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Response Surface Method for Simultaneous Separation of Non-Steroidal Anti-Inflammatory Drugs and Tetracyclines in Water Samples using Online Solid Phase Extraction-Liquid Chromatography

Norizat Yaakub[1], Nor Suhaila Mohamad Hanapi[1,*], Nurzaimah Zaini[1], Noorfatimah Yahaya[2], Wan Nazihah Wan Ibrahim[1] and Ahmad Lutfi Anis[3]

[1]School of Chemistry and Environment, Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia

[2]Integrative Medicine Cluster, Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia, Bertam, 13200 Kepala Batas, Penang, Malaysia

[3]Faculty of Applied Sciences, Universiti Teknologi MARA, 94300 Kota Samarahan, Sarawak, Malaysia

*****Corresponding author (e-mail: norsuhaila979@uitm.edu.my)

The solid online phase extraction-liquid chromatography (SPE-LC) method was developed and validated for the simultaneous determination of non-steroidal anti-inflammatory drugs (NSAIDs) and Tetracyclines (TCs) from water samples. The selected NSAIDs and TCs were diclofenac (DIC), naproxen (NAP), acetaminophen (ACE) and oxytetracycline (OTC), doxycycline (DOC). Several essential parameters of valve switching time, acidified water and flow rate were optimised using the Box-Behnken Design (BBD) of Response Surface Methodology (RSM). Results were achieved under optimised conditions of 0.71 min valve switching time, 0.1 % acidified water and 0.78 mL min^{-1} flow rate of the mobile phase. Under optimised conditions, the method shows good linearities with the coefficient of determination (R^2) in the range of 0.9990-0.9997 and low limits of detection (LOD) ≤ 0.73 mg L^{-1}. The method was applied to the analysis of water samples with good relative recoveries in the range 94.4% - 108.3% and relative standard of deviation (RSD) ≤ 3.67 (n=3). The proposed method showed good selectivity and was sensitive to the simultaneous extraction of NSAIDs and TCs. Hence, it can be concluded that the online SPE LC is rapid, simple and has high extraction efficiencies.

Keywords: Online solid phase extraction-liquid chromatography; Response Surface Methodology; Box-Behnken Design; non-steroidal anti-inflammatory; tetracyclines; water samples

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Pharmaceutical drugs (PDs) are synthetic or natural chemical compounds aimed to prevent, fight and cure an infectious or non-infectious disease in humans or animals. Due to their increasing demand and production, PDs residues have emerged as a known contaminant around the globe, especially in water matrices with the concentration in range ng/L to µg/L [1-2]. Improper household disposal of drugs, industrial and healthcare sewage and inefficient treatment of wastewater in treatment plants are among the sources of drug contaminants [3]. This residue may bioaccumulate and become a potential risk to aquatic biota and other living organisms (including humans) by bio-magnification through trophic level via consumption and development of resistant pathogens [4].

Non-steroidal anti-inflammatory drugs (NSAIDs) and tetracyclines (TCs) are among the most common PDs in clinical and veterinary medicine. NSAIDs are a class of pharmaceuticals with the largest global market size in 2020, amounting to more than USD80 billion [2]. On the other hand, TCs production and usage are estimated to be up to 200,000 tonnes annually [5]. Many reports have described the works of measuring NSAIDs and TCs in various water matrices [6-8]. For example, Gilart and co-workers [9] investigated the concentration of NSAIDs in effluent and influent wastewater and reported four NSAIDs, namely naproxen, fenoprofen, diclofenac and ibuprofen, with concentrations ranging between 0.05 - 20.17 µg L^{-1}. Wang and co-workers [10] detected three dominant tetracycline antibiotics (i.e. tetracycline, oxytetracycline and doxycycline) with concentrations ranging between 11.16-56.09 ng L^{-1} in the Yangtze River that serves as the source of drinking water.

In recent years, solid phase extraction (SPE) has been the most widely used extraction method for the pre-treatment of PDs residue in the environment due to its high specificity and good repeatability [11]. Many reported works concluded SPE provides high extraction recoveries and analyte pre-concentrations [12-13].

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However, SPE revealed some drawbacks, such as disposable cartridge waste and high usage of organic solvents [14]. In line with current technological development, SPE can be performed in online mode and coupled with chromatographic analysis. This online technique offers many advantages compared to offline mode. For example, it avoids the loss of analytes due to evaporation during sample transfer from extraction to detection processes. Additionally, the technique is simple and provides a more effective extraction for monitoring selected PDs from water samples [15-16].

The process of optimisation for the isolation of pharmaceutical drugs in water samples using online SPE may include several factors influencing extraction efficiency. One factor at a time (OFAT) and design of experimental (DOE) are mainly applied during optimisation. However, the OFAT method alone is time-consuming because of the large number of experiments and the likelihood of inaccurate results due to its inability to identify significant interactions between variables [17-18]. Comparatively, DOE is an alternative strategy to obtain optimum conditions. This method is highly accurate, less time-consuming due to the reduction in the number of experiments, and can determine which variable affects the most while giving valid conclusions over a wide range of experimental conditions [18].

DOE, such as the Response Surface Methodology (RSM) with Box-Behnken design (BBD), is a tool in chemometrics that applies multivariate optimisation methods. The three-level design approach in BBD offers an advantage by changing all levels of variables simultaneously during optimisation, being able to estimate the number of parameters of the quadratic model, constructing sequential designs and noting any lack of fit of the model [19].

This study aims to determine selected NSAIDs (i.e. Diclofenac, Naproxen and Acetaminophen) and Antibiotics (i.e. Oxytetracycline and Doxycycline) simultaneously using online SPE-LC in water samples. In addition, RSM experimental design with BBD was applied for the development and validation studies of analytical performance.

MATERIALS AND METHODS

Chemical Reagents

Selected NSAIDs and tetracyclines standards, namely diclofenac (DIC), naproxen (NAP), acetaminophen (ACE), oxytetracycline (OTC) and doxycycline (DOC) and formic acid as organic acid were purchased from Sigma-Aldrich and used without further purification. Methanol (MeOH) and acetonitrile (ACN) with high-performance liquid chromatography (HPLC) grade utilised as solvent were purchased from Merck (Darmstadt, Germany). Ultra-pure water produced by Barnstead Nanopure (Thermo Scientific) was used.

Preparation of Standard and Sample Collection

Each stock solution of 1000 mg L^{-1} of NSAIDs and TCs was prepared separately in methanol and stored in amber glass bottles at -20 ^{o}C prior to the experiment. For TCs stock solution, the amber bottle was wrapped with aluminium foil to avoid photo-degradation. In addition, each individual working standard (10 mg L^{-1}) was prepared in methanol prior to analysis to prevent the analyte from decomposition.

Tap water samples were collected from a laboratory in Universiti Teknologi MARA (UiTM) Shah Alam, Selangor, Malaysia. Collected water samples were filtered using Whatman 0.45 µm Glass Fiber (Whatman International Ltd Maidstone, England) to remove colloidal particles and stored in a freezer at 4°C until analysis.

Online Solid Phase Extraction-liquid Chromatography

Analysis of NSAIDs and TCs in water samples were performed using an automated online SPE HPLC Dionex Ultimate 3000 system (Sunnyvale, CA, USA). The system was equipped with a 10.2 mL loop autosampler, left and right dual gradient pump, solvent rack degasser, thermostat column, online SPE column of IonPac RFIC Guard (4.0 x 50 mm) (Thermo Scientific, USA), an analytical column of Acclaim PolarAdvantage II (5 µm, 120 Å, 4.6mm x 150 mm) (Thermo Scientific, USA), and a diodearray detector (DAD). All data obtained were processed using Chromeleon^{TM} Software v.6.8 (Dionex).

The method comprises four steps of sample loading, clean-up, elution and LC separation under a programmable 6-port and 2-position switching valve. The column temperature was set at 40 ^{o}C. Next, 10 µL of water sample was injected into the flow system using an auto-sampler syringe. The switching valve positioned the SPE column to load the sample together with the conditioning solution (acidified water). A washing solvent was used for flushing out unwanted matrix from the sample while retaining target analytes on the SPE column.

The valve was switched back to connect the SPE column to the analytical column immediately after washing or cleaning up. Gradient elution mobile phase composition between mobile phase A (0.1% formic acid in ultrapure water) and mobile phase B (acetonitrile) carried the analytes from the SPE column to the analytical column with a flow rate of 0.78 mL/ min. The gradient started with 15% of mobile phase B for 0.5 min, increased to 60% from 0.5-17.0 min to 100% from 17.0-18.0 min, remained at 100% until 26.0 min and then decreased to initial composition. The conditioning solution will then equilibrate the

SPE column to be ready for the next sample loading. Extraction and separation of selected NSAIDs and TCs were analysed within 30 min.

Detection Method

Selected NSAIDs and TCs were observed simultaneously for acetaminophen (ACE) at 230 nm, naproxen (NAP) at 254 nm and diclofenac (DIC), oxytetracycline (OTC) and doxycycline (DOC) at 270 nm using a Diode Array Detector (DAD). Identification was based on retention time and ultraviolet (UV) spectrum corresponding to the maximum absorption value of each analyte.

Experimental Design

To obtain the optimum conditions for the simultaneous extraction of selected NSAIDs and TCs, a Box-Behnken Design (BBD) of Response Surface Methodology (RSM) was employed to optimise three independent variables, namely the flow rate of the mobile phase, the valve switching time for transfer, and the acidified water with formic acid. The experimental design was generated using Design-Expert version 6.0.4 (Stat-Ease Software) for regression analysis.

Validation of Analytical Method

The validation of online SPE-LC was assessed to ensure that the analytical procedure is reliable and fit for the intended purpose. Linearity (R^2), precision, accuracy, the limit of detection (LOD) and limit of quantification (LOQ) were calculated from the data obtained. Linear regression of the calibration curve was used to determine LOD and LOQ. The precision of the method was expressed in terms of relative standard deviation (RSD %) and accuracy in terms of relative recovery (i.e. % relative recovery).

RESULTS AND DISCUSSION

Experimental Design using BBD

BBD is a useful experimental design to investigate the effects of several variables simultaneously with maximum efficiency, which requires fewer design points than a full factorial Central Composite Design (CCD) [20]. The final number of experiments generated involved three factors and three levels, and the results obtained are presented in Table 1.

The three optimised conditions for extraction of three selected NSAIDs and two selected TCs using online SPE-LC were 0.71 min valve switching time, 0.1% of acidified water and 0.78 mL min^{-1} of flow rate with the desirability of 0.971. Equation (1) is the regression equation for a fitted model, where Y is the response (total peak area) of target analytes, A is the valve switching time, B is the percentage of acidified water, and C is the flow rate.

$$Y = 139.65-0.42A - 0.54B + 0.32C - 1.79A^2 - 5.01B^2 - 3.04C^2 + 0.46AB - 1.75AC-0.078BC \quad \text{(Eq.1)}$$

Table 1. Box-Behnken Design (BBD) for the analysis of NSAIDs and TCs.

Run	*Valve Switchingtime (min)*	*Acidified water (%)*	*Flow rate (mL/min)*	*Total Peak Area of NSAIDs andTCs (mAu*min)*
1	1.00	0.10	0.50	136.51
2	0.75	0.15	0.50	130.44
3	0.75	0.15	1.00	130.97
4	0.75	0.10	0.75	139.30
5	1.00	0.15	0.75	132.03
6	0.50	0.10	1.00	136.61
7	0.75	0.05	1.00	132.90
8	1.00	0.05	0.75	131.49
9	1.00	0.10	1.00	133.99
10	0.75	0.10	0.75	139.99
11	0.50	0.05	0.75	134.58
12	0.50	0.10	0.50	132.53
13	0.75	0.05	0.50	132.06
14	0.50	0.15	0.75	133.28

Table 2. Analysis of variance (ANOVA) for the Regression Models.

Source of Variation	*Sum of Squares*	*DF*	*Mean Square*	*F Value*	*P Value*	
Regression	112.54	9	12.50	10.58	0.0183	Significant
A	1.43	1	1.43	1.21	0.3334	
B	2.32	1	2.32	1.96	0.2337	
C	0.80	1	0.80	0.68	0.4569	
A^2	10.27	1	10.27	8.69	0.0421	
B^2	80.28	1	80.28	67.92	0.0012	
C^2	29.65	1	29.65	25.08	0.0074	
AB	0.85	1	0.85	0.72	0.4451	
AC	12.25	1	12.25	10.36	0.0323	
BC	0.024	1	0.024	0.020	0.8935	
Residual	4.73	4	1.18			
Lack of Fit	4.49	3	1.50	6.29	0.2833	Not significant
Pure Error	0.24	1	0.24			
Total	117.27	13				

DF= 9, F= 10.58 and P= 0.0183

Analysis of Variance

Analysis of variance (ANOVA) and regression analysis was used to assess the significance of variables presented (P-values), the sum of squares, mean square, lack of fit (F-values) and degree of freedom (DF). The statistical significance of the model was determined using ANOVA analysis. Multi-linear regression was applied to the results of BBD. The effects of the independent variables, which are valve switching time, acidified water and flow rate, were evaluated by second-order (quadratic) equations. The analysed data are presented in Table 2.

The results show the statistical significance of the second-order equation and regression for the selected NSAIDs and TCs. The P-value of 0.0183 indicated there is only a 1.83 % chance that a model F-value this large could occur due to noise. The "Lack of Fit F-value" of 6.29 implied that the Lack of Fit is not significant relative to the pure error. There is a 28.33 % statistical probability that a "Lack of Fit F-value" could be observed (P-value=0.2833). Non-significant lack of fit is good because the model needs to be fit. The reliability of the fitted model was proven by the high F-values and the low P-values.

Table 3 represents the summary of the analysis of variance (ANOVA) regression model for the quadratic response model for NAP, DIC, ACE, OTC and DOC. R^2 represents the quality of fit of the quadratic polynomial model, and the value shows the relationship between the predicted and actual values. The calculated R^2 value was 0.9597 (Table 3), indicating a good-fitted model and significance for the extraction of selected NSAIDs and TCs [21].

The parity plot in Figure 1 shows that the actual and predicted values have a strong correlation where points around the diagonal line indicate a good fit for the model. The average difference is less than 1, showing minimal differences between the actual and predicted values. The Pareto chart in Figure 2 shows visualised main effects of variables in extraction to the total peak areas of selected NSAIDs and TCs. The most influent response either from single variable A (valve switching time), B (acidified water) or C (flow rate) or a combination of different variables (AC, AB or BC) or a combination of the same variables (A2, B2 or C2) seen under this figure.

The variable that affected the extraction of selected NSAIDs and TCs to total peak areas the most, as shown in the Pareto chart, was acidified water, B2. Extraction of selected NSAIDs and TCs readily ionised in water may need an H^+ ion. Acidified water supplied H^+ to neutralise those analytes and increase the efficiency of selected NSAIDs and TCs to adsorb onto SPE sorbent during extraction.

Table 3. ANOVA Analysis of selected NSAIDs and TCs.

Transform	*Model*	*Lack of Fit*	*DF*	*R-square*	*Equation*
Square Root	Quadratic Significant	Not Significant	9	0.9597	Sqrt (Total PeakArea) = 139.65-0.42A - 0.54B + 0.32C - 1.79A^2 - 5.01B^2 - 3.04C^2 + 0.46AB - 1.75AC - 0.078BC

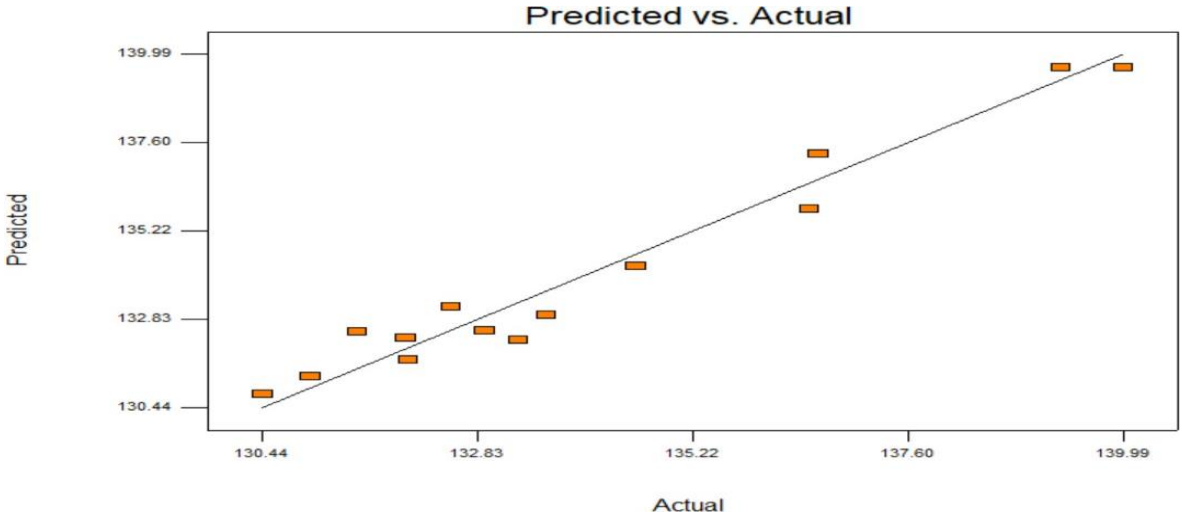

Figure 1. The parity plot between predicted and actual (experimental) values for selected NSAIDs and TCs

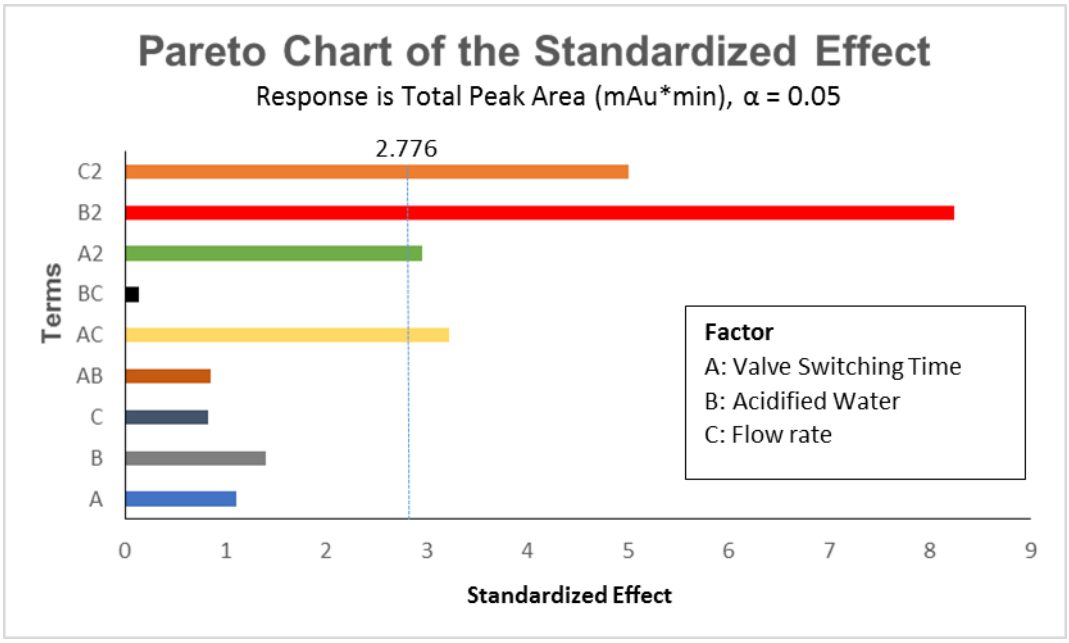

Figure 2. Pareto chart of the standardised effect in Online SPE-LC

Response Contour Plot

In this work, BBD was used to investigate the effects of valve switching time, acidified water and flow rate on the peak area of selected NSAIDs and TCs in the form of three-dimensional (3D) plots. The illustration in Figure 3 (a) - (c) shows the main and interactive effects of independent variables on the response variables for selected NSAIDs and TCs.

The interaction between the percentage of acidified water and valve switching time at a constant flow rate is shown in Figure 3 (a). The total peak area was low at low acidified water and valve switching time. However, it was increased when acidified water and valve switching time were gradually increased. At 0.1 % of acidified water and 0.71 min for valve switching time, the highest peak area of analytes obtained was 139.67 mAu*min. However, the peak area gradually decreased at 0.15 % and 1.00 min for valve switching time.

NSAIDs in an acidic solution and TCs with three values of pKa, which are present in an acidic solution with sufficient protons (H^+), may increase the adsorbent onto extraction reverse phase sorbent and LC column. However, if the NSAIDs and TCs are in a basic solution, they may dissociate into conjugate bases due to insufficient proton (H^+). As a result, ionisation will occur and reduced peak area.

Figure 3 (b) shows the interaction between the valve switching time and flow rate while the percentage of acidified water was kept constant. The total peak area was low at a low solvent flow rate and low valve switching time but increased when both factors increased gradually. The lowest peak area of analytes (132.95 mAu*min) was obtained at 1.00 min of valve switching time and 1.00 mL min^{-1} solvent flow rate.

A sample with 10 µL was injected using an autosampler into the system, and a mobile phase with a flow rate of 0.5 mL min^{-1} carrying the analytes. When the valve switching time was set at 0.5 min, the peak area for all analytes was low compared to the 0.71 min valve switching time. Meanwhile, at 1.00 min valve switching time, the peak area of analytes gradually decreased, which may be due to an increase in flow rate and less time for analytes to be embedded onto the sorbent.

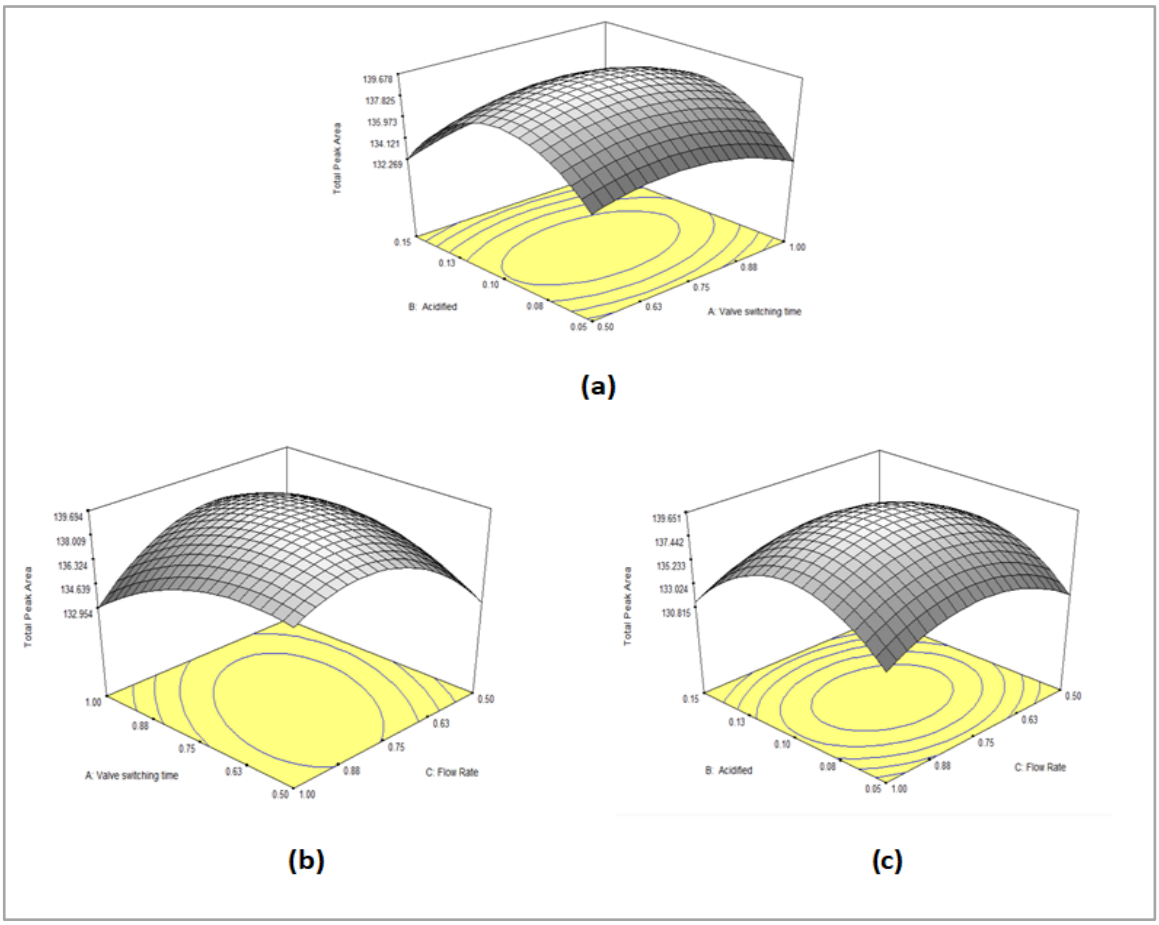

Figure 3. RSM 3D Contour Plots for Online SPE-LC. Legends: (a) Valve Switching Time and Acidified Water (b) Valve Switching Time and Flow rate (c) Acidified Water and Flow Rate against Total Peak Area of selected NSAIDs and TCs

Table 4. Validation data of Online SPE-LC Method for Selected NSAIDs and TCs in Tap Water Samples.

Sample	*Analyte*	*Linear range (mg L^{-1})*	*Coefficient of determination (R^2)*	*LOD, (mg L^{-1})*	*LOQ (mg L^{-1})*
Tap water	ACE[a]	0.5-10	0.9993	0.29	0.99
	NAP[a]	0.5-10	0.9990	0.28	0.92
	DIC[a]	1-10	0.9995	0.56	1.86
	OTC[b]	1-10	0.9997	0.50	1.52
	DOC[b]	1-10	0.9991	0.73	2.45

[a] =NSAIDs and [b] =TCs

Figure 3 (c) shows the interaction between the percentage of acidified water and flow rate while valve switching time was kept constant. At low acidified water percentage and flow rate of the mobile phase, the total peak area was low (130.87 mAu*min). The highest peak area of analytes (139.65 mAu*min) was found at 0.78 mL min^{-1} for the mobile phase flow rate.

Method Validation of Online SPE-LC

Validation of Online SPE-LC was conducted after optimisation for linearity, precision and relative recoveries. Five (5) concentrations of the standard mixture in the range 0.5 to 10 mg L^{-1} (ACE and NAP) and 1 to 10 mg L^{-1} (DIC, OTC and DOC) with three (3) replicates to the generated calibration curve. Table 4 shows validation data of the online SPE-LC method of selected NSAIDs and TCs in the tap water samples.

A high coefficient of determination (R^2=0.9990-0.9997) was obtained for each analyte's linear curve during validation. LOD of the method was calculated using a linear regression equation to show the sensitivity of the method, and results were in the range of 0.28 - 0.73 mg L^{-1}. Table 5 shows the percentage recovery by spiking tap water samples to give final concentrations of 1.5 and 7.0 mg L^{-1}. The results show good percentage recoveries in the 94.4 %-108.3 % range. These results indicate that the developed online SPE-LC method is a simple, highly sensitive and green extraction method which could potentially be used in the chemical laboratory for routine analysis of water samples.

Table 5. Validation data of Online SPE-LC Method of Selected NSAIDs and TCs from Spiked Water Samples.

Analyte	*Spiked concentration (mg L^{-1})*	*Tap Water*	
		Relative recovery (%)	*RSD (n=3)*
ACE[a]	1.5	100.3	1.89
	7.0	98.7	0.19
NAP[a]	1.5	101.9	3.67
	7.0	95.1	0.47
DIC[a]	1.5	108.3	1.37
	7.0	95.8	1.13
OTC[b]	1.5	99.6	0.78
	7.0	94.4	0.18
DOC[b]	1.5	101.5	2.24
	7.0	96.5	1.62

[a] =NSAIDs and [b] =TCs

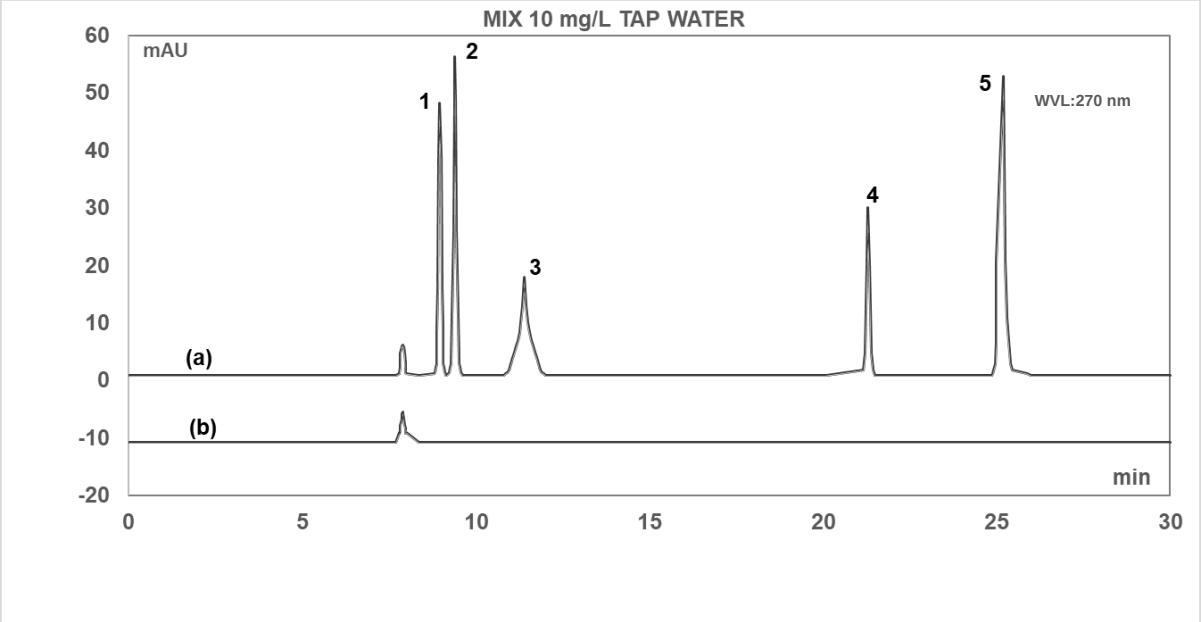

Figure 4. Chromatograms of (a) spiked and (b) unspiked tap water samples in 10 mg L^{-1} of mixed selected NSAIDs and TCs under optimum conditions of valve switching time 0.71 min, 0.1% acidified water and flow rate 0.78 mL/min, for 1. OTC, 2. ACE, 3. DOC, 4. NAP, and 5. DIC.

Table 6. Comparison study of the determination of NSAIDs and TCs by online SPE with other published methods.

Analysis method	*Sorbent*	*Type of sample*	*Linear range (ng/mL)*	*LOD (ng/mL)*	*Recoveries (%)*	*Ref.*
DLLME-UHPLC-MS	DCM/MeOH/ ACN	Wastewater/River water	0.04 - 200	0.006 - 0.091	76.77 - 99.97	[23]
SPE-LC-MS	Oasis HLB	Wastewater/Surface water	10.0-1000	0.001 - 0.06	50.0 - 116.0	[24]
SPE-LC-DAD	Oasis HLB	Wastewater	1.5 - 5.0	0.01 - 0.96	71.0 - 103.0	[25]
Online-SPE-LC-DAD	C_{18}	Tap water	500 - 10000	280-730	94.4 - 108.3	This work

Figure 4 shows the chromatograms of spiked and unspiked tap water samples in 10 mg L^{-1} of mixed selected NSAIDs and TCs. The chromatograms revealed that all analytes were successfully extracted and separated from the tap water samples. Besides that, DOC also displayed a broader peak compared to other analytes, which could be due to interference as TCs are metal chelators, thus affecting the extraction performance [22]. Table 6 is summarised the comparison between the efficiency of the developed online-SPE method for NSAIDs and TCs with previously reported methods in terms of linear range, LODs, and percentage recoveries.

CONCLUSIONS

In conclusion, online SPE-LC has been successfully developed to analyse selected NSAIDs and TCs in water samples rapidly. The selected NSAIDs and TCs were diclofenac (DIC), naproxen (NAP), acetaminophen (ACE), oxytetracycline (OTC) and doxycycline (DOC). Simultaneous determination of NSAIDs and TCs using Online SPE combined with liquid chromatography has the potential to improve extraction efficiency by shortening the extraction and analysis time. The investigation using BBD of RSM for three parameters shows optimum conditions of 0.71 min for valve

switching time, 0.1 % for acidified water, and 0.78 mL min^{-1} flow rate of the mobile phase. The optimum parameters were used for further analysis, and successfully extracted selected NSAIDs and TCs from real water samples. The method was validated with good linearities (R^2) in the range of 0.9990-0.9997. The method was applied for the analysis of tap water samples with good relative recoveries in the range of 94.4 %-108.3 %. The results from this work show that the Online SPE-LC method is a rapid and efficient technique for extracting and separating NSAIDs and TCs in water samples.

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