ABSTRACT

Objective: This study was intended to optimize reversed-phase high-performance liquid chromatography (RP-HPLC) method for the determination of Tartrazine (TAR) and Auramin O (AUO) in powder drinks using experimental design of central composite design (CCD) approach.

Methods: TAR and AUO in powder drink product has same properties, therefore both analytes were analysed using C18 column (XBridge Shield RP 18 250 mm x 4.6 mm i.d., 5 µm) using Shimadzu LC 20 AD chromatograph equipped with photo-diode array (PDA) detector at 300-650 nm. Some factors responsible for RP-HPLC separation of TAR and AUO including the concentration of buffer, the ratio of mobile phase and flow rate were optimized using CCD. The responses evaluated were peak area, retention time, and tailing factor. The mobile phase used was acetonitrile and ammonium acetate buffer, and acetonitrile composition was optimized at 84-86% for separation of TAR and AUO, delivered at a flow rate of 0.8–1.2 ml/min, using ammonium acetate buffer at 19-21 mmol.

Results: CCD showed that separation of TAR and AUO was influenced by flow rate, the ratio of acetonitrile and ammonium acetate concentration. These factors affected significantly to retention time, peak area, and tailing factor. The optimal condition obtained based on CCD was flow rate of 1.2 ml/min, the ratio of acetonitrile 86%, and ammonium acetate concentration of 19 mmol.

Conclusion: CCD can be used to get optimum condition for analysis of TAR and AUO in powder drink product.

Keywords: Tartrazine, Auramin O, HPLC, CCD, Powder drink

INTRODUCTION

Tartrazine (TAR) is one of the synthetic dyes food additives (BTP) that are permitted to be used on food products to improve the appearance, colour, and texture of foods [1]. Tartrazine (TAR) is azo dyes and auramin O (AUO) is diphenyl methane dyes. The chemical structures of TAR and AUO were shown in fig. 1. TAR is allowed in a food product with a certain maximum value limit. AUO is one of the synthetic dyes that are prohibited to be used in food products. Several studies in various countries have shown that there are cases of counterfeiting of traded food products, including counterfeiting of added dyes because illegal synthetic dyes are cheaper than legal food colouring. Because of its similarity colour, TAR can be replaced by AUO [2].

The synthetic colorants including TAR and AUO are suspected to be unhealthy and unsafe substances for humans, and as a consequence, synthetic colorants became perceptible as undesirable or harmful by consumers [3, 4]. Therefore, the synthetic colours have been the subject of numerous toxicological investigations and their values are established by national and international legislation, especially for their use in food, drinks, drugs and cosmetics [5, 6]. TAR is dangerous if used over safety limits and AUO can cause toxicity and even death for consumers. Therefore, analytical methods capable of detecting and quantifying TAR and AUO must be developed in order to ensure the food safety. Among analytical methods, reversed phase HPLC with a variety of detectors was available for analysis of TAR and AUO.

Fig. 1: The chemical structures of Tartrazine (TAR) and Auramine O (AUO)
Simultaneous determination of TAR and AUO has been carried out by Rohman et al. [7] using HPLC, however, there is no further studies related to simultaneous analysis of two synthetic dyes. Most quantitative analysis of synthetic dyes based on the type of legal or non-illegal colouring agents. The similar solubility of TAR and AUO poses a challenge in the separation of these two dyes simultaneously due to the proximity of polarity and the possibility of large counterfeiting which has the same colour when used in food products. Some methods have been reported for determination of TAR and AUO individually which included thin layer chromatography (TLC) [8], HPLC with the detector of photo-diode array [9-11], liquid chromatography-mass spectrometry (LCMS) [8] and FTIR spectroscopy [12]. The optimization method of HPLC for the simultaneous analysis of TAR and AUO using experimental design is very interesting. Experimental design is a tool having the ability to reveal possible interactions between variables, while saving time and simplifying work [13, 14]. Experimental designs have been widely used to determine the optimum conditions for chromatographic separation in the field of food and pharmaceuticals. Purba et al. [15] have optimized HPLC conditions to determine Acid Orange 7 and Sudan II in blusher products based on response surface methodology using box behenken design (BBD) approach. In this study, reversed-phase high-performance liquid chromatography (RP-HPLC) using experimental design of central composite design (CCD) approach was optimized for the separation of Tartrazine (TAR) and Auramin O (AUO) in powder drinks.

MATERIALS AND METHODS

Powder drink products were obtained from local markets in Yogakarta. Reference standards of Tartrazine (CI 19140, Control Number: 110397), Auramin O (CI 41000, Control Number: B0114315) were acquired from the national agency of drug and food control (NADFC) of Republic of Indonesia. All solvents used for the mobile phase were of HPLC grade and obtained from E. Merck (Darmstadt, Germany). Aquabidest was obtained from Elmaro (Indonesia).

Preparation of reference standards

An approximately of 5.00 mg of each TAR and AUO was accurately weighed using analytical balance (Metler Toledo MX5) with the sensitivity of 0.1 mg, added with 0.5 ml of each standard solutions (TAR and AUO), added with 3 ml aquabidest, sonicated for 5 min, and with added with aquabidest to volume 10 ml. The solution was filtered with PTFE 0.45 µm. The solution was injected into HPLC system.

HPLC instrumentation

TAR and AUO were analysed using chromatograph of Shimadzu LC 20AD chromatograph equipped with photo-diode array (PDA) (Shimadzu LC 20AD, M20A PDA Detector) at the wavelength of 300-650 nm. Separation of analytes was performed using C18 column (XBridge Shield RP 18 250 mm x 4.6 mm i.d., 5 µm). The mobile phase was modified from the method of determining Tartrazine [10], used water as solvent, the composition of acetonitrile was optimized at 84-86% for separation TAR and AUO, delivered at a flow rate of 0.8-1.2 ml/min, using ammonium acetate buffer at 19-21 mmol.

Experimental design using CCD

The most relevant multivariate techniques is response surface methodology (RSM), an optimization based on fit of the polynomial equation to data experiment. Symmetrical design of RSM, namely central composite design (CCD) and box behenken design (BBD) are frequently used in HPLC method optimization because they can resolve HPLC separation-related problems which the number of factors is higher than 2 [16,17]. CCD and BBD differs in the selection of experimental point, variables number, as well as number of run and block. Central composite design (CCD), which is a widely used form of RSM, encompasses the advantages of factorial design [16]. The factors (independent variables) evaluated were flow rate of mobile phase (X1), the ratio of acetonitrile for separation of TAR and AUO (X2), and ammonium acetate buffer concentration (X3). While the responses or dependent variables evaluated included retention time TAR and AUO (Y1 and Y2), peak area TAR and AUO (Y3 and Y4), tailing factor TAR and AUO (Y5 and Y6).

<table>
<thead>
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<th>Std</th>
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<td>Conc. ACN (%) (X2)</td>
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Conc. = concentration; TAR = tartrazine; AUO = Auramine O; ACN = acetonitrile.
RESULTS AND DISCUSSION

Quantitative analysis of different dyes is most often performed in reversed phase (RP) or ion pair (IP) systems, while is usually based on measurements via UV–VIS detection, especially using diode array detector (DAD). The similar polarity between TAR and AUO might be copied by isocratic elution method, therefore the separation of TAR and AUO is in one condition. Reversed phase (C18) column retained TAR and AUO in high concentration of non-polar solvent, therefore an experimental design approach was used. Central-composite design (CCD) was used for HPLC separation of TAR and AUO. CCD was performed using 20 runs, applying 3 independent variables (factors) namely flow rate (X1), ratio of acetonitrile for separation of TAR and AUO (X2), and ammonium acetate buffer concentration (X3) along with response variables of retention time TAR (Y1), retention time AUO (Y2), peak area TAR (Y3), peak area AUO (Y4), tailing factor TAR (Y5) and tailing factor AUO (Y6). CCD using these factors and responses resulted during optimization were compiled in table 1. Based on analysis of variance (ANOVA) results, the equation obtained using X1, X2, and X3 as independent variables with the retention time of TAR (Y1) as the response was:

\[ Y_1 = -0.065364 - 7.77684X_1 - 0.143565X_2 + 0.0118803X_3 + 0.001875X_1^2 - 0.000625X_2^2 + 2.29397X_3^2 + 0.00872X_1X_2 - 0.001402X_1X_3 \]

\[ (Adj. R^2 = 0.9954) \] (Eq.1).

The statistic results revealed that adjusted \( R^2 \) obtained was > 0.8 acceptable [18], exhibiting that the experimental model was a good fit using polynomial equation. The difference between predicted \( R^2 \) with the adjusted \( R^2 \) in all responses was less than 0.2. Eq.1 informed that variables of flow rate (mL/min) (X1), ratio of ACN (%) (X2), and ammonium acetate concentration (X3) have a negative effect on the retention time of TAR.

The variables of X1, X2, and X3 had a quadratic form of X3 contributed significantly for response of Y1 (P<0.05) based on one way ANOVA results. The variables of X3 affected negatively, meaning that the increased levels of flow rate (X1) would decrease the retention time of TAR (decreased sensitivity), while the increased ratio of acetonitrile (X2) and ammonium acetate concentration (X3) could increase the retention time of TAR. Contour plot of retention time of TAR along with 3D surface graph was shown in fig. 2.

Similarly, the equation for retention time AUO (Y2) using multiple linear regression were:

\[ Y_2 = -41.79701 - 10.59627X_1 + 1.14694X_2 + 0.430479X_3 - 0.01875X_1^2 - 0.00625X_2^2 + 2.29397X_3^2 + 0.00872X_1X_2 - 0.001402X_1X_3 \]

\[ (Adj. R^2 = 0.9961) \] (Eq.2).

The contour plot along with with 3D surface graph of retention time of AUO was shown in fig. 3. Statistic parameter of Y2 revealed adjusted \( R^2 \) (Adj. \( R^2 \)) was > 0.8 acceptable [17, 18] exhibiting that the experimental model was a good fit using the polynomial equation. Based on ANOVA results from variables of X1, X2, and X3, the quadratic form of X3 contributed significantly for the response of Y2 (P<0.05). The variables of X1 affected negatively, meaning that the increased levels of flow rate (X1) would decrease the retention time of AUO (decreased sensitivity), while the increased ratio of acetonitrile (X2) and ammonium acetate concentration (X3) could increase the retention time of AUO.

FIG 2: The contour plot of retention time (in minute) of tartrazine (TAR) [A] and 3D surface graph of retention time of TAR [B] as a results of variables of flow rate (mL/min), concentration of acetonitrile (%), and ammonium acetate concentration

FIG 3: The contour plot of retention time of Auramine O (AUO) [A] and 3D surface graph of retention time of AUO [B] as a results of variables of flow rate (mL/min), the concentration of acetonitrile (%), and ammonium acetate concentration
Eq. 3 revealed the response of peak area TAR (Y3). The statistic results for Y3 informed that adj R² was >0.8. The variables of X1, X2, X3 and X4, linear form of X1 and quadratic form of X2 and X3 contributed significantly for the response of Y3 (P<0.05). The variables of X1, X2 and X3 affected positively, meaning that the increased levels of flow rate (ml/min) (X1), acetonitrile (%) (X2) and ammonium acetate concentration (mM) (X3) would increase peak area of TAR (increased sensitivity).

\[
Y_3 = -8.39256 \times 10^8 - 2.27267 \times 10^6 X_1 + 1.88030 \times 10^7 X_2 + 4.55925 \times 10^6 X_3 - 12551.25000 X_1 X_2 - 1677.50000 X_1 X_3 - 1010.50000 X_2 X_3 + 2.7207 \times 10^5 X_1^2 - 1.10407 \times 10^5 X_2^2 - 1.1881 \times 10^5 X_3^2 \quad (\text{Adj. R}^2 \text{of} \ 0.8836) \quad (\text{Eq. 3})
\]

Eq. 4 showed the correlation between response of peak area of Auramine O (AUO) and independent variables of X1, X2, and X3 along with its interaction. The statistic results for Y4 showed that Adj. R² obtained was in the acceptable limits [18]. The ANOVA results revealed that variables of X1, X2, X3, and X4, quadratic form of X1 contributed significantly for the response of Y4 (P<0.05). Based on ANOVA results in variables of X1, X2, X3, and X4, quadratic form of X1 and X2 contributed significantly for the response of Y4 (P<0.05).

\[
Y_4 = 2.64137 \times 10^7 - 1.93302 \times 10^7 X_1 + 1.41257 \times 10^5 X_2 - 1.18438 \times 10^6 X_3 - 51320.00000 X_1 X_2 - 60133.75000 X_1 X_3 - 14398.50000 X_2 X_3 + 339.03771 X_1^2 + 0.032298 X_2^2 + 0.026218 X_3^2 \quad (\text{Adj. R}^2 \text{of} \ 0.9956) \quad (\text{Eq. 4})
\]

Eq. 5 and 6 corresponded to the response of tailing factor of TAR (Y5) and AUO (Y6). The statistic results for Y5 revealed that Adj. R² obtained was <0.8, which was not acceptable. Based on ANOVA results variables of X1, X2, X3, and X4, quadratic form of X1, X2, and X3 contributed significantly for the response of Y5 (P<0.05).

\[
Y_5 = 254.92078 - 4.62826 X_1 - 5.59046 X_2 - 1.44966 X_3 + 0.033 X_1 X_2 - 0.001875 X_1 X_3 + 0.004875 X_2 X_3 + 0.907344 X_1^2 + 0.032228 X_2^2 + 0.026218 X_3^2 \quad (\text{Eq. 5})
\]

\[
Y_6 = -352.13095 + 1.84260 X_1 + 7.89106 X_2 + 1.64942 X_3 + 0.007500 X_1 X_2 + 0.006250 X_1 X_3 + 0.007500 X_2 X_3 + 1.13995 X_1^2 - 0.046305 X_2^2 - 0.039765 X_3^2 \quad (\text{Adj. R}^2 \text{of} \ 0.2471) \quad (\text{Eq. 6})
\]

Fig. 4 and fig. 5 showed the contour plot along with 3D surface graph of tailing factor of TAR and AUO.

Central composite design using three optimum factors namely flow rate (1.2 ml/min), ratio of acetonitrile (86 %) and ammonium acetate concentration (19 mmol) was successfully used to get an optimum condition of HPLC method for analysis of TAR and AUO in powder drink samples. The HPLC chromatogram obtained using this condition was shown in Fig.6. It is clear that both TAR and AUO were clearly separated using optimum condition suggested by CCD.
CONCLUSION

CCD design can be used to get optimum condition for analysis of TAR and AUO in powder drink product. The optimum conditions suggested for separation TAR and AUO based on CCD was the mobile phase containing ACN 86% with a flow rate of 1.2 ml/min, with ammonium acetate buffer concentration of 19 mmol.

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AUTHORS CONTRIBUTIONS

ADL conducted research activity, compiled data, and prepared manuscript. AR and SM designed research activities, prepared manuscript and made critical thinking on the manuscript.

CONFLICT OF INTERESTS

The authors have declared “no conflicts of interest with respect to the research, authorship, and/or publication of this article”.

REFERENCES