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Optimization of Extraction Procedure for Determination of Caffeine Residue in Water

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Abstract
Extraction procedure for determination of caffeine in water with aided experimental design was successfully optimized. Three variables, namely pH sample, flow rate and elution volume were tested during this study using 2^3 full factorial of central composite design. An optimum working condition was suggested at pH sample (6.4), flow rate (1.2 ml/min) and elution volume of caffeine from sorbent is 9.5 ml of methanol. Detection and quantification limits were obtained at 0.02 and 0.25 ng/ml respectively. Good recovery was calculated within the range of 80.9 – 93.7 % at three levels of concentration. Repeatability and reproducibility value show this method to be reliable for routine analysis with low RSD < 10 %. Concentration levels of caffeine in real samples were recorded in the range of 0.64 – 59.01 ng/ml. Hierarchical cluster analysis grouped all stations into three clusters at D_{link}/D_{max} x 100 < 15. Wastewater discharge from cafeteria or residential area was a solid reason linked to the presence of high caffeine during this study.

Keywords: Experimental design, Freshwater, Physcoactive drug, Response surface, Stimulant

1. Introduction
Caffeine ($C_8H_{10}N_4O_2$) is a xanthine alkaloid compound. It acts as a mild central nervous system stimulant, myocardial stimulant and smooth-muscle relaxant [1]. It is widely used in beverage, food, pharmaceutical and cosmetic industries. A non-metabolized form excreted though human urine may reach in the range of 0.5 to 3 % [2]. Caffeine has high solubility (13.5 g/l), negligible volatility, and stable under variable environmental conditions [3, 4]. It is well-documented to be present in wastewater influent or effluent [5–7], river [3, 8], lake [9], groundwater [10, 11] and seawater [4]. It is known to be a good anthropogenic marker for degradation water quality by wastewater since it is highly consumed by humans [12]. In Malaysia, the occurrence of caffeine in the aquatic environment has been depicted in few literatures such as wastewaters [6, 13, 14] and river [13].Separation technique of caffeine in water preferably used solid phase extraction [2, 15, 16]. The usage of sorbents like hydrophilic-lipophilic balance [17, 18], non-polar C18, [5, 8, 19] and mix mode cation exchange [20, 21] has been well-documented in previous studies. High Performance Liquid Chromatography (HPLC) is most widely used for quantitative or qualitative determination of caffeine. It is not limited to detection in water but also in beverage [22], herbal product [23], urine [24], saliva and plasma [25]. Detection limits were achieved by using HPLC that varies depending on separation technique, sample matrices, column type and selection of mobile phase. HPLC is still a good choice in pharmaceutical residue determination especially involving compounds that

do not require derivatization step prior to analysis. The aim of this study is to optimize the condition of extraction procedure for determination of caffeine residue in water. An experimental design was applied in order to evaluate interactions between variables.

2. **Experimental**

2.1.*Chemical and reagents*

Caffeine standard was purchased from Sigma-Aldrich (St. Louis, USA). Methanol, hexane, ethyl acetate (liquid chromatography grade) and formic acid were purchased from Merck (Darmstadt, Germany). Sodium acetate and acetic acid solution for preparing buffer solution was purchased from R & M Chemical (Edmonton, Canada). Ammonium hydroxide 30 % was supplied by J.T. Baker (Gross-Gerau, Germany). Deionized water was obtained from EasypureRodi (Barnstead, USA). Sorbent Oasis MCX 6cc (150 mg) was purchased from Waters (Milford, USA). Stock solution was prepared by diluting approximate caffeine standard into methanol at level concentration of 1000 µg/ml. Working solution was then prepared by diluting a series of low level concentrations of standard solution. Buffer solution (sodium acetic solution) was prepared by dissolving an appropriate amount of sodium acetate in acetic acid.

2.2 *Optimization procedure*

Three variables, namely pH sample, flow rate and elution volume were subjected to optimization in this study. To ascertain the main effect and interactions between variables, a 2^3 full factorial of central composite design was generated using Minitab software (Stat Inc. USA). A total of 20 experiments were conducted in this study. Quadruplicate of four central points was added to estimate experimental error and satisfaction rotate-ability was set at $\alpha = \pm 1.633$. The descriptive of variables studied and design matrix are shown in Table 1 below.

Table 1 Full factorial design matrix

	Code	**-1.63**	**-1**	**0**	**+1**	**+1.63**
pH sample	A	5.5	6	7	8	9.5
Flow rate (ml/min)	B	1.2	2	4	8	10
Elution volume (ml)	C	2.5	4	6	8	9.5

Main effects were visualized using optimization plots and data were evaluated by analysis of variance (ANOVA, F test) to distinguish significant levels. Surface and contour plot was illustrated to show interaction between variables. In general, extraction procedure was started by conditioning sorbent using 5 ml hexane, 5 ml methanol, 5 ml ethyl acetate and 10 ml deionized water (pH 5.5). Water miscible solvent was used to provide wet surface (active site) of sorbent in order to maintain high retention. [26]. Water samples (500 ml) after adjusted at required pH were then loaded onto sorbent at desired flow rate. Sorbent was then washed using 0.75 % formic acid in deionized water (10 ml). Caffeine was then eluted using 0.75 % ammonium hydroxide in methanol. An ammonium hydroxide was added to provide maximum reverse phase retention, efficient to elute basic compounds in unionized form [26]. The amount of methanol as elution volume depends on the design matrix. Concentrated sample was then placed under gentle nitrogen stream before being reconstituted with 0.5 ml methanol.

2.3 *Method validation*

Linearity of established method was determined by plotting calibration curve of series concentrations within range of 15 to 200 µg/ml (n = 7). Detection and quantification limits were determined using formula equation 1 based signal to noise ratio on 3:1 and 10:1 respectively. Recovery, repeatability and reproducibility measurements were determined by using three levels of concentrations (1.5, 25 and 50 µg/ml). Standard solution was spiked into deionized water for all validation works.

$$\text{Limit of Detection (S/N)} = 2H/h \quad (1)$$

H is defined as peak height of targeted analyte, while h is determined as peak height of signal noise over a distance equal to 20 times the width at half height h. Limit of quantification was then calculated as 10 times of LOD value [27].

2.4 *Chromatographic separation*
The residue of caffeine was determined by using High Performance Liquid Chromatography equipped with UV detector (HPLC–UV). Chromolith® Performance RP-18e (4.6 x 10 mm, 5µm) column was used in this study. The description of instrument setting is summarized in Table 2 below.

Table 2 HPLC setting for analysis of caffeine

	Setting
Injection volume (µL)	20
Mobile phase (mL)	100 % Methanol
Retention time (min)	2.00
Run time (min)	3.00
Flow rate ($mLmin^{-1}$)	1.40
Wavelength (nm)	273

2.5 *Analysis of real samples*
Water samples were collected twice in October 2015 involving five stations along water channel (Alur Ilmu) in the main campus of Universiti Kebangsaan Malaysia and two stations located at Langat River which is connected to Alur Ilmu outlet (Table 3). Sample was collected using 500 ml glass bottle, placed in cool box and transferred to laboratory for further analysis. The pH of water samples was measured in situ using multiprobe sensor instrument (YSI550, USA). In the laboratory, sample was filtered using membrane filter 0.45 µm to remove suspended particulates. Hierarchical agglomerative cluster analysis (Ward method) was performed to discriminate the level of pollution between stations. Data output was also generated using Minitab software (Stat Inc. USA).

Table 3 Description of sampling location in this study

Station	**Longitude**	**Latitude**
Before Cafeteria FST	101.781537 E	2.922975 N
After Cafeteria FST	101.781801 E	2.923580 N
Eko-Niaga (Student Centre)	101.782136 E	2.924971 N
Pusanika (Student Centre)	101.781247 E	2.926025 N
Mosque	101.778397E	2.928981N
Teras Jernang	101.758619 E	2.917894 N
Tangkas River	101.787721 E	2.948516 N

FST: Faculty of Science and Technology

3. **Results and discussion**

3.1 *Optimization condition*
Experimental work based on central composite design, 2^3 was successfully carried out. The second order polynomial equation obtained for the optimized variables is given below:

Peak Area = 683.0 - 95.4A - 51.2B + 237.8C - 120.3A2 - 86.0B2 + 49.4C2 + 68.4AB + 36.2AC - 180.6BC

Only variables elution volume (C) and interaction between (AB, AC) have shown positive linearity of fitted model. According to Joglekar and May [28], to have a good fit of the optimum model, coefficient R^2 should be at

least 0.80. In this study, R^2 was obtained at 0.82. The main effects of individual variables are illustrated in Figure 1. An optimum working condition of extraction procedure was suggested at pH sample (6.4), flow rate (1.2 ml/min) and elution volume is 9.5 ml of methanol. An additional experiment was carried out using suggested optimum condition, in which good agreement was obtained and low relative standard deviation (0.84 %) was calculated between actual and experimental value.

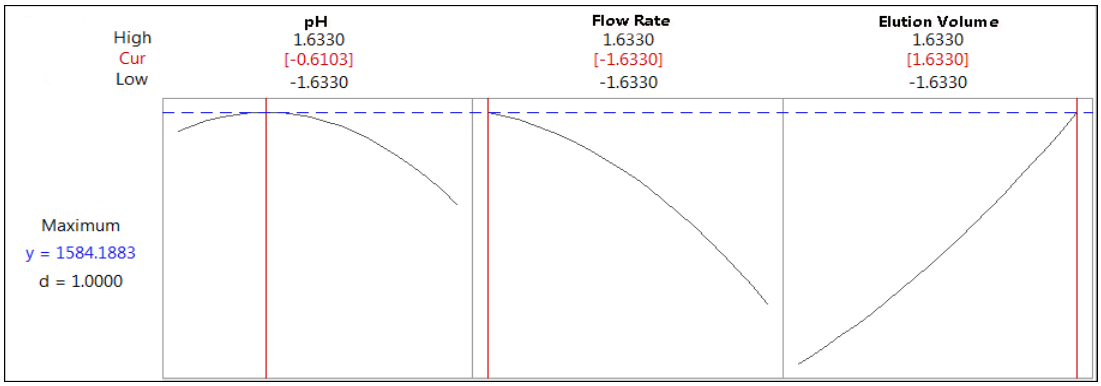

Figure 1 Main effects of individual variables on optimization plot

Variables namely elution volume and interaction term behavior between flow rate and elution volume (BC) contribute significantly ($p < 0.05$) as shown in Table 4. According to Castiglioni et al. [29], the optimum elution condition for Oasis MCX was achieved when using only 2ml of methanol, followed by ammonia and sodium hydroxide. However, caffeine was not included in their list of pharmaceuticals studied. In this study, an amount of methanol (9.5 ml) with 0.75 % ammonium hydroxide was able to give the highest main effect desorption of analyte. Recovery was achieved up to 93.77 % as compared to only 18.72 % when eluting using 2.5 ml of methanol. Another co-factor in related to efficacy elution volume is flow rate of extraction process with p value ANOVA is 0.032. Lowering of flow rate theoretically will enhance analyte to retain on sorbent during loading process of water samples. Hence, factor of elution volume would take over to determine how much organic solvent can elute (desorption) analyte from sorbent. In this study, the flow of water samples or elution solvent was preferably placed at the lowest setting (1.2 ml/min), which gives better signal response of the targeted analyte.

Table 4 Descriptive of Analysis of Variance

Variables	**DF**	**Sum of Square**	**Mean Square**	**F value**	**P value**
Model	9	1230156	136684	4.65	**0.016**
A	1	98304	98304	3.35	0.101
B	1	28325	28325	0.96	0.352
C	1	611094	611094	20.81	**0.001**
A^2	1	184756	184756	6.29	**0.033**
B^2	1	94496	94496	3.22	0.106
C^2	1	31147	31147	1.06	0.330
AB	1	26925	26925	0.92	0.363
AC	1	7539	7539	0.26	0.625
BC	1	187784	187784	6.39	**0.032**
Lack of Fit	4	142188	35547	1.46	0.340
Pure Error	5	122134	24427		
Total	18	1494479			

Bold is significant ($p < 0.05$)

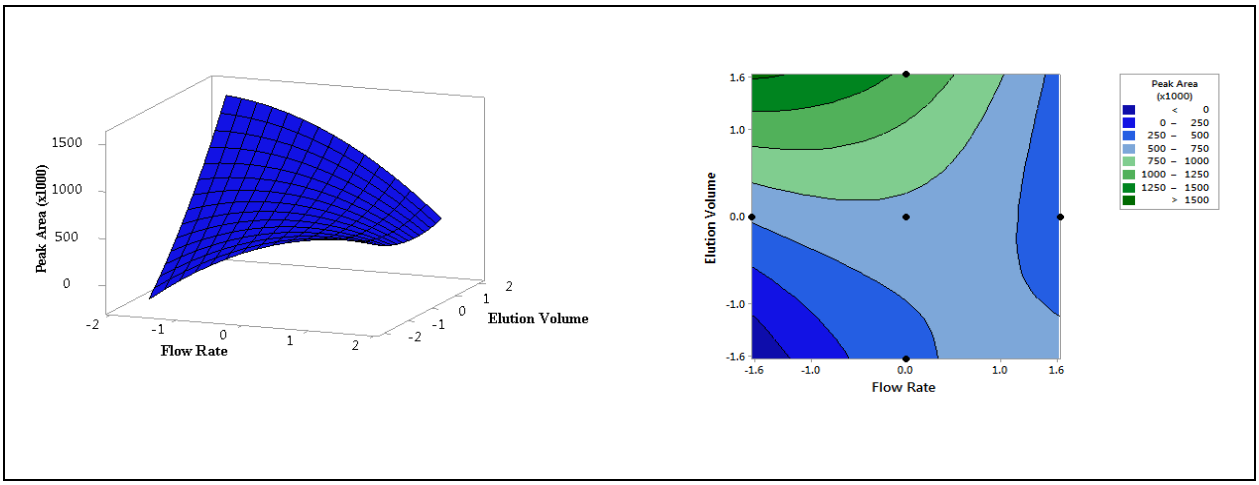

Figure 2 Surface and contour plot for interaction term between flow rate vs elution volume

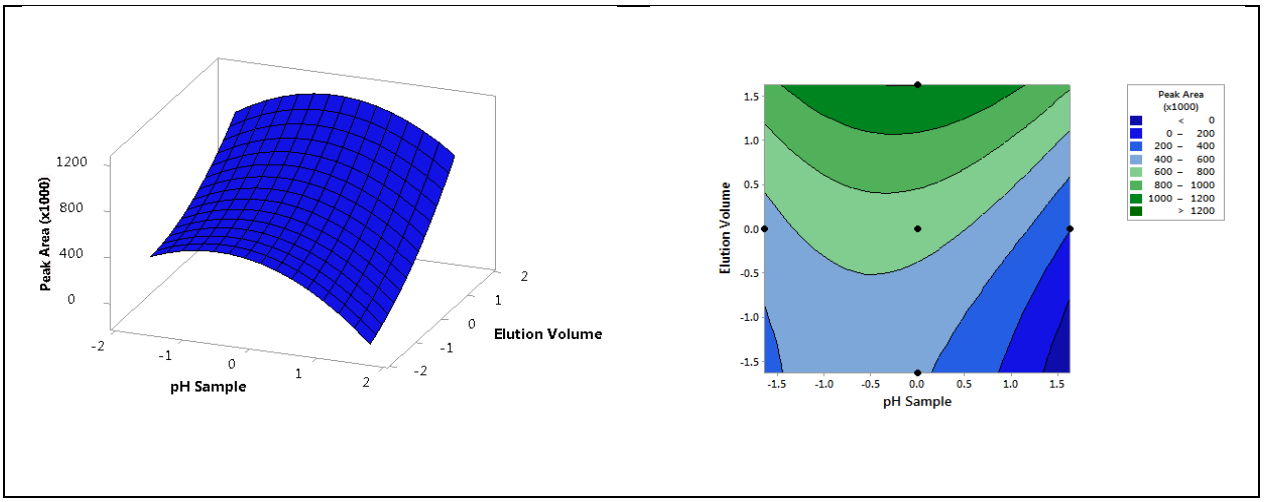

Figure 3 Surface and contour plot for interaction term between pH sample vs elution volume

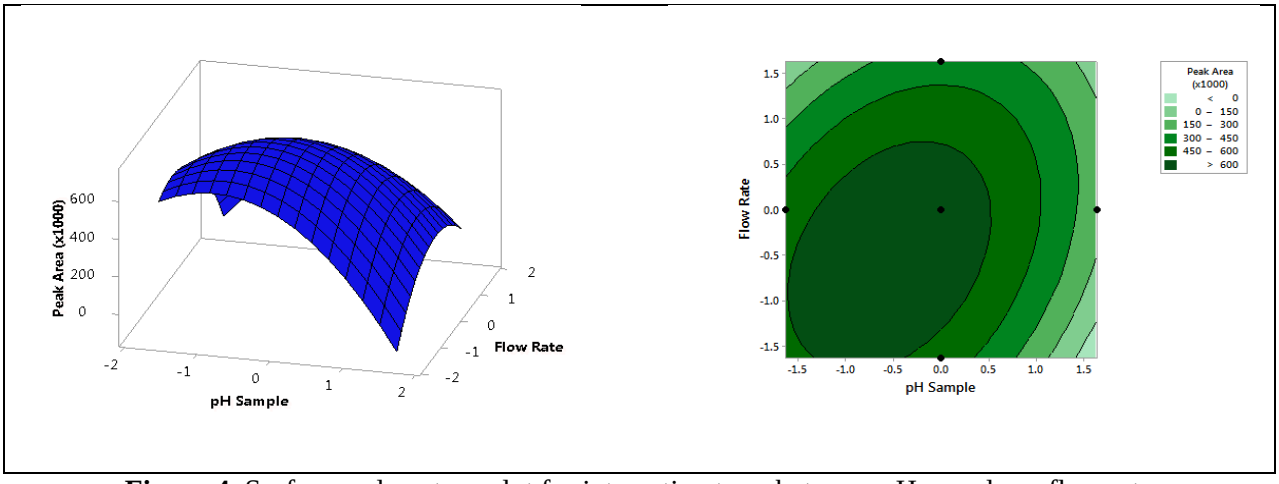

Figure 4. Surface and contour plot for interaction term between pH sample vs flow rate

MCX sorbent is the most suitable for the extraction of basic and neutral compounds from aqueous solution. Sample solution is required to be acidified in order to ionize basic compounds [30]. According to Gros et al. [31], an efficacy of basic compounds extraction using MCX sorbent showed improvement by adjusting sample at low pH values and elute with mixture of ammonia and methanol. In this study, the optimum condition for pH sample was suggested at pH 6.4, but additional experiment was carried out by adjusting pH to 5.5, indicating signal response of peak area remained without significant change ($p > 0.05$). Furthermore, freshwater sample usually recorded the pH value within range 6.5–7.5. Therefore, an analysis of real water sample was decided to prior be acidified to pH 5.5 using buffer solution before further extraction.The interaction between variables was illustrated in Figure 2 – 4 respectively. Interpolation of fitted model expressed that only two-way interaction (flow rate vs elution volume) contributed significantly based on the given design matrix.

3.2 Analytical figure of merit

Validations of analytical figure of merit were also performed in order verify whether the established method is reliable for quantitative analysis. Calibration curves were obtained by plotting the response of peak area versus spiked concentration using seven different levels of concentrations. Coefficient of correlation was achieved at $R^2 = 0.995$. The sensitivity of established method was demonstrated by determining detection and quantification limits. Quantification limit of developed method reached 0.25 ng/ml. It is still reliable for quantitative analysis of water and wastewater since caffeine in literatures was reported to be present in high concentrations (part per billion). Recovery of caffeine spiked in deionized water was achieved in the range of 80.93 – 93.77 %. Better result was obtained compared to the literature, 69.10 % [21]. The bias of intra-day and inter-day precision was recorded at low relative standard deviation, which is less than < 10 %. The validation result is summarized in Table 5 and 6 below.

Table 5. Analytical figure of merit on developed method

	Value obtained
Linearity (n = 7)	$y = 41112x + 611651$ ($R^2 = 0.995$)
Instrument Detection Limit (ng/ml)	3.40
Method Detection Limit (ng/ml)	0.02
Quantification Detection Limit (ng/ml)	0.25

Table 6. Results of recovery and precision test

Concentration (µg/ml)	**Recovery (%)**	**Repeatability % RSD (n = 3)**	**Reproducibility % RSD (n = 5)**
50	46.89 (93.77)	2.49	2.78
25	21.60 (86.40)	2.79	2.88
1.5	1.21 (80.93)	4.49	7.45

Efficacy extraction of caffeine residue in this study was compared to other documented literatures as summarized in Table 7. Detection limits of developed method indicate that it was closed to method performance of HLB sorbents but better than type of sorbent C_{18}. The usage of organic solvent in elution step is much lower than when using HLB sorbent as previously reported by Ismail et al. [6] and Montagner and Jardim [32]. Recoveries value of spiked samples in this study was tolerable with others established methods, which close to the actual value. In comparison to other studies, the drawback of this method is require more time to extract sample since the suggested flow rate was slow at rate 1.2 ml/min.

3.3 Level of Concentration

The developed method for the determination of caffeine residue in water was applied to examine the occurrence of pollutant in the main campus of UKM and Langat River. Stations in main campus are S1 (Before Cafeteria FST Outlet), S2 (Cafeteria FST Outlet), S3 (Ekoniaga – student centre), S4 (Pusanika – student centre), S5

(Mosque), while in Langat River namely S6 (Teras Jernang) and S7 (Tangkas River, a tributary of Langat River).Level of pH in water samples was recorded in the range of 6.03 to 7.71.

Table 7. Comparison of method performance with literature studies

	This Study	**Alawi et al. [5]**	**Ismail et al. [6]**	**Montagner and Jardim [32]**
Sorbent type	MCX	C_{18}	HLB and C_{18}	HLB
Flow rate (ml/min)	1.2	3	1.0	10
Elution volume (ml)	9.5	8	15	12
Detection limit (ng/ml)	0.02	0.6	0.01	0.01
Quantification limit (ng/ml)	0.25	1.9	0.04	0.03
Recovery range (%)	80.9 – 93.7	92.6 – 109	87.4 –104.6	74 – 102

Range concentrations of caffeine residue present in water are as the following: S1 (1.24 – 13.2 ng/ml), S2 (12.28 – 15.80 ng/ml), S3 (57.88 – 59.01 ng/ml), S4 (0.64 – 17.96 ng/ml), S5 (1.72 – 9.40 ng/ml), S6 (15.32 – 21.80 ng/ml) and S7 (17.56 – 20.32 ng/ml). Mean concentrations of caffeine in all stations were calculated to be 3.92 ng/ml (S1), 14.24 ng/ml (S2), 58.44 ng/ml (S3), 9.28 ng/ml (S4), 5.56 ng/ml (S5), 18.56 ng/ml (S6) and 18.92 ng/ml (S7). The chromatogram of caffeine in real sample was shown in Figure 5.

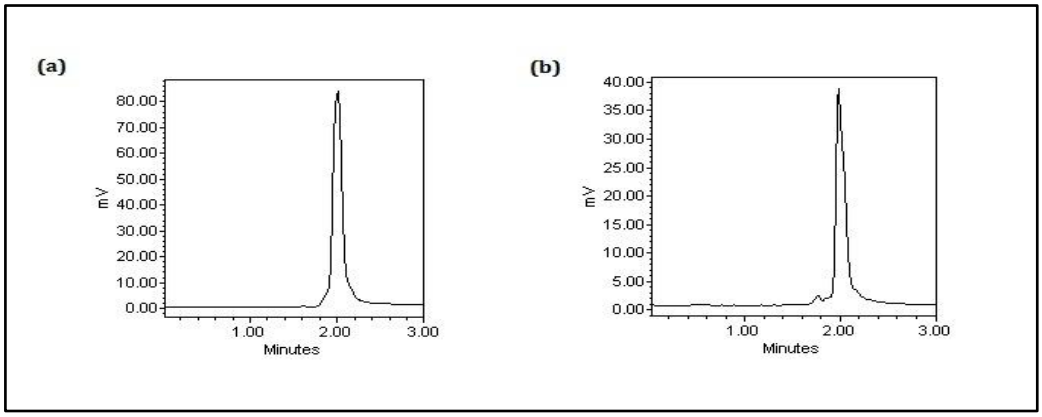

Figure 5. The chromatogram of caffeine in (a) spiked sample and (b) freshwater sample

Cluster analysis generated a dendrogram grouping seven sampling stations into three groups at $(D_{link}/D_{max}) \times 100 < 15$ as illustrated in Figure 6.

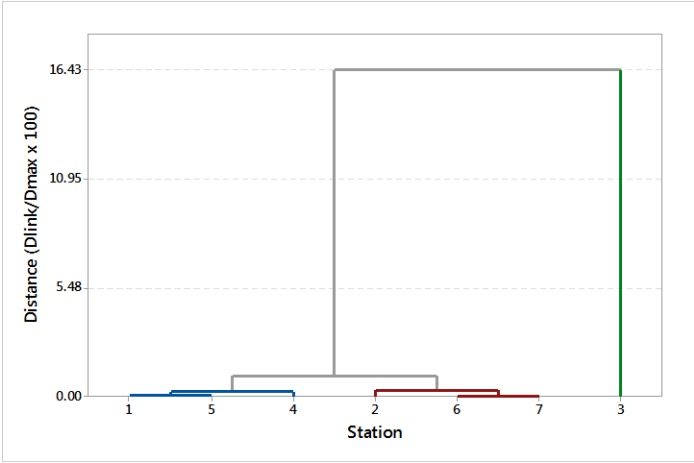

Figure 6. Dendrogram of clustering the site similarities

Cluster I consisted of station S1, S4 and S5, while cluster II and III were represented by station S2, S6, S7 and S3 respectively. Cluster II was recognized as the point source of pollution. Stations located close to cafeterias or residential area (Teras Jernang and Sungai Tangkas villages) received input from direct wastewater discharge. Cluster I was depicted as non-point sources or may have dilution effect from station S2 (Cafeteria FST Outlet). Cluster III explained about the potential accumulation of caffeine in water. No additional point source was observed in the vicinity of the station except flow from station S2 and it was believed to be due to very low water flow (stagnant) in this area. Statistical analysis of one-way ANOVA indicated that the level of pollutant between stations was significantly different at $p < 0.05$.

Conclusions

Extraction procedure for determination of caffeine residue in water was successful optimized. Variable namely elution volume shows the highest main effect in design matrix of experimental works. Interaction term between flow rate and elution volume has shown the most significant ($p< 0.05$) influence on extraction procedure. A good fit model was obtained with desirability function achieved at $d = 1.00$ and ANOVA, $p = 0.016$. Validation tests on developed method prove that it is suitable and reliable for routine analysis which is good recovery, sensitive and precise. Despite the limit of detection of this method only achieve at sub part per billion, it was believe also associate to limitation of instrument which difficult to challenge more advance tools. The drawback of time consuming has to overcome in further work. Analysis of real samples showed the level concentration of caffeine obtained was within the range of 0.64 – 59.01 ng/ml. Source of pollutant coming from direct discharge of wastewater through cafeteria outlet and residential area was identified.

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