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Central Composite Design for Optimizing Derivatization of Metformin and Guanylurea in Water Samples Detected by Gas Chromatography - Mass Spectrometry

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Abstract: Emerging pollutants (EPs) are chemicals produced by human and industrial activities such as pharmaceuticals. Metformin, a widely used first-line oral drug for type 2 diabetes, is difficult to be metabolized inside the human body and thus becomes an emerging environmental contaminant with growing concerns. Guanylurea is metformin's biotransformation product. To help better track the occurrence of the two compounds in the environments, the improvement of methods for metformin and guanylurea analysis is necessary. Derivatization of metformin and guanylurea is the key pre-treatment procedure before the analysis of metformin and guanylurea by gas chromatography-mass spectrometry (GC-MS). Central Composite design (CCD), a statistical design of experiments (DOE) methodology, was applied to identify the impact of factors affecting the derivatization reactions of metformin and guanylurea. The four factors included within the CCD modelling are temperature (70-90ºC), reacting time (40-70minutes), solvent (acetonitrile, 1,4-dioxane), and ratio (0.5-1.5:1) of reagent to target component. Buformin and N-methyl-bis(trifluoroacetamide) were used as the internal standard and derivatization reagent, respectively. The optimal conditions for enhancing the sensitization of metformin and guanylurea derivatization performance were obtained.

1.Introduction

Emerging pollutants (EPs) are chemicals produced by human and industrial activities and formed from the release of lots of chemical products, such as pharmaceuticals, personal care products, food additives, laundry detergents, preservatives, dyes, paints, pesticides, and contrast media (Grassi, et al, 2012). Metformin and guanylurea are defined as EPs due to the widely usage of metformin, the incomplete treatment of metformin in wastewater treatment plants (WWTPs), and its low biodegradation capacity in the environment (Niemuth and Klaper, 2018). Metformin could play an endocrine disruptor in juvenile fathead minnows (Crago et al., 2016; Niemuth and Klaper, 2015), and could affect the food chain after its discharge into the aquatic system (Eggen and Lillo, 2012). Guanylurea could have an anti-mitotic effect to inhibited root growth of onions (Turno et al., 1960). The implementation of monitoring programmes has been recommended to assess the environmental persistency of metformin and guanylurea because the

occurrence of metformin and guanylurea in the aquatic environment has been frequently reported with increased concentrations (Markiewicz et al., 2017a, b).

Metformin ($C_4H_{11}N_5$, 165.63 g mol^{-1}), a biguanide in chemical classification, is one of the most effective oral drugs for type 2 diabetes (Chaudhury et al., 2017; Cho et al., 2018; IDF, 2015). Metformin is prescribed within 119 countries of the world and more than 200 million people are taking metformin for controlling glucose (Kyzas et al., 2015; WHO, 2016). However, the pharmacokinetics of metformin in rats indicated that 92–100% of metformin taken by rats could not be metabolized by body and was directly discharged after usage (Gabr et al., 2017). This resulted in the existence of metformin in both urine and feces samples. Guanylurea ($C_2H_6N_4O$, 102.10 g mol^{-1}) is the main biodegraded transformation product formed from metformin through an aerobic biodegradation process in sewage (Tisler and Zwiener, 2018).

Methods for the detection of metformin and guanylurea have been developed in recent years based on chromatography (Brack et al. 2015; Goedecke et al., 2017; Majidano and Khuhawar, 2012; Trindade et al., 2018; Ucakturk, 2013; USEPA, 2007). Goedecke et al. (2017) reported that the gas chromatography-mass spectrometry (GC-MS) method could detect low concentrations of metformin in water samples. Derivatization of metformin and guanylurea is the key pre-treatment procedure before the analysis of metformin and guanylurea by GC-MS. Since metformin and guanylurea are non-volatile biguanides, the direct volatilization during GC–MS analysis could degrade these non-volatile biguanides if without proper derivatization treatment. However, the derivatization performance still needs to be enhanced to achieve sensitive and accurate detection of low concentrations of metformin and guanylurea in wastewater and surface water samples by GC-MS.

Design of experiments (DOE) can help to evaluate the configurations of basic experimental design and material alternatives, to select parameters of design for working well with different field conditions, to obtain the key parameters influencing performance of design, and to optimize the ideal results of models. Thus, designing experiments is helpful to effectively achieve computer simulations or laboratory experiments (Lye, 2002). There are many different types of experimental statistical design techniques (Lye, 2018) and the response surface methodology (RSM), has been identified as one of the most useful design for optimizing the ideal conditions of a process. RSM containing mathematical and statistic techniques is used to model and analyze a response of interest affected by several variables and to optimize the response. Based on RSM, the optimization function could be obtained including the maximum, minimum, or target to a set of results. Curvature response surface can be determined under the RSM. Central Composite design (CCD) is a popular RSM design to fit the second order model. CCD is generally constructed with a fractional factorial part of Resolution V following star or axial points with couple center points (Lye, 2018). In this study, derivatization of metformin and guanylurea has been optimized via CCD.

Previous studies using GC-MS for determination of metformin in aqueous samples were summarised (Tao et al., 2018). Various studies have implemented the derivatization of metformin using one-factor-at-a-time (OFAT) (Goedecke et al., 2017; Majidano and Khuhawar, 2012; Ucakturk, 2013), but no relevant studies using CCD for the system optimization. Therefore, in this study, CCD was employed to develop a model for evaluating factors influencing the derivatization of metformin and guanylurea. The four factors included within the CCD modelling are temperature (70-90ºC), reacting time (40-70minutes), solvent (acetonitrile, 1,4-dioxane), and ratio (0.5-1.5:1) of reagent to target component (Goedecke et al., 2017; Majidano and Khuhawar, 2012; Ucakturk, 2013). Through evaluating the impact of each factor, the optimal conditions for enhancing the sensitization of metformin and guanylurea derivatization performance were obtained.

2. Methodology

The factors selected include temperature, reaction time, solvents and ratio between the reagent and target chemical (Table 1). The reagent is important to metformin achieve derivatization reaction for preparing procedure of GC-MS method. The reagents applied (Goedecke et al., 2017; Majidano and Khuhawar, 2012; Ucakturk, 2013) were methylglyoxal (MGo), N-methyl-bis(trifluoroacetamide) (MBTFA), N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA), N-methyl-N (trimthylsilyl)trifluoroacetamide (MSTFA), and MSTFA/imidazole. Buformin and diphenylamine (IS) were respectively used as internal standard for derivatization (Goedecke et al., 2017; Ucakturk, 2013). Based on the references (Goedecke

et al., 2017; Majidano and Khuhawar, 2012; Ucakturk, 2013), MBTFA and buformin were selected as the derivatization reagent and internal standard, respectively, in this study.

Table 1. Range of factors in previous derivatization reactions

Derivatization reagent	Temperature	Time	Solvent	Reference
MGo	90°C	30 minutes	pH 7.5 buffer	Majidano and Khuhawar (2012)
MBTFA, MTBSTFA, MSTFA, MSTFA/imidazole	80°C	60 minutes	none	Ucakturk (2013)
MBTFA	60°C	60 minutes	acetonitrile	Goedecke et al. (2017)

Guanylurea has never been detected with metformin using the GC-MS method. At the initial exploratory stage, OFAT experiment was used to determine the range of three numeric factors. Four chemicals were involved in the derivatization reaction, which are two emerging pollutants (metformin and guanylurea), one internal standard for indicating derivatization reaction (buformin), and one reagent (MBTFA).

2.1 Determination of the Factor Levels

Different temperatures, ratios, and solvents were tested by OFAT. The data of OFAT is not shown (available upon request). The selected high and low levels of the four factors are illustrated in Table 2. In addition, the value of alpha was set as 1.5 instead of 1.68179 ($k<6$) because the ratio of reagent and each target chemical should be geometric progression.

Table 2. High and low levels of four examined factors

Name	Factor	High	Low	Center	-alpha	+alpha
Temperature(°C)	A	90	70	85	65	95
Time (minutes)	B	70	40	55	32.5	77.5
Ratio	C	0.5:1	1.5:1	0.75:1	0.25:1	1.75:1
Solvent (categoric)	D	1,4-dioxane	Acetonitrile	NA	NA	NA

2.2 Responses

The responses were the ratios of peak areas between guanylurea and metformin generated by GC-MS analysis. The column applied is HP-5ms of Agilent (30m X 0.25μm X 0.25mm). The volume of injected sample was 1μL. The initial temperature in the GC oven was set at 80°C, then raised to 110°C at a rate of 6°C min^{-1}. Then following temperature rouse to 210°C with a rate of 15°C min^{-1}. Finally, the temperature was up to 230°C with a rate of 20°C min^{-1}. The flow rate of helium was at 1.0 mL min^{-1}. The injector and detector temperatures were 250 and 280°C. The total run time of the GC-MS analysis was 12.67 minutes.

2.3 Experimental Procedure

Preparation of stock solutions is the initial procedure of the experiment. The standard solutions of buformin, metformin and guanylurea were prepared by dissolving an appropriate amount of the substances in methanol to reach a concentration of about 1 mg mL^{-1}. The standard solutions were stored at 4°C in the dark. During the derivatization reaction, there are several steps involved. Firstly, 10 μL standard solutions of buformin, metformin and guanylurea should be added into a vial. The solutions are dried by nitrogen gas. A certain amount of reagent MBTFA (ratio of reagent to target chemicals from 0.5:1 to 1.5 :1) will be added into the vial following 100 μL solvent to the residue. Water bath is set at a certain temperature (70-90°C) for a defined time interval minute (40-70 minutes). Afterwards, the vial is taken out of the water bath and a certain amount of solvent is added up to 1 mL for GC-MS measurement.

3. Results and Discussions

3.1 Results of CCD

Design-Expert® (State-Ease, 2018) software in version 11 was employed to design and analyze the data. Using the CCD design, 40 runs were conducted to determine the effect of the four factors on performance of derivatization. For three numeric factors, 20 runs including 6 runs of center point were involved. Then, one categoric factor will double the 20 runs to 40 runs. The response equals to the peak area of guanylurea / the peak area of metformin (response=G/M). All the responses were illustrated in Table 3.

Table 3. The design and input responses

Run	Factor 1 A: Temperature	Factor 2 B: Time	Factor 3 C: Ratio	Factor 4 D: Solvent	Response G/M
1	80	77.5	1.00	dioxane	0.46
2	90	40.0	1.50	dioxane	0.56
3	70	70.0	1.50	acetonitrile	0.33
4	70	70.0	0.50	dioxane	0.25
5	80	55.0	1.00	dioxane	0.33
6	80	55.0	1.00	dioxane	0.35
7	90	40.0	0.50	dioxane	0.45
8	80	55.0	1.00	acetonitrile	0.25
9	80	55.0	1.00	acetonitrile	0.21
10	70	70.0	1.50	dioxane	0.41
11	70	40.0	1.50	acetonitrile	0.31
12	80	55.0	1.00	dioxane	0.37
13	80	55.0	1.00	dioxane	0.37
14	80	55.0	1.75	dioxane	0.51
15	90	40.0	0.50	acetonitrile	0.28
16	80	55.0	1.00	acetonitrile	0.29
17	70	40.0	0.50	dioxane	0.23
18	65	55.0	1.00	dioxane	0.33
19	80	55.0	1.00	acetonitrile	0.28
20	70	40.0	0.50	acetonitrile	0.21
21	90	70.0	1.50	dioxane	0.57
22	80	32.5	1.00	acetonitrile	0.27
23	90	70.0	0.50	dioxane	0.53
24	80	55.0	1.75	acetonitrile	0.48
25	80	55.0	1.00	acetonitrile	0.36
26	70	40.0	1.50	dioxane	0.46
27	80	55.0	0.25	dioxane	0.19
28	90	70.0	1.50	acetonitrile	0.48
29	90	70.0	0.50	acetonitrile	0.36
30	80	77.5	1.00	acetonitrile	0.42
31	80	32.5	1.00	dioxane	0.32
32	90	40.0	1.50	acetonitrile	0.55
33	95	55.0	1.00	acetonitrile	0.50
34	65	55.0	1.00	acetonitrile	0.26
35	70	70.0	0.50	acetonitrile	0.19
36	80	55.0	1.00	dioxane	0.38
37	95	55.0	1.00	dioxane	0.64
38	80	55.0	1.00	acetonitrile	0.35
39	80	55.0	0.25	acetonitrile	0.12
40	80	55.0	1.00	dioxane	0.47

The quadratic process was ordered with backwards selection. The ANOVA table was illustrated in Table 4. The effect A, B, C, D, and A^2 are significant because of their p-values less than 0.05. The values of R^2 and

adjusted R^2 are 0.8626 and 0.8143, respectively, (Table 4) and show both a good fit and satisfactory predictive accuracy.

Table 4. The ANOVA Results

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	0.5070	5	0.1014	42.78	< 0.0001
A-Temperature	0.1962	1	0.1962	82.79	< 0.0001
B-Time	0.0102	1	0.0102	4.30	0.0457
C-Ratio	0.1918	1	0.1918	80.93	< 0.0001
D-Solvent	0.0706	1	0.0706	29.77	< 0.0001
A^2	0.0382	1	0.0382	16.11	0.0003
Residual	0.0806	34	0.0024		
Lack of Fit	0.0523	24	0.0022	0.7707	0.7134
Pure Error	0.0283	10	0.0028		
Cor Total	0.5876	39			
R^2	0.8628				
Adjusted R^2	0.8143				

In addition, all the assumptions of regression such as normality of residuals, constancy of variance, and lack of fit, etc. were fulfilled. The necessary diagnostic plots for verifying the ANOVA reliable were illustrated in Figure 1. The points scatter a linear trend around the indicate line in normal plot of residuals and predicted versus actual plot in Figure 1-(a) & (b). In the normal plot of residual, although the points scattering shows a very slight “S-shape” pattern, the residuals actually appear fairly normal in this case. The points scatter randomly well in residual versus run plot (Figure 1-(d)) indicating the residuals conditions of this assumptions satisfied. The points randomly scatter close to the linear in residual versus predicted plot (Figure 1-(c)) indicating the good fit and no consistent under or over the predicted model.

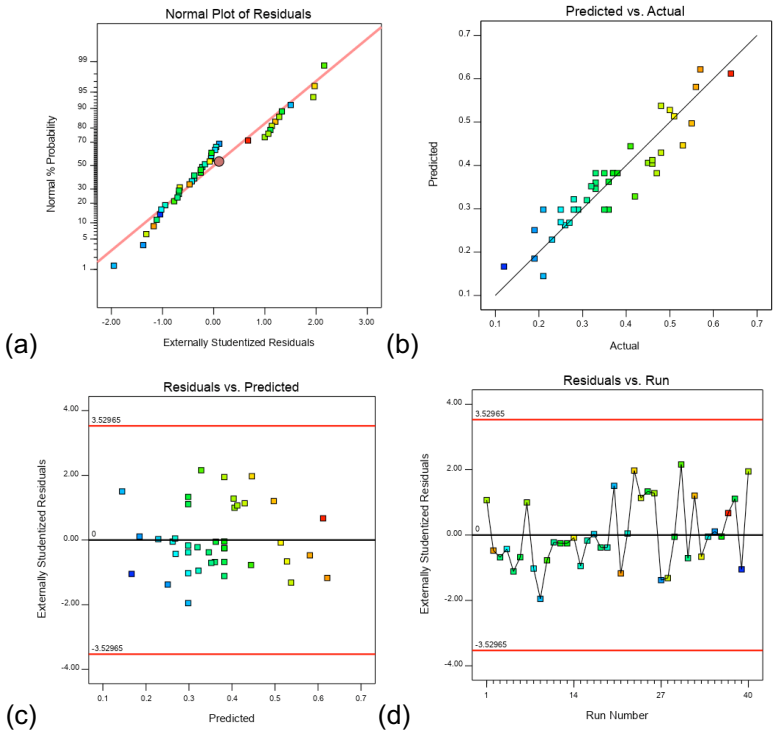

Figure 1. Diagnostic plots, (a) normal plots of residuals, (b) predicted and actual plot, (c) residuals and predicted plot, (d) residuals vs runs plot

3.2 CCD Data Analysis

All the ANOVA assumptions were reasonably fitted with the second-order polynomial model in form of coded factor scale is defined in Equation 1. The Equation 1 can be converted to actual factor scale to give Equation 2 & 3 due to different solvents. In the coded model, the temperature (A) and ratio (C) are similar magnitude effects on the efficiency of the response (G/M). High level of temperature and ratio can increase the responses obeying expected assumption.

[1] $G/M = 0.3401 + 0.0886A + 0.0202B + 0.0876C + 0.042D + 0.043A^2$

[2] $G/M = 2.09298 - 0.05998\ \text{Temperature} + 0.001347\ \text{Time} + 0.1752\ \text{Ratio} + 0.00043\ \text{Temperature}^2$ (in solvent acetonitrile)

[3] $G/M = 2.17798 - 0.05998\ \text{Temperature} + 0.001347\ \text{Time} + 0.1752\ \text{Ratio} + 0.00043\ \text{Temperature}^2$ (in solvent 1,4-dioxane)

This is the first study that discovers the relationships between the factors in derivatization reaction of metformin and guanylurea for the GC-MS method. In this design, it is found that the responses of G/M display linear with factor time and ratio and curvature shape with factor temperature. The reason is probably the selection of the levels of these numeric factors.

The temperature is the first important factor to the derivatization reaction because the reaction is sensitive to the temperature (Goedecke et al., 2017; Ucakturk, 2013). The range of temperature was detected from 20 to 60°C for derivatization reaction of metformin by Goedecke et al. (2017) because the boiling point of acetonitrile is 82°C. The results showed that the responses of metformin were increased with increasing temperatures (Goedecke et al., 2017). The temperature at 80°C was used to do the derivatization reaction of metformin without solvent by Ucakturk (2013). In two reviewed studies (Goedecke et al., 2017; Ucakturk, 2013), the range of temperature were tested from 20 to 80°C with or without solvent acetonitrile. Thus, temperature was tested from 60 to 90°C without solvents in the previous OFAT experiments because of the boiling point of acetonitrile. The results indicated that metformin had good performance with increasing temperatures no matter with or without the solvent. However, guanylurea did not perform well without a solvent, thus, solvent should be involved in the derivatization reaction. In addition, responses of metformin and guanylurea detectability increased with solvents due to increasing temperatures based on the results of previous OFAT experiments. Thus, the range of temperature has been confined with the following range of 70-90°C as documented in Table 2.

Due to the bad performance of guanylurea without a solvent in the derivatization reaction, the selection of solvents was operated through comparing the boiling point and chemical polarity. Acetonitrile has been used in reviewed study (Goedecke et al., 2017). Two more solvents were selected to test, such as 1,4-dioxane, and toluene. The boiling points of 1,4-dioxane and toluene are 101.1°C and 110.6 °C, respectively (ATSDR, 2012). The results of previous OFAT experiment revealed that 1,4-dioxane performed good at responding to metformin and guanylurea in the derivatization reaction at 80°C with ratio at 1:1 for 60 minutes. Note that toluene does not solve polar chemical very well. Thus, 1,4-dioxane and acetonitrile are selected as solvents.

The ratio of reagent to target chemicals is another important factor to affect the response of derivatization reaction. The previous OFAT experiments tested the range of ratio at 80°C and 60 minutes with or without the solvent, acetonitrile. The range of ratio was selected from 0.5:1 to 1.5:1. The results of previous experiments showed that the high ratio could affect the derivatization reaction to obtain better responses of metformin and guanylurea. The reaction time has been detected at 30 minutes, 60 minutes, and 90 minutes by Ucakturk (2013) for derivatization reaction of metformin. The highest response of metformin was obtained at 60 minutes (Ucakturk, 2013). Hence, the range of reaction time is selected from 40 minutes to 70 minutes.

The effect of individual variances can be better understood visually through graphs illustrated in Figure 2. It is evident that temperature and ratio have higher increasing slopes indicating the importance of these two factors in determining the response of G/M. The significant curvature trend of factor A (Time) matches to

the model. The slope of time is the flattest of all the slopes which means that time minimally affects the response of G/M. There is an obvious increase in the responses of G/M via changing solvents from acetonitrile to 1,4-dioxane showing that 1,4-dioxane displays a good performance in the derivatization reaction.

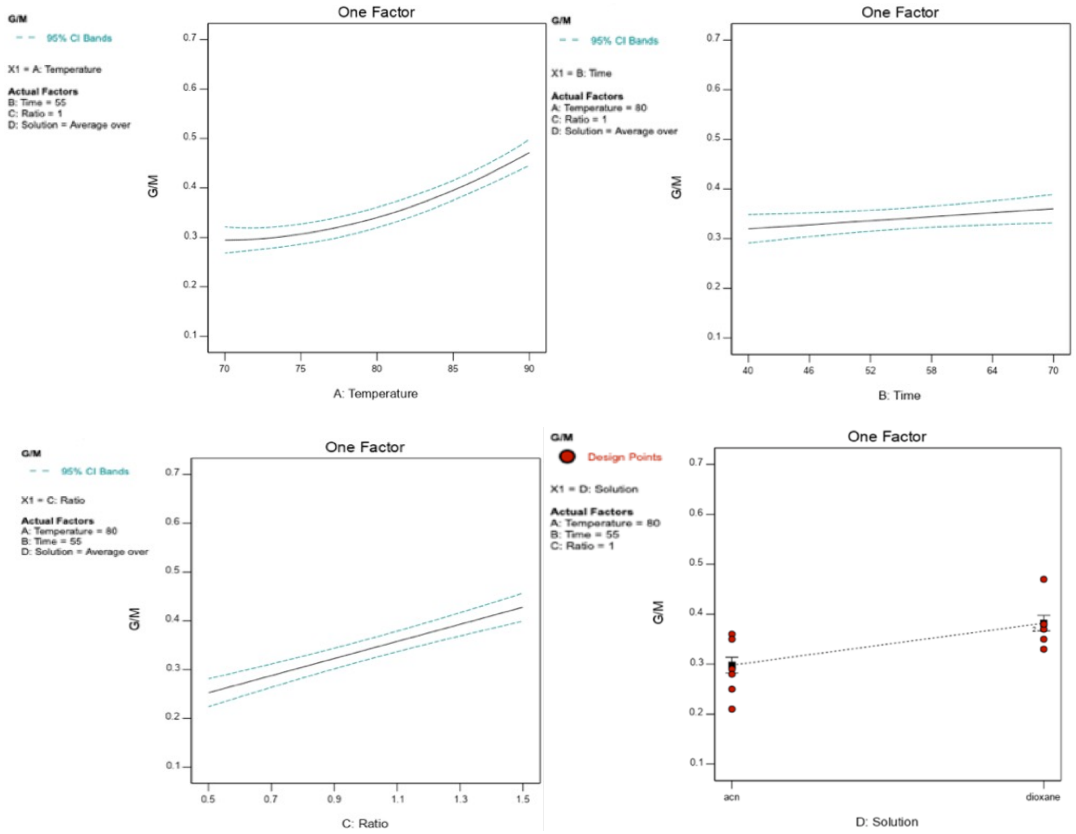

Figure 2. Model graphs of important factors

Overall, interaction plots are unavailable because there are no significant interactions between the four factors. The interactions between the four factors are respectively illustrated in three-dimensional response surface plots (Figure 3). In Figure 3-(a) & (b), it is obvious that the responses of G/M in solvent 1,4-dioxane are much higher than the responses in solvent acetonitrile. In addition, the responses of G/M at high ratios are much higher than the responses at low ratios in Figure 3-(c) & (d).

All the factors were set in range to optimize the maximum response. The maximum result of G/M was 0.622 with desirability at 0.964, and the temperature, time, ratio, and solvent were 90°C, 70 minutes, 1.5:1, and 1,4-dioxane respectively. In addition, the shorter reaction time, the better performance of GC-MS anlysis. The factor of time was selected to be minimum while other factors were set in range. The optimal result with minimum time (40 minutes) was that G/M equals to 0.581 with desirability at 0.942, and temperature, ratio, and solvent were 90°C, 1.5:1, and 1,4-dioxane, respectively.

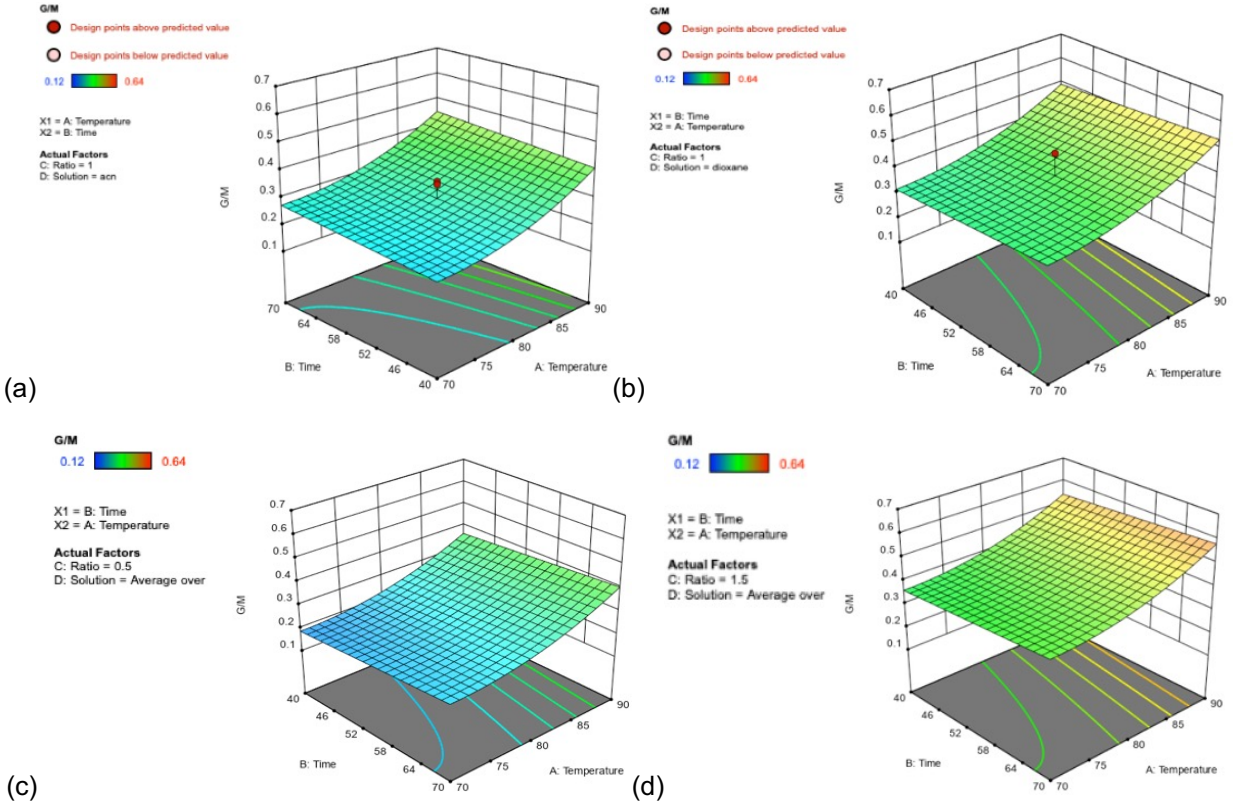

Figure 3. 3-D response surface plot of temperature and time in different solvents and ratios, (a) acetonitrile, (b) 1,4-dioxane, (c) ratio=0.5:1, (d) ratio=1.5:1

3.3 Model Validation

Validation procedure is necessary to ultimately test a model, and in this procedure, points will be selected within the range of factors but not at specified test levels. Two additional samples have been used for model validation. In addition, four runs with optimal conditions of derivatization reaction have also been done to confirm whether the model is an adequate representation of the experiment. One replication of the optimal condition with all factors in range and three replications of the optimal condition with minimum time have been performed. All the predicted results for two additional runs and four optimized runs were illustrated in Table 5 which includes the predicted results as well. The results show that the responses of validation run, optimal run, and prediction agree with the error of the experiment. The values fall within the 95% prediction interval, indicating a valid model.

Table 5. Validation runs comparison

Run	Temperature (°C)	Time (minute)	Ratio	Solvent	Response (G/M)	Prediction (G/M)	95% PI Low	95% PI High
1	75	60	1.25:1	Acetonitrile	0.3078	0.3151	0.2122	0.4180
2	75	50	0.75:1	1,4-dioxane	0.2673	0.2980	0.1951	0.4009
Optimal-1	90	70	1.5:1	1,4-dioxane	0.5727	0.6220	0.5142	0.7289
Optimal-2	90	40	1.5:1	1,4-dioxane	0.4803	0.5811	0.4738	0.6885
Optimal-3	90	40	1.5:1	1,4-dioxane	0.4914	0.5811	0.4738	0.6885
Optimal-4	90	40	1.5:1	1,4-dioxane	0.4909	0.5811	0.4738	0.6885

4.Conclusions

Experimental results indicate that it is possible to design a model for performing the behavior of derivatization reaction. The CCD performs well with the application of DOE in this study. The developed quadratic model provides a reasonable fit and has successfully predicted the results of validation experiments. Based on these results, it is clear that increasing temperature and ratio will be helpful to obtain effective derivatization reaction. In addition, the reaction time has been determined as lowest effect to the derivatization reaction which is good to the preparing procedure of GC-MS method, because the rapid sample preparation can cut short the sample detection time. However, there exists a limitation of the range of temperature due to the boiling point of solvent acetonitrile this procedure a low point in the curvature of temperature factor plot. In addition, the model indicates that 1,4-dioxane is better than acetonitrile as a solvent in the derivatization reaction which means that in the future work higher temperature can be used because of the high boiling point of 1,4-dioxane. The validation tests the quadratic prediction model. The responses of two validation trials and four optimization trial which have been predicted by the model equation to match up to the actual results of experiments.

Further research for optimizing the derivatization reaction will be investigated in solvent 1,4-dioxane with three numeric factors including temperature, reaction time, and ratio due to the limitation of boiling point of acetonitrile. OFAT actually cannot provide information about the interactions between these three numeric factors because these previous experiments have been performed before knowing about the DOE statistical techniques. Thus, 2-level full factorial design will be used to discover the interactions between the three numeric factors. Optimal design will be employed to discover the highest response of the derivatization reaction.

This study would help to improve the analysis method for detecting emerging environmental contaminants and understanding the situation of emerging pollutants in the environmental system. In addition, the strategy of controlling emerging contaminants can be benefit through developing sensitive detection methodology.

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