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Response Surface Methodology used in the Optimization of RP-HPLC Condition for separation of Carmine and Rhodamine B

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ABSTRACT

Carmine (CAR) is one of the permitted coloring agents used in cosmetics, whereas Rhodamine B (RDB) is prohibited as a coloring agent. Due to its similarity color, CAR is frequently replaced by RDB. The objective of this study was to optimize reversed phase high performance liquid chromatography (RP-HPLC) using the experimental design approach based on response surface methodology of Central Composite Design (CCD) for the separation and analysis of CAR and RDB in lipstick products. Some factors (independent variables) responsible for RP-HPLC separation including pH of buffer phosphate (X_1), the acetonitrile ratio (X_2), flow rate of mobile phase (X_3), and column temperature (X_4) were investigated. On the other hand, the responses (dependent variables) evaluated were resolution between CAR and RDB (Y_1), tailing factor of CAR (Y_2), tailing factor of RDB (Y_3), retention time of CAR (Y_4), retention time of RDB (Y_5), peak area of CAR (Y_6) and peak area of RDB (Y_7). CCD showed that separation of CAR and RDB was influenced by those independent variables (factors). The optimum predicted conditions for separation of CAR and RDB based on statistical results was pH buffer of 3.4, ACN 55%, flow rate of 1.1 mL/min and column temperature of 35°C with desirability of 1. Both CAR and RDB were clearly separated using optimum condition, as suggested by CCD. The developed techniques were effective for optimizing chromatographic separation, therefore, the time consumption and large number of running during RP-HPLC analysis could be hindered.

Keywords: Carmine, Rhodamine B, RP-HPLC, central composite design, house hold product.

INTRODUCTION

The use of coloring agent in many cosmetic products such as lipsticks, eye shadows, eyeliners, blushers, and nail polishes is common, especially for woman. Most of the cosmetics products contain one or more coloring agents (dyes) in order to provide the desired colors (Weisz *et al*., 2018). The use of coloring agents in cosmetics are mainly to increase acceptability, to identify the cosmetics products as well as to fit the standard preparations and products stability (Allam and Kumar, 2011). The dyes used in cosmetics products are subject to a wide range of restrictions from the main regulatory authorities such as US Food and Drug Administration (FDA) in the United States of America, the European Commission (EC) in the European Union (EU), and the Ministry of Health, Labor and Welfare (MHLW) in Japan. In Indonesia, the restriction of coloring agents in cosmetics was regulated by The National Agency of Drug and Food Control, Republic of Indonesia (Nohynek *et al*., 2010). Carmine (CI 75470) [CAR] is one of the permitted coloring agents used in cosmetics including lipsticks, but in some susceptible people, the contact of the human body with CAR can produce allergic reactions, sensitization or photosensitization (DiCello *et al*., 1999; Miyakawa *et al*., 2017). In addition, Rhodamine B (CI 45170) [RDB], is prohibited as a coloring agent in cosmetic products in the United States, EU, and Indonesia

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(Gagliardi *et al.,* 1996; Tatebe *et al.*, 2014). Due to its similarity color, CAR is frequently replaced by RDB (Kanekar and Khale, 2014). The chemical structures of CAR and RDB (Figure 1). As a consequence, analytical methods capable of identifying and quantifying of CAR and RDB must be developed and optimized in order to ensure the cosmetic safety.

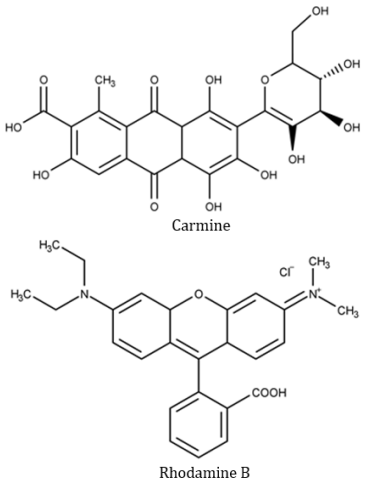

Figure 1. The chemical structures of carmine (CI 75470) and Rhodamine B (CI 45170)

Reversed phase-high performance liquid chromatography (RP-HPLC) with detectors of mass spectrometry with several ionization techniques (Sun *et al*., 2007; Feng *et al*., 2011; Li *et al*., 2013) and photo-diode array (Rastogi *et al*., 1997; Miranda-Bermudez *et al*., 2014) has been widely reported for the analysis of coloring agents. However, the developed RP-HPLC method during the optimization of HPLC condition used the conventional method. Experimental design has been used as tools for method optimization in HPLC, especially for simultaneous component such as CAR and RDB. The experimental design has been widely used to optimize conditions for chromatographic separation in analytical chemistry because it has the ability to reveal possible interactions among variables so that it is able to save time and to simplify work (Bezerra *et al*., 2008). A simple method using Response Surface Method was developed for the extraction and determination of coloring pigments of CAR in cochineals (*Dactylopius coccus* Costa). Two-level factorial design was used in order to optimize the solvent extraction parameters of temperature, time, methanol concentration in the extractant mixture, and the number of extractions (Gonzalez *et al*., 2002). Single factor experiment was also applied to optimize the experimental conditions. Wang *et al.* (2015) used various parameters to achieve the best extraction efficiency. Through the single factor tests, the authors selected four factors (pH, volume, flow rate and eluent flow rate). In the present study, RP-HPLC was optimized using experimental design approach based on response surface methodology using Central Composite Design (CCD). The Experimental design allows predicting the optimum condition of RP-HPLC in short experiment and time (Shaji and Shah, 2016). This design can determine correlation between factors and responses (output) that are resulted in the experiment process.

MATERIALS AND METHODS

The reference standards of carmine (CAR) and Rhodamine B (RDB) were obtained from the national agency of drug and food control (NADFC), Republic of Indonesia. All solvents used for mobile phase were of HPLC grade and obtained from E. Merck (Darmstadt, Germany). Aquabidest was obtained from Ikapharmindo (Indonesia).

Preparation of reference standards

For preparation of stock solution, an approximately of 5.00mg of each CAR and RDB was accurately weighed using analytical balance (Metler Toledo MX5) with sensitivity of 0.01mg and was added into volumetric flask 50mL and dissolved with mobile phase until volume. This solution was then used for preparing calibration curve.

HPLC instrumentation

CAR and RDB were analyzed using chromatograph of Shimadzu LC 20AD equipped with photo-diode array (PDA) (Shimadzu LC 20AD, M20A PDA Detector) at wavelength 245-600nm. The separation was achieved using Cosmosil C_{18} column (250mm x 4.6mm i.d., 5µm). The mobile phase used was acetonitrile-phosphate buffer pH 3.4 (55:45 v/v) delivered isocratically at flow rate of 1.1mL/min, using column temperature at 35°C.

Experimental design using CCD

The experimental design based on response surface methodology (RSM) using central composite design (CCD) is used during HPLC method optimization because RSM can resolve HPLC separation-related problems which the number of factors is higher than 2 (Siregar *et al*., 2018). In this study, the optimization of RP-HPLC separation of CAR and RDB was studied using four factors (independent variables), i.e.

Table I. Central Composite design using dependent variables of variation pH Buffer phosphate (X_1), ratio of acetonitrile (X_2), flow rate (X_3) and column temperature (X_4) with response variables of resolution (Y_1), tailing factor of carmine (Y_2), tailing factor rhodamin B (Y3), retention time carmine (Y_4), retention time rhodamine B (Y_5), peak area carmine (Y_6), and peak area rhodamin B (Y7) used in HPLC optimization for separation of carmine and rhodamine B.

Std.	Run	Dependent Variabel				Response						
						Carmine			Rhodamine B			
		X1	X2	X3	X4	Y4	Y6	Y2	Y5	Y7	Y3	Y1
12	1	2.8	55	1.1	35	2.587	83143	1.5	7.949	215190	0.98	7.851
20	2	3.1	52.5	1	32.5	1.931	71231	1.43	5.757	188954	0.93	7.314
10	3	3.1	52.5	1.2	32.5	2.121	76629	1.46	5.566	199772	0.98	6.638
16	4	3.1	57.5	1	32.5	2.114	76082	1.5	5.892	198471	1.03	6.758
21	5	3.1	57.5	1.2	32.5	2.033	69597	1.54	4.827	176922	1.03	6.088
1	6	3.1	52.5	1	37.5	2.064	69271	1.54	6.404	177065	0.98	7.667
6	7	3.1	52.5	1.2	37.5	2.105	75758	1.51	6.007	198351	1.05	6.782
5	8	3.1	57.5	1.2	37.5	2.115	76279	1.52	5.866	199356	1.06	6.454
22	9	3.1	57.5	1	37.5	2.3	78499	1.52	6.164	208283	1.06	6.426
8	10	3.4	55	1.1	30	1.99	66778	1.59	6.545	173590	1.02	7.541
29	11	3.4	55	0.9	35	2.292	80427	1.43	7.025	223066	0.96	7.626
30	12	3.4	55	1.1	35	2.165	73136	1.6	6.103	187292	0.97	7.069
7	13	3.4	55	1.1	35	2.108	75925	1.5	5.931	198834	1.04	6.649
9	14	3.4	55	1.1	35	2.119	77036	1.49	5.94	198970	1.03	6.636
13	15	3.4	55	1.1	35	1.924	70034	1.45	5.871	187293	0.96	7.137
14	16	3.4	55	1.1	35	2.386	81311	1.59	5.709	210718	1.06	5.746
4	17	3.4	55	0.9	35	2.303	81668	1.55	5.256	226995	1.01	5.467
19	18	3.4	55	1.3	35	2.299	81780	1.55	5.289	227509	0.99	5.674
23	19	3.4	50	1.1	35	1.798	64012	1.48	4.854	168374	1.01	6.244
28	20	3.4	60	1.1	35	2.38	79865	1.59	7.939	207136	1.02	7.952
3	21	3.4	55	1.1	40	1.989	67234	1.55	4.73	175039	1.06	5.532
11	22	3.7	52.5	1	32.5	2.429	82229	1.57	5.612	214289	1.05	5.793
15	23	3.7	52.5	1.2	32.5	2.143	77114	1.47	7.955	196954	0.98	9.064
27	24	3.7	57.5	1.2	32.5	2.107	74396	1.49	5.401	217591	1.02	6.501
17	25	3.7	57.5	1	32.5	2.307	80444	1.42	6.956	223812	0.93	7.884
25	26	3.7	52.5	1	37.5	1.919	70172	1.51	4.437	189234	0.98	5.486
18	27	3.7	57.5	1	37.5	1.922	70165	1.52	4.498	189214	1	5.206
26	28	3.7	57.5	1.2	37.5	2.119	75974	1.6	4.456	202080	1.08	4.792
2	29	3.7	52.5	1.2	37.5	2.575	92772	1.49	7.015	241244	1.03	6.956
24	30	4	55	1.1	35	2.11	76113	1.52	6.068	198029	1.05	6.776

pH buffer phosphate (X_1), ratio of acetonitrile (X_2), flow rate (X_3) and column temperature (X_4). While, the responses (dependent variables) evaluated were resolution between CAR and RDB (Y_1), tailing factor of CAR (Y_2), tailing factor of RDB (Y_3), retention time of CAR (Y_4), retention time of RDB (Y_5), peak area of CAR (Y_6) and peak area of RDB (Y_7).

Data analysis

RSM using CCD together with statistical parameters was carried out using Design-Expert version 8.0.4.1. Factors were considered to affect the responses significantly if the value of coefficient of determination (R^2) ≥ 0.8 and Adjusted R^2 value > 0.8. The difference between Predicted R^2 with the Adjusted R^2 must be less than 0.2. The statistical test of independent t-test was used for comparing results obtained from CCD and from the actual experiments.

RESULTS AND DISCUSSION

RP-HPLC is a method of choice for analysis of CAR and RDB simultaneously due to its capability to provide the separation between CAR and RDB with acceptable sensitivity.

Some factors affected HPLC separation of analytes, therefore, it is a need to optimize HPLC condition to get optimum separation with desired requirement. The general desired requirement are lower value tailing factor and retention time and higher values of resolution and peak area (Prabaningdyah *et al*., 2017). Conventionally, the optimization is performed using OVAT (One Variable in One Time) approach. OVAT is simple, however, this approach failed to identify the interaction among factors, as a consequence, the experimental design based on response surface methodology was employed to overcome this problem (Setyawan *et al*., 2018). One of commonly RSM approach used in HPLC separation is Box-Behnken Design (BBD), as used by Prabandiyah *et al.* (2018) for the optimization of HPLC separation among curcuminoids and Setyawan *et al.* (2018) for the separation of catechins and gallates.

CCD was performed using 30 runnings, employing 4 independent variables (factors), namely pH buffer phosphate (X_1), the acetonitrile ratio (X_2), flow rate (X_3), and column temperature (X_4). The responses (dependent variables) observed were resolution (Rs) between CAR and RDB (Y_1), tailing factor of CAR (Y_2), tailing factor of RDB (Y_3), retention time of CAR (Y_4), retention time of RDB (Y_5), peak area of CAR (Y_6) and peak area of RDB (Y_7). CCD using these factors and responses resulted during optimization (Table I).

Response on resolution (Y1)

Based on analysis of variance (ANOVA) results, the equation obtained using X_1, X_2, X_3, and X_4 as independent variables with resolution of peaks between CAR and RDB (Y_1) as response was:

$\sqrt{Y1} =$ 7.34 - 0.09 X1 - 0.08 X2 - 0.27 X3 + 0.01 X4 (R^2adjusted = 0.9747) (**Eq.1**)

Based on the above equation, the higher resolution (Y_1) could be obtained by increasing column temperature (X_4) and decreasing pH buffer phosphate (X_1), acetonitrile ratio (X_2), and flow rate (X_3). The increasing column temperature has not increased Y_1 significantly, but the decreasing flow rate has decreased significantly. P-values less than 0.05 indicated that model terms are significant. In this case X_1, X_2, X_3 and X_4 are significant model terms. The Predicted R^2 of 0.9678 is in reasonable agreement with the Adjusted R^2 of 0.9747. Factors (independent variables) significantly affected the responses (dependent variables) if $R^2 \geq 0.8$. The difference between Predicted R^2 with the Adjusted R^2 must be less than 0.2. The contour plot of resolution of CAR and RDB and 3D surface graph as a result of variables of pH buffer phosphate, ratio of acetonitrile, flow rate and column temperature (Figure 2). The lower acetonitrile ratio, the lower resolution.

Response of tailing factor carmine (Y2)

Based on ANOVA results, the equation obtained for the quadratic model using X_1, X_2, X_3, and X_4 as independent variables with tailing factor of CAR (Y2) was:

$Y_2 = 12.34 - 2.98X_1 - 0.23X_2 + 3.59X_3 - 0.09X_4 + 0.03X_1X_2 - 0.02X_1X_3 + 0.01X_1X_4 - 0.05X_2X_3 + 0.001X_2X_4 + 0.01X_3X_4 + 0.12X1^2 + 0.001X2^2 - 0.43X3^2 - 0.001X4^2$ (Adjusted $R^2 = 0.9306$) (**Eq.2**)

Based on the above equation, the lower response of tailing factor CAR (Y_2) could be obtained by decreasing flow rate (X_3) and increasing pH buffer phosphate (X_1), acetonitrile ratio (X_2), and column temperature (X_4). The decreasing flow rate has increased Y_2 significantly, but the increasing other variables has not decreased significantly. The statistical results revealed that adjusted coefficient of determination (Adj. R^2) obtained was > 0.8, which was within the acceptable limits (R^2>0.8). This indicated that experimental model was good fit using polynomial equations. Based on ANOVA results, the variables of X_1, X_2 and X_4 as well as interaction between $X_1.X_2$; X_1X_4; $X_2.X_3$ and $X_2.X_4$ and quadratic form of X_1 and X_2 contributed significantly for response of Y_2 ($P < 0.05$). Figure 3 exhibited the contour plot of tailing factor of CAR and 3D surface graph as a results of variables of X_1, X_2, X_3, and X_4. The lower acetonitrile ratio and the higher pH buffer phosphate, the lower tailing factor for carmine (Figure 3).

Response tailing factor Rhodamine B (Y_3)

The equation obtained using X_1, X_2, X_3, and X_4 as independent variables with tailing factor of RDB (Y_3) was linear model, in the form of:

$Y_3 = 0.88 - 0.05X_1 + 0.01X_2 - 0.03X_3 - 0.006X_4$ (Adjusted $R^2 = 0.4867$) (**Eq.3**).

Based on the above equation, X_1, X_2, X_3, and X_4 has not influenced the response of tailing factor RDB (Y_3) significantly. The Eq. 3 corresponded to the response of tailing factor of RDB (Y_3). The statistic results for Y_3 revealed that Adj. R^2 obtained was < 0.8, which was not acceptable. Based on ANOVA results, the variables of X_3 has no interaction with response of Y_3 (P Value > 0.05).

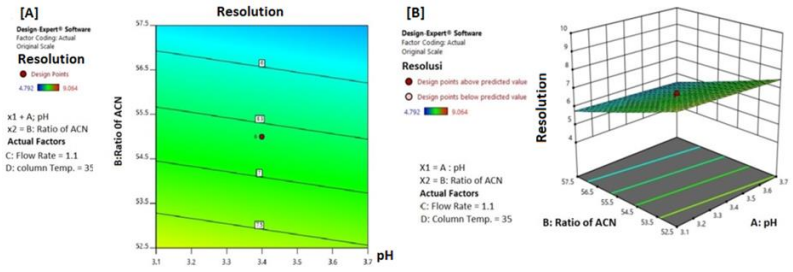

Figure 2. The contour plot of resolution of Carmine and Rhodamine B [A] and 3D surface graph [B] as a results of variables of pH Buffer phosphate, ratio of acetonitrile, flow rate and column temperature.

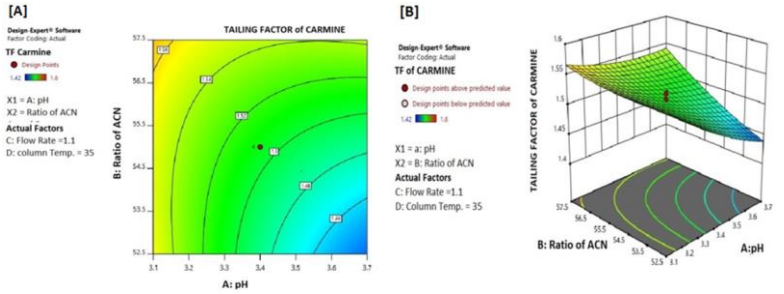

Figure 3. The contour plot of tailing factor of Carmine [A] and 3D surface graph [B] as a results of variables of pH Buffer phosphate (X1), ratio of acetonitrile (X2), flow rate (X3) and column temperature (X4).(X1), ratio of acetonitrile (X2), flow rate (X3) and column temperature (X4).

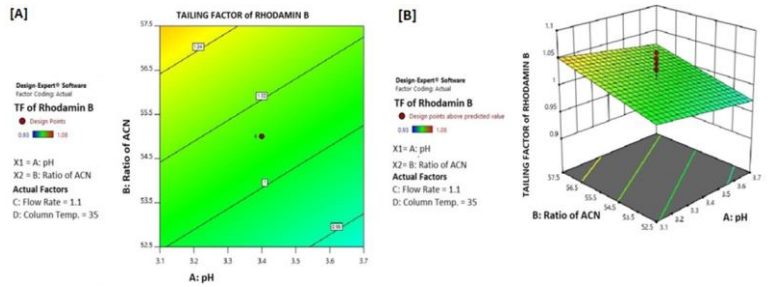

Figure 4. The contour plot of tailing factor of Rhodamine B [A] and 3D surface graph [B] as a results of variables of pH Buffer phosphate (X1), ratio of acetonitrile (X2), flow rate (X3) and column temperature (X4)(X1), ratio of acetonitrile (X2), flow rate (X3) and column temperature (X4).

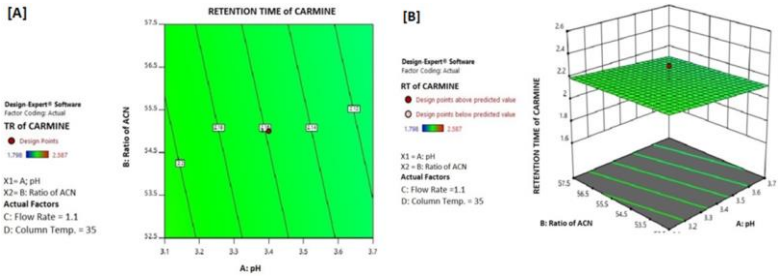

Figure 5. The contour plot of retention time of carmine [A] and 3D surface graph [B] as a results of variablesresult of variables of pH Buffer phosphate (X1), ratio of acetonitrile (X2), flow rate (X3) and column temperature (X4).

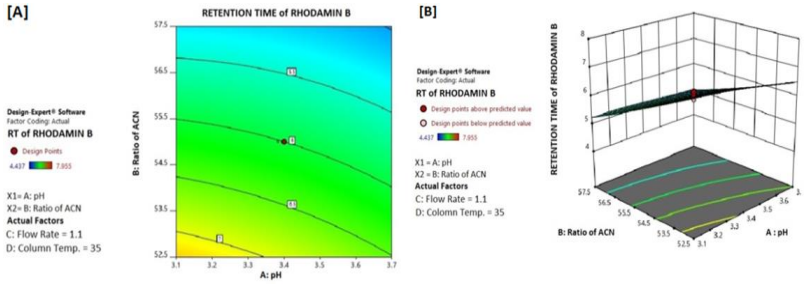

Figure 6. The contour plot of retention time of Rhodamine B [A] and 3D surface graph [B] as a result of pH buffer phosphate (X1), ratio of acetonitrile (X2), flow rate (X3) and column temperature (X4).

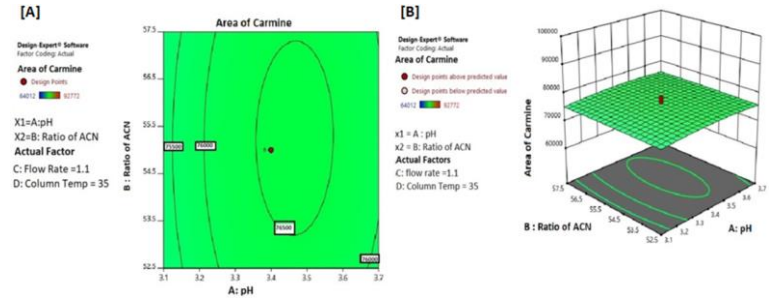

Figure 7. The contour plot of peak area of Rhodamine B [A] and 3D surface graph [B] as a result of variables of pH buffer phosphate (X1), ratio of acetonitrile (X2), flow rate (X3) and column temperature (X4).

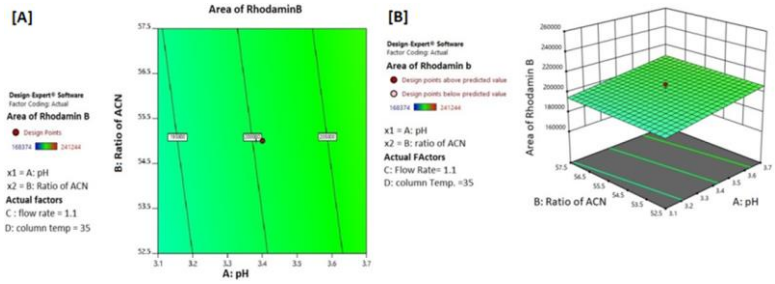

Figure 8. The contour plot of peak area of Rhodamine B [A] and 3D surface graph [B] as a results of variables of pH Buffer phosphate (X1), ratio of acetonitrile (X2), flow rate (X3) and column temperature (X4).

The contour plot of tailing factor of RDB [A] and 3D surface graph [B] as a results of variables of pH Buffer phosphate, ratio of acetonitrile, flow rate and column temperature (Figure 4). Based on the Eq. 3, pH buffer phosphate (X_1) have higher influence for resolution than others. The lower acetonitrile ratio and the higher pH buffer phosphate, the lower tailing factor for RDB (Figure 4).

Response of retention time of Carmine (Y_4)

Based on ANOVA results, the equation obtained for the linear relationship between X_1, X_2, X_3, and X_4 as independent variables with retention time of CRM (Y4) was:

$$Y_4 = 4.84 - 0.15X_1 - 0.001X_2 - 1.98X_3 + 0.007X_4 \quad \text{(Adjustable } R^2 = 0.9152\text{)} \quad \textbf{(Eq.4)}$$

Based on the above equation, the lower retention time of CAR (Y_4) can be obtained by increasing flow rate. The increasing column temperature has not significantly increased Y4. Based on ANOVA results, the statistic results for Y_4 informed that adj.R^2 was > 0.8. The variables of X_1, X_2, X_3, and X_4 was in the linear form and contributed significantly for response of Y_4 ($P<0.05$). Figure 5

exhibited the contour plot of retention time of CAR and 3D surface graph as a result of variables of X_1, X_2, X_3, and X_4. The change of acetonitrile ratio and pH buffer phosphate did not influence to retention time of CAR significantly (Figure 5).

Response of retention time of Rhodamine B (Y_5)

Based on ANOVA results, the equation obtained using X1, X2, X3, and X4 as independent variables with retention time RDB (Y_5) as response was:

$Y_5 = 106.29 - 6.29X_1 - 2.47X_2 - 33.42X_3 + 0.63X_4 + 0.15X_1X_2 + 1.54X_1X_3 - 0.007X_1X_4 + 0.48X_2X_3 + 0.003X_2X_4 - 0.02X_3X_4 - 0.65X1^2 + 0.009X2^2 - 1.26X3^2 - 0.01X4^2$ (Adjustable $R^2 = 0.9884$) (**Eq.4**).

Based on the above equation, the lower retention time of RDB (Y_5) can be obtained by increasing flow rate (X_3). The increasing X_1 and X_2 also lower Y_5. The column temperature (X_4) has not significantly increased Y_5. The statistical results revealed that adjusted coefficient of determination (Adj. R^2) obtained was > 0.8, which was within the acceptable limits (R^2>0.8). This indicated that experimental model was fit using polynomial equations (Sadhukan *et al*., 2016). The variables of X_1, X_2 and X_3 as well as interaction between $X_1.X_2$ and $X_2.X_3$ and quadratic form of X_1, X_2 and X_4 contributed significantly for response of Y_5 ($P < 0.05$). Figure 6 exhibited the contour plot of retention time of RDB and 3D surface graph as a results of variables of X_1, X_2, X_3, and X_4. Based on Fig.6, the higher acetonitrile ratio and the higher pH buffer phosphate, the lower retention time of RDB.

Response of peak area of carmine (Y_6)

Based on ANOVA results, the equation obtained for the relationship between X_1, X_2, X_3, and X_4 as independent variables with peak area of CAR (Y_6) was:

$Y_6 = -1.43E+05 + 62098.49X_1 + 4976.03X_2 - 1.77E+05X_3 + 5970.04X_4 + 27.92X_1X_2 + 22814.58X_1X_3 - 643.25X_1X_4 - 813.75X_2X_3 - 35.83X_2X_4 + 433.75X_3X_4 - 9553.59X1^2 - 26.45X2^2 + 29667.71X3^2 - 30.05X4^2$ (Adjustable $R^2 = 0.9592$) **(Eq. 6)**

Based on the above equation, the higher peak area of CAR (Y_6) can be obtained by increasing pH buffer phosphate (X_1) and decreasing flow rate (X_3). The variables of X_3 as well as interaction between X_1 and X_3 ($X_1.X_3$) and quadratic form of X_1 contributed significantly for response of Y_6 ($P<0.05$). Figure 7 exhibited the contour plot of retention time of CAR and 3D surface graph as a results of variables of X_1, X_2, X_3, and X_4. The change of acetonitrile ratio and pH buffer phosphate did not influence the retention time of CAR significantly.

Response of peak area of Rhodamine B (Y_7)

Eq. 7 revealed the response of peak area of RDB (Y_7) describing the relationship between X_1, X_2, X_3, and X_4 as independent variables with peak area of RDB (Y7).

$Y_7 = 2.88E+05 + 23156.39X_1 + 401.1X_2 - 1.82E+05X_3 + 345.93X_4$ (Adjusted $R^2 = 0.9788$) **(Eq.7)**

Based on the above equation, the higher peak area of RDB (Y_7) can be obtained by increasing pH buffer phosphate (X_1) and decreasing flow rate (X_3). The statistic results for Y_7 informed that adj. R^2 was > 0.8. The variables of X_1 and X_3 contributed significantly for response of Y_3 ($P<0.05$). The variables of X_1 and X_3 affected positively, meaning that the increased levels of pH Buffer (X_1) and flow rate (X_3) would increase peak area of RDB (increased sensitivity). The contour plot along with along with 3D surface graph of peak area of RDB was shown in Figure 8. The change of acetonitrile ratio and pH buffer phosphate did not influence the peak area of RDB.

Based on Eq.7, among four independent variables, pH buffer phosphate (X_1) and flow rate (X_4) has significant influence on the responses (Y). The positive effect of increasing pH buffer phosphate (X_1) was decreasing tailing factor CAR (Y_2) and increasing peak area of CAR (Y_6). The negative effects of higher value of flow rate are decreasing resolution (Y_1), peak area of CAR (Y_6), and peak area of RDB (Y_7) and increasing tailing factor of CAR (Y_2), whereas the positive effect is decreasing the retention time of CAR and RDB. Based on the Figure 2-7, the decreasing acetonitrile ratio and the increasing pH buffer phosphate have some positive effects, such as higher value of resolution and the lower value of tailing factor of CAR and tailing factor of RDB.

The optimum predicted conditions for separation of CAR and RDB based on the statistical results was as follows: pH buffer 3.4, ACN 55%, flow rate of 1.1 mL/min and column temperature of 35^{o}C with desirability of 1. It means that 100% data can be described by selected model, and the desired response would be reached easily. The HPLC chromatogram obtained using this optimum condition was shown in Figure 9. It is clear that CAR and RDB were clearly separated using RP-HPLC

using optimum condition, as suggested by the experimental design of central composite design (CCD).

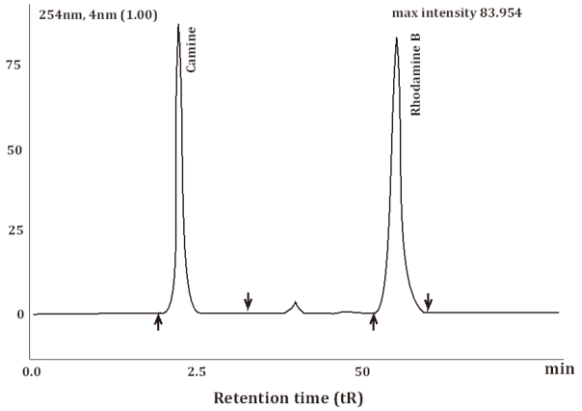

Figure 9. The separation of carmine and rhodamine B using the optimized RP-HPLC condition as suggested by central composite design (CCD). See text for optimum condition of RP-HPLC.

CONCLUSION

Central composite design (CCD) showed that separation of CAR and RDB was influenced by pH buffer phosphate, ratio of acetonitrile, flow rate and column temperature. The optimum predicted conditions for separation of CAR and RDB was pH buffer 3.4, ACN 55%, flow rate of 1.1 mL/min and column temperature of 35^{o}C with desirability of 1. CAR and RDB were clearly separated using optimum condition, as suggested by CCD.

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