



## Optimization of C<sub>18</sub>-Cellulose Triacetate Thin Film for Analysis of Caffeine Residue in Water

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This study reports the optimal condition of an extraction method for caffeine residue analysis in water. C<sub>18</sub> was impregnated with cellulose triacetate by using a solution casting method to produce a thin film. Optimization work was performed based on a 2<sup>3</sup>-full factorial central composite design, which was subjected to salt addition, extraction time, and stirring rate as the main parameters. The optimum condition suggested by the model was as follows; salt addition (0.6 %, m/v), extraction time (11 min) and stirring rate (300 rpm). The generated model and 2-way interaction were significant at  $p < 0.05$ . Detection and quantification limits of the developed method were calculated at 0.06 and 0.21 ng/mL, respectively. The thin film displayed exceptional recovery (83.90-98.50 %) and repeatability (7.71-12.40 % RSD) at two levels of concentration.

**Keywords:** Caffeine, Water, Cellulose triacetate, Thin film.

### INTRODUCTION

Caffeine is a biologically-active drug that acts as a stimulant which can be very useful for medication but may be harmful when the dosage is very high [1]. The global average consumption of caffeine is estimated to range between 80 and 400 mg per person per day [2]. Caffeine is rapidly metabolized and approximately from 0.5 to 10 % is excreted through urine and feces [3]. In water, caffeine is highly soluble (13.5 g/L), and moderately stable in natural water, despite being vulnerable to biological degradation. The stability of caffeine in environment is caused by resonance stability [4]. In surface waters, caffeine has half-life ranging from 5.3 to 24 h [5].

Caffeine is an excellent tracer because its detection in aquatic system indicates human waste source [2,6,7]. Conventionally, the residue was extracted using solid phase extraction. Packing materials, such as C<sub>18</sub>, hydrophilic-lipophilic balance or cation exchange [8-10], are universal sorbents for the intended purpose. Recovery and repeatability aspects also attain satisfying levels. However, the main drawbacks related to solid phase extraction are high consumption of organic solvent and disposal of the cartridge after single use. Indeed, when the thick coating is applied, analytes are likely to be carried over to the next extraction [11].

Thin film microextraction (TFME) is fibre-SPME with varying sorbent phase geometry, but functions under a similar principle [12]. In TFME, the extraction phase used is a sheet of flat film with a high surface area-to-volume ratio. Based on that arrangement, as the volume of the extraction phase increases, the thickness of the coating stays constant or even thinner. The technology of TFME has been developed to address limiting uptake rate and capacity [13]. Thin film microextraction is suitable for extraction of trace analytes since it takes up a short extractive phase [14]. There are two settings in TFME, namely thin film brushes and thin film membrane. As for this study, thin film membrane made of C<sub>18</sub> and cellulose triacetate (CTA) was prepared. Later, the membrane was attached to a cotter pin and was directly placed in the samples for extraction procedures.

### EXPERIMENTAL

Caffeine of high purity standard, hydrochloric acid and sodium hydroxide (analytical grade) were purchased from Sigma-Aldrich, St. Louis, USA. Organic solvents (methanol and chloroform) were purchased (HmbG Chemicals, Germany). Commercial Bondesil C<sub>18</sub> 40 µm and cellulose triacetate (CTA) were purchased from Agilent Technologies, California and Santa Cruz Biotechnology Inc., Texas, respectively.

**Preparation of C<sub>18</sub>-CTA thin film:** A thin film was prepared by initially weighing 0.04 g of CTA and placing it in a 4 mL vial. Next, 2 mL of chloroform was added into the vial. The chloroform solution of CTA was left for at least 5 h to ensure that it had completely dissolved. Later, 0.01 g of C<sub>18</sub> was added to CTA-chloroform solution, which had been poured on a petri dish. The mixture solution was sonicated for 2 min to ensure that the film was homogenous and had uniform distribution. The solution was left at room temperature for 2 h until it dried completely. The internal diameter for the thin film was 66 mm. In order to avoid thin film from tearing, polyvinyl self-adhesive reinforcement O-ring was applied.

**Extraction procedure:** Three parameters, namely salt addition (X<sub>1</sub>), extraction time (X<sub>2</sub>) and stirring rate (X<sub>3</sub>) were subjected to optimization in this study. In order to ascertain the main effect and the interactions between the selected parameters, a 2<sup>3</sup>-full factorial of a central composite design was generated with Statistica software version 10 (Statsoft, Witzhausen). A total of 20 experimental runs were carried out in this study. Quadruplicate of four central points was added to estimate experimental error, while the satisfaction rotate-ability was set at  $\alpha = \pm 1.68$ . The description of variables studied, and the design matrix are presented in Table-1. A second order polynomial equation was then fitted to the data *via* multiple regression procedure.

TABLE-1  
EXPERIMENTAL VARIABLES AND THEIR  
LEVELS FOR THE CENTRAL COMPOSITE DESIGN

Independent variables	Symbol	Code levels				
		- $\alpha$	-1	0	+1	+ $\alpha$
Salt addition (% m/v)	X <sub>1</sub>	0.6	1.0	1.5	2.0	2.34
Extraction time (min)	X <sub>2</sub>	7	10	15	20	23
Stirring rate (rpm)	X <sub>3</sub>	132	200	300	400	468

The quality of fit of polynomial model equation was expressed by the coefficient of determination R<sup>2</sup>, whereas its statistical significance was determined *via* F-test. The significance of the regression coefficient was tested by using *t*-test. The extraction efficiency of the method was evaluated as the mean of peak height of triplicate successive injections (n = 3).

In general, C<sub>18</sub>-CTA composite thin film was conditioned by immersing it into 10 mL methanol within 30 s to activate the sorbent surface. The water sample was adjusted to the desired pH 9 with 0.1 M NaOH. Standard solution of caffeine was spiked into deionized water sample at a concentration level of 6 ng/mL. The saline solution contains sodium chloride; 1 mL was added to the sample solution. Next, the sample (80 mL) was transferred into a 100 mL screw cap vial. A piece of film was placed inside the same vial. Cotter pin was applied as external holders to support and to maintain the flat surface of the film. Later, the solution was stirred at the desired rate (X<sub>3</sub>) and the extraction time was set depending on the required time (X<sub>2</sub>). After that C<sub>18</sub>-CTA film was removed and transferred into a new safe lock vial. The targeted analyte was desorbed from the film by using 50  $\mu$ L of methanol *via* sonication for at least 10 minutes. The extract was filtered with a 0.45  $\mu$ m nylon syringe. Lastly, the extract (20  $\mu$ L) was introduced to HPLC coupled with SPD-10A UV detector (Shimadzu) for final analysis.

**Method validation:** The efficiency of method performance was evaluated based on linearity, precision, accuracy, reusability, limit of detection (LOD) and limit of quantitation (LOQ). A series of caffeine standard solution that ranged from 10 ng/mL to 500 ng/mL was used to construct a calibration curve. Recovery test was performed at two concentration levels, 12.5 ng/mL and 25 ng/mL, of spiked standard solution. Intra-day assay (repeatability) and inter-day (reproducibility) were performed using two concentration levels, 12.5 ng/mL and 25 ng/mL, respectively. Detection and quantification limits were calculated by using linear regression and signal-to-ratio methods at 3:1 (LOD) and 10:1 (LOQ), respectively. The lowest concentration spiked was 6 ng/mL and triplicate analysis was performed. Reusability assay involved reapplication of used thin film for the next extraction. Standard solution was also spiked at two levels of concentration.

**Analysis of real sample:** Water sample was taken from one location (coordinate N 5°17'03.3" E 103°10'13.5") at Ibai river, Terengganu, Malaysia. The sample was collected by using 1000 mL glass bottle, placed in a cool box, and transferred to a laboratory for further analysis. The pH of water samples measured *in-situ* was pH 7.45. In the laboratory, the samples were filtered by using a 0.45  $\mu$ m membrane filter to remove suspended particulates prior to extraction procedure.

## RESULTS AND DISCUSSION

**Optimum condition:** Experimental work based on central composite design was successfully carried out. The final optimum condition was calculated by using optimizer plot, which allowed compromising amongst various responses (Fig. 1). This function sought a combination of factor levels that jointly optimized a set of responses by satisfying the requirements for each response in the set. The second order polynomial equation obtained for the optimized variables is given by eqn. 1:

$$\begin{aligned} \text{Peak height} = & 102.04 - 24.99X_1 - 3.21X_2 + \\ & 4.21X_3 - 7.59X_1^2 - 18.02X_2^2 - 21.10X_3^2 + \\ & 17.09X_1X_2 + 0.84X_1X_3 - 5.59X_2X_3 \end{aligned} \quad (1)$$

Variables, namely stirring rate (X<sub>3</sub>) and interaction between (X<sub>1</sub>X<sub>2</sub>, X<sub>2</sub>X<sub>3</sub>) displayed positive linearity of the fitted model. A positive sign in front of the terms indicates synergistic effect, whereas a negative sign reflects antagonistic effect [15]. A *p*-value of 0.001 signified that the model terms were significant. In this study, R<sup>2</sup> was obtained at 0.83, explaining 83 % of the variability in response could be explained by the model. R<sup>2</sup> adjusted was calculated at 0.72. According to Joglekar and May [16] in order to have a good fit of the optimum model, coefficient R<sup>2</sup> should be at least 0.80. The R<sup>2</sup> coefficient in this study ensured satisfactory adjustment of the quadratic model to the experimental data. R<sup>2</sup> adjusted had corrected the R<sup>2</sup> value of the sample size or the number of terms in the model. The R<sup>2</sup> adjusted was lower than the R<sup>2</sup> value. Desirability 1 is for maximum, whereas desirability 0 is for non-desirable situations or minimum. In this study, desirability was recorded at 0.98. The desirability function is commonly applied to evaluate the qualitative or quantitative response *via* simple and quick transformation of multiple responses for a measurement [17]. The coefficient of variation refers to a measure of reproducibility.

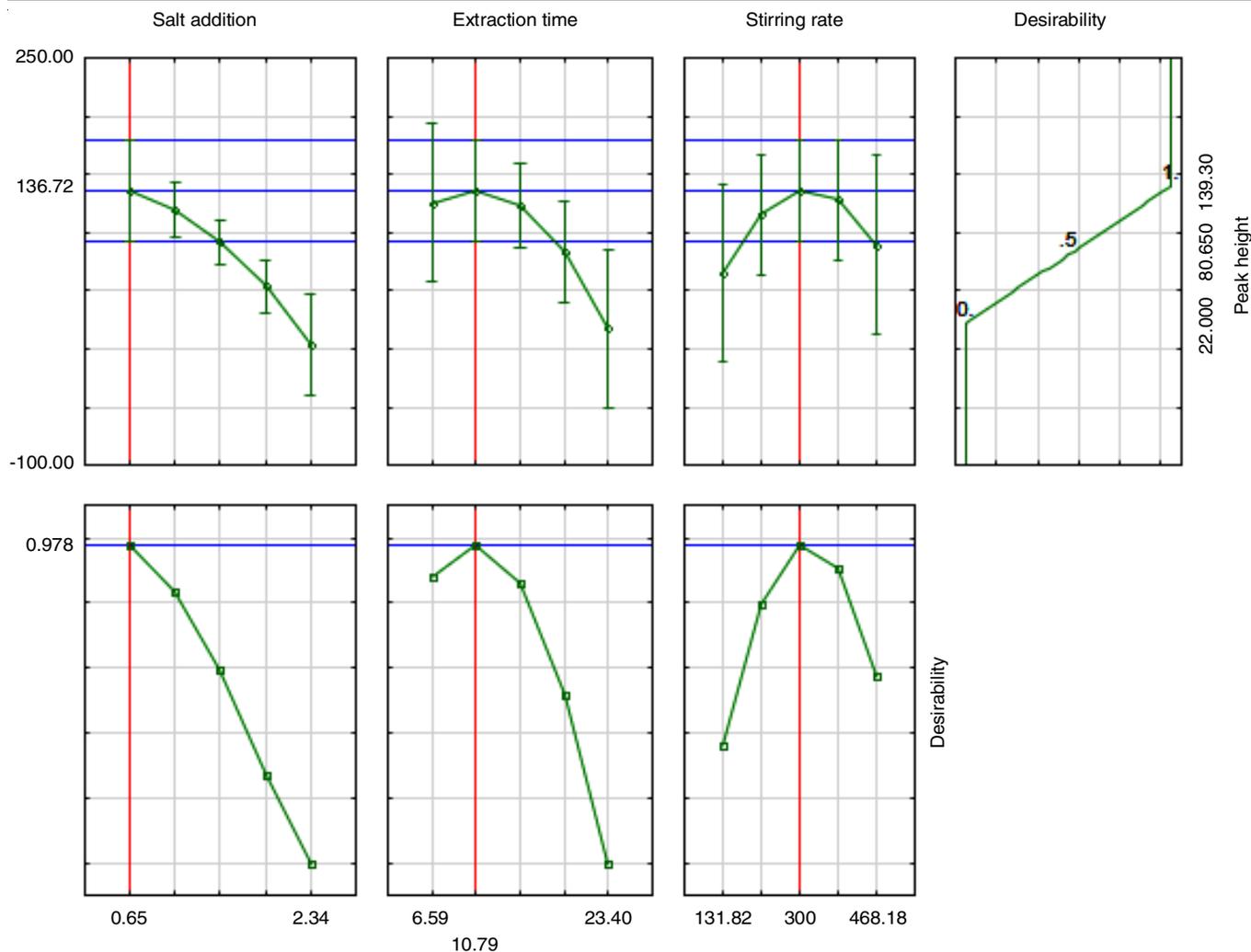


Fig. 1. Optimizer plots for studied variables

bility of the model, whereby a model is considered reasonably reproducible if the value is below 10 %. The coefficient of variation (CV) for the suggested model was 1.72 %.

Optimum working condition of extraction procedure as suggested by the model is listed as follows: salt addition (0.6 % m/v), extraction time (10.7 min) and stirring rate (300 rpm) (Fig. 1). An additional experiment was conducted by using the suggested optimum condition, in which good agreement was obtained and low relative standard deviation (3.01 %) was achieved between the actual and experimental values. A *p*-value below 0.05 in ANOVA test (Table-2) and Pareto chart (Fig. 2) signified that the statistical significance of an effect at 95 % confidence level. The variable with the largest effect was the linear term of salt addition ( $X_1$ ), followed by quadratic effects of extraction time ( $X_2^2$ ), and stirring rate ( $X_3^2$ ). For interaction term between salt addition *vs.* extraction time ( $X_1X_2$ ), it was remarkably sensitive to minor alterations in both variables. The significant factors were ranked based on the value of F-ratio. In this study, the ranking is as follows:  $X_1 > X_3 > X_2 > X_1X_2$ . The "Lack of Fit (LOF) *p*-value" implied the LOF is not significant relative to pure error. The LOF value was recorded at 0.64.

Normal probability illustrated in Fig. 3a plot shows that the data adhered to a straight line, which indicated normal

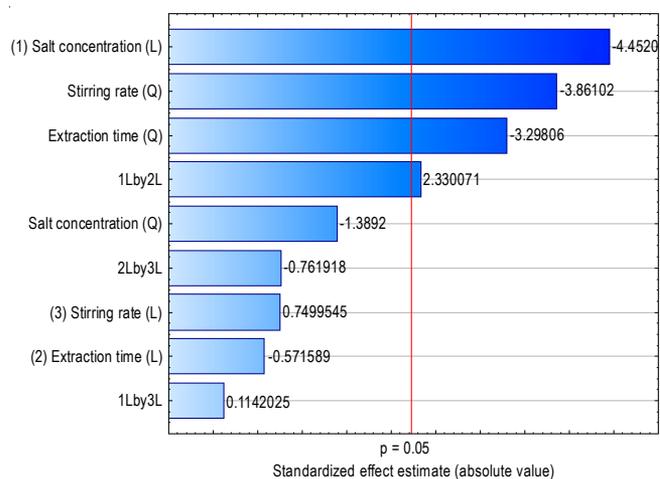


Fig. 2. Standardized effect of variables on maximum response of peak height

distribution and no evidence of non-normality, skewness, outlier, or undefined variable. The point cluster around the diagonal line reflected the optimal fit of the model, since the deviation between experimental and predicted values was minimal. This supports the adequacy of the least-squares fit. Indeed, it allows one to use response surface as a predictive tool to determine the optimum condition. Residuals *versus* predicted values

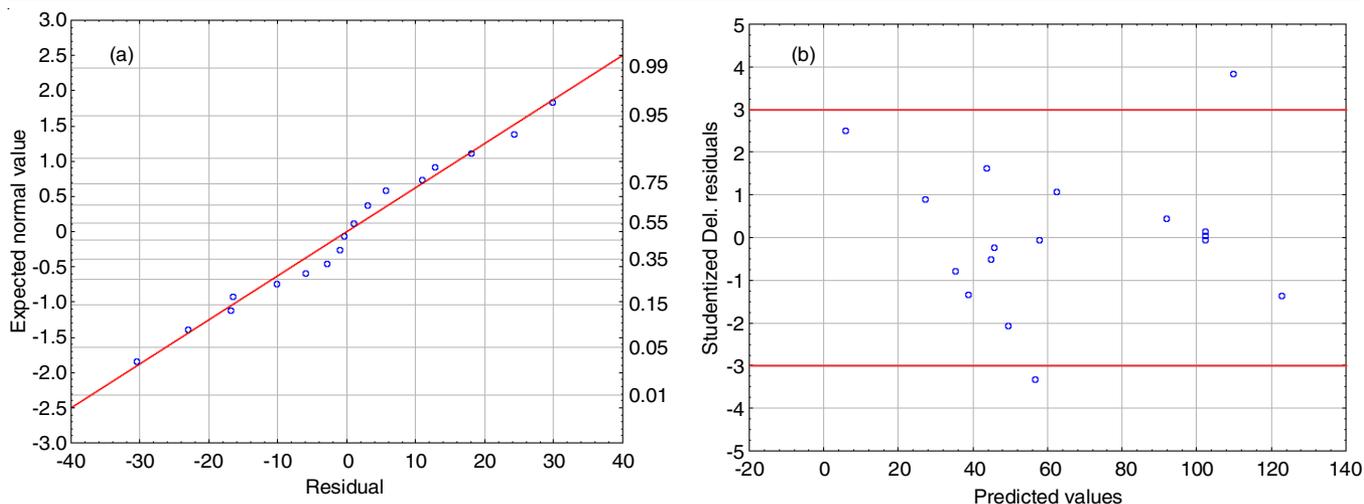
Fig. 3. (a) Normal probability plot and (b) Residuals *versus* fitted data set

TABLE-2  
ANALYSIS OF VARIANCE (ANOVA) FOR THE  
SECOND-ORDER REGRESSION MODEL

	SS	df	MS	F-value	P > F
(X <sub>1</sub> ) Salt addition (L)	8527.77	1	8527.77	19.82	0.001**
Salt addition (Q)	830.31	1	830.31	1.92	0.194
(X <sub>2</sub> ) Extraction time (L)	140.56	1	140.56	0.32	0.580
Extraction time (Q)	4679.79	1	4679.79	10.87	0.008**
(X <sub>3</sub> ) Stirring rate (L)	241.98	1	241.97	0.56	0.470
Stirring rate (Q)	6413.74	1	6413.74	14.90	0.003**
1 L by 2 L	2335.86	1	2335.86	5.42	0.042*
1 L by 3 L	5.61	1	5.61	0.01	0.911
2 L by 3 L	249.76	1	249.76	0.58	0.463
Error	4302.38	10	430.23		
Total SS	26150.67	19			

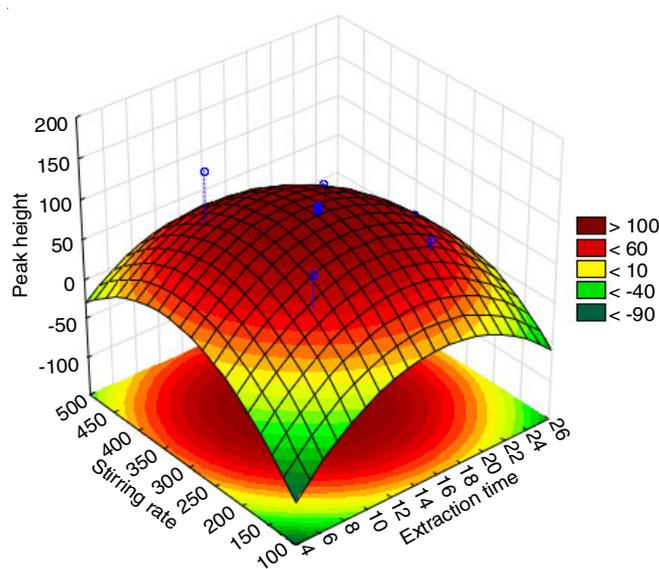
\*P < 0.05; \*\*P < 0.01

portrayed in Fig. 3b depict the consistency of the variance in errors through equal scatter of the residual data close to 0 value on the y-axis.

3D response surface plots were obtained for a given pair of factors at fixed and optimal values of other variables. The curvatures of these plots indicated the interaction between the variables. Interactive effects are essential for true optimization, instead of the single factor-at-a time method. The maximum predicted value is indicated by the surface confined in the smallest ellipse in the contour diagram.

A controlled parameter to enhance the kinetic process (stirring rate) is known to have an impact upon extraction efficiency. It controls the thickness of the boundary layer. In this study, thermodynamic equilibrium phase was achieved when higher speed had been introduced during the extraction process. Theoretically, the higher the stirring rate, the faster is the mass transfer between aqueous phase and membrane. The contribution of stirring rate to reduce extraction time seemed very low and negligible, as it only reached 0.95 %. The best kinetic rate obtained in this study seemed higher than that reported in previous work (200 rpm), in which the extraction phase involved 15 mL vial [18]. It is noteworthy to highlight that when sample size increased (80 mL), the kinetic rate also

increased to overcome the boundary layer that exists between the aqueous and the membrane. The flux of analytes in the region outside the boundary layer is directly controlled by agitation, whereas in the boundary layer surrounding the device is controlled by diffusion [19]. The response surface of interaction terms is illustrated in Figs. 4-6.

Fig. 4. 3D response of interaction term between extraction time (X<sub>2</sub>) *vs.* stirring rate (X<sub>3</sub>)

The effect of increasing the ionic strength of water sample was evaluated by adding NaCl (0.6-2.34 %, w/v) into the water sample. The scale salt addition was sensitive towards method efficiency. Minimal contact was required to reach equilibrium, which reduced the solubility of analytes in the aqueous phase and enhanced their migration towards solid-phase thin-film. The percentage of contribution for salt addition *vs.* extraction time accounted for 8.93 %. The interaction term between salt addition and extraction time exhibited significant variance.

Membrane extraction is an equilibrium-based extraction procedure and therefore, a time-dependent process. A thin film with a large surface area-to-volume ratio results in enhancement

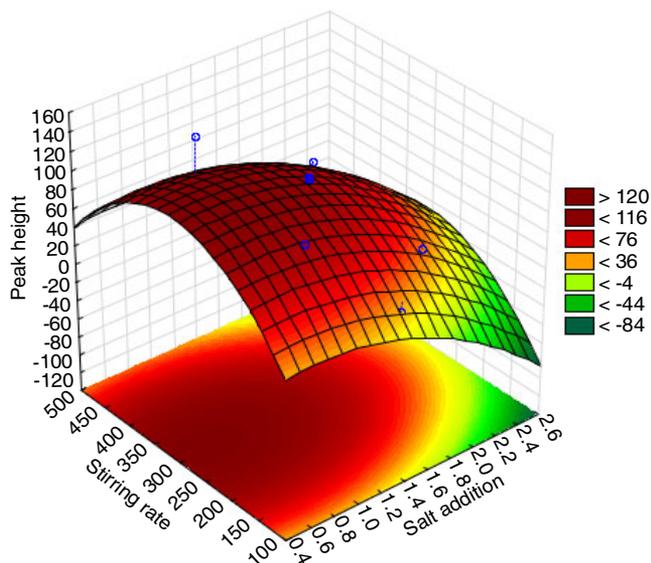


Fig. 5. 3D response of interaction term between salt addition ( $X_1$ ) vs. stirring rate ( $X_3$ )

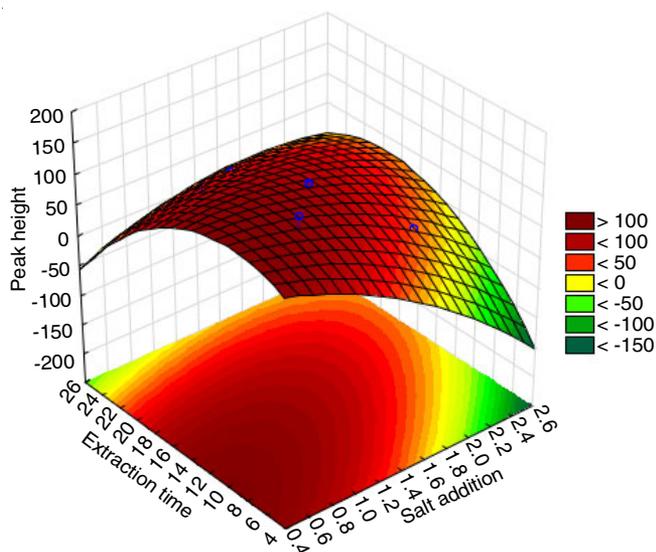


Fig. 6. 3D response of interaction term between salt addition ( $X_1$ ) vs. extraction time ( $X_2$ )

of the extraction efficiency without sacrificing extraction time [13]. The extraction efficiency was found optimum at 10.7 min and after that it slowly decreased as observed in the curve. The time required to reach the equilibrium phase was reduced when the thin film was positioned to fit the end of the cotter pin, when compared to previous work [18] that demanded 30 min for the intended purpose. As for routine analysis, it appeared difficult to get the exact time of the suggested condition. In the next analysis, batch experiments (11 and 13 min,  $n = 3$ ) were performed to determine the robustness of the extraction method. The  $t$ -test analysis revealed that the calculated concentration obtained did not differ statistically, in which recoveries were achieved at 81.68 % (11 min) and 83.4% (13 min), respectively. Therefore, the time for extraction was set at 11 min.

Under optimal conditions, the model predicted a maximum response of 138.31. In order to compare the predicted result with the practical value, experimental re-checking was performed by using this optimal condition. A mean value of 131 (94.73%)

obtained from the real experiments validated the developed model. The developed empirical model was reasonably accurate, while the peak heights recorded for the actual values during confirmation runs were within the 95 % prediction interval that ranged from 89.74 to 186.89.

**Analytical figure of merit:** The linear dynamic range was obtained by plotting the response of peak height *versus* spiked concentration by using five different concentration levels. Linearity range achieved at a satisfactory level at  $R^2$  was 0.996. The sensitivity of the developed method was demonstrated by determining LOD and LOQ, which were 0.06 ng/mL and 0.21 ng/mL, respectively. Meanwhile, LOD and LOQ based on S/N ratio were 0.014 ng/mL and 0.048 ng/mL, respectively. The strategy to fix the position of thin film had led to lower detection limit, when compared to previous work [18] (0.13 ng/mL).

Recoveries were calculated at 83.90 and 98.50 % when caffeine standards were spiked at 12.5 ng/mL and 25 ng/mL, respectively. The calculated values fell within the acceptable range of 80-110 % based on AOAC guidelines for concentration below 100 ng/mL. The relative standard deviation values for repeatability of intra- and inter-day were 7.71-11.63 % and 9.5-12.4 %, respectively. The calculated value was below 15 % RSD, in which the maximum acceptable value had been based on AOAC guidelines. Therefore, the method performance is considered good analytical work.

The reusability test revealed that performance of thin film reduced in which only 46.26-49.17 % recovery of spiked concentration was obtained ( $n = 3$ ). Since sonication technique desorbs analytes from the sorbent, it is believed that the high energy could have affected the surface area of the thin film. Therefore, it can be concluded that the produced thin film had remained a single-use sorbent.

**Concentration levels of caffeine:** Caffeine residue was found in a water sample at 45 pg/80 mL (Fig. 7). In comparison, sample from same location was extracted using commercial Oasis HLB solid phase extraction, which gave a concentration level at 32.80 pg/80 mL. The presence of caffeine residue in river water is believed due to the discharge input from human anthropogenic activities. In fact, the sampling location is close to human settlement area. In the next experiment, fortified sample with standard 25 ng/mL showed that the relative recovery at 94.20-97.10 % revealed the minimum matrix effect.

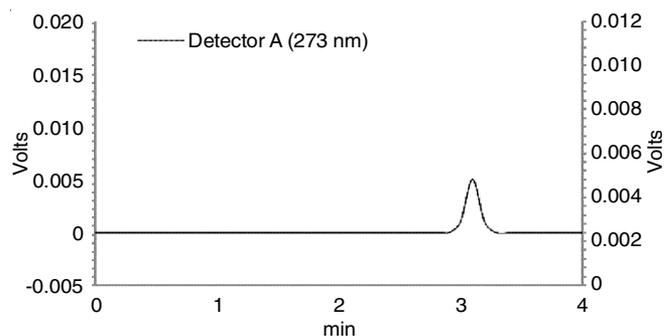


Fig 7. Chromatogram of caffeine detected in water samples

## Conclusion

The optimal condition suggested by the polynomial model had been successfully applied to determine caffeine residue

in water sample analysis. Variables, namely salt addition, extraction time and stirring rate, significantly contributed when interacted with other variables. The developed model possesses several advantages, including dynamic linear range as well as high recovery and repeatability. Despite the good analytical figure of merits reported in this study, an attempt to reuse thin film appeared to be unsuccessful as the extraction performance dropped to below 50 % even after an extraction. The developed method has been proven to display good performance for analysis of real environmental waters.

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#### CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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