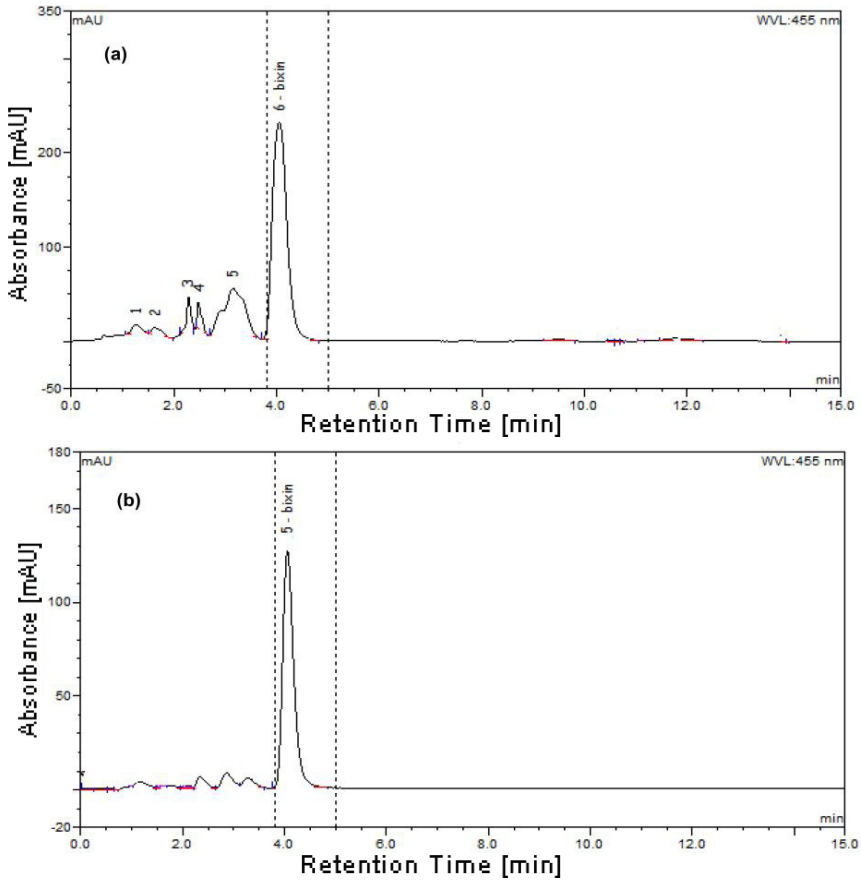


Fig. 8. HPLC chromatogram of annatto extract obtained by ASE (a) and annatto extract obtained by usual method (b) with use of cyclohexane:acetone (3:2)

4. Conclusion

Based on the methodology described by FAO/WHO and HPLC analysis, the difference in percentage of acetone shows significant effects on bixin extraction yields by accelerated solvent extraction. Acetone is a good extraction solvent for bixin as it breaks down cell walls and is miscible with bixin. Acetone show also the best resolution for analysis of annatto extracts by UV-Vis spectrophotometry. However, use of 100 % acetone decrease bixin extraction yields. Therefore, mixtures of cyclohexane and acetone are beneficial to obtain a polarity range in order to obtain

the optimum solvent ratio for bixin extraction. In this study, optimum bixin extraction yield was obtained by using cyclohexane:acetone (60 % : 40 %) solution.

Acknowledgements

The authors thank the financial support of the Indonesian Directorate General of Higher Education (DIKTI) and Tanjungpura University, West Kalimantan, Pontianak, Indonesia.

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