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RSM-Optimization Approach for Dispersive Micro Solid Phase Extraction of Tetracycline Antibiotics in Water

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A dispersive micro solid phase extraction (D-µ-SPE) method was developed using C_{18} for the extraction and pre-concentration of tetracycline antibiotics (TCs) in water samples prior to analysis by high performance liquid chromatography - ultraviolet/diode array detector (HPLC-UV/DAD). The selected TCs residues were tetracycline (TC), oxytetracycline (OTC) and doxycycline (DOC). A Central Composite Design (CCD) of Response Surface Methodology (RSM) was used to optimize the mass of sorbent and extraction time. Results showed that the coefficient of determination, R^2, for the extraction of the selected TCs was 0.9980, indicating a well fit model. The optimum conditions obtained from the extraction analysis were 75 mg sorbent mass and 30 min extraction time. Under these optimum conditions, good linearities were obtained over the range of 0.1-10 mg L^{-1} with R^2 values of 0.9990-0.9997 and a detection limit of 0.053-0.099 mg L^{-1}. The method was successfully applied to river and tap water samples. The developed method proved to be simple, rapid, and reliable, with good extraction efficiencies for the detection of antibiotics in water samples.

Key words: Dispersive Micro Solid Phase Extraction; Response Surface Methodology; Central Composite Design; tetracycline antibiotics; water samples

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Antibiotics are natural or semisynthetic compounds frequently used to treat bacterial infections in humans and farm animals [1]. Most antibiotic residues found in water are usually not fully absorbed by the human body, thus they are excreted as urine and faeces into the environment [2]. However, the overconsumption of antibiotics can lead to resistance because of their long residence time in the environment. In addition, sewage treatment plants may not effectively remove antibiotic residues [3]. Levels of tetracycline antibiotics (TCs) have been measured in tap water with median concentrations between 0.089 ng/mL and 0.0064 ng/mL [4]. In China, the level of antibiotics such as oxytetracycline (OTC) and TCs have been reported in the range of 0.16–5.7 and 0.7– 65.2 µg L^{-1}, respectively [5].

Analysis of TCs and OTCs requires very extensive sample pre-treatment to remove major interferences and analyte enrichment [6]. Various sample preparation techniques based on extraction using sorbent materials have been proposed, including solid phase extraction (SPE), which is widely used for the isolation and pre-concentration of pharmaceutical analytes [7]. Instead of conventional solid phase extraction (SPE), dispersive micro SPE (D-µ-SPE) has been used to increase the efficiency of interaction between the sorbent and the analyte [8].

Dispersive-µ-SPE is an interesting approach developed by Anastassiades and co-workers in 2003 [9]. It is commonly known as QuEChERS, an acronym for its main features: quick, easy, cheap, effective, rugged, and safe. It involves the extraction of analytes from a homogenized sample using an acetonitrile and salt solution and the separation of the supernatant from the D-SPE media [10].

Compared to conventional SPE, dispersive SPE is a more efficient and user-friendly alternative technique that uses solid phase dispersion in a sample matrix as its fundamental principle. The close contact achieved by the analyte-adsorbent interaction favours the kinetics of the sorption, which increases the efficiency of the overall process, resulting in a shorter extraction time [11].

More recently, dispersive micro-solid phase extraction (D-µ-SPE) has been developed as a simple and miniaturized modification of DSPE that can be

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applied to extract and enrich nonsteroidal anti-inflammatory drugs (NSAIDs) [12], pesticides [13], polycyclic aromatic hydrocarbons (PAHs) [14], heavy metal ions [15] and bisphenol A [16].

The integrated effect of the mass of sorbent and extraction time can be determined by employing Response Surface Methodology (RSM). This may be achieved by employing a Central Composite Design (CCD) to construct three-dimensional (3D) surface and contour plots.

In this study, the RSM and CCD approaches were employed in dispersive micro SPE using HPLC-DAD to extract selected antibiotics in water samples. This method was expected to extract tetracycline antibiotics (oxytetracyclines, tetracycline and doxycycline) rapidly and efficiently.

MATERIALS AND METHODS

Chemical Reagents

All tetracycline antibiotics (TCs): tetracycline (TC), doxycycline (DOC) and oxytetracycline (OTC) of high purity grade (>99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other solvents such as HPLC grade methanol (MeOH), acetonitrile (ACN), HPLC grade water, hydrochloric acid (HCl) 37% and formic acid (HCOOH) 98% were also purchased from Merck (Darmstadt, Germany). Nitrogen for drying was high purity grade (> 99/9998%) and supplied by Tehran Gas Co. (Tehran, Iran). Octadecylsilane (C_{18}) and primary-secondary amine (PSA) were purchased from Materials (Shanghai, China) and ultrapure water was obtained from a Millipore Milli-Q system (Molsheim, France).

Preparation of Stock and Standard Solution

The individual stock solutions of OTC, TC and DOC were prepared separately in HPLC grade methanol to a final concentration of 1000 mg L^{-1}. A standard mixture of 100 mg L^{-1} was prepared by diluting 10 mL of each stock solution with methanol to a final volume of 100 mL. A series of working standard solutions were prepared by dilution in methanol before analysis to prevent the decomposition of analytes. Spiked water samples were prepared by adding 1 mL of 10 mg L^{-1} standard solutions into 9 mL of deionized water to obtain a final concentration of 1 mg L^{-1}. All standard solutions were stored in amber glass bottles at 4 °C when not in use.

Sample Collection

Tap water samples were collected from a laboratory in Universiti Teknologi MARA (UiTM) Shah Alam, Selangor, Malaysia, while river water samples were collected from Hulu Langat, Selangor, Malaysia. The collected samples were filtered through 0.45 µm nylon filter paper to remove any suspended particles. Samples were kept in polyethylene bottles and stored at 4 °C prior to the analysis process.

Dispersive-Micro-Solid Phase Extraction (D-µ-SPE) Procedure

Fifty milligrams of octadecylsilane C_{18} were dispersed into 10 mL of an aqueous sample (50 mL, pH 7) in a centrifuge tube. The mixture was stirred by a magnetic stirrer for 15 min. The sorbent was then separated from the solution by centrifugation at a speed of 4000 RPM for 5 min, and the supernatant was discarded. The desorption solvent (methanol) was added into the centrifuge tube and stirred for another 15 min. The mixture then underwent centrifugation at the same speed and duration. The solvent was decanted and evaporated to 1 mL under a gentle stream of nitrogen gas. One milligram of the extracted analyte was transferred to a 1 mL glass vial. From that vial, 10 µL was injected into the HPLC system. Figure 1 shows a schematic diagram of the D-µ-SPE procedure.

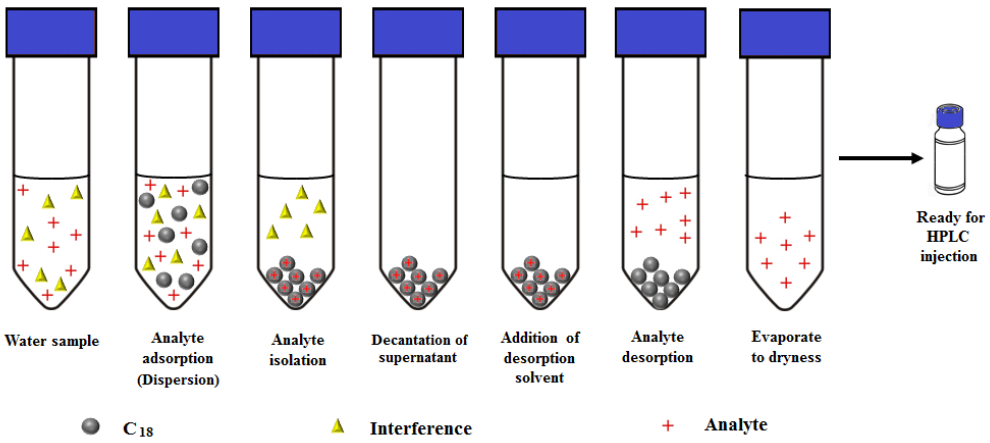

Figure 1. Schematic diagram of the D-µ-SPE procedure

Table 1. Coded values of variables for the experimental design

Factor	Parameter	Coded Level of Variables				
		−α	-1	0	+1	+ α
A	Mass of sorbent (mg)	39.64	50	75	100	110.36
B	Extraction time (minutes)	1.72	10	30	50	58.28

Chromatographic Conditions

The targeted analytes were determined using an Agilent HPLC system (Agilent Technologies, USA) with a reverse phase Zorbax SB-C18 column (2.1 mm × 100 mm × 3.5 µm). The system applied gradient elution between the acidified ultrapure water and acetonitrile over time, with a starting ratio of A%: B% (95:5) where the percentage of A gradually decreased while B gradually increased. At the end of the process, the percentage of both mobile phases were equal, 50%, respectively. An ultraviolet-DAD detector was used at 270 nm.

Experimental Design

To obtain the optimum conditions for the simultaneous extraction of TCs, a Central Composite Design (CCD) of Response Surface Methodology (RSM) was employed to optimize two independent variables, mass of sorbent and extraction time. The experimental design was generated using Design-Expert version 6.0.4 (Stat Ease Software) for regression analysis with the coded level of selected factors (−α, −1, 0, +α, +1) as shown in Table 1.

Validation of Analytical Method

The validation of D-µ-SPE was assessed for linearity (R^2), limit of detection (LOD), limit of quantification (LOQ), precision and accuracy to ensure that the analytical procedure was reliable and fit for the intended purpose. LOD and LOQ were calculated based on linear regression of the calibration curve. Precision was expressed in terms of relative standard deviation (RSD %) and accuracy (% relative recovery).

RESULTS AND DISCUSSION

Experimental Design using CCD

CCD is a useful method to investigate the effects of several variables influencing the responses, reducing the number of experimental trials and increasing efficiency by varying them simultaneously [17]. A set of experiments consisting of 13 runs were generated with a design matrix consisting of five levels of two factors. The experimental results are shown in Table 2.

Optimization of the extraction of 3 types of tetracyclines using D-µ-SPE-LC yielded two conditions, 75 mg sorbent and 30 min extraction time. The regression equation of the fitted model is given in Equation 1, where Y is the response (total peak area) of target analytes, A is the mass of sorbent and B is extraction time.

$$Y = 25.49 + 0.25A + 0.018B - 8.99A2 - 9.64B2 - 0.70AB \quad \text{(Eq. 1)}$$

Table 2. Central Composite Design (CCD) for the analysis of tetracyclines

Run	***Mass of sorbent (mg)***	***Extraction time (min)***	***Total Peak Area (mAu*min)***
1	110.36	30.00	7.942
2	75.00	30.00	25.583
3	75.00	1.72	6.543
4	50.00	50.00	7.062
5	75.00	30.00	25.492
6	100.00	50.00	6.373
7	39.64	30.00	7.527
8	100.00	10.00	7.549
9	75.00	30.00	26.09
10	75.00	58.28	6.335
11	50.00	10.00	5.451
12	75.00	30.00	25.146
13	75.00	30.00	25.114

Table 3. Results obtained from regression and ANOVA analysis

Source of variation	*Sum of squares*	*DF*	*Mean square*	*F value*	*P value*	
Regression	1072.88	5	214.58	1215.09	<0.0001	Significant
A	0.50	1	0.50	2.82	0.1370	
B	2.480E-003	1	2.48E-003	0.014	0.9090	
A2	562.82	1	562.82	3187.12	<0.0001	
B2	646.80	1	646.80	3662.68	<0.0001	
AB	1.94	1	1.94	11.00	0.0128	
Residual	1.24	7	0.18			
Lack of fit	0.61	3	0.20	1.29	0.3922	Not significant
Pure error	0.63	4	0.16			
Total	1074.12	12				

Analysis of Variance

Analysis of variance (ANOVA) and regression analysis were used to assess the significance of variables (P-values), the sum of squares, mean square, lack of fit test (F-values) and degree of freedom (DF). The statistical significance of the model was defined via an ANOVA analysis. Multi-linear regression was applied to the results of the CCD. The effect of independent variables, which are mass of sorbent and extraction time were evaluated by a second-order equation (quadratic). The data is presented in Table 3.

The results show the statistical significance of the second-order equation and regression for all analytes. The obtained P-value was < 0.0001, which indicated the significance of the regression model [18]. The "Lack of Fit F-value" of 1.29 implied the lack of fit was not significant relative to the pure error. The P value obtained indicated that there was a 39.22 % statistical probability that the large "Lack of Fit F-value" could be observed. The high F-values and low P-values proved the reliability of the fitted model.

Table 4 presents the summary of the analysis of variance (ANOVA) regression model for the response quadratic model for OTCs, TCs and DOCs. The value of R^2 showed an acceptable relationship between the predicted and actual values. As shown in Table 3, the R^2 value calculated for the extraction of tetracyclines was 0.9988, and the adjusted R^2 was 0.9980 indicating a well fit model and significance. The closer the value of R^2 to unity, the better the empirical model fits the actual data [19]. The coefficient of variation (CV) of 1.86 %, which was less than 10%, indicates that the model is reproducible. The prediction residual errors sum square (PRESS) value of 0.18, which was low, also supported the model.

The parity plot shows a satisfactory correlation between the actual and predicted values, where the points are clustered around the diagonal line, indicating the model was a good fit (Figure 2). The differences between experimental and predicted values were small, with an average difference of less than 1.

The main effects of the variables were visualized using a Pareto chart, as shown in Figure 3. According to the chart, the extraction time, B2 had the largest influence on the normalized peak area, which affected the extraction efficiencies of TCs the most. In SPE, extraction time is an important optimization parameter for efficient extraction, as a suitable extraction time should be sufficient for all analytes to be extracted from the samples without being back-extracted.

Table 4. Summary of ANOVA results

Transform	*Model*	*Lack of fit*	*DF*	*R-square*	*Equation*
Square root	Quadratic Significant	Not Significant	5	0.9988	Y = 25.49 + 0.25A + 0.018B – 8.99A2 – 9.64B2 – 0.70AB

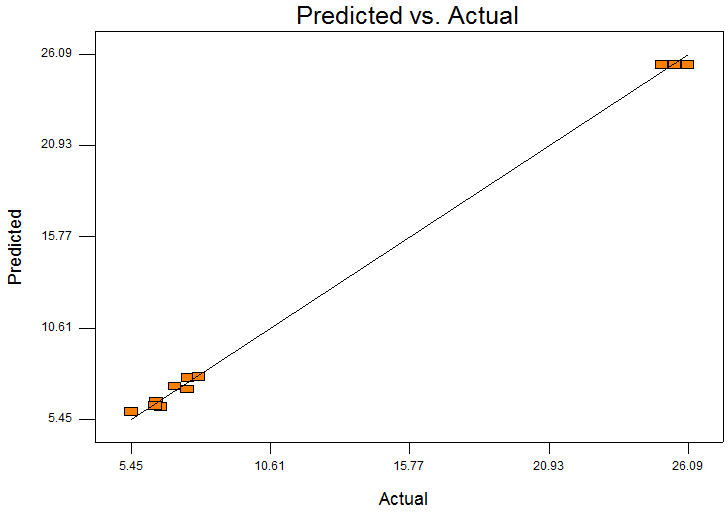

Figure 2. The parity plot between predicted and experimental values

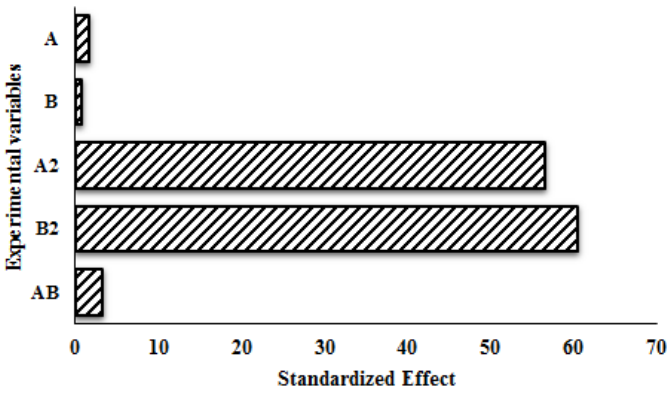

Figure 3. Pareto chart of the main effects of variables in D-µ-SPE-LC (A: Mass of sorbent; B: Extraction time)

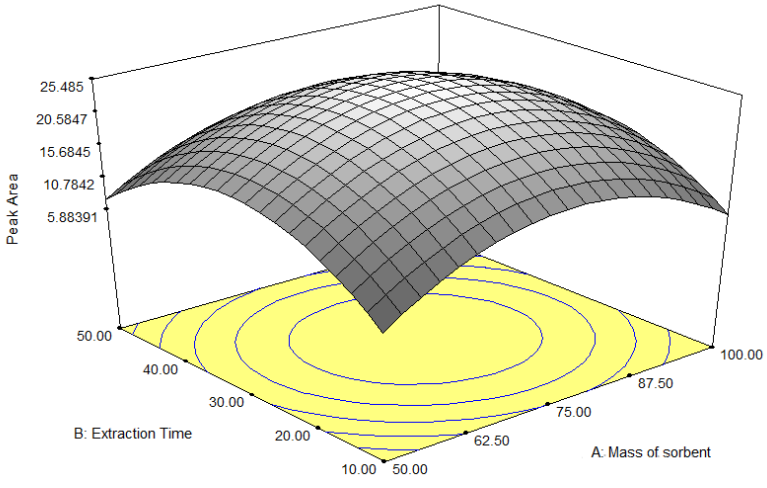

Figure 4. RSM 3-D surface and contour plots for the mass of sorbent and extraction time

Response Contour Plot

The results of the CCD experiments were further visualized in the form of three-dimensional (3D) surface and contour plots. RSM was used to investigate the integrated effect of the mass of sorbent and extraction time in the form of 3D plots. As illustrated in Figure 4, the mass of sorbent and variables acted in parallel, which considerably influenced the response or peak area.

The lowest peak area of analytes (5.451 mAu*min) was obtained with 50 mg sorbent and an extraction time of 10.0 min. The highest peak area of analytes (26.09 mAu*min) was obtained with 75 mg sorbent and 30.0 min extraction time. The increase in the amount of C_{18} used, from 50-75 mg, resulted in the peak area increasing. However, the usage of sorbent over 75 mg led to a decrease in the peak area. Similarly, as the extraction time increased from 0-30 min, extraction efficiencies also increased due to increased peak area. The peak area was highest at 30 min because there was sufficient time for equilibrium to be achieved between the sample matrix and C_{18} sorbent. The efficiency gradually decreased after 30 min of extraction, as the targeted analytes may have been extracted back into the sample matrices from the sorbent during this time [20]. Zhou et al. reported a shorter separation time of 23 min in their analysis of antibiotics [21]. However, our method has been optimized with a longer run time of 30 min to allow more compounds to be extracted for better separation and detection. Therefore, the optimum mass of sorbent (75 mg) and extraction time (30 min) were chosen for the subsequent experiment.

Method Validation and Analytical Performance

The optimization of D-µ-SPE-LC was validated for linearity, precision, and relative recoveries. A calibration curve was generated using five (5) concentrations of the standard mixture in the range of 0.1 to 10 mg L^{-1} with three replicates. Table 5 shows the validation data of the D-µ-SPE-LC method for TCs in tap and river water samples.

Linear curves for each analyte were obtained with excellent coefficients of determination (R^2 = 0.9990-0.9997). The sensitivity of the method, expressed as the LOD, was determined via a linear regression method, and the results were in the range of 0.053-0.099 mg L^{-1}. The percentage recovery study was done by spiking the river water samples to give final concentrations of between 0.1 and 10 mg L^{-1}. The results showed good percentage recoveries in the range of 75.4 % to 99.6 % (Table 6). Hence, the D-µ-SPE-LC method proved to be a simple, sensitive, selective and green extraction method that has the potential to be used in a laboratory for water sample analysis.

Table 5. Quantitative results of D-µ-SPE-LC of TCs in tap water and river water samples

Sample	*Analyte*	*Linear Range (mg/L)*	*Coefficient of Determination*	*LOD (mg/L)*	*LOQ (mg/L)*
Tap Water	OTC	0.1-10	0.9991	0.095	2.51
	TC	0.1-10	0.9990	0.099	0.94
	DOC	0.1-10	0.9992	0.089	3.47
River Water	OTC	0.1-10	0.9996	0.062	1.04
	TC	0.1-10	0.9997	0.053	1.53
	DOC	0.1-10	0.9994	0.076	1.63

Table 6. Relative recovery studies for D-µ-SPE-LC of TCs from spiked water samples.

Analyte	*Spiked concentration (mg L^{-1})*	*Tap Water*		*River Water*	
		Relative Recovery (%)	*RSD*	*Relative Recovery (%)*	*RSD*
OTC	0.1	80.4	2.34	76.2	1.87
	10	99.6	0.60	97.6	0.55
TC	0.1	83.3	1.22	75.4	1.85
	10	95.3	1.74	97.0	0.77
DC	0.1	86.8	1.43	83.8	4.66
	10	97.2	3.67	96.0	0.30

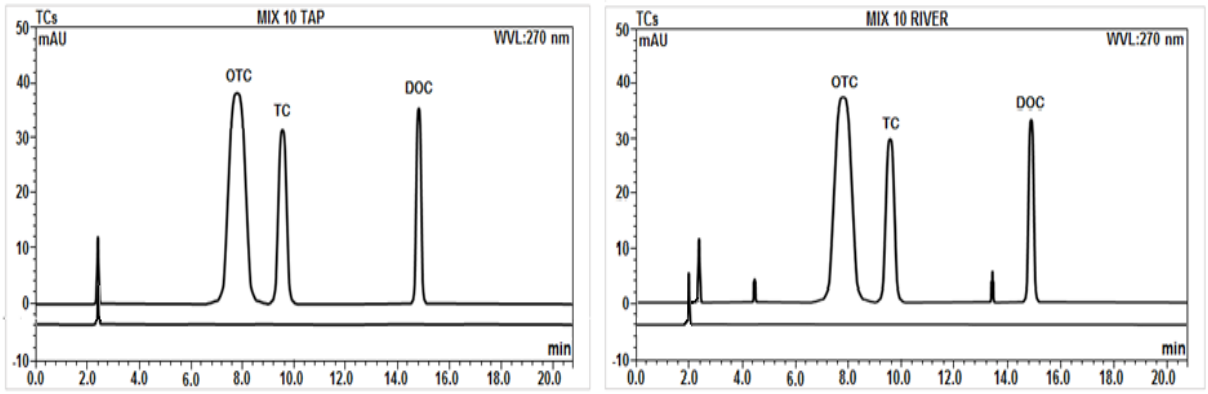

Figure 5. HPLC chromatogram of TCs in spiked and unspiked samples of (a) tap water and (b) river water

Table 7. Comparison study of the determination of TCs by SPE with other published methods.

Analysis method	*Sorbent*	*Type of sample*	*Linear range (µg L^{-1})*	*LOD (µg L^{-1})*	*Recoveries (%)*	*Ref.*
µ-SPE-HPLC-UV	PET-GO nanofiber	Honey	10–5000	15.3	89–94	[23]
MSPE-DLLME	Fe_3O_4@SiO_2@GO-β-CD	Water	10–200	1.8–2.9	71-122	[24]
SPE-HPLC	Graphene	Milk	20–1000	10-20	82-104	[25]
MSPE-UPLC–TUV	Fe_3O_4@SiO_2@FeO	Tap, river	0.133–333	0.027-0.107	91-105	[26]
D-µ-SPE-LC	C_{18}	Tap, river	100-10000	53-99	80-100	This work

The chromatograms of spiked tap water and river water samples in 10 mg L^{-1} of mixed TCs are shown in Figures 5(a) and 5(b), respectively. The chromatograms revealed that all analytes were successfully extracted and separated from both water samples. It can also be observed that the peak for OTC was broader than the other TC peaks. There is a possibility that there was some interference as TCs are metal chelators, and this may affect the extraction [22].

Comparison with other Reported Methods

The efficiency of the developed D-µ-SPE method for TCs was compared with previously reported methods in terms of linear range, LODs, and percentage recoveries. The comparison is summarized in Table 7.

CONCLUSION

In conclusion, this study proved that dispersive micro solid phase extraction coupled with liquid chromatography (D-µ-SPE-LC) could be optimized for sorbent mass and reaction time using the response surface method. 75 mg of sorbent mass and 30 min extraction time were the optimum conditions that achieved the highest peak area. This method was successfully applied to extract tetracycline antibiotics with good relative recoveries (80-100 %).

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The authors declare that they have no conflict of interest.

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