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RESEARCH ARTICLE

Optimization of Solvent Systems for Color Fractionation of *Monascus* Pigment

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ABSTRACT - *Monascus* fungi are unique among the various microorganisms that produce edible pigments, which consist of red, orange, and yellow. These pigments are typically present in a mixture. Hence, pigment separation is needed. This study aims to optimize solvent systems that can effectively fractionate the pigments. The optimization was carried out using column chromatography and the data was analyzed using Central Composite Design (CCD). The interaction of the solvent systems ethyl acetate : formic acid : acetic acid : water was optimized. The optimal solvent system obtained was 98 : 14 : 12 : 28 (vol/vol.) for ethyl acetate : formic acid : acetic acid : water, respectively. The red and yellow pigment obtained were 2.42 mg and 0.93 mg, respectively. Based on the finding, 72.2% of the total *Monascus* pigments was red while remaining was yellow.

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1.0 INTRODUCTION

A food colorant can affect the human's psychology on how the food will taste [1–3]. A comprehensive review on the psychological impact of color on consumer expectations and behavior in relation to the sensory experiences also has been conducted [4, 5]. Color is used to recover the original color of the food product [1], which faded during processing due to exposure to light, air, temperature, and storage conditions [2, 3]. Therefore, food coloring is in higher demand. Consequently, people are producing synthetic colorant. Synthetic colorants may synthesize chemically, such as extracted from coal tar or highly purified oil products [3]. However, chemically synthesized synthetic colorant resulted in hazardous effects on human health, such as allergic reactions, mutagenicity, and carcinogenicity [6]. These factors have raised the demand for naturally occurring edible coloring produced by microorganism [7]. In addition, edible coloring made from microorganisms has many advantages, including the ability to produce efficiently under controlled physicochemical conditions, good harvest quality, ease to scale up, and independence from the capricious nature [8].

The use of *Monascus* pigments for food is currently covered by more than 50 patents in Japan, United States, France and Germany [9]. *Monascus* fungi can produce various potentially useful metabolites including both primary metabolites, such as ethyl alcohol, acids, esters, and other flavoring compounds, and secondary metabolites, such as pigments, lovastatin (monacolin), and antimicrobial agents [10]. Furthermore, the *Monascus* pigment also has significant medical potential as it contains numerous bioactive metabolites. *Monascus* pigment can produce at least six azaphilone pigments, such as two types of yellow (monascin and ankaflavine), two types of orange (rubropunctatin and monascorubrine), and two types of red (rubropunctamine and monascorubramine) [11]. Pigment components can be separated using various types of chromatography [12] or capillary electrophoresis (CE).

This study aims to optimize the solvent systems that can separate the *Monascus* pigments using column chromatography (CC). The data was then analyzed using Central Composite design (CCD) via Design Expert software.

2.0 METHODS AND MATERIAL

2.1 Extraction and Estimation of Pigments

About 0.25g of fermented substrate with *Monascus* was extracted using 60% ethanol at three different temperatures; 30, 40 and 50 °C in the incubator shaker for 1 hour at 200 rpm. Next, the substrate was filtered using filter paper (Whatman No. 1 filter paper, Whatman, England). Then, the spectra absorption analysis was performed at 400 nm and 500 nm wavelength for yellow and red pigment, respectively, using UV-Spectrophotometer (Hitachi U-1900) [13]. Sample then was completely dried using rotary evaporator under 78 °C (Rotavapor R-210, Buchi, Switzerland) to measure the mass of the extracted pigments.

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2.2 Thin Layer Chromatography

The preparative thin layer chromatography (TLC) silica plate (12 cm x 10 cm) was used. The solvent systems that being choose for this analysis were ethyl acetate: formic acid: acetic acid: water (100 : 11 :11: 26, vol/vol) [13], n-Hexane: ethyl acetate (7 : 3) [14], n-Hexane : acetone (1:1) [15]. The separation pattern and the retention factor (R_f) value of the spots developed on TLC plate was calculated using Eq. (1).

$$R_f = \text{Distance travelled by component}/\text{Distance travelled by solvent} \quad (1)$$

The best solvent system that managed to separate the red and yellow pigment individually was selected to optimize in the column chromatography.

2.3 Experimental Design

The experimental work was conducted based on the design generated by central composite design (CCD) using Design Expert 7.0 software. The solvent system that demonstrated superior separation on TLC has been selected as the factors to be optimized in CCD. Four factors for the solvent system were choose for the column chromatography, that is ethyl acetate, formic acid, acetic acid and water. The level of the factors is presented in Table 1. First, the column was pre-eluted with 5 ml of selected solvent system as in Table 3. Next, approximately 0.1 mL of the pigments was then loaded on the top of the column. Later, the remaining solvent was added slowly and the elution process began. The separated color was then collected at the bottom of the column. The collected liquid was dried using rotary evaporator [16]. Finally, the mass of the dried sample was measured. The experiments were run in triplicate.

Table 1. Independent variables of the solvent system and their levels

Independent variable	**Factor level**				
	-2	-1	0	1	2
Ethyl Acetate (mL)	90	95	100	105	110
Formic Acid (mL)	1	6	11	16	21
Acetic Acid (mL)	1	6	11	16	21
Water (mL)	16	21	26	31	36

3.0 RESULTS AND DISCUSSION

3.1 Effect of Temperature on Extraction

Extraction at different temperature were investigated on the dried fermented substrate of *Monascus*. The fermented substrate was extracted at different temperature from 30 to 50 °C, as shown in Table 2. According to many researchers, the wavelength of 500 nm indicated red pigment [7, 17], while 400 nm is yellow pigment [18]. It is interesting to note that, the optimum extraction temperature for both yellow and red was different, 30 °C and 40 °C, respectively. At 500 nm, the absorbance was highest at 40 °C, which resulted 0.430 AU. On the other hand, at 400 nm, the highest absorbance obtained at 30 °C with 0.423 AU. These finding indicated that 40 °C and 30 °C are the optimum kinetic energy that the bond can withstand and maximum red and yellow pigments are produced, respectively.

Higher temperatures usually increase the solute's solubility, which should enhance the extraction process. As the temperature increases, the viscosity of the solvent is decreased and the solute's diffusivity is elevated, which would be projected to maximize the pigments' diffusivity within the solid pores. As a result, increase the extraction rate [19]. However, the current finding resulted contrary, where at 50 °C, the *Monascus* pigments resulted the lowest color absorbance for both 400 nm and 500 nm wavelengths. It was because, when the temperature becomes too high for a certain period, it can cause the forces between the structures of the *Monascus* pigments molecule begin to break. Thus, the pigment in the sample being degraded. This behavior also observed by Daud et al. [19], where the pigments extracted were denatured when high temperature was used.

Table 2. The color intensity for each temperature at 400 nm and 500 nm

Temperature (°C)	**Absorbance (AU/g)**	
	400 nm	500 nm
30	0.432 ± 0.032	0.387 ± 0.006
40	0.397 ± 0.011	0.450 ± 0.009
50	0.227 ± 0.016	0.256 ± 0.008

3.2 Analysis of Color Fractionation Using Thin Layer Chromatography

Thin layer chromatography (TLC) is a chromatographic technique used to separate the components of a mixture using a thin stationary phase supported by an inert backing. All the solvent systems of ethyl acetate: formic acid: acetic acid:

water (100 : 11 :11: 26, vol/vol), n-Hexane: ethyl acetate (7 : 3), n-Hexane : acetone (1:1), shows a different separation of the pigment due to the different polarity of the solvent system. Thus resulted different R_f values. Though, the R_f value for the yellow pigments were almost similar for all of the solvent system (R_f = 0.78-0.82). On the other hand, for solvent system using n-hexane : acetone (1:1), only yellow spots are obtained with R_f of 0.82, while the red pigments are not moving up of the plate. These are due to the more polar structure of the red pigment. The yellow pigment, which are less polar structure was weakly adsorbed and travels along the TLC plate. Thus, the least polar pigment is best solved in a non-polar solvent and will thus have a largest running distance.

In contrast, with solvent system ethyl acetate : formic acid : acetic acid : water (100:11:11:26, vol/vol), red pigments were obtained at R_f 0.52-0.72. At this stage, the solvent system is highly polar and attracted the polar compound to absorb and travels on the plate. While, for solvent system n-hexane : ethyl acetate (7:3, vol/vol), the red pigment were obtained at R_f 0.00-0.32. And some of pigments were remained at the bottom line of the TLC and did not move up.

Thus, the basis for separation is the polarity. When considering the polarity of mobile phase, the polarity should always be relatively less than the stationary phase [20]. Thus, when choosing an organic compound as a mobile phase, the polarity of the organic solvent should be concerned. A high value of R_f value indicated a high hydrophobicity of the compound. A high R_f for the yellow pigment suggest that it is more hydrophobic than the red pigment.

The polarity of the solvent system was a follows: ethyl acetate: formic acid: acetic acid: water > n-hexane: ethyl acetate > n-hexane : acetone. Thus, the solvent system of ethyl acetate: formic acid: acetic acid: water, was further optimized via Central Composite Design (CCD).

3.3 Optimization of Color Fractionation

Table 3 shows the solvent system generated by Central Composite Design (CCD). For factors involved which were ethyl acetate (A), formic acid (B), acetic acid (C), and water (D).

Table 3. Experimental data for CCD optimization

Run	Factor				Response	
	A (mL)	B (mL)	C (mL)	D (mL)	Mass of Dry Red Colour (mg)	Mass of Dry Yellow Colour (mg)
1	100.00	11.00	11.00	16.00	2.31	1.04
2	95.00	6.00	16.00	21.00	2.32	1.09
3	90.00	11.00	11.00	26.00	2.33	1.12
4	105.00	16.00	6.00	21.00	2.30	1.03
5	95.00	16.00	16.00	31.00	2.39	0.97
6	95.00	6.00	16.00	31.00	2.31	1.08
7	95.00	6.00	6.00	21.00	2.28	1.1
8	110.00	11.00	11.00	26.00	2.32	1.07
9	95.00	16.00	16.00	21.00	2.35	1.03
10	100.00	11.00	1.00	26.00	2.26	0.98
11	105.00	16.00	6.00	31.00	2.31	1.10
12	105.00	16.00	16.00	21.00	2.36	0.94
13	100.00	21.00	11.00	26.00	2.36	0.94
14	105.00	6.00	16.00	31.00	2.28	1.06
15	100.00	11.00	11.00	36.00	2.33	0.99
16	95.00	16.00	6.00	21.00	2.31	1.02
17	105.00	6.00	6.00	21.00	2.28	0.94
18	100.00	11.00	11.00	26.00	2.41	0.96
19	105.00	16.00	16.00	31.00	2.34	0.93
20	105.00	6.00	6.00	31.00	2.29	1.05
21	100.00	11.00	11.00	26.00	2.41	0.96
22	100.00	11.00	21.00	26.00	2.33	0.93
23	95.00	6.00	6.00	31.00	2.31	1.03
24	95.00	16.00	6.00	31.00	2.37	0.96
25	105.00	6.00	16.00	21.00	2.30	0.95
26	100.00	1.00	11.00	26.00	2.31	1.12
27	100.00	11.00	11.00	26.00	2.42	0.84

Note: A: Ethyl acetate, B: Formic acid, C: Acetic acid, D: Water

3.4 Statistical Analysis of Red and Yellow Pigment

Table 4 shows the Analysis of variance (ANOVA) table of mass of dry red and yellow pigment obtained from CCD. The Fisher's F-values of 21.12 and 4.58 with relatively low p-values of <0.0001 and 0.006 were obtained for red and

yellow pigment, respectively. The p-value was used as a tool to check the significance of each model terms, which is desirable to indicate whether the terms in the model have a significant effect on the response [21]. The smaller the p-value (< 0.0500), the larger the significance of the term was. As in Table 4, the p-values for the pigments were <0.01, thus, the red and yellow pigments models were significant. On the other hand, the "lack of fit" p-values of the model were 0.1698 and 0.9157, for red and yellow, respectively, implied that the lack of fit is not significant relative to the pure error. The non-significant lack of fit is good as this shows that the suggested model equation fits well with the experimental results.

Factors A, B, C, D, AD, CD, A^2, B^2, C^2, D^2 were significant model terms for red pigment. Whereas, factors B, AD, A^2, B^2, D^2 were significant for yellow pigment. The goodness of fit was evaluated by the R-squared (R^2) value. The R^2 value near to 1 indicated that the experimental data are closed to the fitted regression line. The R^2 obtained for red and yellow were 0.9610 and 0.8422, respectively. The values suggested that the model could predict 96.10% and 84.22% of the variability for red and yellow, respectively.

Table 4. The Fisher's F-value, p-values and R^2 values of red and yellow pigments

Source	Red pigment		Yellow pigment	
	F Value	p-value Prob > F	F Value	p-value Prob > F
Model	21.12	<0.0001[a]	4.58	0.0060 [a]
A	10.96	0.0062 [a]	3.41	0.0897
B	57.97	<0.0001[a]	10.91	0.0063 [a]
C	31.67	0.0001[a]	1.85	0.198
D	5.37	0.039 [a]	0.0094	0.924
AB	0.657	0.4332	3.63	0.08
AC	4.56E-14	1	3.19	0.099
AD	8.05	0.0149 [a]	8.16	0.0145 [a]
BC	4.11	0.0654	3.19	0.099
BD	2.63	0.1308	1.42	0.257
CD	5.92	0.0316 [a]	0.0142	0.90
A^2	68.41	<0.0001[a]	23.8	0.0004 [a]
B^2	53.80	<0.0001[a]	9.56	0.0093 [a]
C^2	122.76	<0.0001[a]	1.06	0.323
D^2	76.37	<0.0001[a]	7.18	0.021 [a]
Residual				
Lack of Fit	5.28	0.1698	0.31	0.9157
Pure Error	6.667E-003		8.267E-003	
R^2	0.9610		0.8422	

[a] Significant, A: Ethyl acetate, B: Formic acid, C: Acetic acid, D: Water

Figure 1 shows a graphical representation of the predicted (mathematically calculated) and actual (experimental) plot of model for red and yellow pigments. The predicted values of red and yellow are quite similar to the experimental values. It demonstrated that the regression model has strong correlation between the model prediction and its experimental results. Thus, the developed regression model is reliable.

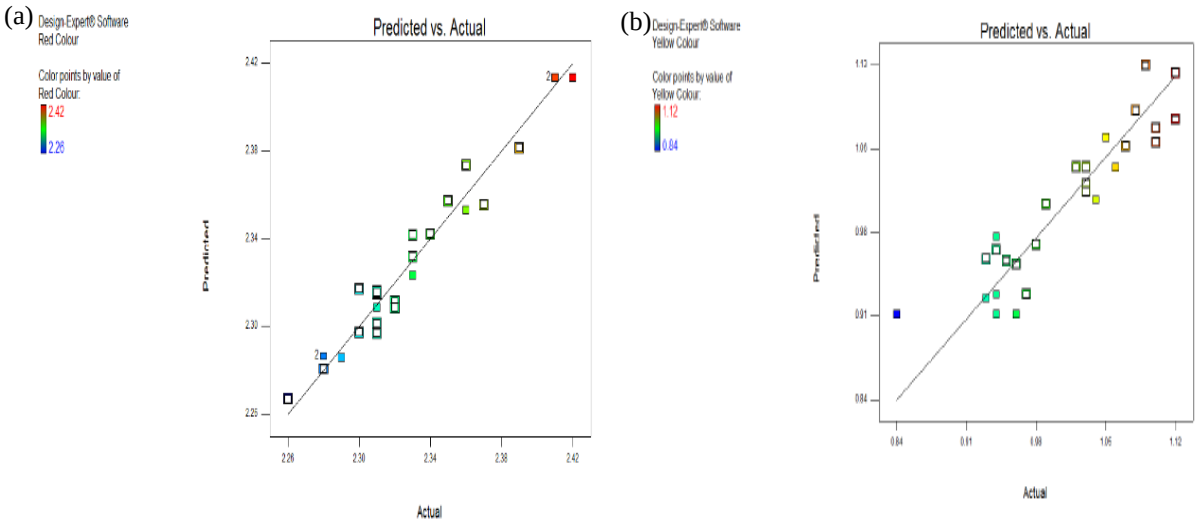

Figure 1. The actual versus predicted plot for (a) mass of dry red color and (b) mass of dry yellow color

3.5 Responses Surface Analysis of Red Pigment

Figure 2 shows the three-dimensional (3D) surface of the factors (i.e. ethyl acetate (A), acetic acid (C) and water (D)) to the red pigments. Interaction between water (D) and ethyl acetate (A), and between acetic acid (C) and water (D) managed to optimize the color separation. Both interactions involved water, DA (Figure 2 a) and CD (Figure 2 b). Water had been stated as the most polar solvent [19] due to the uneven distribution of electron density and ability to form hydrogen bonds with other polar molecules. In this study, water interact with the acetic acid and ethyl acetate, which are both moderately polar solvents. These interactions resulted in changed in the solvent's overall polarity making it more suitable for attracting and separating the polar compounds, including the red pigment. The addition of water effectively modifies the solvent system's properties, making it more conducive to chromatographic separation and to isolate the red pigment more effectively during the chromatography process.

During elution process in column chromatography, the non-polar yellow pigment eluted through the column much faster compared to the polar red pigment. While the red pigment adhered to the silica gel. The polar solvents system designed slowly moved the red pigment along the column. Highest red pigment obtained at water 27 ml and ethyl acetate 99 ml, with fixed formic acid:acetic acid, 11:11 (v/v) (Figure 2 a). Further increased or decreased the water and ethyl acetate ratio did not improve the separation, in fact reduced the red yield. On the other hand, the interaction of water and acetic acid resulted the highest red pigment at 27 ml and 12 ml, respectively, with fixed ethyl acetate : water (100:11, vol/vol). Further increased or decreased the water and acetic acid did not rise the separation, nonetheless reduced the red yield (Figure 2 b). A similar finding was also reported by Schwarz et al. (2003) [22] and Degenhardt et al. (2000) [23]. They successfully separated acylated anthocyanins pigment using ethyl acetate/water with acidified 0.1% trifluoroacetic acid (TFA) via high-speed counter-current chromatography.

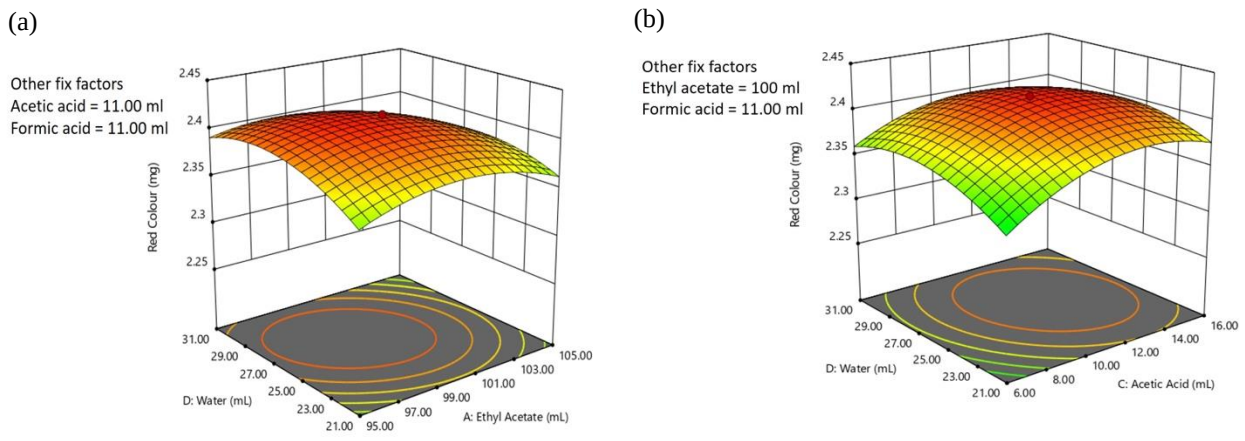

Figure 2**.** 3D response surface (a) interaction of ethyl acetate and water on mass of red color and (b) interaction of acetic acid and water on mass of red color

3.6 Validation Test

To validate the aforementioned results, validation experiment was performed under the optimum conditions. The predicted optimal values obtained for solvent system were 98 : 14 : 12 : 28 (vol/vol) of ethyl acetate : formic acid : acetic acid : water, respectively. The red pigment obtained was 2.42 mg, while yellow was 0.93 mg. The predicted value and the experimental value were compared and the percentage error 2.62 %. was calculated. The percentage error obtained was within acceptable limits of 10 %. This demonstrated that the good correlation between the predicted and measured values of these experiments.

4.0 CONCLUSION

As conclusion, 40 °C is the optimum temperature for red *Monascus* pigments extraction. The best solvent system for thin layer chromatography was ethyl acetate : formic acid : acetic acid : water (100: 11 : 11 : 26, vol/vol.), which resulted good separation between red and yellow pigments. Based on the optimization study, the optimal solvent system obtained was at 98 : 14 : 12 : 28 (vol/vol), ethyl acetate : formic acid : acetic acid : water, respectively. The optimization of the solvent system demonstrates a practical approach for enhancing the efficiency of pigment separation techniques, contributing to the advancement of analytical methods in pigment research.

5.0 CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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