BUKTI KORESPONDENSI

Judul Artikel : Separation and analysis of *Monascus* yellow pigments produced on durian seed substrate

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Penulis : ***Srianta, I.**, Nugerahani, I. and Ristiarini, S**.**

No	Perihal	Tanggal
1	Bukti konfirmasi submit artikel beserta cover letter, formulir submission, dan artikel yang di-submit	10 Januari 2020
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1

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10 Januari 2020

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Short communication manuscript submission

srianta ignatius <srianta2601@gmail.com> 10 Januari 2020 pukul 23.06
Kepada: Food Research <foodresearch.my@outlook.com>

Dear Prof. Son Radu
Editor in Chief of Food Research

Please kindly find the attached files of cover letter, submission form and manuscript titled "separation and analysis of monascus yellow pigment produced on durian seed substrate" (Authors: Ignatius Srianta, Susana Ristiarini and Ira Nugerahani) for your consideration and possible publication in Food Research journal.,

I am looking forward to being informed the evaluation process

Best regards
Srianta

3 lampiran

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Manuscript Type *(Please Bold)*	Original Article Review **Short Communication** Technical Notes
Authors	Ignatius Srianta, Susana Ristiarini and Ira Nugerahani
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January 10, 2019

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Dear Sir,

COVER LETTER FOR MANUSCRIPT SUBMISSION TO FOOD RESEARCH

Please include your manuscript title and your significant results and impact of your research to be considered for publication in Food Research.

On behalf of my colleagues, I wish to submit the attached short communication manuscript titled "Separation and analysis of Monascus yellow pigments produced on durian seed substrate" for your consideration and possible publication in the Food Research journal.

The manuscript is on separation of Monascus yellow pigments produced on durian seed substrate with solid state fermentation, and the pigments analysis by using spectrophotometry, thin layer chromatography (TLC) and liquid chromatography-mass spectrometry (LC-MS).

We produced the pigments with *Monascus purpureus* M9 on durian seed according to our previous method. The Monascus yellow pigments was separated on a silica gel column, eluted with ethyl acetate:ethanol:water=90:25:4. This method was developed in our laboratory. The profile and composition of the Monascus yellow pigment were investigated.

The study on the separation and analysis of Monascus yellow pigments produced on durian seed substrate has never been carried out before. The Monascus yellow pigments contained several yellow pigment compounds that potential as functional natural food colorant. These would give benefit to your journal and the readers.

I also would like to suggest 4 potential reviewers as mentioned in the manuscript submission form.

I thank you for considering this manuscript. Correspondence should be addressed to Dr. Ignatius Srianta, email: srianta_wm@yahoo.com . I look forward to being updated on the progress of the manuscript in the evaluation process.

Your sincerely,

Dr. Ignatius Srianta
Department of Food Technology
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Indonesia 60625,

Separation and analysis of Monascus yellow pigment produced on durian seed substrate

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Abstract

This research was aimed to separate and analysis of monascus yellow pigment (MYP) with chromatography. MYP was in a mixture with red and orange pigments. MYP separation was performed in a silica gel column with eluent of ethyl acetate:ethanol:water=90:25:4. The separated MYP then subjected to analysis by using spectrophotometer, TLC, LC-PDA and LC-MS/MS. The results revealed that the separated MYP has maximum absorption at 386 nm. Five MYP compounds detected by using LC-MS/MS, 2 of them were well known yellow pigment compounds, monascin and ankaflavin. Monascin and yellow II were the major pigment compounds in the MYP. The MYP is being further studied for the antidiabetic bioactivity.

Keywords: monascus, yellow pigment, durian seed, separation, analysis

1. Introduction

Monascus pigments are secondary metabolites of Monascus fungi biosynthesized through polyketide pathway. It has been used as natural food colourant, food supplement, and in traditional medicine. The monascus pigments consists of many pigment compounds which are categorized into 3 groups i.e. red, orange and yellow pigments. Six well known pigment compounds are monascorubramine and rubropunctamine (red pigments), monascorubrin and rubropunctatin (orange pigments), monascin and ankaflavin (yellow pigments) (Feng *et al*., 2012; Srianta *et al*., 2014; Agboyibor *et al*., 2018). Until 2011, 39 new pigment compounds have been identified (Feng *et al*., 2012).

Rice is commonly used as solid state fermentation substrate in monascus pigments production. Non-rice substrates have been developed in the monascus pigment e.g. corn, adlay, sorghum, dioscorea, jackfruit seed and durian seed (Pattanagul *et al*., 2007; Babitha *et al*., 2007; Hsu *et al*., 2011; Srianta *et al*., 2012; Srianta *et al*., 2016). Our previous studies revealed that *Monascus purpureus* produced red, orange and yellow pigments. Exploration of monascus yellow pigments is still limited. MYP is

not only potential as natural colorant for biscuit, mayonaisse, wheat noodle, etc. (Srianta *et al*., 2014), but also as functional food ingredient and in medicine because MYP has various possitive health effects i.e. antioxidant, antidiabetes and anticancer (Hsu *et al*., 2011; Chen and Wu, 2016). Monascin and ankaflavin have antitumor activity (Akihisa *et al*., 2005; Su *et al*., 2005), anti-obesity activity, anti-atherosclerosis effect (Wang *et al*., 2013; Liu *et al*., 2018), improve memory and learning ability (Lee *et al*., 2015). Chen and Wu (2016) show that MYPs have promising potential for application in food and pharmaceutical industries based on their bioactivities.

Silica gel has been applied in monascus pigments mixture separation through thin layer chromatography and column chromatography systems with various eluents i.e. chloroform, methanol, benzene, acetone and petroleum ether (Kim *et al*., 2006; Vidyalakhsmi *et al*., 2009; Hsu *et al*., 2011; Lee *et al*., 2011). In practice, ethyl acetate, ethanol and water are often more preferred for food and pharmaceutical processing compared to other solvents due to their safety and affordability. HPLC is utilized to analyze various monascus pigments bacause its high sensitivity and multiple detection systems including ultraviolet, fluorescent, photodiode array and mass spectrometer detectors (Feng *et al*., 2012).

This research was aimed to separate and analysis MYP produced on durian seed substrate. This is the first investigation in MYP separation using ethyl acetate, ethanol and water.

2. Materials and Methods

2.1 Microorganism, substrate and chemicals

Monascus purpureus M9 culture was used in the solid state fermentation on durian seed substrate. The culture was mantain periodically on Potato Dextrose Agar. Durian seed was obtained from a durian processing unit in Surabaya. The durian seed is by product of the food industry, and was used as the SSF substrate. All chemicals were chromatographic and analytical grades.

2.2 Pigments production through solid state fermentation with durian seed substrate

Pigments production was performed through solid state fermentation on durian seed substrate according to our previous research (Srianta *et al*., 2012). After peeling, cutting, soaking, sterilization at 121oC for 10 minutes and cooling, the durian seed substrate was inoculated with *Monascus purpureus* starter culture, and then incubated at 30^{o}C for 14 days. The fermented matter was dried at 45^{o}C for 24 hours, ground and sieved.

2.3 Monascus pigment separation

The monascus pigment was extracted with soxhletation method in ethanolic solvent at ratio of 1:50 for 2 hours. The extract was then evaporated in a rotary evaporator. The evaporated extract was subjected to separation process. MYP separation was performed in a column chromatography (10 mm x 500 mm) with silica gel 60. The column was packed with silica gel by slurry method, then pre eluted with ethyl acetate:ethanol:water=90:25:4. The extract was put into the column and eluted with ethyl acetate:ethanol:water=90:25:4. The obtained fractions were subjected to analysis by using TLC and spectrophotometer. The MYP fraction was analyzed the pigment composition by using LC-MS/MS.

2.4 Pigment analysis

The separated pigments were subjected to the TLC analysis according to Nimnoi and Lumyong (2011) with modification, on silica gel F60 plate, with ethyl acetate:ethanol:water at 90:25:4. The separated MYP was scanned in a range of wavelength 380-600 nm with UV-Vis spectrophotometer

(Shimadzu UV-1900, Japan). Pigment composition analysis was conducted by using LC-MS/MS (Shimadzu, Kyoto, Japan) with Q1 scan positive mode and SIM positive mode. The extract was filtered through 0.2 um PTFE filter membrane, put on to the auto sampler. Pigments were separated on a Cosmosil 5 C18-MS-II (4.6 i.d. x 150 mm) column, with an isocratic program of mobile phase consist of acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid (60:40 vol/vol), flow rate of 0.5 mL/min; oven temperature of 40^{o}C, run time for 30 min. Pigment compounds were detected by MS/MS using electro-spray ionization in the positive ion mode, scan range: m/z 100 to 1000.

3. Results and Discussion

In the pigments separation by using column chromatography, 4 fractions (F1 F2, F3 and F4) derived with different color. The first fraction was MYP target, while other fractions were orange and red pigments. The MYP has the highest retention factor, followed by F2, F3 and F4. Those fractions and pigments profile on TLC shown in Figure 1. Those profiles describe that the MYP was the pigments with the lowest polarity among the others. Although in the chemical structure of monascus pigments are similar each other which consist of β-ketoacid and chromophore, MYP compounds contain less polar groups than those of red pigments. The presence of $–NH_3$ group can increase the polarity of red pigments.

Figure 2 show the fractions scanning by using spectrophotometer at 380-700 nm. F1, the MYP, has a λ max at 386 nm that was in yellow spectrum. F3 and F4 have 2 peaks in orange and red spectrum. Those reflect that contain a mixture of orange and red pigments. This is the first investigation on the pigment compounds composition of MYP by using the LC-MS/MS. The LC-MS chromatograms and pigment composition of the extracts are presented in Figure 3 and Table 1, respectively. Five MYP compounds were detected by using LC-MS/MS i.e. monascin, ankaflavin, xanthomonasin B, monascopyridine B and Yellow II. Monascin and Yellow II were the major pigment compounds in the MYP with relative amount of 59,81% and 27,80%. The MYP composition depends on the pigment composition in MFDS, extraction and separation processes.

Pigments in MFDS is produced through a complex polyketide biosynthesis pathway in the Monascus fungi, but still unclear and controversy. Some researchers proposed that only orange pigments (rubropunctatin and monascorubramine) were biosynthetic, and the others were transformed from them chemically. In orange pigment Rubropunctatin biosynthesis, it consist of β-ketide pathway to form acetate and malonate and another pathway to form n-hexanoyl acetyl residue. Yellow pigments can be transformed from orange pigments via oxidation. Our previous research revealed that solvent type affected the monascus pigment composition. Ethanolic extract contain higher MYP than those in the mixture solvent of ethanol and water. This research used ethanol as the extraction solvent. In the separation process, the chromatographic system used has successfully separated the MYP from the red and orange pigments.

Monascin, the major MYP compound in this research, has attracted for their strong bioactivities such as anticancer, antitumor, antidiabetic, antioxidative stress, antiinflammatory, antiobesity and antiatherosclerosis activities, also improve memory and learning ability. On the other hand, there is no report on the yellow II bioactivity. In conclusion, the separated MYP has great potential as functional ingredient in food and pharmaceuticals. The MYP is being further studied for the antidiabetic bioactivity.

4. Conclusion

The MYP has been successfully separated in a column chromatography packed with silica gel 60 and eluent of ethyl acetate:ethanol:water=90:25:4. Five MYP compounds have been detected by using LC-MS/MS i.e. monascin, ankaflavin, xanthomonasin B, monascopyridine B and yellow II. Monascin and yellow II were the major pigment compounds in the MYP.

Conflict of Interest

Authors declare no conflict of interest.

Acknowledgments

This work was supported by Direktorat Riset dan Pengabdian Masyarakat, Direktorat Jenderal Penguatan Riset dan Pengembangan, Kementerian Riset, Teknologi, dan Pendidikan Tinggi, Republik Indonesia through the scheme of Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT) 2019 [grant number 200X/WM01.5/N/2019].

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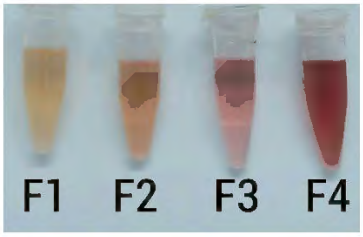
Figure 1. Picture of 4 separated fractions of Monascus pigments (A) and their TLC chromatogram (B). F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.



(A)

(B)

Figure 2. Scanning results of separated Monascus pigment fractions by using spectrophotometer. F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.

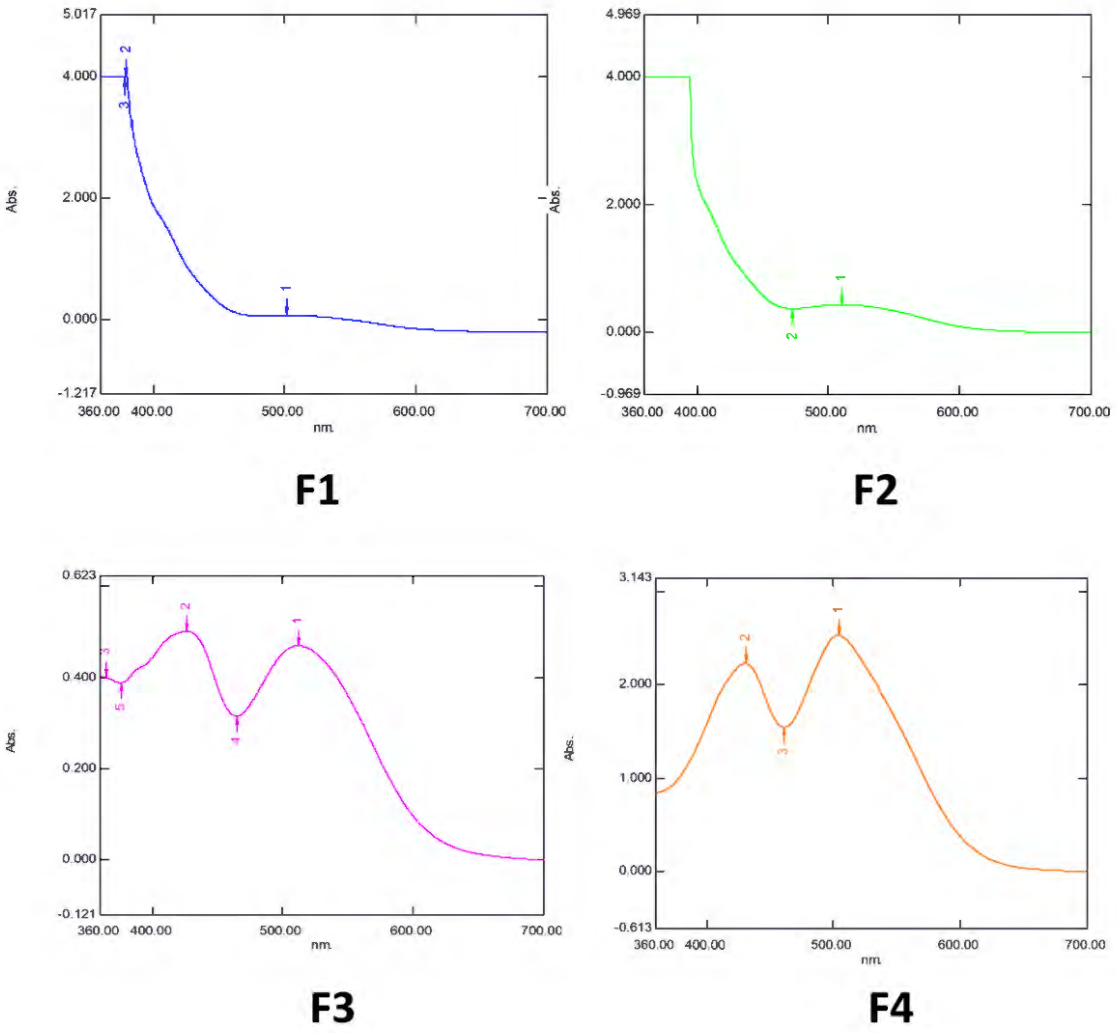
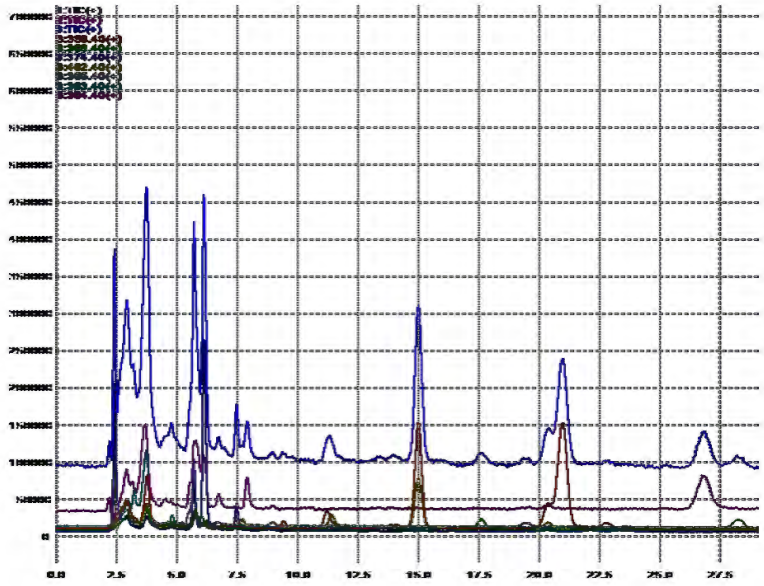
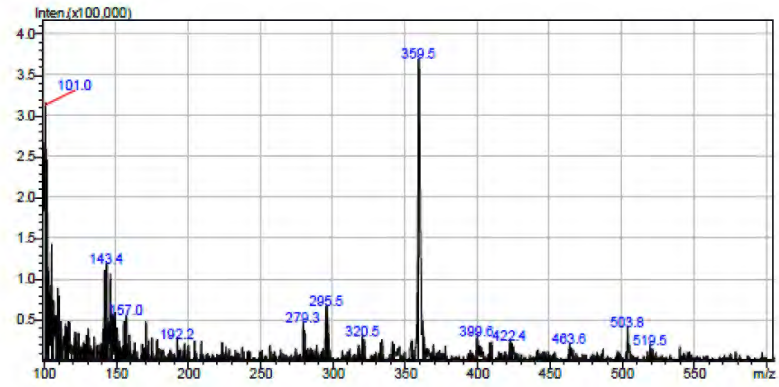


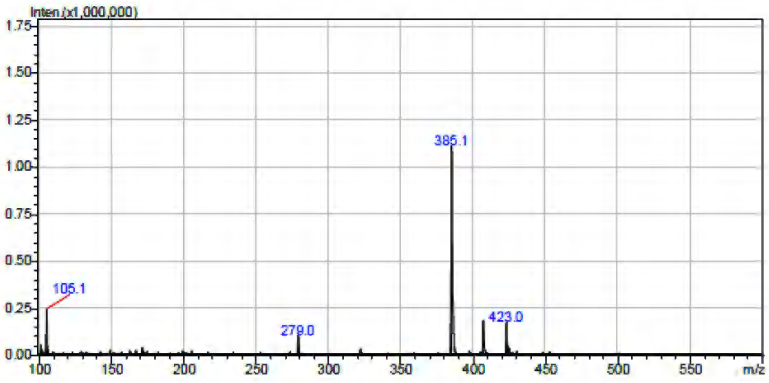
Figure 3. LC-MS analysis results: Total Ion Chromatogram of MYP (A) and representatives of chromatogram of major MYP monascin (B) and yellow II (C).



(A)



(B)



(C)

Table 1. MYP composition

MYP compound	Content (%)
Monascin	59.81
Ankaflavin	6.32
XanthomonasinA	n.d.
XanthomonasinB	0.67
Monascopyridin A	n.d.
Monascopyridin B	5.39
Yellow II	27.80

n.d. = not detected

2

Bukti konfirmasi respon penerimaan artikel beserta letter to author

15 Januari 2020

Gmail

srianta ignatius <srianta2601@gmail.com>

FR-2020-020

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Dear Prof. Son Radu
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Please kindly find the attached files of cover letter, submission form and manuscript titled "separation and analysis of monascus yellow pigment produced on durian seed substrate" (Authors: Ignatius Srianta, Susana Ristiarini and Ira Nugerahani) for your consideration and possible publication in Food Research journal.,

I am looking forward to being informed the evaluation process

Best regards
Srianta

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15th January 2020

Authors: Srianta, I., Nugerahani, I. and Ristiarini, S.

Manuscript title: Separation and analysis of Monascus yellow pigment produced on durian seed substrate

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3

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7 Februari 2020

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1 message

Food Research <foodresearch.my@outlook.com> Fri, 7 Feb 2020 at 23:55
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Dear Srianta,

Manuscript FR-2020-020 entitled " Separation and analysis of Monascus yellow pigment produced on durian seed substrate " which you submitted to Food Research, has been reviewed. The comments of the reviewer(s) are included in the attached file.

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Because we are trying to facilitate timely publication of manuscripts submitted to Food Research, your revised manuscript should be submitted before or by 8th March 2020. If it is not possible for you to submit your revision by this date, please let us know.

Once again, thank you for submitting your manuscript to Food Research and I look forward to receiving your revised manuscript.

Sincerely,
Professor Dr. Son Radu
foodresearch.my@outlook.com

Chief Editor, Food Research

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	A (Excellent)	B	C	D	E (Worst)
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1.	**Title** *"Separation and analysis of Monascus yellow pigment produced on durian seed substrate "* *This title reflects the article*	
2.	**Abstract** *This research was aimed to separate and analyse Monascus yellow pigment with chromatography. Monascin and yellow II were the major pigment compounds in MYP.*	
3.	**Keywords** *Monascus, yellow pigment, durian seed*	
4.	**Introduction** *The introduction is concise with sufficient references to the topic.*	
5.	**Research design/Methodology** *Materials and Methods are well described and probably reproducible*	
6.	**Data Analysis** *The results are well presented and discussed. The main results is that monascin and yellow II were the major pigments in MYP. The indication of further studies to evaluate antidiabetic bioactivity is a little prematurate and useless.*	
7.	**Conclusion** *The conclusion is short and is a clear summary of the study*	
8.	**References**	
9.	**English Proficiency** *It has really to be improved and corrected by an English-speaking person.*	

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10	**Additional comments/suggestions by the reviewer about the article** *It's a pity not to have information on the pigment bioactivity.*	

Overall Evaluation

Please choose one.

Accept		**Major Revision**	
Minor Revision	**X**	**Reject**	

This article is relevant as it identified two yellow pigments produced by Monascus on an unusual substrate, the durian seed. It should be accepted if English proficiency is improved.

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MANUSCRIPT EVALUATION FORM

Date : 15th January 2020

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Title of Manuscript : Separation and analysis of Monascus yellow pigment produced on durian seed substrate

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Evaluation Criteria	Grade				
	A (Excellent)	B	C	D	E (Worst)
1. Appropriateness of Contents	A				
2. Originality of Topic	A				
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4. Research Methodology	A				
5. Data Analysis	A				
6. Relevance to the Journal	A				

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1.	**Title** *It should reflect the article*	Fine
2.	**Abstract** *Background, Aim, Methodology and Conclusion*	Fine, just in once sentence add the significance of your study to the field of Food Research
3.	**Keywords** *Min. 3 and Max. 6*	Fine
4.	**Introduction** *Concise with sufficient background*	Fine
5.	**Research design/Methodology** *Clearly described and reproducible*	Fine
6.	**Data Analysis** *Results well presented and discussed*	Fine
7.	**Conclusion** *A clear summary of the study*	Please revisit [see attached document]
8.	**References** *References should follow the journal's format*	Good relevant set of references
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8.	**References** *References should follow the journal's format*	Minor revision on the writing the citation and references. Writing the journal's name is italicized. A space in one reference is one.
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Separation and analysis of Monascus yellow pigment produced on durian seed substrate

Abstract

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Keywords: monascus, yellow pigment, durian seed, separation, analysis

1. Introduction

Monascus pigments are secondary metabolites of Monascus fungi biosynthesized through polyketide pathway. It has been used as natural food colourant, food supplement, and in traditional medicine. The monascus pigments consists of many pigment compounds which are categorized into 3 groups i.e. red, orange and yellow pigments. Six well known pigment compounds are monascorubramine and rubropunctamine (red pigments), monascorubrin and rubropunctatin (orange pigments), monascin and ankaflavin (yellow pigments) (Feng *et al*., 2012; Srianta *et al*., 2014; Agboyibor *et al*., 2018). Until 2011, 39 new pigment compounds have been identified (Feng *et al*., 2012).

Rice is commonly used as solid state fermentation substrate in monascus pigments production. Non-rice substrates have been developed in the monascus pigment e.g. corn, adlay, sorghum, dioscorea, jackfruit seed and durian seed (Pattanagul *et al*., 2007; Babitha *et al*., 2007; Hsu *et al*., 2011; Srianta *et al*., 2012; Srianta *et al*., 2016). Our previous studies revealed that *Monascus purpureus* produced red, orange and yellow pigments. Exploration of monascus yellow pigments is still limited. MYP is not only potential as natural colorant for biscuit, mayonaisse, wheat noodle, etc. (Srianta *et al*., 2014), but also as functional food ingredient and in medicine because MYP has various possitive health effects i.e. antioxidant, antidiabetes and anticancer (Hsu *et al*., 2011; Chen and Wu, 2016). Monascin and ankaflavin have antitumor activity (Akihisa *et al*., 2005; Su *et al*., 2005), anti-obesity activity, anti-atherosclerosis effect (Wang *et al*., 2013; Liu *et al*., 2018), improve memory and learning ability (Lee *et al*., 2015). Chen and Wu (2016) show that MYPs have promising potential for application in food and pharmaceutical industries based on their bioactivities.

Silica gel has been applied in monascus pigments mixture separation through thin layer chromatography and column chromatography systems with various eluents i.e. chloroform, methanol, benzene, acetone and petroleum ether (Kim *et al*., 2006; Vidyalakhsmi *et al*., 2009; Hsu *et al*., 2011; Lee *et al*., 2011). In practice, ethyl acetate, ethanol and water are often more preferred for food and pharmaceutical processing compared to other solvents due to their safety and affordability. HPLC is utilized to analyze various monascus pigments ~~bacause~~because its high sensitivity and multiple detection systems including ultraviolet, fluorescent, photodiode array and mass spectrometer detectors (Feng *et al*., 2012).

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2. Materials and Methods

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The monascus pigment was extracted with soxhletation method in ethanolic solvent at ratio of 1:50 for 2 hours. The extract was then evaporated in a rotary evaporator. The evaporated extract was subjected to separation process. MYP separation was performed in a column chromatography (10 mm x 500 mm) with silica gel 60. The column was packed with silica gel by slurry method, then pre eluted with ethyl acetate:ethanol:water=90:25:4. The extract was put into the column and eluted with ethyl acetate:ethanol:water=90:25:4. The obtained fractions were subjected to analysis by using TLC and spectrophotometer. The MYP fraction was analyzed the pigment composition by using LC-MS/MS.

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The separated pigments were subjected to the TLC analysis according to Nimnoi and Lumyong (2011) with modification, on silica gel F60 plate, with ethyl acetate:ethanol:water at 90:25:4. The separated MYP was scanned in a range of wavelength 380-600 nm with UV-Vis spectrophotometer (Shimadzu UV-1900, Japan). Pigment composition analysis was conducted by using LC-MS/MS (Shimadzu, Kyoto, Japan) with Q1 scan positive mode and SIM positive mode. The extract was filtered through 0.2 um PTFE filter membrane, put on to the auto sampler. Pigments were separated on a Cosmosil 5 C18-MS-II (4.6 i.d. x 150 mm) column, with an isocratic program of mobile phase consist of acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid (60:40 vol/vol), flow rate of 0.5 mL/min; oven temperature of 40°C, run time for 30 min. Pigment compounds were detected by MS/MS using electro-spray ionization in the positive ion mode, scan range: m/z 100 to 1000.

3. Results and Discussion

In the pigments separation by using column chromatography, 4 fractions (F1 F2, F3 and F4) derived with different color. The first fraction was MYP target, while other fractions were orange and red pigments. The MYP has the highest retention factor, followed by F2, F3 and F4. Those fractions and pigments profile on TLC shown in Figure 1. Those profiles describe that the MYP was the pigments with the lowest polarity among the others. Although in the chemical structure of monascus pigments are similar each other which consist of β-ketoacid and chromophore, MYP compounds contain less

polar groups than those of red pigments. The presence of $-NH_3$ group can increase the polarity of red pigments.

Figure 2 shows the fractions scanning by using spectrophotometer at 380-700 nm. F1, the MYP, has a λ max at 386 nm that was in yellow spectrum. F3 and F4 have 2 peaks in orange and red spectrum. Those reflect that contain a mixture of orange and red pigments. This is the first investigation on the pigment compounds composition of MYP by using the LC-MS/MS. The LC-MS chromatograms and pigment composition of the extracts are presented in Figure 3 and Table 1, respectively. Five MYP compounds were detected by using LC-MS/MS i.e. monascin, ankaflavin, xanthomonasin B, monascopyridine B and Yellow II. Monascin and Yellow II were the major pigment compounds in the MYP with relative amount of 59,81% and 27,80%. The MYP composition depends on the pigment composition in MFDS, extraction and separation processes.

Pigments in MFDS is produced through a complex polyketide biosynthesis pathway in the Monascus fungi, but still unclear and controversy. Some researchers proposed that only orange pigments (rubropunctatin and monascorubramine) were biosynthetic, and the others were transformed from them chemically. In orange pigment Rubropunctatin biosynthesis, it consist of β-ketide pathway to form acetate and malonate and another pathway to form n-hexanoyl acetyl residue. Yellow pigments can be transformed from orange pigments via oxidation. Our previous research revealed that solvent type affected the monascus pigment composition. Ethanolic extract contain higher MYP than those in the mixture solvent of ethanol and water. This research used ethanol as the extraction solvent. In the separation process, the chromatographic system used has successfully separated the MYP from the red and orange pigments.

Monascin, the major MYP compound in this research, has attracted for their strong bioactivities such as anticancer, anti-tumor, anti-diabetic, antioxidative stress, anti-inflammatory, anti-obesity and showed anti-~~atherosclerosis~~ atherogenic activities, also improve memory and learning ability. On the other hand, there is no report on the yellow II bioactivity. **In conclusion??**, the separated MYP has great potential as functional ingredient in food and pharmaceuticals. The MYP is being further studied for the anti-diabetic bioactivity.

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4. Conclusion ??

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The MYP has been successfully separated in a column chromatography packed with silica gel 60 and eluent of ethyl acetate:ethanol:water=90:25:4. Five MYP compounds have been detected by using LC-MS/MS i.e. monascin, ankaflavin, xanthomonasin B, monascopyridine B and yellow II. Monascin and yellow II were the major pigment compounds in the MYP.

Conflict of Interest

Authors declare no conflict of interest.

Acknowledgments

This work was supported by Direktorat Riset dan Pengabdian Masyarakat, Direktorat Jenderal Penguatan Riset dan Pengembangan, Kementerian Riset, Teknologi, dan Pendidikan Tinggi, Republik Indonesia through the scheme of Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT) 2019 [grant number 200X/WM01.5/N/2019].

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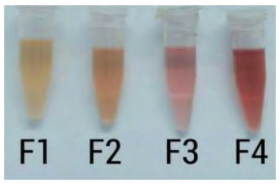
Figure 1. Picture of 4 separated fractions of Monascus pigments (A) and their TLC chromatogram (B). F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.



(A)

(B)

Figure 2. Scanning results of separated Monascus pigment fractions by using spectrophotometer. F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.

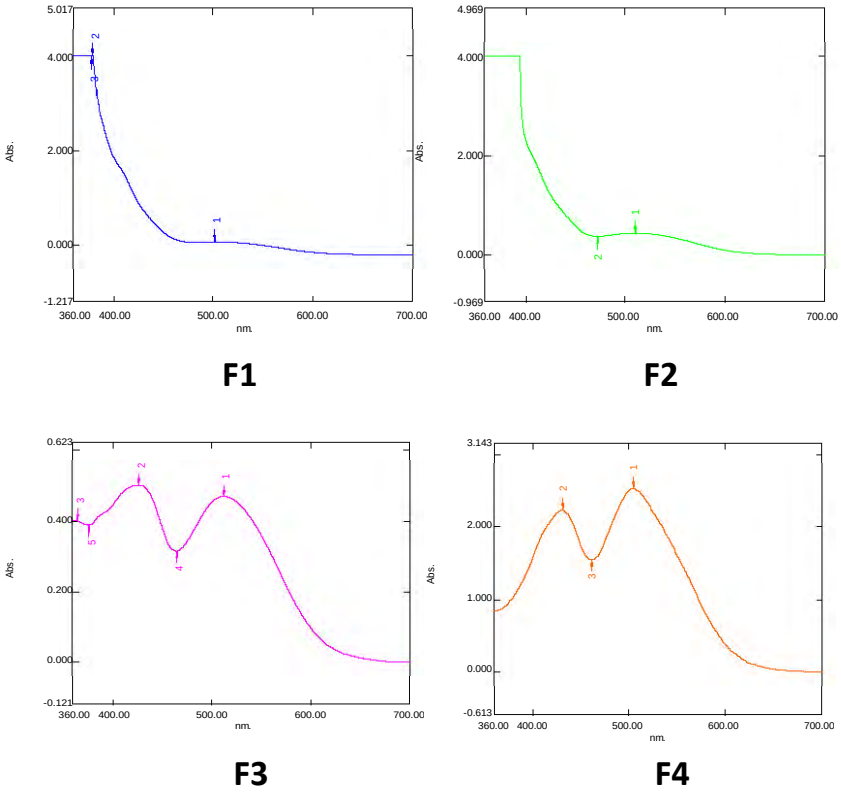
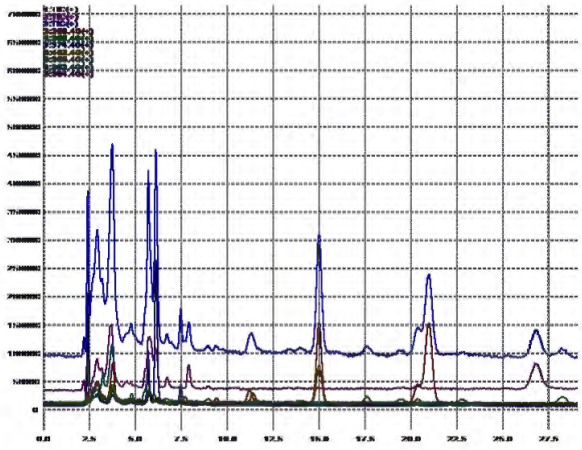
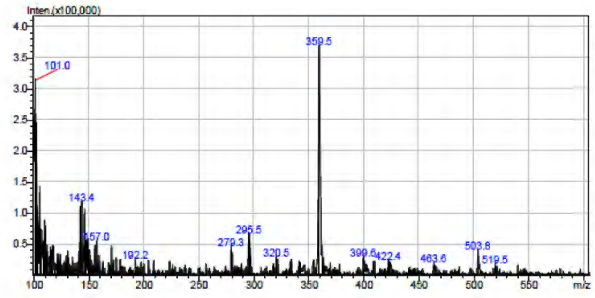


Figure 3. LC-MS analysis results: Total Ion Chromatogram of MYP (A) and representatives of chromatogram of major MYP monascin (B) and yellow II (C).



(A)



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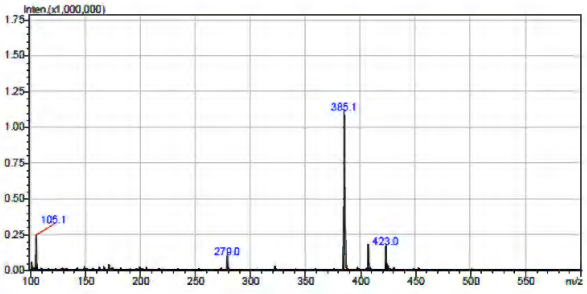

(C)

Table 1. MYP composition

MYP compound	Content (%)
Monascin	59.81
Ankaflavin	6.32
XanthomonasinA	n.d.
XanthomonasinB	0.67
Monascopyridin A	n.d.
Monascopyridin B	5.39
Yellow II	27.80

n.d. = not detected

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Pigments in MFDS is produced through a complex polyketide biosynthesis pathway in the Monascus fungi, but still unclear and controversy. Some researchers proposed that only orange pigments (rubropunctatin and monascorubramine) were biosynthetic, and the others were transformed from them chemically. In orange pigment Rubropunctatin biosynthesis, it consist of β-ketide pathway to form acetate and malonate and another pathway to form n-hexanoyl acetyl residue. Yellow pigments can be transformed from orange pigments via oxidation. Our previous research revealed that solvent type affected the monascus pigment composition. Ethanolic extract contain higher MYP than those in the mixture solvent of ethanol and water. This research used ethanol as the extraction solvent. In the separation process, the chromatographic system used has successfully separated the MYP from the red and orange pigments.

Monascin, the major MYP compound in this research, has attracted for their strong bioactivities such as anticancer, antitumor, antidiabetic, antioxidative stress, antiinflammatory, antiobesity and antiatherosclerosis activities, also improve memory and learning ability. On the other hand, there is no report on the yellow II bioactivity. In conclusion, the separated MYP has great potential as functional ingredient in food and pharmaceuticals. The MYP is being further studied for the antidiabetic bioactivity.

4. Conclusion

The MYP has been successfully separated in a column chromatography packed with silica gel 60 and eluent of ethyl acetate:ethanol:water=90:25:4. Five MYP compounds have been detected by using LC-MS/MS i.e. monascin, ankaflavin, xanthomonasin B, monascopyridine B and yellow II. Monascin and yellow II were the major pigment compounds in the MYP.

Conflict of Interest

Authors declare no conflict of interest.

Acknowledgments

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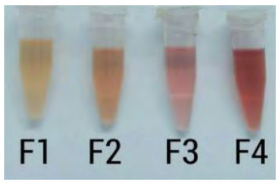
Figure 1. Picture of 4 separated fractions of Monascus pigments (A) and their TLC chromatogram (B). F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.



(A)

(B)

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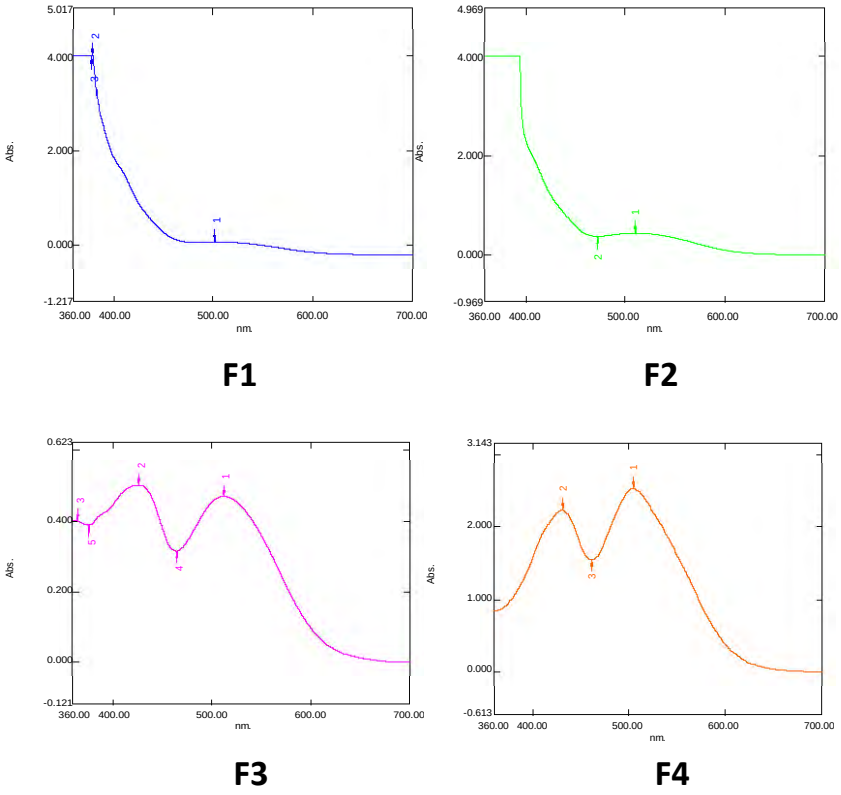
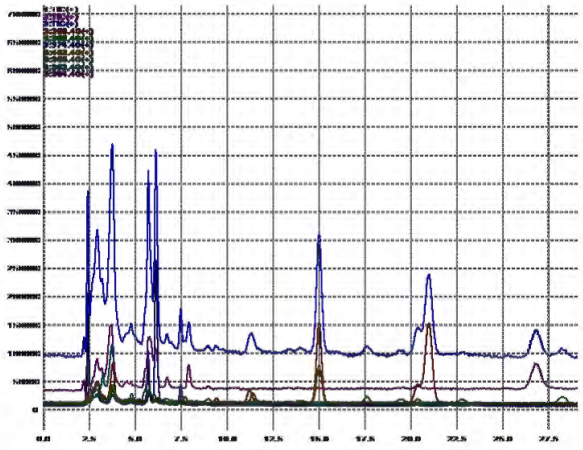
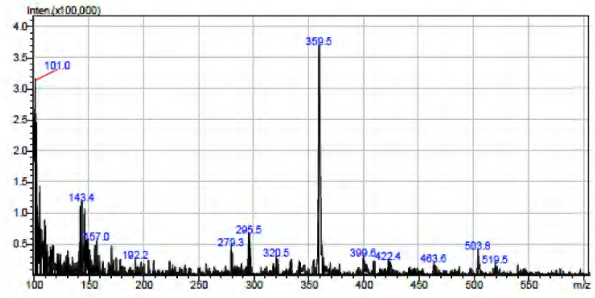


Figure 3. LC-MS analysis results: Total Ion Chromatogram of MYP (A) and representatives of chromatogram of major MYP monascin (B) and yellow II (C).



(A)



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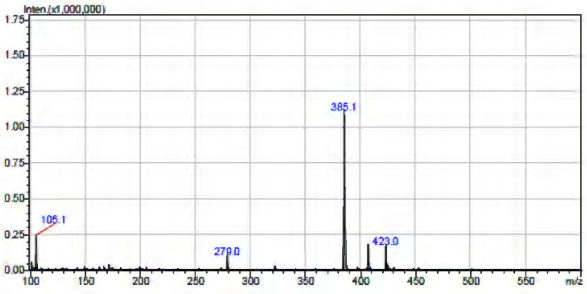

(C)

Table 1. MYP composition

MYP compound	Content (%)
Monascin	59.81
Ankaflavin	6.32
XanthomonasinA	n.d.
XanthomonasinB	0.67
Monascopyridin A	n.d.
Monascopyridin B	5.39
Yellow II	27.80

n.d. = not detected

Separation and analysis of Monascus yellow pigment produced on durian seed substrate

Abstract

This research was aimed to separate and analysesis of Mmonascus yellow pigment (MYP) with chromatography. MYP was in a mixture with red and orange pigments. MYP separation was performed in a silica gel column with eluent of ethyl acetate:ethanol:water=90:25:4. The separated MYP were then subjected to analysis by using spectrophotometer, TLC, LC-PDA and LC-MS/MS. The results revealed that the separated MYP has a maximum absorption at 386 nm. On five MYP compounds detected by using LC-MS/MS, 2 of them were well known yellow pigment compounds, monascin and ankaflavin. Monascin and yellow II were the major pigment compounds in the MYP. The MYP is being further studied for the antidiabetic bioactivity.

Keywords: Mmonascus, yellow pigment, durian seed, separation, analysis

1. Introduction

Monascus pigments are secondary metabolites of Monascus fungi biosynthesized through polyketide pathway. It has been used as natural food colourant, food supplement, and in traditional medicine. MThe monascus pigments consists of many pigment compounds which are categorized into 3 groups i.e. red, orange and yellow pigments. Six well known pigment compounds are monascorubramine and rubropunctamine (red pigments), monascorubrin and rubropunctatin (orange pigments), monascin and ankaflavin (yellow pigments) (Feng *et al*., 2012; Srianta *et al*., 2014; Agboyibor *et al*., 2018). Until 2011, 39 new pigment compounds have been identified (Feng *et al*., 2012).

Rice is commonly used as solid state fermentation substrate in monascus Monascus pigments production. Non-rice substrates have been developed in the monascus Monascus pigment e.g. corn, adlay, sorghum, dioscorea, jackfruit seed and durian seed (Pattanagul *et al*., 2007; Babitha *et al*., 2007; Hsu *et al*., 2011; Srianta *et al*., 2012; Srianta *et al*., 2016). Our previous studies revealed that *Monascus purpureus* produced red, orange and yellow pigments. Exploration of monascus Monascus yellow pigments is still limited. MYP is not only potential as natural colorant for biscuit, mayonnaisse, wheat noodle, etc. (Srianta *et al*., 2014), but also as functional food ingredient and in medicine because MYP has various possitive health effects i.e. antioxidant, antidiabetes and anticancer (Hsu *et al*., 2011; Chen and Wu, 2016). Monascin and ankaflavin have antitumor activity (Akihisa *et al*., 2005; Su *et al*., 2005), anti-obesity activity, anti-atherosclerosis effect (Wang *et al*., 2013; Liu *et al*., 2018), improve memory and learning ability (Lee *et al*., 2015). Chen and Wu (2016) show that MYPs have promising potential for application in food and pharmaceutical industries based on their bioactivities.

Silica gel has been applied in monascus Monascus pigments mixture separation through thin layer chromatography and column chromatography systems with various eluents i.e. chloroform, methanol, benzene, acetone and petroleum ether (Kim *et al*., 2006; Vidyalakhsmi *et al*., 2009; Hsu *et al*., 2011; Lee *et al*., 2011). In practice, ethyl acetate, ethanol and water are often more preferred for food and pharmaceutical processing compared to other solvents due to their safety and affordability. HPLC is utilized to analyze various monascus Monascus pigments beacause its high sensitivity and multiple detection systems including ultraviolet, fluorescent, photodiode array and mass spectrometer detectors (Feng *et al*., 2012).

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This research was aimed to separate and analyseis MYP produced on durian seed substrate. This is the first investigation in MYP separation using ethyl acetate, ethanol and water.

2. Materials and Methods

2.1 Microorganism, substrate and chemicals

Monascus purpureus M9 culture was used in the solid state fermentation on durian seed substrate. The culture was maintain periodically on Potato Dextrose Agar. Durian seed was obtained from a durian processing unit in Surabaya (Indonesia). The durian seed is a by product of the food industry, and was used as the SSF substrate. All chemicals were chromatographic and analytical grades.

2.2 Pigments production through solid state fermentation with durian seed substrate

Pigments production was performed through solid state fermentation on durian seed substrate according to our previous research (Srianta *et al*., 2012). After peeling, cutting, soaking, sterilization at 121oC for 10 minutes and cooling, the durian seed substrate was inoculated with *Monascus purpureus* starter culture, and then incubated at 30°C for 14 days. The fermented matter was dried at 45°C for 24 hours, ground and sieved.

2.3 Monascus pigment separation

The monascus pigment was extracted with soxhletation method in ethanolic solvent at ratio of 1:50 for 2 hours. The extract was then evaporated in a rotary evaporator. The evaporated extract was subjected to separation process. MYP separation was performed in a column chromatography (10 mm x 500 mm) with silica gel 60. The column was packed with silica gel by slurry method, then pre eluted with ethyl acetate:ethanol:water=90:25:4. The extract was put into the column and eluted with ethyl acetate:ethanol:water=90:25:4. The obtained fractions were subjected to analysis by using TLC and spectrophotometer. The MYP fraction was analyzed the pigment composition by using LC-MS/MS.

2.4 Pigment analysis

The separated pigments were subjected to the TLC analysis according to Nimnoi and Lumyong (2011) with modification, on silica gel F60 plate, with ethyl acetate:ethanol:water at 90:25:4. The separated MYP was scanned in a range of wavelength 380-600 nm with UV-Vis spectrophotometer (Shimadzu UV-1900, Japan). Pigment composition analysis was conducted by using LC-MS/MS (Shimadzu, Kyoto, Japan) with Q1 scan positive mode and SIM positive mode. The extract was filtered through 0.2 um PTFE filter membrane, put on to the auto sampler. Pigments were separated on a Cosmosil 5 C18-MS-II (4.6 i.d. x 150 mm) column, with an isocratic program of mobile phase consist of acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid (60:40 vol/vol), flow rate of 0.5 mL/min; oven temperature of 40°C, run time for 30 min. Pigment compounds were detected by MS/MS using electro-spray ionization in the positive ion mode, scan range: m/z 100 to 1000.

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3. Results and Discussion

In the pigments separation by using column chromatography, 4 fractions (F1 F2, F3 and F4) derived with different color. The first fraction was MYP target, while other fractions were orange and red pigments. The MYP has the highest retention factor, followed by F2, F3 and F4. Those fractions and pigments profile on TLC shown in Figure 1. Those profiles describe that the MYP was the pigments with the lowest polarity among the others. Although in the chemical structure of Mmonascus pigments are similar each other which consist for all of them of β-ketoacid and chromophore, MYP

compounds contain less polar groups than those of red pigments. The presence of $-NH_3$ group can increase the polarity of red pigments.

Figure 2 show the fractions scanning by using spectrophotometer at 380-700 nm. F1, the MYP, has a λ max at 386 nm that was in yellow spectrum. F3 and F4 have 2 peaks in orange and red spectrum. Those reflect that contain a mixture of orange and red pigments. This is the first investigation on the pigment compounds composition of MYP by using the LC-MS/MS. The LC-MS chromatograms and pigment composition of the extracts are presented in Figure 3 and Table 1, respectively. Five MYP compounds were detected by using LC-MS/MS i.e. monascin, ankaflavin, xanthomonasin B, monascopyridine B and Yellow II. Monascin and Yellow II were the major pigment compounds in the MYP with relative amount of 59,81% and 27,80%. The MYP composition depends on the pigment composition in MFDS, extraction and separation processes.

Pigments in MFDS is produced through a complex polyketide biosynthesis pathway in the Monascus fungi, but it is still unclear and controversy. Some researchers proposed that only orange pigments (rubropunctatin and monascorubramine) were biosynthetic, and the others were transformed from them chemically. In orange pigment r~~R~~ubropunctatin biosynthesis, it consist of β-ketide pathway to form acetate and malonate and another pathway to form n-hexanoyl acetyl residue. Yellow pigments can be transformed from orange pigments via oxidation. Our previous research revealed that solvent type affected the M~~m~~onascus pigment composition. Ethanolic extract contain higher MYP than those in the mixture solvent of ethanol and water. This research used ethanol as the extraction solvent. In the separation process, the chromatographic system used has successfully separated ~~the~~ MYP from the red and orange pigments.

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Conflict of Interest

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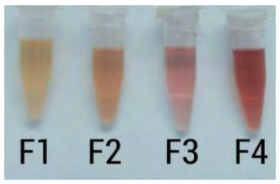

(A)

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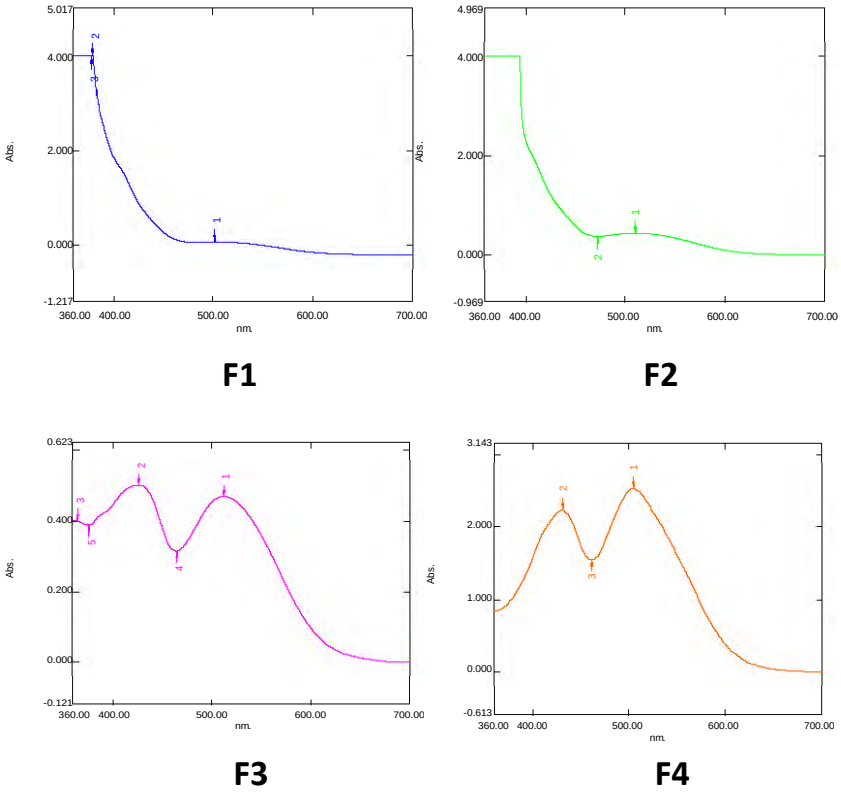

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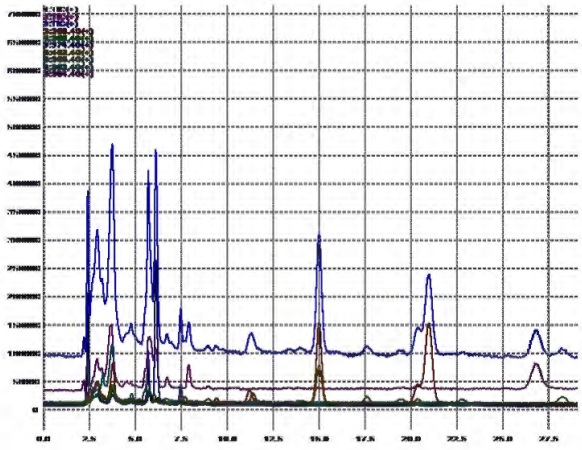

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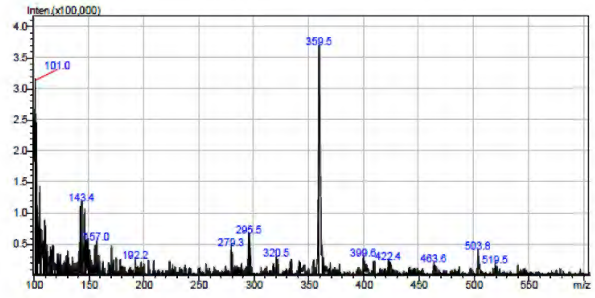

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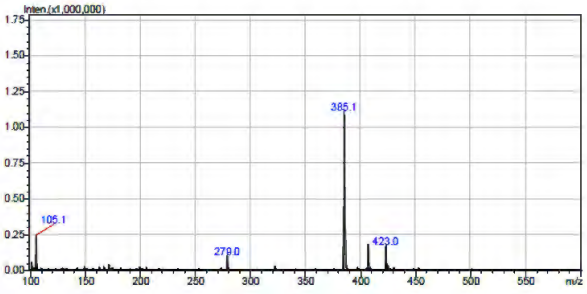

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n.d. = not detected

4

Bukti konfirmasi submit revisi artikel beserta respon kepada reviewer dan artikel yang di-resubmit

2 Maret 2020

Re: FR-2020-020 (Revised)

Dari: srianta_wm@yahoo.com

Kepada: foodresearch.my@outlook.com

Tanggal: Senin, 2 Maret 2020 pukul 08.00 GMT+7

Dear Prof. Son Radu

Please kindly find the attached files of FR-2020-020_revised version and response to reviewers comments. Thank you for your kind attention

Best regards
Srianta

> On Feb 7, 2020 23:55, Food Research <foodresearch.my@outlook.com> wrote:
>
> > Dear Srianta,
> >
> > Manuscript FR-2020-020 entitled " Separation and analysis of Monascus yellow pigment produced on durian seed substrate " which you submitted to Food Research, has been reviewed. The comments of the reviewer(s) are included in the attached file.
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> > The reviewer(s) have recommended publication, but also suggest some revisions to your manuscript. Therefore, I invite you to respond to the reviewer(s)' comments and revise your manuscript. Once the revised manuscript is prepared, please send it back to me for further processing.
> >
> > Because we are trying to facilitate timely publication of manuscripts submitted to Food Research, your revised manuscript should be submitted before or by 8th March 2020. If it is not possible for you to submit your revision by this date, please let us know.
> >
> > Once again, thank you for submitting your manuscript to Food Research and I look forward to receiving your revised manuscript.
> >
> > Sincerely,
> > Professor Dr. Son Radu
> > foodresearch.my@outlook.com
> >
> > Chief Editor, Food Research

FR-2020-020_Revised version.docx
196.8kB

FR-2020-020_Response to The Reviewers Comments.docx
16.6kB

Response to The Reviewers Comments
FR-2020-020

No.	Section	Page	Line	Reviewer Comment	Response
1.	Abstract	1	1	Analysis Monascus	It has been revised: Analyse *Monascus*
		1	3	The separated MYP then subjected to analysis by using spectrophotometer, TLC, LC-PDA and LC-MS/MS.	It has been modified: The separated MYP were then subjected to analysis by using spectrophotometer, TLC, LC-PDA and LC-MS/MS.
		1	4	Five MYP compounds detected by using LC-MS/MS, 2 of them were well known yellow pigment compounds, monascin and ankaflavin	It has been modified: On five MYP compounds detected by using LC-MS/MS, 2 of them were well known yellow pigment compounds, monascin and ankaflavin
		1	5	Monascin and yellow II were the major pigment compounds in the MYP.	It has been revised: Monascin and yellow II were the major pigment compounds in MYP.
		1	6	Reviewer suggestion: "The MYP is being further studied for the antidiabetic bioactivity" to be deleted	It has been deleted
2.	Introduction	1	3	The monascus pigments	It has been revised: *Monascus* pigments
		1	6,7,9,13, 15	monascus pigments	It has been revised: *Monascus* pigments
		1	10	mayonaisse	It has been revised: mayonnaise
		1	10	possitive	It has been revised: positive
		1	11	anti-obesity	It has been revised: antiobesity activity
		1	15	because its high	It has been modified: because of its high
		2	16	analysis	It has been revised: analyse
3,	Material and Methods	2	1	solid state	It has been revised: solid-state
		2	2	mantain	It has been revised: maintain
		2	3	Durian seed was obtained from a durian processing unit in Surabaya	It has been modified: Durian seed was obtained from a durian processing unit in Surabaya (Indonesia)
4.	Result and Discussion	2	1	In the pigments separation by using column	It has been modified: In the pigments separation by using column chromatography, 4

				chromatography, 4 fractions (F1 F2, F3 and F4) derived with different color	fractions (F1 F2, F3 and F4) derived from different color
		2	5	monascus pigments	It has been revised: *Monascus* pigments
		3	5	Although in the chemical structure of *Monascus* pigments are similar each other which consist of β-ketoacid and chromophore, MYP compounds contain less polar groups than those of red pigments.	It has been modified: Although the chemical structure of *Monascus* pigments consist for all of them of β-ketoacid and chromophore, MYP compounds contain less polar groups than those of red pigments.
		3	13	The MYP composition depends on the pigment composition in MFDS	It has been revised: The MYP composition depends on the pigment composition in *Monascus* Fermented Durian Seed (MFDS)
		3	16	Rubropunctatin	It has been revised: rubropunctatin
		3	18	monascus pigments	It has been revised: *Monascus* pigments
		3	21	Monascin, the major MYP compound in this research, has attracted for their strong bioactivities such as anticancer, anti-tumor, anti-diabetic, antioxidative stress, anti-inflammatory, anti-obesity and anti-atherosclerosis activities, also improve memory and learning ability.	It has been modified: Monascin, the major MYP compound in this research, is attractive for its strong bioactivities such as anticancer, antitumor, antidiabetic, antioxidative stress, antiinflammatory, antiobesity and showed antiatherogenic activities, and also improves memory and learning ability.
		3	23, 24	Reviewer suggestion: “In conclusion, the separated MYP has great potential as functional ingredient in food and pharmaceuticals. The MYP is being further studied for the antidiabetic bioactivity” to be deleted	It has been deleted
	English			It has really to be	It has been done

	proficiency			improved and corrected by an English-speaking person	

Separation and analysis of Monascus yellow pigment produced on durian seed substrate

Abstract

This research was aimed to separate and analyse *Monascus* yellow pigment (MYP) with chromatography. MYP was in a mixture with red and orange pigments. MYP separation was performed in a silica gel column with eluent of ethyl acetate:ethanol:water=90:25:4. The separated MYP were then subjected to analysis by using spectrophotometer, TLC, LC-PDA and LC-MS/MS. The results revealed that the separated MYP has a maximum absorption at 386 nm. On five MYP compounds detected by using LC-MS/MS, 2 of them were well known yellow pigment compounds, monascin and ankaflavin. Monascin and yellow II were the major pigment compounds in MYP.

Keywords: *Monascus*, yellow pigment, durian seed, separation, analysis

1. Introduction

Monascus pigments are secondary metabolites of Monascus fungi biosynthesized through polyketide pathway. It has been used as natural food colourant, food supplement, and in traditional medicine. *Monascus* pigments consists of many pigment compounds which are categorized into 3 groups i.e. red, orange and yellow pigments. Six well known pigment compounds are monascorubramine and rubropunctamine (red pigments), monascorubrin and rubropunctatin (orange pigments), monascin and ankaflavin (yellow pigments) (Feng *et al*., 2012; Srianta *et al*., 2014; Agboyibor *et al*., 2018). Until 2011, 39 new pigment compounds have been identified (Feng *et al*., 2012).

Rice is commonly used as solid state fermentation substrate in *Monascus* pigments production. Non-rice substrates have been developed in the *Monascus* pigment e.g. corn, adlay, sorghum, dioscorea, jackfruit seed and durian seed (Pattanagul *et al*., 2007; Babitha *et al*., 2007; Hsu *et al*., 2011; Srianta *et al*., 2012; Srianta *et al*., 2016). Our previous studies revealed that *Monascus purpureus* produced red, orange and yellow pigments. Exploration of *Monascus* yellow pigments is still limited. MYP is not only potential as natural colorant for biscuit, mayonnaise, wheat noodle, etc. (Srianta *et al*., 2014), but also as functional food ingredient and in medicine because MYP has various positive health effects i.e. antioxidant, antidiabetes and anticancer (Hsu *et al*., 2011; Chen and Wu, 2016). Monascin and ankaflavin have antitumor activity (Akihisa *et al*., 2005; Su *et al*., 2005), antiobesity activity, anti-atherosclerosis effect (Wang *et al*., 2013; Liu *et al*., 2018), improve memory and learning ability (Lee *et al*., 2015). Chen and Wu (2016) show that MYPs have promising potential for application in food and pharmaceutical industries based on their bioactivities.

Silica gel has been applied in *Monascus* pigments mixture separation through thin layer chromatography and column chromatography systems with various eluents i.e. chloroform, methanol, benzene, acetone and petroleum ether (Kim *et al*., 2006; Vidyalakhsmi *et al*., 2009; Hsu *et al*., 2011; Lee *et al*., 2011). In practice, ethyl acetate, ethanol and water are often more preferred for food and pharmaceutical processing compared to other solvents due to their safety and affordability. HPLC is utilized to analyze various *Monascus* pigments because of its high sensitivity and multiple detection systems including ultraviolet, fluorescent, photodiode array and mass spectrometer detectors (Feng *et al*., 2012).

This research was aimed to separate and analyse MYP produced on durian seed substrate. This is the first investigation in MYP separation using ethyl acetate, ethanol and water.

2. Materials and Methods

2.1 Microorganism, substrate and chemicals

Monascus purpureus M9 culture was used in the solid-state fermentation on durian seed substrate. The culture was maintain periodically on Potato Dextrose Agar. Durian seed was obtained from a durian processing unit in Surabaya (Indonesia). The durian seed is a by product of the food industry, and was used as the SSF substrate. All chemicals were chromatographic and analytical grades.

2.2 Pigments production through solid state fermentation with durian seed substrate

Pigments production was performed through solid state fermentation on durian seed substrate according to our previous research (Srianta *et al*., 2012). After peeling, cutting, soaking, sterilization at 121oC for 10 minutes and cooling, the durian seed substrate was inoculated with *Monascus purpureus* starter culture, and then incubated at 30^{o}C for 14 days. The fermented matter was dried at 45^{o}C for 24 hours, ground and sieved.

2.3 Monascus pigment separation

The monascus pigment was extracted with soxhletation method in ethanolic solvent at ratio of 1:50 for 2 hours. The extract was then evaporated in a rotary evaporator. The evaporated extract was subjected to separation process. MYP separation was performed in a column chromatography (10 mm x 500 mm) with silica gel 60. The column was packed with silica gel by slurry method, then pre eluted with ethyl acetate:ethanol:water=90:25:4. The extract was put into the column and eluted with ethyl acetate:ethanol:water=90:25:4. The obtained fractions were subjected to analysis by using TLC and spectrophotometer. The MYP fraction was analyzed the pigment composition by using LC-MS/MS.

2.4 Pigment analysis

The separated pigments were subjected to the TLC analysis according to Nimnoi and Lumyong (2011) with modification, on silica gel F60 plate, with ethyl acetate:ethanol:water at 90:25:4. The separated MYP was scanned in a range of wavelength 380-600 nm with UV-Vis spectrophotometer (Shimadzu UV-1900, Japan). Pigment composition analysis was conducted by using LC-MS/MS (Shimadzu, Kyoto, Japan) with Q1 scan positive mode and SIM positive mode. The extract was filtered through 0.2 um PTFE filter membrane, put on to the auto sampler. Pigments were separated on a Cosmosil 5 C18-MS-II (4.6 i.d. x 150 mm) column, with an isocratic program of mobile phase consist of acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid (60:40 vol/vol), flow rate of 0.5 mL/min; oven temperature of 40^{o}C, run time for 30 min. Pigment compounds were detected by MS/MS using electro-spray ionization in the positive ion mode, scan range: m/z 100 to 1000.

3. Results and Discussion

In the pigments separation by using column chromatography, 4 fractions (F1 F2, F3 and F4) derived from different color. The first fraction was MYP target, while other fractions were orange and red pigments. The MYP has the highest retention factor, followed by F2, F3 and F4. Those fractions and pigments profile on TLC shown in Figure 1. Those profiles describe that MYP was the pigments with the lowest polarity among the others. Although the chemical structure of *Monascus* pigments

consist for all of them of β-ketoacid and chromophore, MYP compounds contain less polar groups than those of red pigments. The presence of $-NH_3$ group can increase the polarity of red pigments.

Figure 2 show the fractions scanning by using spectrophotometer at 380-700 nm. F1, the MYP, has a λ max at 386 nm that was in yellow spectrum. F3 and F4 have 2 peaks in orange and red spectrum. Those reflect that contain a mixture of orange and red pigments. This is the first investigation on the pigment compounds composition of MYP by using the LC-MS/MS. The LC-MS chromatograms and pigment composition of the extracts are presented in Figure 3 and Table 1, respectively. Five MYP compounds were detected by using LC-MS/MS i.e. monascin, ankaflavin, xanthomonasin B, monascopyridine B and Yellow II. Monascin and Yellow II were the major pigment compounds in the MYP with relative amount of 59,81% and 27,80%. The MYP composition depends on the pigment composition in *Monascus* Fermented Durian Seed (MFDS), extraction and separation processes.

Pigments in MFDS produced through a complex polyketide biosynthesis pathway in the Monascus fungi, but it is still unclear and controversy. Some researchers proposed that only orange pigments (rubropunctatin and monascorubramine) were biosynthetic, and the others were transformed from them chemically. In orange pigment rubropunctatin biosynthesis, it consist of β-ketide pathway to form acetate and malonate and another pathway to form n-hexanoyl acetyl residue. Yellow pigments can be transformed from orange pigments via oxidation. Our previous research revealed that solvent type affected the *Monascus* pigment composition. Ethanolic extract contain higher MYP than those in the mixture solvent of ethanol and water. This research used ethanol as the extraction solvent. In the separation process, the chromatographic system used has successfully separated MYP from the red and orange pigments.

Monascin, the major MYP compound in this research, is attractive for its strong bioactivities such as anticancer, antitumor, antidiabetic, antioxidative stress, antiinflammatory, antiobesity and showed antiatherogenic activities, and also improves memory and learning ability. On the other hand, there is no report on the yellow II bioactivity.

4. Conclusion

The MYP has been successfully separated in a column chromatography packed with silica gel 60 and eluent of ethyl acetate:ethanol:water=90:25:4. Five MYP compounds have been detected by using LC-MS/MS i.e. monascin, ankaflavin, xanthomonasin B, monascopyridine B and yellow II. Monascin and yellow II were the major pigment compounds in MYP.

Conflict of Interest

Authors declare no conflict of interest.

Acknowledgments

This work was supported by Direktorat Riset dan Pengabdian Masyarakat, Direktorat Jenderal Penguatan Riset dan Pengembangan, Kementerian Riset, Teknologi, dan Pendidikan Tinggi, Republik Indonesia through the scheme of Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT) 2019 [grant number 200X/WM01.5/N/2019].

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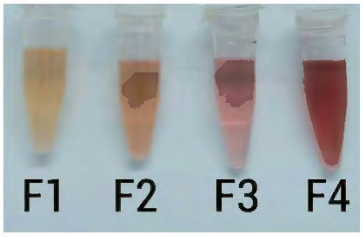
Figure 1. Picture of 4 separated fractions of Monascus pigments (A) and their TLC chromatogram (B). F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.



(A)

(B)

Figure 2. Scanning results of separated Monascus pigment fractions by using spectrophotometer. F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.

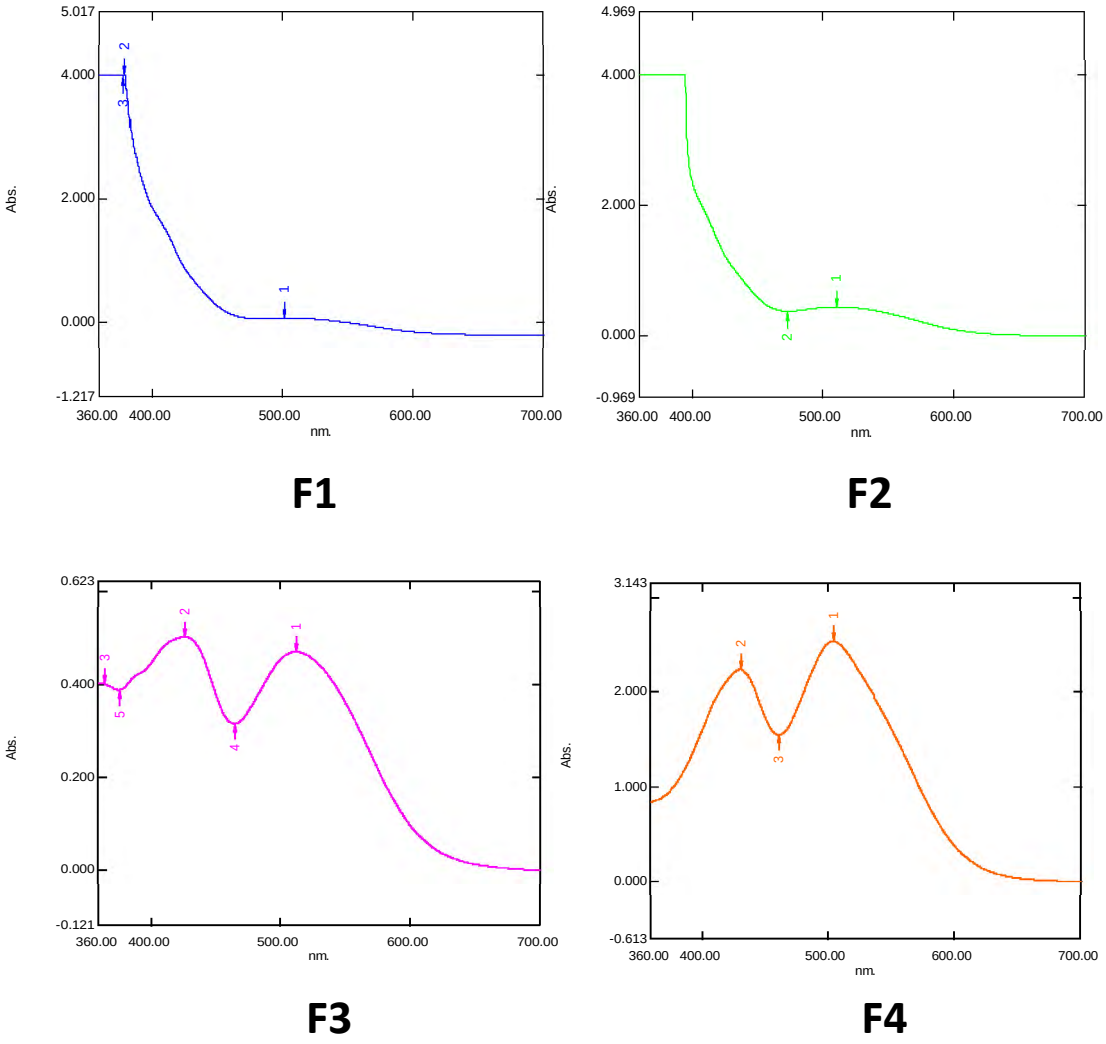
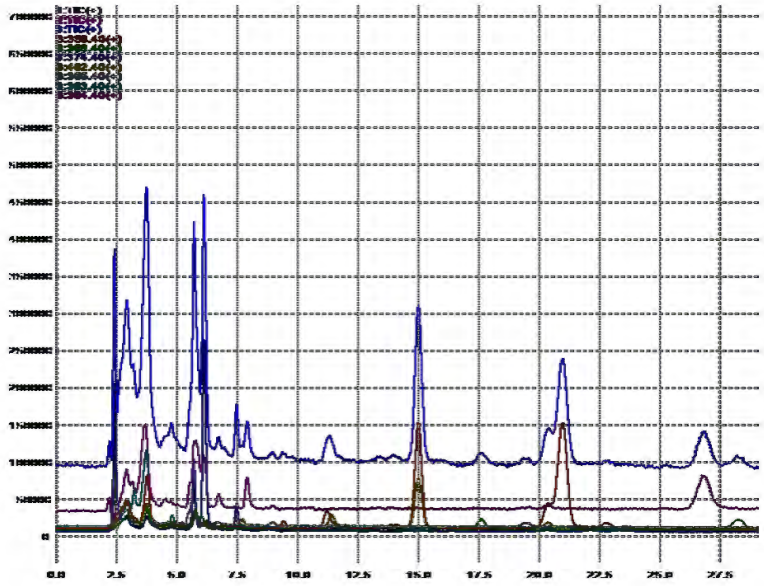
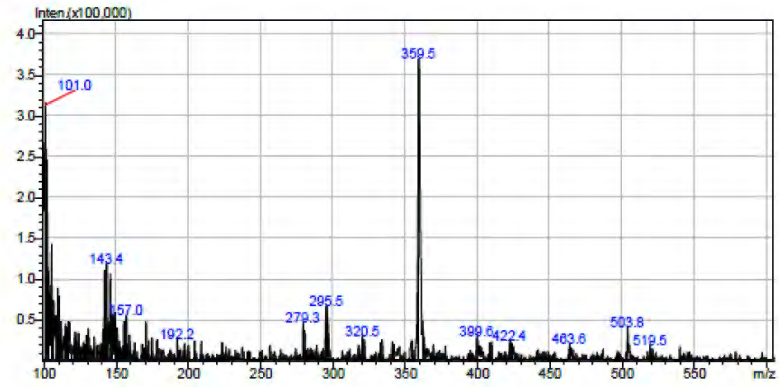


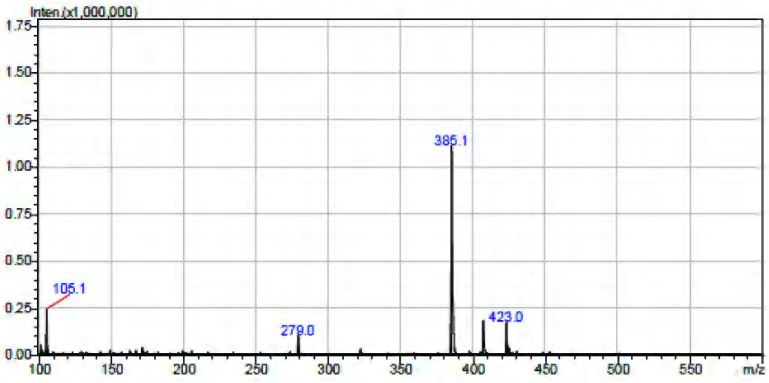
Figure 3. LC-MS analysis results: Total Ion Chromatogram of MYP (A) and representatives of chromatogram of major MYP monascin (B) and yellow II (C).



(A)



(B)



(C)

Table 1. MYP composition

MYP compound	Content (%)
Monascin	59.81
Ankaflavin	6.32
XanthomonasinA	n.d.
XanthomonasinB	0.67
Monascopyridin A	n.d.
Monascopyridin B	5.39
Yellow II	27.80

n.d. = not detected

5

Bukti konfirmasi artikel diterima dan acceptance letter

7 Maret 2020

Re: FR-2020-020 - Decision on your manuscript

Dari: Food Research (foodresearch.my@outlook.com)

Kepada: srianta_wm@yahoo.com

Tanggal: Sabtu, 7 Maret 2020 pukul 11.52 GMT+7

Dear Prof Srianta,

It is a pleasure to accept your manuscript for publication in Food Research journal. Please refer to the attachment for your acceptance letter. I will contact you again once the galley proof is ready for viewing and approval.

Thank you for your fine contribution. We look forward to your continued contributions to the Journal.

Sincerely,
Dr. Vivian New
Editor
Food Research

From: srianta_wm@yahoo.com <srianta_wm@yahoo.com>
Sent: Monday, 2 March, 2020 9:00 AM
To: Food Research <foodresearch.my@outlook.com>
Subject: Re: FR-2020-020 (Revised)

Dear Prof. Son Radu

Please kindly find the attached files of FR-2020-020_revised version and response to reviewers comments. Thank you for your kind attention

Best regards
Srianta

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Because we are trying to facilitate timely publication of manuscripts submitted to Food Research, your revised manuscript should be submitted before or by 8th March 2020. If it is not possible for you to submit your revision by this date, please let us know.

Once again, thank you for submitting your manuscript to Food Research and I look forward to receiving your revised manuscript.

Sincerely,
Professor Dr. Son Radu
foodresearch.my@outlook.com

Chief Editor, Food Research

FR-2020-020 Acceptance Letter.pdf
31.1kB

FOOD
RESEARCH

7th March 2020

Dear Prof Dr Srianta, I.,

ACCEPTANCE LETTER

Food Research, is pleased to inform you that the following manuscript has been accepted for publication in Food Research journal.

Manuscript Title	:	Separation and analysis of Monascus yellow pigment produced on durian seed substrate
Authors	:	Srianta, I., Nugerahani, I. and Ristiarini, S.

We thank you for your fine contribution to the Food Research journal and encourage you to submit other articles to the Journal.

Yours sincerely,

Professor Dr. Son Radu
Chief Editor
Food Research

6

Bukti konfirmasi permintaan klarifikasi beberapa poin di artikel sebelum masuk tahap produksi

23 Maret 2020

Re: FR-2020-020 - Article Production

Dari: Food Research (foodresearch.my@outlook.com)

Kepada: srianta_wm@yahoo.com

Tanggal: Senin, 23 Maret 2020 pukul 16.25 GMT+7

Dear Prof Srianta,

Manuscript ID: FR-2020-020
Manuscript Title: Separation and analysis of Monascus yellow pigment produced on durian seed substrate

Before we can proceed with the article production, I would like to clarify a few points that I have commented in the manuscript. Please refer to the attachment. Please address the issues raised in the comments.

Please use the attached copy to make your revisions as it has been corrected to the Journal's format. Once you have done, kindly revert the copy to me as soon as possible. Please note the faster you respond, the quicker we will process your manuscript.

Thanks & Regards,
Vivian New
Editor
Food Research

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Chief Editor, Food Research

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212.1kB

Separation and analysis of Monascus yellow pigment produced on durian seed substrate

*Srianta, I., Nugerahani, I. and Ristiarini, S.

Department of Food Technology, Faculty of Agricultural Technology, Widya Mandala Catholic University Surabaya, Jalan Dinoyo 42-44 Surabaya, Indonesia 60265

*Corresponding author: srianta_wm@yahoo.com

Article history:

Received: 15 January 2020

Received in revised form: 2 March 2020

Accepted: 5 March 2020

Abstract

This research was aimed to separate and analyse *Monascus* yellow pigment (MYP) with chromatography. MYP was in a mixture with red and orange pigments. MYP separation was performed in a silica gel column with eluent of ethyl acetate: ethanol: water = 90: 25: 4. The separated MYP were then subjected to analysis by using spectrophotometer, TLC, LC-PDA and LC-MS/MS. The results revealed that the separated MYP has a maximum absorption at 386 nm. Out of five MYP compounds detected by using LC-MS/MS, two of them were well known yellow pigment compounds, monascin and ankaflavin. Monascin and yellow II were the major pigment compounds in MYP.

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1. Introduction

Monascus pigments are secondary metabolites of Monascus fungi biosynthesized through the polyketide pathway. It has been used as a natural food colourant, food supplement, and in traditional

medicine. *Monascus* pigments consist of many pigment compounds which are categorized into 3 groups i.e. red, orange and yellow pigments. A total of six well-known pigment compounds are monascorubramine and rubropunctamine (red pigments), monascorubrin and rubropunctatin (orange pigments), monascin and ankaflavin (yellow pigments) (Feng *et al*., 2012; Srianta *et al*., 2014; Agboyibor *et al*., 2018). Until 2011, 39 new pigment compounds have been identified (Feng *et al*., 2012).

Rice is commonly used as solid-state fermentation substrate in *Monascus* pigments production. Non-rice substrates have been developed in the *Monascus* pigment e.g. corn, adlay, sorghum, dioscorea, jackfruit seed and durian seed (Pattanagul *et al*., 2007; Babitha *et al*., 2007; Hsu *et al*., 2011; Srianta *et al*., 2012; Srianta *et al*., 2016). Our previous studies revealed that *Monascus purpureus* produced red, orange and yellow pigments. Exploration of *Monascus* yellow pigments is still limited. MYP is not only a potential natural colorant for biscuit, mayonnaise, wheat noodle, etc. (Srianta *et al*., 2014) but also as functional food ingredient and in medicine, because MYP has various positive health effects i.e. antioxidant, antidiabetes and anticancer (Hsu *et al*., 2011; Chen and Wu, 2016). Monascin and ankaflavin have antitumor activity (Akihisa *et al*., 2005; Su *et al*., 2005), antiobesity activity, anti-atherosclerosis effect (Wang *et al*., 2013; Liu *et al*., 2018), improve memory and learning ability (Lee *et al*., 2015). Chen and Wu (2016) show that MYPs have promising potential for application in food and pharmaceutical industries based on their bioactivities.

Silica gel has been applied in *Monascus* pigments mixture separation through thin layer chromatography and column chromatography systems with various eluents i.e. chloroform, methanol, benzene, acetone and petroleum ether (Kim *et al*., 2006; Vidyalakshmi *et al*., 2009; Hsu *et al*., 2011; Lee *et al*., 2011). In practice, ethyl acetate, ethanol and water are often more preferred for food and pharmaceutical processing compared to other solvents due to their safety and affordability. HPLC is utilized to analyze various *Monascus* pigments because of its high sensitivity and multiple detection systems including ultraviolet, fluorescent, photodiode array and mass spectrometer detectors (Feng *et al*., 2012).

This research was aimed to separate and analyse MYP produced on durian seed substrate. This is the first investigation in MYP separation using ethyl acetate, ethanol and water.

2. Materials and methods

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The monascus pigment was extracted with soxhletation method in ethanolic solvent at a ratio of 1:50 for 2 hrs. The extract was then evaporated in a rotary evaporator. The evaporated extract was subjected to separation process. MYP separation was performed in a column chromatography (10 mm x 500 mm) with silica gel 60. The column was packed with silica gel by slurry method, then pre eluted with ethyl acetate: ethanol: water = 90:25:4. The extract was put into the column and eluted with ethyl acetate: ethanol: water = 90:25:4. The obtained fractions were subjected to analysis by using TLC and spectrophotometer. The MYP fraction was analyzed the pigment composition by using LC-MS/MS.

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The separated pigments were subjected to the TLC analysis according to Nimnoi and Lumyong (2011) with modification, on silica gel F60 plate, with ethyl acetate: ethanol: water at 90:25:4. The separated MYP was scanned in a range of wavelength 380-600 nm with UV-Vis spectrophotometer (Shimadzu UV-1900, Japan). Pigment composition analysis was conducted by using LC-MS/MS (Shimadzu, Kyoto, Japan) with Q1 scan positive mode and SIM positive mode. The extract was filtered through a 0.2 um PTFE filter membrane, put on to the autosampler. Pigments were separated on a Cosmosil 5 C18-MS-II (4.6 i.d. x 150 mm) column, with an isocratic program of mobile phase consists of acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid (60:40 vol/vol), flow rate of 0.5 mL/min; oven temperature of 40°C, run time for 30 min. Pigment compounds were detected by MS/MS using electro-spray ionization in the positive ion mode, scan range: m/z 100 to 1000.

3. Results and discussion

In the separation of the pigments by using column chromatography, 4 fractions (F1 F2, F3 and F4) derived from different color. The first fraction was MYP target, while other fractions were orange and red pigments. The MYP has the highest retention factor, followed by F2, F3 and F4. Those fractions and pigments profile on TLC shown in Figure 1. Those profiles describe that MYP was the pigments with the lowest polarity among the others. Although the chemical structure of *Monascus* pigments consists of all of them of β-ketoacid and chromophore, MYP compounds contain less polar groups than those of red pigments. The presence of $–NH_3$ group can increase the polarity of red pigments.

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Pigments in MFDS produced through a complex polyketide biosynthesis pathway in the Monascus fungi, but it is still unclear and controversy. Some researchers proposed that only orange pigments (rubropunctatin and monascorubramine) were biosynthetic, and the others were transformed from them chemically. In orange pigment rubropunctatin biosynthesis, it consist of the β-ketide pathway to form acetate and malonate and another pathway to form n-hexanoyl acetyl residue. Yellow pigments can be transformed from orange pigments via oxidation. Our previous research revealed that solvent type affected the *Monascus* pigment composition. The ethanolic extract contains higher MYP than those in the mixture solvent of ethanol and water. This research used ethanol as the extraction solvent. In the separation process, the chromatographic system used has successfully separated MYP from the red and orange pigments.

Monascin, the major MYP compound in this research, is attractive for its strong bioactivities such as anticancer, antitumor, antidiabetic, antioxidative stress, antiinflammatory, antiobesity and showed antiatherogenic activities, and also improves memory and learning ability. On the other hand, there is no report on the yellow II bioactivity.

4. Conclusion

The MYP has been successfully separated in a column chromatography packed with silica gel 60 and eluent of ethyl acetate:ethanol:water=90:25:4. Five MYP compounds have been detected by using LC-MS/MS i.e. monascin, ankaflavin, xanthomonasin B, monascopyridine B and yellow II. Monascin and yellow II were the major pigment compounds in MYP.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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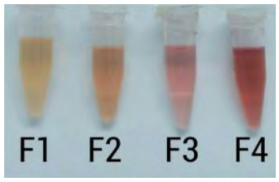
Figure 1. Picture of 4 separated fractions of *Monascus* pigments (A) and their TLC chromatogram (B). F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.



(A)

(B)

Figure 2. Scanning results of separated *Monascus* pigment fractions by using spectrophotometer. F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.

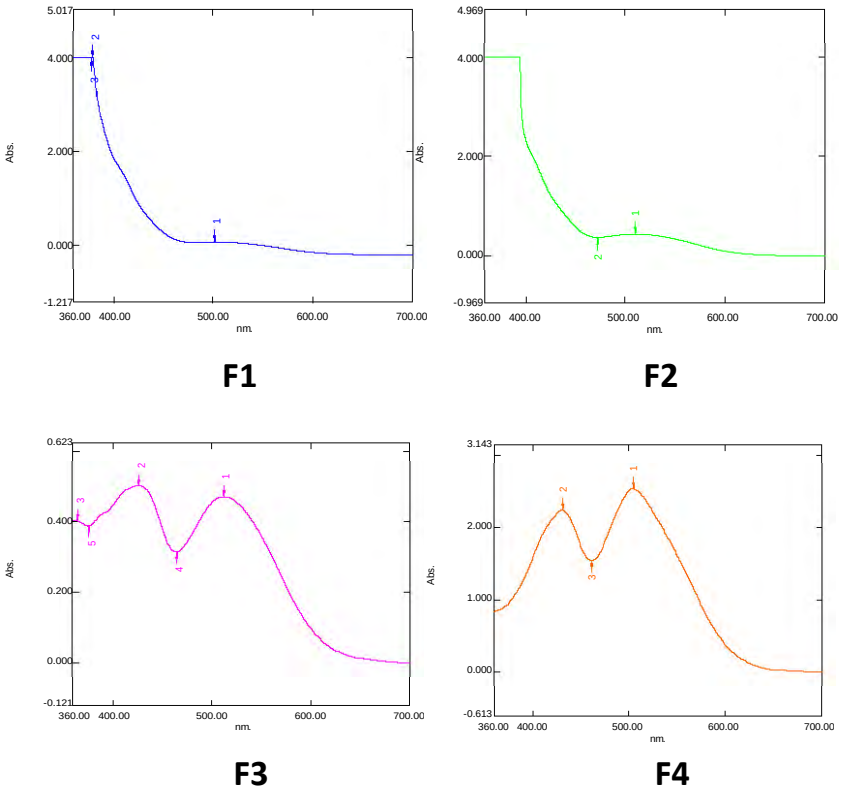
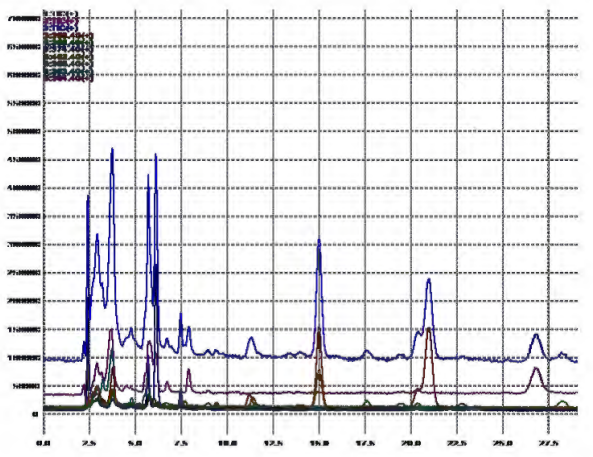
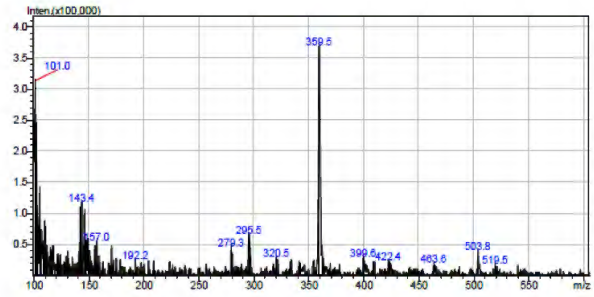


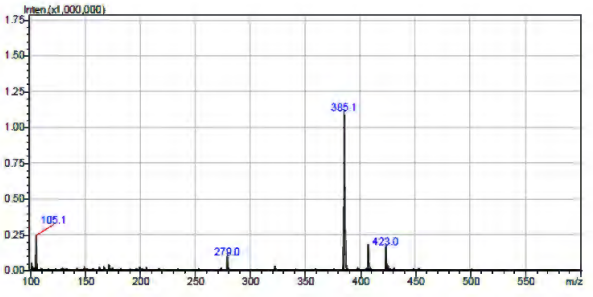
Figure 3. LC-MS analysis results: Total Ion Chromatogram of MYP (A) and representatives of the chromatogram of major MYP monascin (B) and yellow II (C).



(A)



(B)



(C)

Table 1. MYP composition

MYP compound	Content (%)
Monascin	59.81
Ankaflavin	6.32
XanthomonasinA	n.d.
XanthomonasinB	0.67
Monascopyridin A	n.d.
Monascopyridin B	5.39
Yellow II	27.80

n.d. = not detected

7

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24 Maret 2020

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Kepada: foodresearch.my@outlook.com

Tanggal: Selasa, 24 Maret 2020 pukul 01.18 GMT+7

Dear Dr. Vivian
Please kindly the attached file of corrected FR-2020-020. Thank you
Best regards
Srianta

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> Manuscript Title: Separation and analysis of Monascus yellow pigment produced on durian seed substrate
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Separation and analysis of Monascus yellow pigment produced on durian seed substrate

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Abstract

This research was aimed to separate and analyse *Monascus* yellow pigment (MYP) with chromatography. MYP was in a mixture with red and orange pigments. MYP separation was performed in a silica gel column with eluent of ethyl acetate: ethanol: water = 90: 25: 4. The separated MYP were then subjected to analysis by using spectrophotometer, TLC, LC-PDA and LC-MS/MS. The results revealed that the separated MYP has a maximum absorption at 386 nm. Out of five MYP compounds detected by using LC-MS/MS, two of them were well known yellow pigment compounds, monascin and ankaflavin. Monascin and yellow II were the major pigment compounds in MYP.

Keywords: *Monascus*, Yellow pigment, Durian seed, Separation, Analysis

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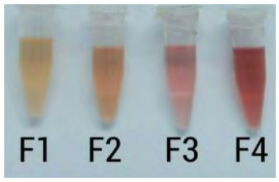
Figure 1. Picture of 4 separated fractions of *Monascus* pigments (A) and their TLC chromatogram (B). F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.



(A)

(B)

Figure 2. Scanning results of separated *Monascus* pigment fractions by using spectrophotometer. F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.

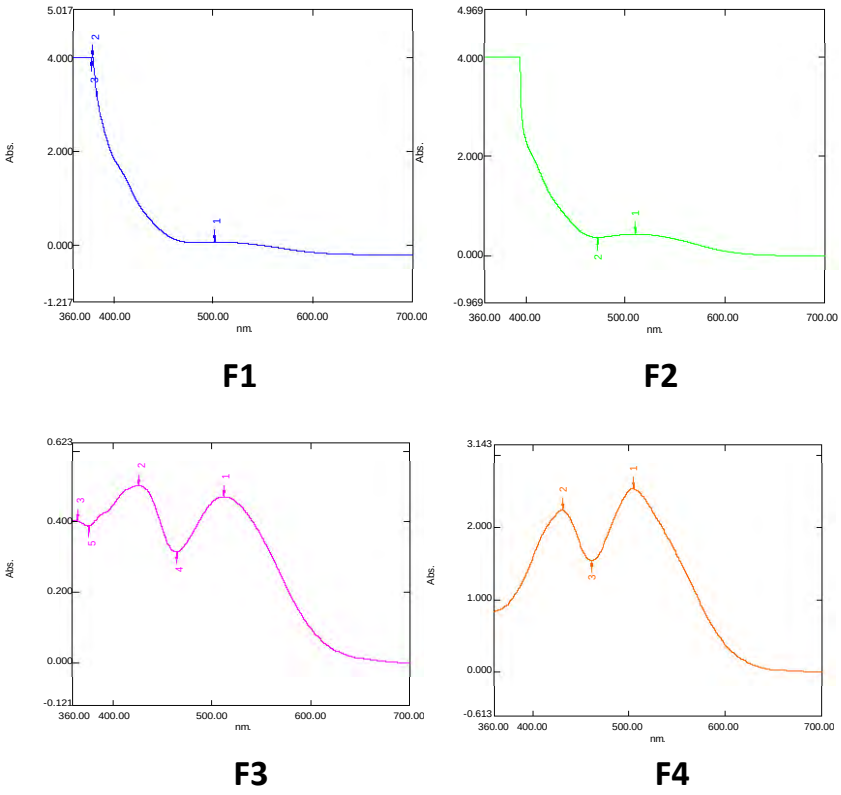
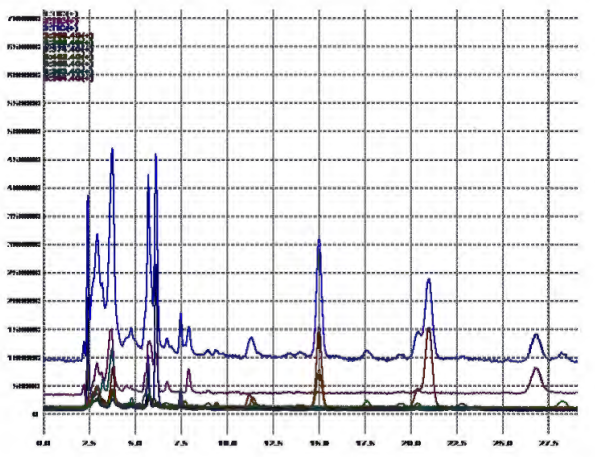
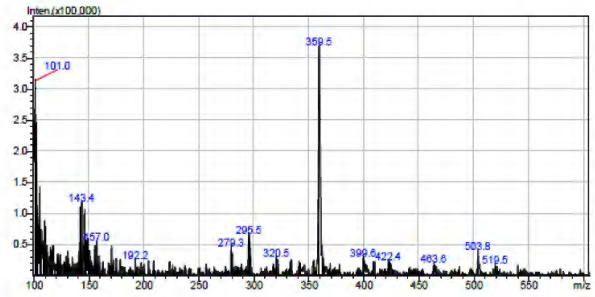


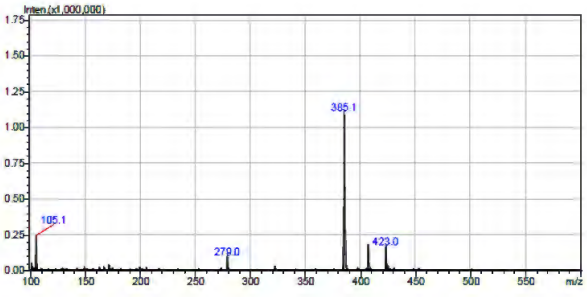
Figure 3. LC-MS analysis results: Total Ion Chromatogram of MYP (A) and representatives of the chromatogram of major MYP monascin (B) and yellow II (C).



(A)



(B)



(C)

Table 1. MYP composition

MYP compound	Content (%)
Monascin	59.81
Ankaflavin	6.32
XanthomonasinA	n.d.
XanthomonasinB	0.67
Monascopyridin A	n.d.
Monascopyridin B	5.39
Yellow II	27.80

n.d. = not detected

8

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24 Maret 2020

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Food Research

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> Because we are trying to facilitate timely publication of manuscripts submitted to Food Research, your revised manuscript should be submitted before or by 8th March 2020. If it is not possible for you to submit your revision by this date, please let us know.
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9

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Once again, thank you for submitting your manuscript to Food Research and I look forward to receiving your revised manuscript.

Sincerely,
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FULL PAPER

Separation and analysis of *Monascus* yellow pigment produced on durian seed substrate

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Abstract

This research was aimed to separate and analyse *Monascus* yellow pigment (MYP) with chromatography. MYP was in a mixture with red and orange pigments. MYP separation was performed in a silica gel column with eluent of ethyl acetate: ethanol: water = 90: 25: 4. The separated MYP were then subjected to analysis by using spectrophotometer, TLC, LC-PDA and LC-MS/MS. The results revealed that the separated MYP has a maximum absorption at 386 nm. Out of five MYP compounds detected by using LC-MS/MS, two of them were well known yellow pigment compounds, monascin and ankaflavin. Monascin and yellow II were the major pigment compounds in MYP.

1. Introduction

Monascus pigments are secondary metabolites of *Monascus* fungi biosynthesized through the polyketide pathway. It has been used as a natural food colourant, food supplement, and in traditional medicine. *Monascus* pigments consist of many pigment compounds which are categorized into three groups i.e. red, orange and yellow pigments. A total of six well-known pigment compounds are monascorubramine and rubropunctamine (red pigments), monascorubrin and rubropunctatin (orange pigments), monascin and ankaflavin (yellow pigments) (Feng *et al*., 2012; Srianta *et al*., 2014; Agboyibor *et al*., 2018). Until 2011, thirty-nine new pigment compounds have been identified (Feng *et al*., 2012).

Rice is commonly used as solid-state fermentation substrate in *Monascus* pigments production. Non-rice substrates have been developed in the *Monascus* pigment e.g. corn, adlay, sorghum, dioscorea, jackfruit seed and durian seed (Pattanagul *et al*., 2007; Babitha *et al*., 2007; Hsu *et al*., 2011; Srianta *et al*., 2012; Srianta *et al*., 2016). Our previous studies revealed that *Monascus purpureus* produced red, orange and yellow pigments. Exploration of *Monascus* yellow pigments is still limited. MYP is not only a potential natural colorant for biscuit, mayonnaise, wheat noodle, etc. (Srianta *et al*., 2014) but also as functional food ingredient and in medicine, because MYP has various positive health effects i.e. antioxidant, antidiabetes and anticancer (Hsu *et al*., 2011; Chen and Wu, 2016). Monascin and ankaflavin have antitumor activity (Akihisa *et al*., 2005; Su *et al*., 2005), antiobesity activity, anti-atherosclerosis effect (Wang *et al*., 2013; Liu *et al*., 2018), improve memory and learning ability (Lee *et al*., 2015). Chen and Wu (2016) show that MYPs have promising potential for application in food and pharmaceutical industries based on their bioactivities.

Silica gel has been applied in *Monascus* pigments mixture separation through thin layer chromatography and column chromatography systems with various eluents i.e. chloroform, methanol, benzene, acetone and petroleum ether (Kim *et al*., 2006; Vidyalakshmi *et al*., 2009; Hsu *et al*., 2011; Lee *et al*., 2011). In practice, ethyl acetate, ethanol and water are often more preferred for food and pharmaceutical processing compared to other solvents due to their safety and affordability. HPLC is utilized to analyze various *Monascus* pigments because of its high sensitivity and multiple detection systems including ultraviolet, fluorescent, photodiode array and mass spectrometer detectors (Feng *et al*., 2012).

This research was aimed to separate and analyse MYP produced on durian seed substrate. This is the first

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investigation in MYP separation using ethyl acetate, ethanol and water.

2. Materials and methods

2.1 Microorganism, substrate and chemicals

Monascus purpureus M9 culture was used in the solid-state fermentation on durian seed substrate. The culture was maintained periodically on Potato Dextrose Agar. Durian seed was obtained from a durian processing unit in Surabaya (Indonesia). The durian seed is a by-product of the food industry and was used as the SSF substrate. All chemicals were chromatographic and analytical grades.

2.2 Pigments production through solid-state fermentation with durian seed substrate

Pigments production was performed through solid-state fermentation on durian seed substrate according to our previous research (Srianta *et al*., 2012). After peeling, cutting, soaking, sterilization at 121°C for 10 mins and cooling, the durian seed substrate was inoculated with *Monascus purpureus* starter culture, and then incubated at 30°C for 14 days. The fermented matter was dried at 45°C for 24 hrs, ground and sieved.

2.3 Monascus pigment separation

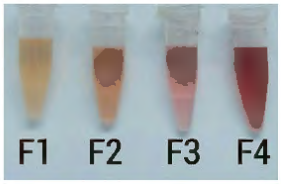
The *Monascus* pigment was extracted with soxhletation method in ethanolic solvent at a ratio of 1:50 for 2 hrs. The extract was then evaporated in a rotary evaporator. The evaporated extract was subjected to separation process. MYP separation was performed in a column chromatography (10 mm x 500 mm) with silica gel 60. The column was packed with silica gel by slurry method, then pre eluted with ethyl acetate: ethanol: water = 90:25:4. The extract was put into the column and eluted with ethyl acetate: ethanol: water = 90:25:4. The obtained fractions were subjected to analysis by using TLC and spectrophotometer. The MYP fraction was analyzed the pigment composition by using LC-MS/MS.

2.4 Pigment analysis

The separated pigments were subjected to the TLC analysis according to Nimnoi and Lumyong (2011) with modification, on silica gel F60 plate, with ethyl acetate: ethanol: water at 90:25:4. The separated MYP was scanned in a range of wavelength 380-600 nm with UV-Vis spectrophotometer (Shimadzu UV-1900, Japan). Pigment composition analysis was conducted by using LC-MS/MS (Shimadzu, Kyoto, Japan) with Q1 scan positive mode and SIM positive mode. The extract was filtered through a 0.2 um PTFE filter membrane, put on to the autosampler. Pigments were separated on a Cosmosil 5 C18-MS-II (4.6 i.d. x 150 mm) column, with an isocratic program of mobile phase consists of acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid (60:40 vol/vol), flow rate of 0.5 mL/min; oven temperature of 40°C, run time for 30 min. Pigment compounds were detected by MS/MS using electro-spray ionization in the positive ion mode, scan range: m/z 100 to 1000.

3. Results and discussion

In the separation of the pigments by using column chromatography, 4 fractions (F1 F2, F3 and F4) with different colors have been derived. The first fraction was MYP target, while other fractions were orange and red pigments. The MYP has the highest retention factor, followed by F2, F3 and F4. Those fractions and pigments profile on TLC shown in Figure 1. Those profiles describe that MYP was the pigments with the lowest polarity among the others. Although the chemical structure of *Monascus* pigments consists of all of them of β-ketoacid and chromophore, MYP compounds contain less polar groups than those of red pigments. The presence of $–NH_3$ group can increase the polarity of red pigments.



(A)

(B)

Figure 1. Picture of 4 separated fractions of *Monascus* pigments (A) and their TLC chromatogram (B). F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.

Figure 2 shows the fractions scanning by using spectrophotometer at 380-700 nm. F1, the MYP, has a λ max at 386 nm that was in yellow spectrum. F3 and F4 have 2 peaks in the orange and red spectrum. Those reflect that contain a mixture of orange and red pigments. This is the first investigation on the pigment compounds composition of MYP by using the LC-MS/MS. The LC-MS chromatograms and pigment composition of the extracts are presented in Figure 3 and Table 1, respectively. Five MYP compounds were detected by using LC-MS/MS i.e. monascin, ankaflavin,

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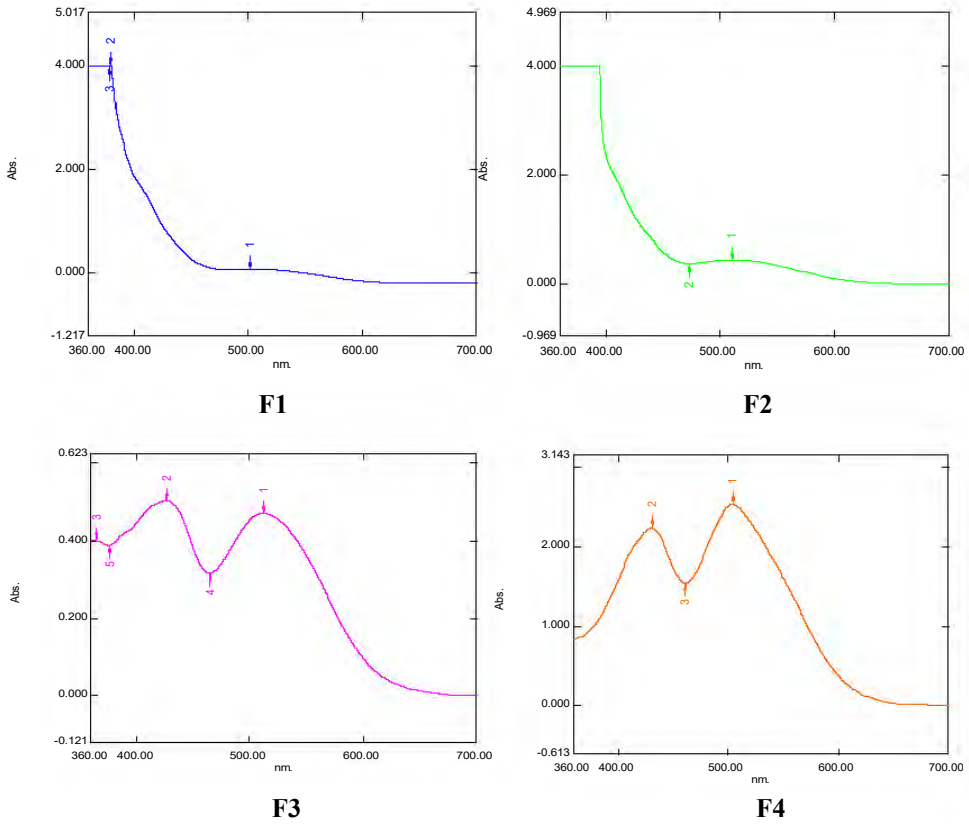

Figure 2. Scanning results of separated *Monascus* pigment fractions by using spectrophotometer. F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.

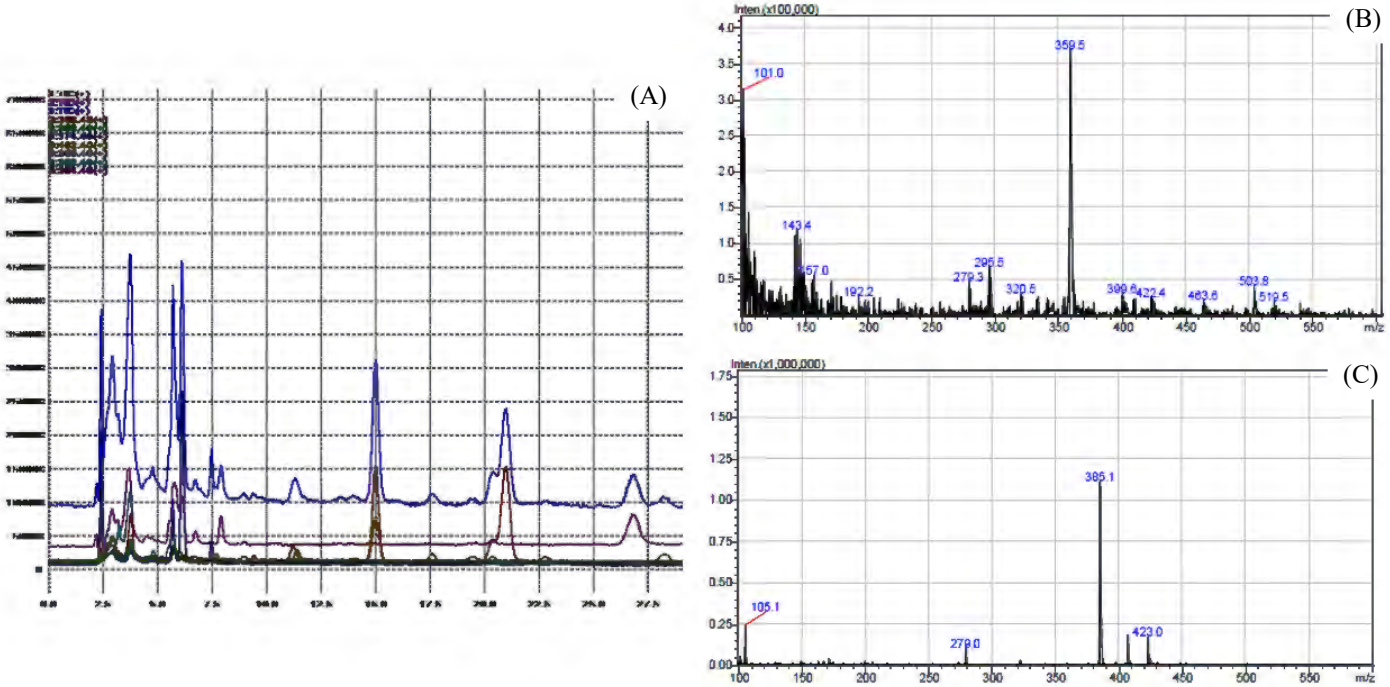

Figure 3. LC-MS analysis results: Total Ion Chromatogram of MYP (A) and representatives of the chromatogram of major MYP monascin (B) and yellow II (C).

Table 1. MYP composition

MYP compound	Content (%)
Monascin	59.81
Ankaflavin	6.32
XanthomonasinA	n.d.
XanthomonasinB	0.67
Monascopyridin A	n.d.
Monascopyridin B	5.39
Yellow II	27.80

n.d. = not detected

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xanthomonasin B, monascopyridine B and Yellow II. Monascin and Yellow II were the major pigment compounds in the MYP with the relative amount of 59.81% and 27.80%. The MYP composition depends on the pigment composition in *Monascus* Fermented Durian Seed (MFDS), extraction and separation processes.

Pigments in MFDS produced through a complex polyketide biosynthesis pathway in the *Monascus* fungi, but it is still unclear and controversy. Some researchers proposed that only orange pigments (rubropunctatin and monascorubramine) were biosynthetic, and the others were transformed from them chemically. In orange pigment rubropunctatin biosynthesis, it consist of the β-ketide pathway to form acetate and malonate and another pathway to form n-hexanoyl acetyl residue. Yellow pigments can be transformed from orange pigments via oxidation. Our previous research revealed that solvent type affected the *Monascus* pigment composition. The ethanolic extract contains higher MYP than those in the mixture solvent of ethanol and water. This research used ethanol as the extraction solvent. In the separation process, the chromatographic system used has successfully separated MYP from the red and orange pigments.

Monascin, the major MYP compound in this research, is attractive for its strong bioactivities such as anticancer, antitumor, antidiabetic, antioxidative stress, antiinflammatory, antiobesity and showed antiatherogenic activities, and also improves memory and learning ability. On the other hand, there is no report on the yellow II bioactivity.

4. Conclusion

The MYP has been successfully separated in a column chromatography packed with silica gel 60 and eluent of ethyl acetate:ethanol:water=90:25:4. Five MYP compounds have been detected by using LC-MS/MS i.e. monascin, ankaflavin, xanthomonasin B, monascopyridine B and yellow II. Monascin and yellow II were the major pigment compounds in MYP.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by Direktorat Riset dan Pengabdian Masyarakat, Direktorat Jenderal Penguatan Riset dan Pengembangan, Kementerian Riset, Teknologi, dan Pendidikan Tinggi, Republik Indonesia through the scheme of Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT) 2019 [grant number 200X/WM01.5/N/2019].

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Separation and analysis of *Monascus* yellow pigment produced on durian seed substrate

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Abstract

This research was aimed to separate and analyse *Monascus* yellow pigment (MYP) with chromatography. MYP was in a mixture with red and orange pigments. MYP separation was performed in a silica gel column with eluent of ethyl acetate: ethanol: water = 90: 25: 4. The separated MYP were then subjected to analysis by using spectrophotometer, TLC, LC-PDA and LC-MS/MS. The results revealed that the separated MYP has a maximum absorption at 386 nm. Out of five MYP compounds detected by using LC-MS/MS, two of them were well known yellow pigment compounds, monascin and ankaflavin. Monascin and yellow II were the major pigment compounds in MYP.

1. Introduction

Monascus pigments are secondary metabolites of *Monascus* fungi biosynthesized through the polyketide pathway. It has been used as a natural food colourant, food supplement, and in traditional medicine. *Monascus* pigments consist of many pigment compounds which are categorized into three groups i.e. red, orange and yellow pigments. A total of six well-known pigment compounds are monascorubramine and rubropunctamine (red pigments), monascorubrin and rubropunctatin (orange pigments), monascin and ankaflavin (yellow pigments) (Feng *et al*., 2012; Srianta *et al*., 2014; Agboyibor *et al*., 2018). Until 2011, thirty-nine new pigment compounds have been identified (Feng *et al*., 2012).

Rice is commonly used as solid-state fermentation substrate in *Monascus* pigments production. Non-rice substrates have been developed in the *Monascus* pigment e.g. corn, adlay, sorghum, dioscorea, jackfruit seed and durian seed (Pattanagul *et al*., 2007; Babitha *et al*., 2007; Hsu *et al*., 2011; Srianta *et al*., 2012; Srianta *et al*., 2016). Our previous studies revealed that *Monascus purpureus* produced red, orange and yellow pigments. Exploration of *Monascus* yellow pigments is still limited. MYP is not only a potential natural colorant for biscuit, mayonnaise, wheat noodle, etc. (Srianta *et al*., 2014) but also as functional food ingredient and in medicine, because MYP has various positive health effects i.e. antioxidant, antidiabetes and anticancer (Hsu *et al*., 2011; Chen and Wu, 2016). Monascin and ankaflavin have antitumor activity (Akihisa *et al*., 2005; Su *et al*., 2005), antiobesity activity, anti-atherosclerosis effect (Wang *et al*., 2013; Liu *et al*., 2018), improve memory and learning ability (Lee *et al*., 2015). Chen and Wu (2016) show that MYPs have promising potential for application in food and pharmaceutical industries based on their bioactivities.

Silica gel has been applied in *Monascus* pigments mixture separation through thin layer chromatography and column chromatography systems with various eluents i.e. chloroform, methanol, benzene, acetone and petroleum ether (Kim *et al*., 2006; Vidyalakshmi *et al*., 2009; Hsu *et al*., 2011; Lee *et al*., 2011). In practice, ethyl acetate, ethanol and water are often more preferred for food and pharmaceutical processing compared to other solvents due to their safety and affordability. HPLC is utilized to analyze various *Monascus* pigments because of its high sensitivity and multiple detection systems including ultraviolet, fluorescent, photodiode array and mass spectrometer detectors (Feng *et al*., 2012).

This research was aimed to separate and analyse MYP produced on durian seed substrate. This is the first

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investigation in MYP separation using ethyl acetate, ethanol and water.

2. Materials and methods

2.1 Microorganism, substrate and chemicals

Monascus purpureus M9 culture was used in the solid-state fermentation on durian seed substrate. The culture was maintained periodically on Potato Dextrose Agar. Durian seed was obtained from a durian processing unit in Surabaya (Indonesia). The durian seed is a by-product of the food industry and was used as the SSF substrate. All chemicals were chromatographic and analytical grades.

2.2 Pigments production through solid-state fermentation with durian seed substrate

Pigments production was performed through solid-state fermentation on durian seed substrate according to our previous research (Srianta *et al*., 2012). After peeling, cutting, soaking, sterilization at 121°C for 10 mins and cooling, the durian seed substrate was inoculated with *Monascus purpureus* starter culture, and then incubated at 30°C for 14 days. The fermented matter was dried at 45°C for 24 hrs, ground and sieved.

2.3 Monascus pigment separation

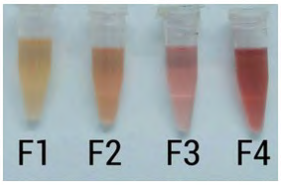
The *Monascus* pigment was extracted with soxhletation method in ethanolic solvent at a ratio of 1:50 for 2 hrs. The extract was then evaporated in a rotary evaporator. The evaporated extract was subjected to separation process. MYP separation was performed in a column chromatography (10 mm x 500 mm) with silica gel 60. The column was packed with silica gel by slurry method, then pre eluted with ethyl acetate: ethanol: water = 90:25:4. The extract was put into the column and eluted with ethyl acetate: ethanol: water = 90:25:4. The obtained fractions were subjected to analysis by using TLC and spectrophotometer. The MYP fraction was analyzed the pigment composition by using LC-MS/MS.

2.4 Pigment analysis

The separated pigments were subjected to the TLC analysis according to Nimnoi and Lumyong (2011) with modification, on silica gel F60 plate, with ethyl acetate: ethanol: water at 90:25:4. The separated MYP was scanned in a range of wavelength 380-600 nm with UV-Vis spectrophotometer (Shimadzu UV-1900, Japan). Pigment composition analysis was conducted by using LC-MS/MS (Shimadzu, Kyoto, Japan) with Q1 scan positive mode and SIM positive mode. The extract was filtered through a 0.2 um PTFE filter membrane, put on to the autosampler. Pigments were separated on a Cosmosil 5 C18-MS-II (4.6 i.d. x 150 mm) column, with an isocratic program of mobile phase consists of acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid (60:40 vol/vol), flow rate of 0.5 mL/min; oven temperature of 40°C, run time for 30 min. Pigment compounds were detected by MS/MS using electro-spray ionization in the positive ion mode, scan range: m/z 100 to 1000.

3. Results and discussion

In the separation of the pigments by using column chromatography, 4 fractions (F1 F2, F3 and F4) with different colors have been derived. The first fraction was MYP target, while other fractions were orange and red pigments. The MYP has the highest retention factor, followed by F2, F3 and F4. Those fractions and pigments profile on TLC shown in Figure 1. Those profiles describe that MYP was the pigments with the lowest polarity among the others. Although the chemical structure of *Monascus* pigments consists of all of them of β-ketoacid and chromophore, MYP compounds contain less polar groups than those of red pigments. The presence of $-NH_3$ group can increase the polarity of red pigments.



(A)

(B)

Figure 1. Picture of 4 separated fractions of *Monascus* pigments (A) and their TLC chromatogram (B). F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.

Figure 2 shows the fractions scanning by using spectrophotometer at 380-700 nm. F1, the MYP, has a λ max at 386 nm that was in yellow spectrum. F3 and F4 have 2 peaks in the orange and red spectrum. Those reflect that contain a mixture of orange and red pigments. This is the first investigation on the pigment compounds composition of MYP by using the LC-MS/MS. The LC-MS chromatograms and pigment composition of the extracts are presented in Figure 3 and Table 1, respectively. Five MYP compounds were detected by using LC-MS/MS i.e. monascin, ankaflavin,

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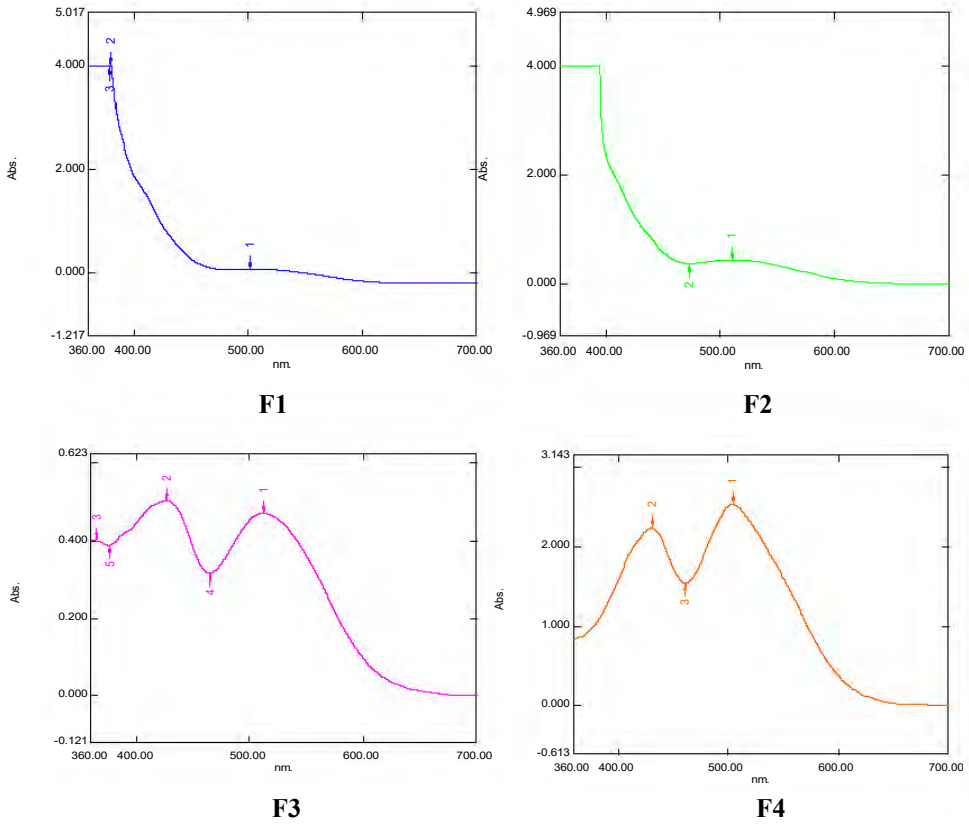

Figure 2. Scanning results of separated *Monascus* pigment fractions by using spectrophotometer. F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.

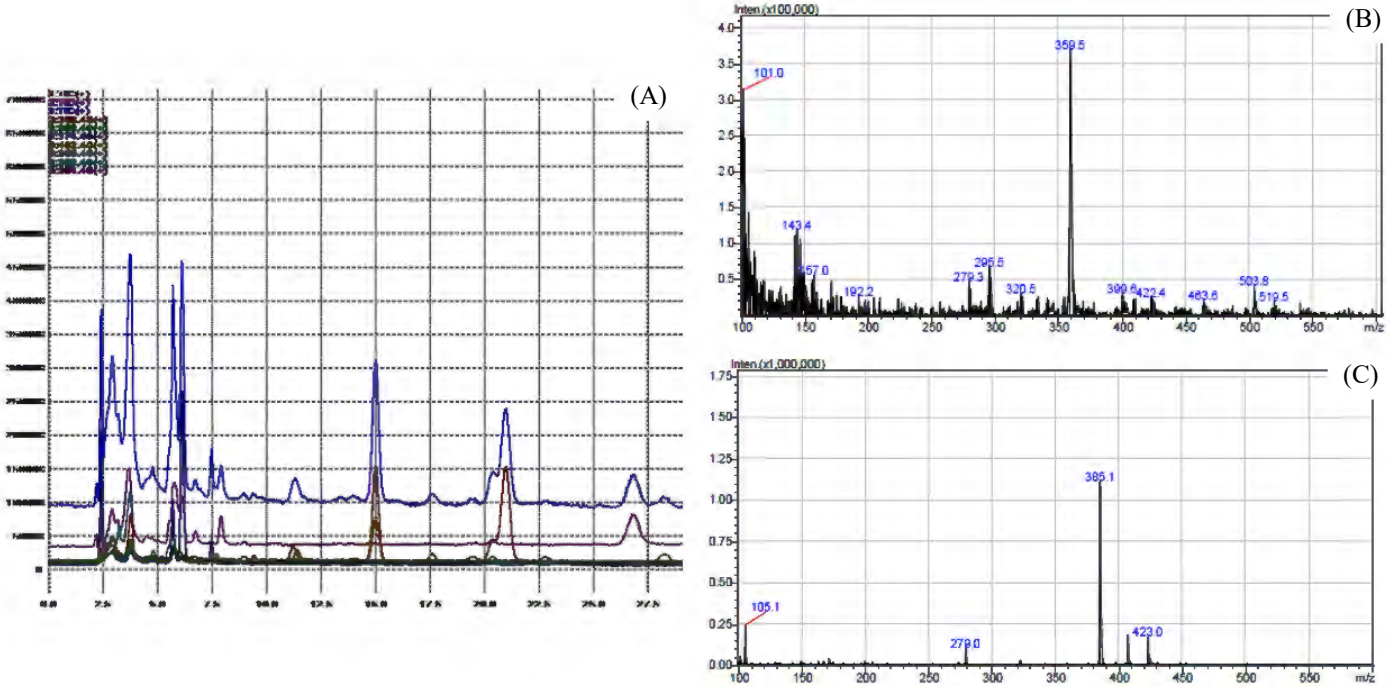

Figure 3. LC-MS analysis results: Total Ion Chromatogram of MYP (A) and representatives of the chromatogram of major MYP monascin (B) and yellow II (C).

Table 1. MYP composition

MYP compound	Content (%)
Monascin	59.81
Ankaflavin	6.32
XanthomonasinA	n.d.
XanthomonasinB	0.67
Monascopyridin A	n.d.
Monascopyridin B	5.39
Yellow II	27.80

n.d. = not detected

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xanthomonasin B, monascopyridine B and Yellow II. Monascin and Yellow II were the major pigment compounds in the MYP with the relative amount of 59.81% and 27.80%. The MYP composition depends on the pigment composition in *Monascus* Fermented Durian Seed (MFDS), extraction and separation processes.

Pigments in MFDS produced through a complex polyketide biosynthesis pathway in the *Monascus* fungi, but it is still unclear and controversy. Some researchers proposed that only orange pigments (rubropunctatin and monascorubramine) were biosynthetic, and the others were transformed from them chemically. In orange pigment rubropunctatin biosynthesis, it consist of the β-ketide pathway to form acetate and malonate and another pathway to form n-hexanoyl acetyl residue. Yellow pigments can be transformed from orange pigments via oxidation. Our previous research revealed that solvent type affected the *Monascus* pigment composition. The ethanolic extract contains higher MYP than those in the mixture solvent of ethanol and water. This research used ethanol as the extraction solvent. In the separation process, the chromatographic system used has successfully separated MYP from the red and orange pigments.

Monascin, the major MYP compound in this research, is attractive for its strong bioactivities such as anticancer, antitumor, antidiabetic, antioxidative stress, antiinflammatory, antiobesity and showed antiatherogenic activities, and also improves memory and learning ability. On the other hand, there is no report on the yellow II bioactivity.

4. Conclusion

The MYP has been successfully separated in a column chromatography packed with silica gel 60 and eluent of ethyl acetate:ethanol:water=90:25:4. Five MYP compounds have been detected by using LC-MS/MS i.e. monascin, ankaflavin, xanthomonasin B, monascopyridine B and yellow II. Monascin and yellow II were the major pigment compounds in MYP.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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FOOD RESEARCH

FULL PAPER

Separation and analysis of *Monascus* yellow pigment produced on durian seed substrate

*Srianta, I., Nugerahani, I. and Ristiarini, S.

Department of Food Technology, Faculty of Agricultural Technology, Widya Mandala Catholic University Surabaya, Jalan Dinoyo 42-44 Surabaya, Indonesia 60265

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Abstract

This research was aimed to separate and analyse *Monascus* yellow pigment (MYP) with chromatography. MYP was in a mixture with red and orange pigments. MYP separation was performed in a silica gel column with eluent of ethyl acetate: ethanol: water = 90: 25: 4. The separated MYP were then subjected to analysis by using spectrophotometer, TLC, LC-PDA and LC-MS/MS. The results revealed that the separated MYP has a maximum absorption at 386 nm. Out of five MYP compounds detected by using LC-MS/MS, two of them were well known yellow pigment compounds, monascin and ankaflavin. Monascin and yellow II were the major pigment compounds in MYP.

1. Introduction

Monascus pigments are secondary metabolites of *Monascus* fungi biosynthesized through the polyketide pathway. It has been used as a natural food colourant, food supplement, and in traditional medicine. *Monascus* pigments consist of many pigment compounds which are categorized into three groups i.e. red, orange and yellow pigments. A total of six well-known pigment compounds are monascorubramine and rubropunctamine (red pigments), monascorubrin and rubropunctatin (orange pigments), monascin and ankaflavin (yellow pigments) (Feng *et al*., 2012; Srianta *et al*., 2014; Agboyibor *et al*., 2018). Until 2011, thirty-nine new pigment compounds have been identified (Feng *et al*., 2012).

Rice is commonly used as solid-state fermentation substrate in *Monascus* pigments production. Non-rice substrates have been developed in the *Monascus* pigment production e.g. corn, adlay, sorghum, dioscorea, jackfruit seed and durian seed (Pattanagul *et al*., 2007; Babitha *et al*., 2007; Hsu *et al*., 2011; Srianta *et al*., 2012; Srianta *et al*., 2016). Our previous studies revealed that *Monascus purpureus* produced red, orange and yellow pigments. Exploration of *Monascus* yellow pigments is still limited. MYP is not only a potential natural colorant for biscuit, mayonnaise, wheat noodle, etc. (Srianta *et al*., 2014) but also as functional food ingredient and in medicine, because MYP has various positive health effects i.e. antioxidant, antidiabetes and anticancer (Hsu *et al*., 2011; Chen and Wu, 2016). Monascin and ankaflavin have antitumor activity (Akihisa *et al*., 2005; Su *et al*., 2005), antiobesity activity, anti-atherosclerosis effect (Wang *et al*., 2013; Liu *et al*., 2018), improve memory and learning ability (Lee *et al*., 2015). Chen and Wu (2016) reported that MYPs have promising potential for application in food and pharmaceutical industries based on their bioactivities.

Silica gel has been applied in *Monascus* pigments mixture separation through thin layer chromatography and column chromatography systems with various eluents i.e. chloroform, methanol, benzene, acetone and petroleum ether (Kim *et al*., 2006; Vidyalakshmi *et al*., 2009; Hsu *et al*., 2011; Lee *et al*., 2011). In practice, ethyl acetate, ethanol and water are often more preferred for food and pharmaceutical processing compared to other solvents due to their safety and affordability. HPLC is utilized to analyze various *Monascus* pigments because of its high sensitivity and multiple detection systems including ultraviolet, fluorescent, photodiode array and mass spectrometer detectors (Feng *et al*., 2012).

This research was aimed to separate and analyse MYP produced on durian seed substrate. This is the first

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FULL PAPER

investigation in MYP separation using ethyl acetate, ethanol and water.

2. Materials and methods

2.1 Microorganism, substrate and chemicals

Monascus purpureus M9 culture was used in the solid-state fermentation on durian seed substrate. The culture was maintained periodically on Potato Dextrose Agar. Durian seed was obtained from a durian processing unit in Surabaya (Indonesia). The durian seed is a by-product of the food industry and was used as the SSF substrate. All chemicals were chromatographic and analytical grades.

2.2 Pigments production through solid-state fermentation with durian seed substrate

Pigments production was performed through solid-state fermentation on durian seed substrate according to our previous research (Srianta *et al*., 2012). After peeling, cutting, soaking, sterilization at 121°C for 10 mins and cooling, the durian seed substrate was inoculated with *Monascus purpureus* starter culture, and then incubated at 30°C for 14 days. The fermented matter was dried at 45°C for 24 hrs, ground and sieved.

2.3 Monascus pigment separation

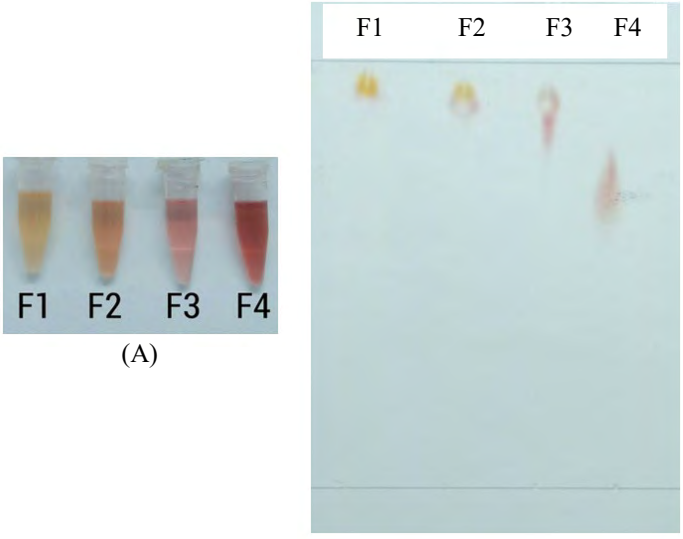
The *Monascus* pigment was extracted with soxhletation method in ethanolic solvent at a ratio of 1:50 for 2 hrs. The extract was then evaporated in a rotary evaporator. The evaporated extract was subjected to separation process. MYP separation was performed in a column chromatography (10 mm x 500 mm) with silica gel 60. The column was packed with silica gel by slurry method, then pre eluted with ethyl acetate: ethanol: water = 90:25:4. The extract was put into the column and eluted with ethyl acetate: ethanol: water = 90:25:4. The obtained fractions were subjected to analysis by using TLC and spectrophotometer. The MYP fraction was analyzed the pigment composition by using LC-MS/MS.

2.4 Pigment analysis

The separated pigments were subjected to the TLC analysis according to Nimnoi and Lumyong (2011) with modification, on silica gel F60 plate, with ethyl acetate: ethanol: water at 90:25:4. The separated MYP was scanned in a range of wavelength 380-600 nm with UV-Vis spectrophotometer (Shimadzu UV-1900, Japan). Pigment composition analysis was conducted by using LC-MS/MS (Shimadzu, Kyoto, Japan) with Q1 scan positive mode and SIM positive mode. The extract was filtered through a 0.2 µm PTFE filter membrane, put on to the autosampler. Pigments were separated on a Cosmosil 5 C18-MS-II (4.6 i.d. x 150 mm) column, with an isocratic program of mobile phase consists of acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid (60:40 vol/vol), flow rate of 0.5 mL/min; oven temperature of 40°C, run time for 30 min. Pigment compounds were detected by MS/MS using electro-spray ionization in the positive ion mode, scan range: m/z 100 to 1000.

3. Results and discussion

In the separation of the pigments by using column chromatography, 4 fractions (F1 F2, F3 and F4) with different colors have been derived. The first fraction was MYP target, while other fractions were orange and red pigments. The MYP has the highest retention factor, followed by F2, F3 and F4. Those fractions and pigments profile on TLC shown in Figure 1. Those profiles describe that MYP was the pigments with the lowest polarity among the others. Although the chemical structure of *Monascus* pigments consists of β-ketoacid and chromophore, MYP compounds contain less polar groups than those of red pigments. The presence of $–NH_3$ group can increase the polarity of red pigments.



(A) (B)

Figure 1. Picture of 4 separated fractions of *Monascus* pigments (A) and their TLC chromatogram (B). F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.

Figure 2 shows the fractions scanning by using spectrophotometer at 380-700 nm. F1, the MYP, has a λ max at 386 nm that was in yellow spectrum. F3 and F4 have 2 peaks in the orange and red spectrum. Those reflect that contain a mixture of orange and red pigments. This is the first investigation on the pigment compounds composition of MYP by using the LC-MS/MS. The LC-MS chromatograms and pigment composition of the extracts are presented in Figure 3 and Table 1, respectively. Five MYP compounds were detected by using LC-MS/MS i.e. monascin, ankaflavin, xanthomonasin B, monascopyridine B and Yellow II.

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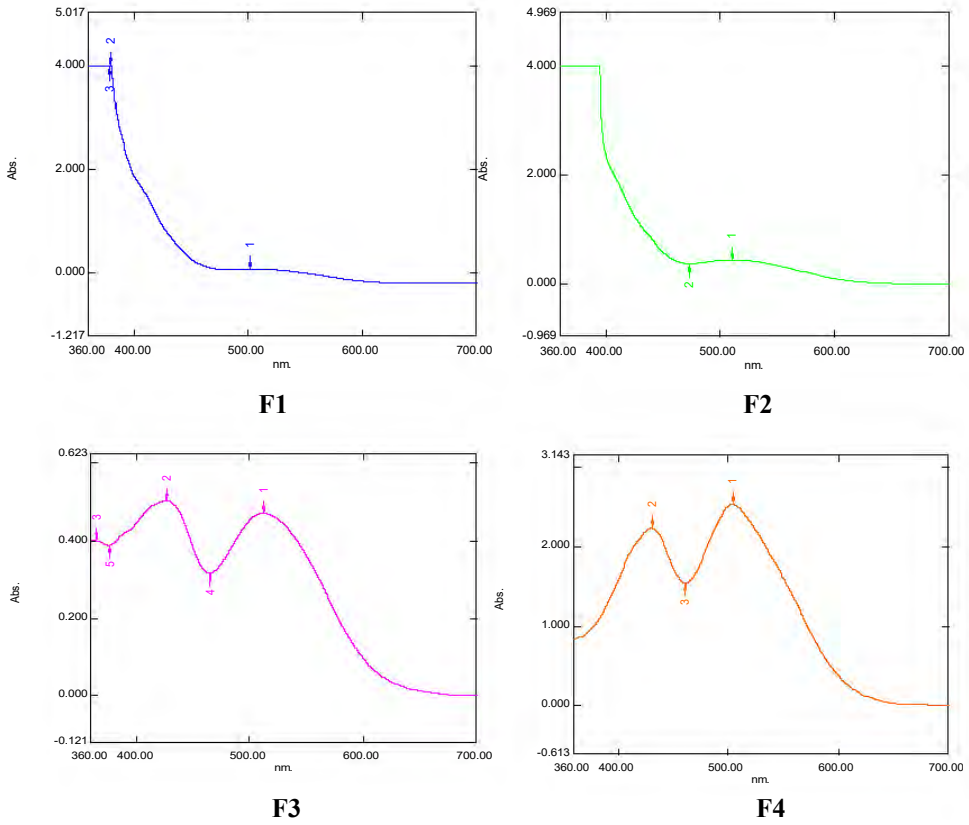

Figure 2. Scanning results of separated *Monascus* pigment fractions by using spectrophotometer. F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.

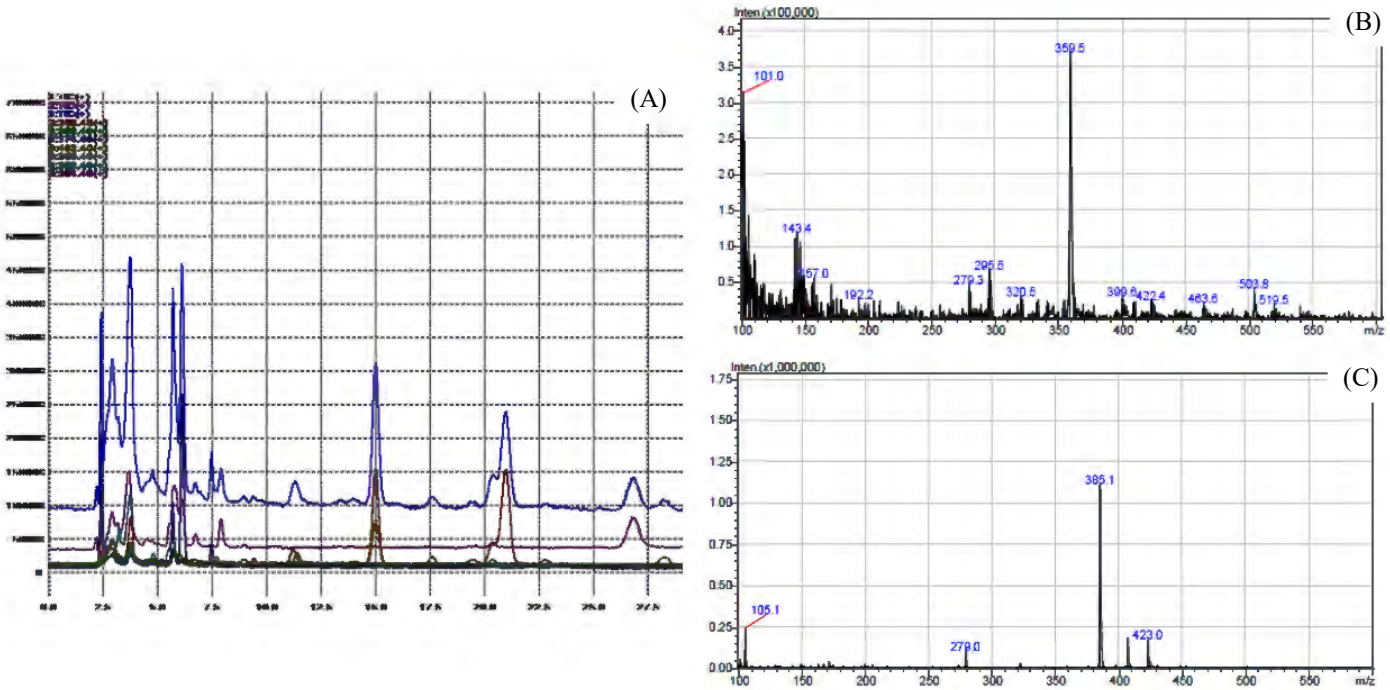

Figure 3. LC-MS analysis results: Total Ion Chromatogram of MYP (A) and representatives of the chromatogram of major MYP monascin (B) and yellow II (C).

Table 1. MYP composition

MYP compound	Content (%)
Monascin	59.81
Ankaflavin	6.32
XanthomonasinA	n.d.
XanthomonasinB	0.67
Monascopyridin A	n.d.
Monascopyridin B	5.39
Yellow II	27.80

n.d. = not detected

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Monascin and Yellow II were the major pigment compounds in the MYP with the relative amount of 59.81% and 27.80%, respectively The MYP composition depends on the pigment composition in *Monascus* Fermented Durian Seed (MFDS), extraction and separation processes.

Pigments in MFDS produced through a complex polyketide biosynthesis pathway in the *Monascus* fungi, but it is still unclear and controversy. Some researchers proposed that only orange pigments (rubropunctatin and monascorubramine) were biosynthetic, and the others were transformed from them chemically. In orange pigment rubropunctatin biosynthesis, it consist of the β-ketide pathway to form acetate and malonate and another pathway to form n-hexanoyl acetyl residue. Yellow pigments can be transformed from orange pigments via oxidation. Our previous research revealed that solvent type affected the *Monascus* pigment composition. The ethanolic extract contains higher MYP than those in the mixture solvent of ethanol and water. This research used ethanol as the extraction solvent. In the separation process, the chromatographic system used has successfully separated MYP from the red and orange pigments.

Monascin, the major MYP compound in this research, is attractive for its strong bioactivities such as anticancer, antitumor, antidiabetic, antioxidative stress, antiinflammatory, antiobesity and showed antiatherogenic activities, and also improves memory and learning ability. On the other hand, there is no report on the yellow II bioactivity.

4. Conclusion

The MYP has been successfully separated in a column chromatography packed with silica gel 60 and eluent of ethyl acetate:ethanol:water=90:25:4. Five MYP compounds have been detected by using LC-MS/MS i.e. monascin, ankaflavin, xanthomonasin B, monascopyridine B and yellow II. Monascin and yellow II were the major pigment compounds in MYP.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by Direktorat Riset dan Pengabdian Masyarakat, Direktorat Jenderal Penguatan Riset dan Pengembangan, Kementerian Riset, Teknologi, dan Pendidikan Tinggi, Republik Indonesia through the scheme of Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT) 2019 [contract number 200X/WM01.5/N/2019].

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Professor Dr. Son Radu
foodresearch.my@outlook.com

Chief Editor, Food Research

14

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1 April 2020

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Food Research 4 (4) : 1135 - 1139 (August 2020)
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FOOD RESEARCH

FULL PAPER

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Durian seed,
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Analysis

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1. Introduction

Monascus pigments are secondary metabolites of *Monascus* fungi biosynthesized through the polyketide pathway. It has been used as a natural food colourant, food supplement, and in traditional medicine. *Monascus* pigments consist of many pigment compounds which are categorized into three groups i.e. red, orange and yellow pigments. A total of six well-known pigment compounds are monascorubramine and rubropunctamine (red pigments), monascorubrin and rubropunctatin (orange pigments), monascin and ankaflavin (yellow pigments) (Feng *et al*., 2012; Srianta *et al*., 2014; Agboyibor *et al*., 2018). Until 2011, thirty-nine new pigment compounds have been identified (Feng *et al*., 2012).

Rice is commonly used as solid-state fermentation substrate in *Monascus* pigments production. Non-rice substrates have been developed in the *Monascus* pigment production e.g. corn, adlay, sorghum, dioscorea, jackfruit seed and durian seed (Pattanagul *et al*., 2007; Babitha *et al*., 2007; Hsu *et al*., 2011; Srianta *et al*., 2012; Srianta *et al*., 2016). Our previous studies revealed that *Monascus purpureus* produced red, orange and yellow pigments. Exploration of *Monascus* yellow pigments is still limited. MYP is not only a potential natural colourant for biscuit, mayonnaise, wheat noodle, etc. (Srianta *et al*., 2014) but also as functional food ingredient and in medicine, because MYP has various positive health effects i.e. antioxidant, antidiabetes and anticancer (Hsu *et al*., 2011; Chen and Wu, 2016). Monascin and ankaflavin have antitumor activity (Akihisa *et al*., 2005; Su *et al*., 2005), antiobesity activity, anti-atherosclerosis effect (Wang *et al*., 2013; Liu *et al*., 2018), improve memory and learning ability (Lee *et al*., 2015). Chen and Wu (2016) reported that MYPs have promising potential for application in food and pharmaceutical industries based on their bioactivities.

Silica gel has been applied in *Monascus* pigments mixture separation through thin layer chromatography and column chromatography systems with various eluents i.e. chloroform, methanol, benzene, acetone and petroleum ether (Kim *et al*., 2006; Vidyalakshmi *et al*., 2009; Hsu *et al*., 2011; Lee *et al*., 2011). In practice, ethyl acetate, ethanol and water are often more preferred for food and pharmaceutical processing compared to other solvents due to their safety and affordability. HPLC is utilized to analyze various *Monascus* pigments because of its high sensitivity and multiple detection systems including ultraviolet, fluorescent, photodiode array and mass spectrometer detectors (Feng *et al*., 2012).

This research was aimed to separate and analyse MYP produced on durian seed substrate. This is the first

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investigation in MYP separation using ethyl acetate, ethanol and water.

2. Materials and methods

2.1 Microorganism, substrate and chemicals

Monascus purpureus M9 culture was used in the solid-state fermentation on durian seed substrate. The culture was maintained periodically on Potato Dextrose Agar. Durian seed was obtained from a durian processing unit in Surabaya (Indonesia). The durian seed is a by-product of the food industry and was used as the SSF substrate. All chemicals were chromatographic and analytical grades.

2.2 Pigments production through solid-state fermentation with durian seed substrate

Pigments production was performed through solid-state fermentation on durian seed substrate according to our previous research (Srianta *et al*., 2012). After peeling, cutting, soaking, sterilization at 121°C for 10 mins and cooling, the durian seed substrate was inoculated with *Monascus purpureus* starter culture, and then incubated at 30°C for 14 days. The fermented matter was dried at 45°C for 24 hrs, ground and sieved.

2.3 Monascus pigment separation

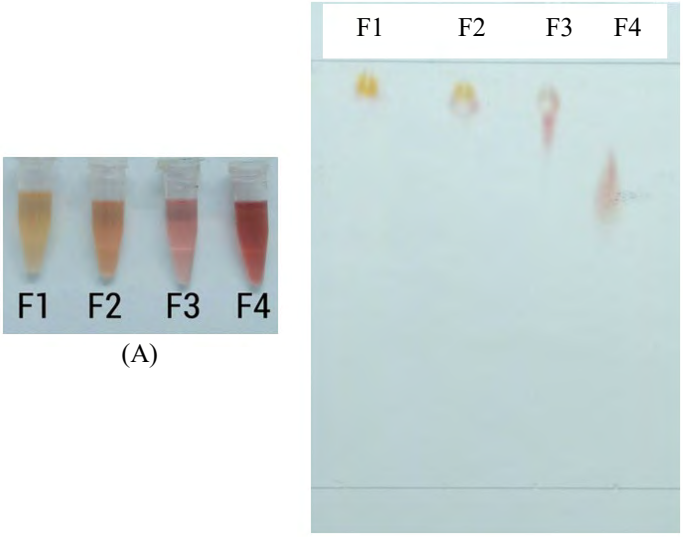
The *Monascus* pigment was extracted with soxhletation method in ethanolic solvent at a ratio of 1:50 for 2 hrs. The extract was then evaporated in a rotary evaporator. The evaporated extract was subjected to separation process. MYP separation was performed in a column chromatography (10 mm x 500 mm) with silica gel 60. The column was packed with silica gel by slurry method, then pre eluted with ethyl acetate: ethanol: water = 90:25:4. The extract was put into the column and eluted with ethyl acetate: ethanol: water = 90:25:4. The obtained fractions were subjected to analysis by using TLC and spectrophotometer. The MYP fraction was analyzed the pigment composition by using LC-MS/MS.

2.4 Pigment analysis

The separated pigments were subjected to the TLC analysis according to Nimnoi and Lumyong (2011) with modification, on silica gel F60 plate, with ethyl acetate: ethanol: water at 90:25:4. The separated MYP was scanned in a range of wavelength 380-600 nm with UV-Vis spectrophotometer (Shimadzu UV-1900, Japan). Pigment composition analysis was conducted by using LC-MS/MS (Shimadzu, Kyoto, Japan) with Q1 scan positive mode and SIM positive mode. The extract was filtered through a 0.2 µm PTFE filter membrane, put on to the autosampler. Pigments were separated on a Cosmosil 5 C18-MS-II (4.6 i.d. x 150 mm) column, with an isocratic program of mobile phase consists of acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid (60:40 vol/vol), flow rate of 0.5 mL/min; oven temperature of 40°C, run time for 30 min. Pigment compounds were detected by MS/MS using electro-spray ionization in the positive ion mode, scan range: m/z 100 to 1000.

3. Results and discussion

In the separation of the pigments by using column chromatography, 4 fractions (F1 F2, F3 and F4) with different colours have been derived. The first fraction was MYP target, while other fractions were orange and red pigments. The MYP has the highest retention factor, followed by F2, F3 and F4. Those fractions and pigments profile on TLC shown in Figure 1. Those profiles describe that MYP was the pigments with the lowest polarity among the others. Although the chemical structure of *Monascus* pigments consists of β-ketoacid and chromophore, MYP compounds contain less polar groups than those of red pigments. The presence of $–NH_3$ group can increase the polarity of red pigments.



(A) (B)

Figure 1. Picture of 4 separated fractions of *Monascus* pigments (A) and their TLC chromatogram (B). F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.

Figure 2 shows the fractions scanning by using spectrophotometer at 380-700 nm. F1, the MYP, has a λ max at 386 nm that was in yellow spectrum. F3 and F4 have 2 peaks in the orange and red spectrum. Those reflect that contain a mixture of orange and red pigments. This is the first investigation on the pigment compounds composition of MYP by using the LC-MS/MS. The LC-MS chromatograms and pigment composition of the extracts are presented in Figure 3 and Table 1, respectively. Five MYP compounds were detected by using LC-MS/MS i.e. monascin, ankaflavin, xanthomonasin B, monascopyridine B and Yellow II.

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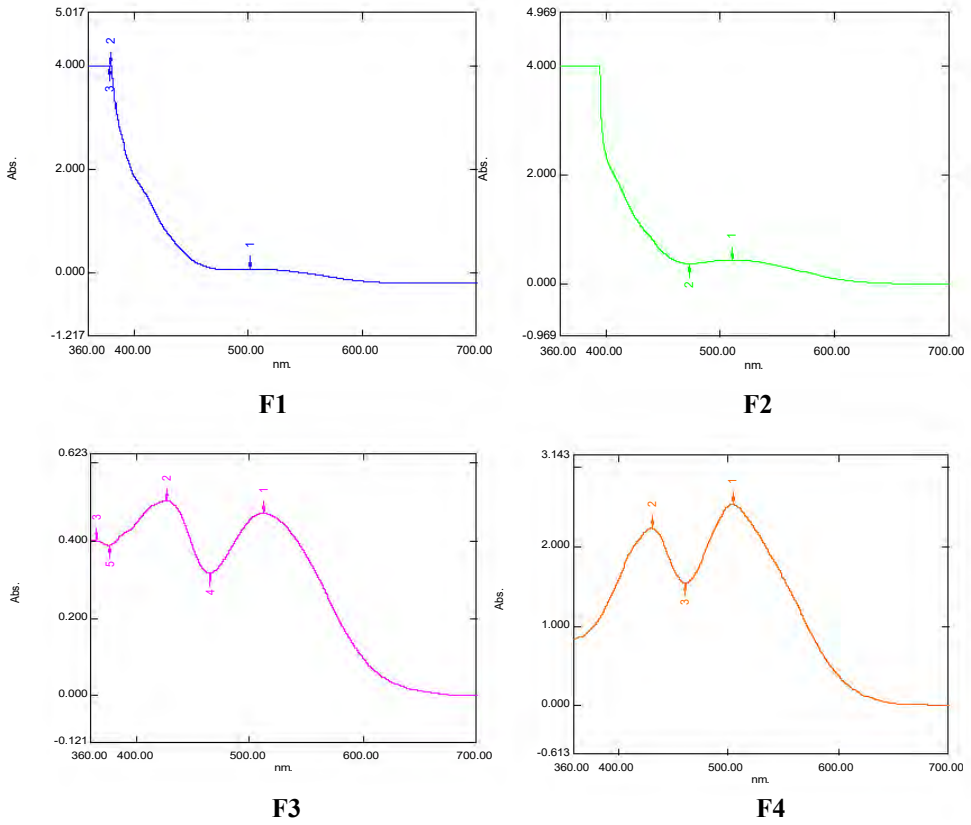

Figure 2. Scanning results of separated *Monascus* pigment fractions by using spectrophotometer. F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.

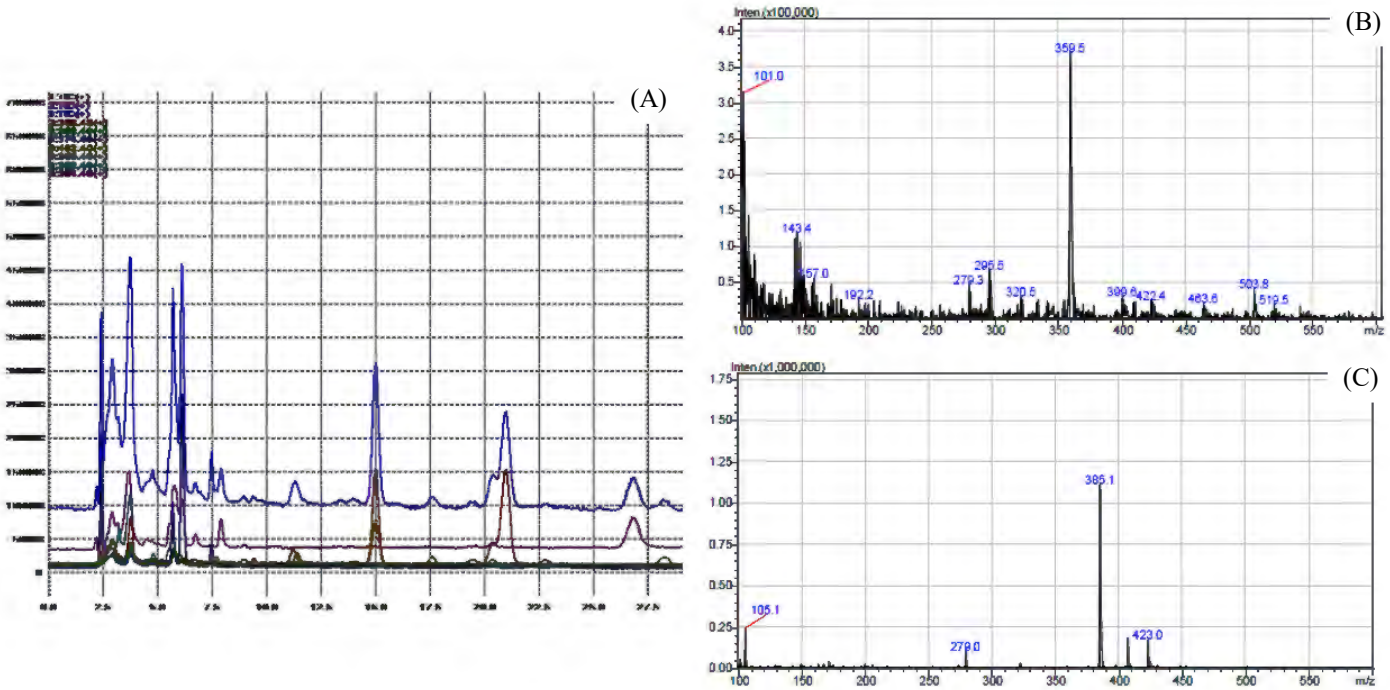

Figure 3. LC-MS analysis results: Total Ion Chromatogram of MYP (A) and representatives of the chromatogram of major MYP monascin (B) and yellow II (C).

Table 1. MYP composition

MYP compound	Content (%)
Monascin	59.81
Ankaflavin	6.32
XanthomonasinA	n.d.
XanthomonasinB	0.67
Monascopyridin A	n.d.
Monascopyridin B	5.39
Yellow II	27.80

n.d. = not detected

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Monascin and Yellow II were the major pigment compounds in the MYP with the relative amount of 59.81% and 27.80%, respectively The MYP composition depends on the pigment composition in *Monascus* Fermented Durian Seed (MFDS), extraction and separation processes.

Pigments in MFDS produced through a complex polyketide biosynthesis pathway in the *Monascus* fungi, but it is still unclear and controversy. Some researchers proposed that only orange pigments (rubropunctatin and monascorubramine) were biosynthetic, and the others were transformed from them chemically. In orange pigment rubropunctatin biosynthesis, it consist of the β-ketide pathway to form acetate and malonate and another pathway to form n-hexanoyl acetyl residue. Yellow pigments can be transformed from orange pigments via oxidation. Our previous research revealed that solvent type affected the *Monascus* pigment composition. The ethanolic extract contains higher MYP than those in the mixture solvent of ethanol and water. This research used ethanol as the extraction solvent. In the separation process, the chromatographic system used has successfully separated MYP from the red and orange pigments.

Monascin, the major MYP compound in this research, is attractive for its strong bioactivities such as anticancer, antitumor, antidiabetic, antioxidative stress, antiinflammatory, antiobesity and showed antiatherogenic activities, and also improves memory and learning ability. On the other hand, there is no report on the yellow II bioactivity.

4. Conclusion

The MYP has been successfully separated in a column chromatography packed with silica gel 60 and eluent of ethyl acetate:ethanol:water=90:25:4. Five MYP compounds have been detected by using LC-MS/MS i.e. monascin, ankaflavin, xanthomonasin B, monascopyridine B and yellow II. Monascin and yellow II were the major pigment compounds in MYP.

Conflict of Interest

The authors declare no conflict of interest.

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