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Article

Development of Methyl Ester Antibody-Based Competitive Indirect ELISA for Quantitative Detection of Mitragynine in Human Urine

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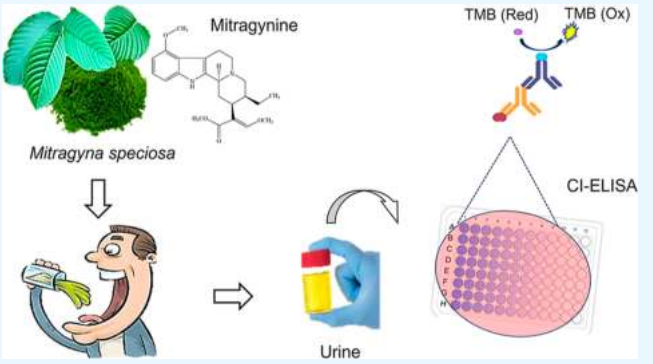
ABSTRACT: Mitragynine is the main psychoactive compound of *Mitragyna speciosa* Korth. (*kratom*). This alkaloid could render psychotropic effects and is often misused as a substitute for commercial drugs. Nowadays, the increasing popularity of *kratom* has led to the development of a rapid and effective detection method. The detection of mitragynine in a biological sample such as urine requires a highly sensitive and specific method due to the complex nature of mitragynine in urine. Enzyme-linked immunosorbent assay (ELISA) is well known as a rapid screening method for biological samples. In this study, a competitive indirect ELISA was successfully developed using MG-22-OCH_3 IgG as a detection antibody for mitragynine in human urine. The mitragynine immunoassay showed a limit of detection and a limit of quantification of 0.412 and 1.25 μg/mL, respectively. The measurement range was between 0.01 and 100.0 μg/mL with a minimal inhibition (IC_{50}) value of 0.152 μg/mL. The developed ELISA was validated using a gold method such as high-performance liquid chromatography−mass spectrometry (HPLC-MS). The percentage of recovery and the coefficient of variation (CV) for the ELISA and LCMS/MS analyses were 84.0−95.70%, 99.20−112.0%, 7.69−9.78%, and 2.86−6.62%, respectively. This indicates that the developed ELISA is a reliable method that can be used as a rapid approach for quantifying mitragynine content in biological samples.



1. INTRODUCTION

Mitragyna speciosa Korth. is a psychoactive plant native to the Southeast Asian region, including Malaysia, Thailand, and Myanmar. It has traditionally been used by natives to relieve muscle pain, diarrhea, hypertension, anti-inflammation and to enhance productivity.[1] However, prolonged consumption of *kratom* can lead to seizure-like symptoms in humans, such as mouth-foaming, aspiration, pneumonia, fever, and hypotension.[2]

Mitragynine (Figure 1) is the major alkaloid in *M. speciosa* leaves. Monitoring the use of this potent compound is crucial for minimizing its abuse, particularly among young individuals who prefer *kratom* over more expensive synthetic drugs. Several chromatographic methods, such as gas chromatography−mass spectrometry (GC−MS),[3] liquid chromatography with linear ion trap-mass spectrometry (LCMS/MS),[4,5] and liquid chromatography with electron spray tandem mass spectroscopy (LC−MS)[6,7] have been widely employed for the detection and quantification of active alkaloids, particularly mitragynine. However, these methods offer sufficient accuracy and sensitivity, they are not suitable for rapid monitoring in routine toxicological screening.

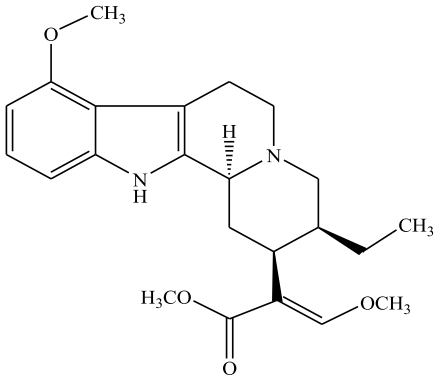


Figure 1. Structural formula of mitragynine.

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In forensic and toxicological applications, a lower limit of detection (LOD) is required to identify trace amounts of mitragynine in complex biological matrices, such as urine. The LOD analysis can vary depending on the sensitivity of the analytical method used. Liquid chromatography technique has been developed for mitragynine detection, with reported LODs of 0.005 μg/mL using ultra-performance liquid chromatography (UPLC),[8] 0.0021 μg/mL using high-performance liquid chromatography-photodiode array,[9] 0.0056 μg/mL using ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS),[4] and 0.47 μg/mL using HPLC-UV.[10] Furthermore, various immunoassays have been developed to detect mitragynine in urine using Competitive Indirect ELISA(CI-ELISA) with different levels of detection sensitivity, such as a LOD of 0.041[11] and 0.015 μg/mL,[12] an IC_{50} value of 0.0012 μg/mL,[13] and a LOD of 32470 μg/mL.[14]

To address the limitations of sophisticated instruments and lengthy analysis, which are not suitable for routine toxicological screening, several rapid methods have been developed for the detection of mitragynine. Over many decades, enzyme-linked immunosorbent assay (ELISA) has been widely used and plays an important role in clinical laboratory analyses. It offers inherent high sensitivity and specificity, high throughput, and robustness for analyzing a wide range of analytes in biological samples. ELISA and other immunoassays enable sensitive detection of antibodies and antigens through highly specific molecular recognition of haptens, including mitragynine.[15]

Despite the advancements in rapid detection methods, there are still inadequacies that need to be addressed. One of the major challenges is the cross-reactivity of antibodies since mitragynine shares similar structures with other alkaloids, such as 7-hydroxymitragynine, speciociliatine, and speciogynine. This potential for false-positive or false-negative results necessitates the enhancement of selectivity and specificity to mitigate cross-reactivity.

Several factors need to be considered to ensure successful production of hapten conjugates with high affinity and specificity antibodies. Factors such as hapten design or modification, carrier selection, coupling method (the coupling of hapten to the carrier molecule), hapten number (the molar ratio of hapten−protein conjugates), and purification of the conjugates affect the quality of the antibodies produced. In our previous research, we successfully developed mitragynine conjugates, including the methyl ester-MG conjugate (Figure 2) for mitragynine polyclonal antibodies, which were employed in the development of an electrochemical immunosensor for the quantification of mitragynine in human urine.[11]

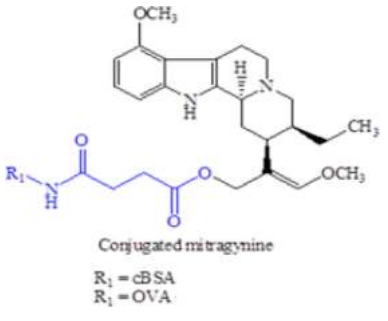

Figure 2. Conjugated methyl ester mitragynine.

The conjugation of the hapten determines the quality of the immunoassay. Additionally, several crucial parameters were optimized, including incubation time, antibody and coating antigen concentrations, the effect of detergent, pH, and salt concentration. These parameters were optimized due to the significant impact of a wide range of external factors on ELISA. The validation of the immunoassay, including the determination of the LOD, limit of quantification (LOQ), precision, recovery, and correlation with the instrumental method, LCMS/MS, was also performed. Therefore, the objective of this study was to develop and optimize a sensitive mitragynine immunoassay using competitive inhibition ELISA for the detection and quantification of mitragynine in human urine.

2. MATERIALS AND METHODS

2.1. Urine Samples. Urine samples positive for mitragynine (n = 10) were collected at the Perlis Contingent Police Headquarters (IPK) in Kangar, Perlis, with the assistance of the Narcotics Crime Investigation Department (NCID) of the Royal Malaysia Police (PDRM), Bukit Aman, Kuala Lumpur. All procedures performed in studies involving human participants were conducted in accordance with the Ethic Committee for Research Involving Human Subject (Ref. No: UPM/TNCPI/RMC/JKEUPM/1.4.18.2).

2.2. Materials and Reagents. All chemicals and reagents used were of analytical grade. The mitragynine stock standard (Chromadex, Irvin, USA) was prepared at a concentration of 1000 μg/mL in methanol from Thermo Fisher Scientific (Waltham, MA). The working standard consisted of mitragynine concentrations of 0.001, 0.01, 0.1, 0.5, 1.0, 5.0, 10.0, and 50.0 μg/mL prepared in 10% methanol. Tween 20, ovalbumin (OVA), Titermax Gold Adjuvant, Freund's Incomplete Adjuvant, and one-step ultra-3,3′,5,5′-tetramethylbenzidine (TMB), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), potassium ferricyanide, $K_3[Fe(CN),$[6] and sodium dihydrogen phosphate (NaH_2PO_4) were purchased from Sigma-Aldrich (St. Louis, MO). Skimmed milk was obtained from Becton Dickinson (Franklin Lakes, NJ). Alkaline phosphatase- and peroxidase-conjugated AffiniPure goat anti-rabbit (H + L) IgGs were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Nunc Maxisorp 96-well microtiter plates were purchased from Thermo Fisher Scientific (Waltham, MA). Ultra-pure water (18.2 MΩ-cm) from a water purification system (ELGA, Lane End, UK) was used to prepare all buffers, including sodium bicarbonate buffer (pH 8.5, 50 mM) and phosphate buffer (pH 7.4, 0.01 M).

2.3. Competitive Indirect ELISA. In our previous study,[16] the generation of anti-mitragynine polyclonal IgG using the mitragynine−cBSA conjugate (i.e., C22-MG-cBSA) was conducted. The purified polyclonal IgG of anti-mitragynine targeting mitragynine conjugated with cationized-bovine serum albumin (i.e., C22-MG-cBSA) was selected as the antigen, following our previously reported method.[17]

The microtiter plate was coated with MG-OVA at a concentration of 0.25 μg/mL, which was pre-determined using the checkerboard ELISA method. The titer of rabbit anti-mitragynine polyclonal antibodies was determined by indirect ELISA, following a previous method,[18] with some modifications. High binding 96-well microtiter plates were coated with mitragynine-OVA (0.25 μg/mL, 100 μL) in PBS (pH 7.4) overnight (16 h) at 4 °C. The next day, the plates were washed three times with PBS and blocked using 5% (w/v) skimmed

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milk in PBS (250 μL) for 2 h at room temperature with constant orbital shaking at 150 rpm. For the competitive step, the mitragynine standard (0.001−50 μg/mL) was incubated separately in 2 mL tubes with the primary antibody (1/1000 v/v; 0.298 μg/mL; 100 μL) and incubated for 2 h at 37 °C. After the incubation step, 100 μL of the solution from each tube was added to the coated and blocked wells and further incubated for 2 h at 37 °C. Next, the secondary antibody, goat anti-rabbit-HRP (Jackson Immuno Research Laboratories, West Grove, PA) (1/2500 v/v; 0.16 μg/mL; 100 μL), was added at a 1/2500 (v/v) dilution and further incubated under similar conditions. Between each incubation, the plates were washed three times with 250 μL of PBS containing 0.01% Tween 20 (PBST). The ELISA reaction was initiated by adding one-step ultra-3,3′,5,5′-tetramethylbenzidine (TMB) (100 μL), and the reaction was developed for 20−30 min in a dark room at room temperature. The reaction was quenched by adding of 0.1 N HCl (100 μL). The absorbance was measured using a Multiskan FC microplate reader (Thermo Scientific, Waltham, MA) at 450 nm. Figure 3 shows the protocol for CI-ELISA for mitragynine.

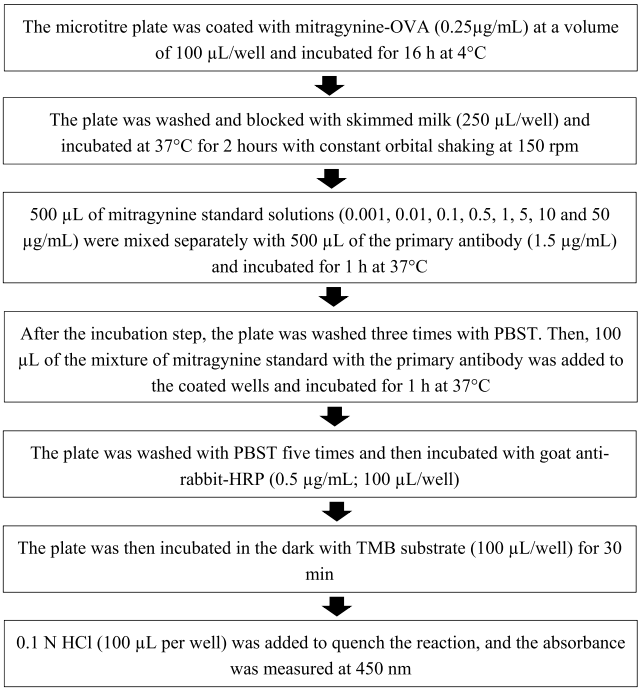

Figure 3. Protocol for CI-ELISA for mitragynine immunoassay.

2.4. Optimization of ELISA Using Response Surface Methodology. The optimum concentrations of the antigen and primary antibodies were determined to be 5.0 and 12.0 μg/mL, respectively, based on checkerboard experiments. Important factors that influence the reaction rate and sensitivity of the mitragynine were optimized using the RSM/Box−Behnken design in Minitab Statistical Software version 16 (Minitab Inc., State College, PA). The variable used in this experiment included the incubation time of primary antibody−mitragynine (X_1), the incubation time of the primary antibody-antigen (target analyte) (X_2), and the organic concentration (methanol) (X_3). The levels of these factors are depicted in Table 1, and the output of the study includes IC_{50} values and A/D values (minimum to maximum value obtained from the four-parameter logistic graph, 4 PL). A total of 30 experimental runs were conducted according to the design, as shown in the Supporting Information (S1). Response surface modeling, statistical data analysis, and optimization condition were performed. The optimal conditions obtained from the variables were verified, and the reliability of the model was validated by conducting experiments and assessing the residual standard error (RSE). The output of this model was expressed by the polynomial regression equation (eq 1), which was used to determine the response as follows

$$y = \beta_0 + \beta_{1\times1} + \beta_{2\times2} + \beta_{3\times3} + \beta_{II\times1^2} + \beta_{22\times2^2} + \beta_{33\times3^2} + \beta_{12\times1\times2} + \beta_{13\times1\times3} + \beta_{23\times2\times3} \quad (1)$$

The optimized conditions obtained from RSM were used to further optimize ELISA parameters, including the effects of pH, the presence of detergent, ionic strength, and pre-incubation of antibody and mitragynine prior to ELISA. The pH values tested were 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0. A detergent concentration of 0.05% Tween 20 was used as the diluent for the primary antibody. Four levels of ionic strength were examined (PBS 1× = 0.163, 2× = 0.326, 4× = 0.652, and 10× = 1.63 μM). Additionally, the effect of pre-incubation of antibody and mitragynine for 1 h at 37 °C was studied. ELISA was performed as described in Section 2.3, with the exception of the incubation time for the standard (free mitragynine) and antigen (mitragynine) with the primary antibody, which was set to 1 h at 37 °C under the optimized conditions. The results were analyzed using a four-parameter log model (4 PL) to determine the IC_{50} value.

2.5. Determination of ELISA Sensitivity. The sensitivity of optimized ELISA was determined by performing the mitragynine standard assay in the range of 0.001−50.0 μg/mL, which allowed to determine the IC_{50} value and assess the assay's sensitivity. Additionally, LOD and LOQ were measured to further evaluate the assay's performance.

LOD represents the lowest analyte concentration that can be reliably distinguished from blank with a specified confidence level, while LOQ is the lowest concentration that can be calculated with acceptable precision and accuracy. The calibration curve was fitted using a non-linear regression with the four-parameter logistic equation (4PL) as shown in eq 2.

Table 1. Levels of the Factors for Optimizing the IC_{50} and A/D Values

factors	symbol	actual level			coded level		
		low	middle	high	low	middle	high
methanol concentration (%)	X_1	5	10	15	−1	0	1
incubation time of primary antibody−mitragynine (minutes)	X_2	30	60	90	−1	0	1
incubation time of primary antibody−antigen (minutes)	X_3	30	60	90	−1	0	1

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ACS Omega 2023, 8, 47412−47426

$$y = \frac{a - d}{1 + \left(\frac{x}{c}\right)^{K}} + d \quad (2)$$

where (y) represents the obtained (nm), (a) and (d) are the maximum and minimum values of responses (nm), respectively, (x) is the concentration at the EC_{50} value (log unit), (c) is the mitragynine concentration, and (k) is the hill slope, a parameter similar to slope (Hill coefficient). EC_{50} refers to the effective concentration that produces a half-maximum response.[19] LOD is calculated as the mean of the blank reading ± 3SD, while LOQ is calculated as the mean of the blank reading ±10SD. These values were then entered into the GraphPad Prism 5 (San Diego, CA), and the interpolated x-value was obtained from the sigmoidal dose−response curve. Based on this value, LOD was determined to be 0.412 μg/mL and LOQ was 1.25 μg/mL.

2.6. Cross-Reactivity. The specificity of the antibody was further tested against mitragynine and structurally similar compounds, including 7-hydroxymitragynine, 16-carboxymitragynine, paynantheine, speciogynine, 8-desmethylmitragynine, corynantheidine, 9-*O*-demethylmitragynine, speciociliatine, gambirine, aricine, and reserpine. All compounds (Sciphaar, Xi'an, China) had a purity of ≥98% purity. To determine the specificity of the assay, cross-reactivity studies were conducted using the mitragynine standard and other structurally related and unrelated compounds. For the evaluation of false-positive results, the cross-reactivity toward non-related compounds (i.e., gambirine, aricine, and reserpine) was chosen, as *kratom* is often consumed in combination with other non-related compounds to enhance its effects. The compounds were selected based on their high potential to cross-react with the anti-mitragynine antibody, with some having similar molecular structures, while others naturally occur alongside mitragynine. The cross-reactivity was performed using CI-ELISA at concentrations of 0.001, 0.01, 0.1, 0.5, 1.0, 5.0, 10.0, and 50.0 μg/mL. The half-maximal inhibitory concentration (IC_{50}) for each compound was determined using GraphPad Prism 5 and a Four Parameter Logistic Curve Fit. IC_{50} is defined as the concentration of a substance that displaces 50% of the antibody-bound HRP. The percentage of cross-reactivity was calculated according to eq 3.

$$\text{Cross} - \text{reactivity}\ (\%) = \frac{IC_{50}\ \text{of mitragynine}}{IC_{50}\ \text{of the tested compound(s)}} \times 100 \quad (3)$$

2.7. Assay Precision and Reproducibility. The consistency of the immunoassay was determined through the intra-assay (precision) and inter-assay (reproducibility) analyses of the immunoassay results. Precision and reproducibility were evaluated using three different plates on the same day and different plates on three different days, performed by two analysts. Before the analysis, blank urine was screened to confirm the absence of mitragynine using HPLC (Waters, Milford, MA, USA). Human urine preparation followed a previous study with some modifications.[20] Blank urine samples were centrifuged at 1500*g* for 15 min at room temperature. The resulting supernatant (1 mL) was collected, diluted with PBS (1:1, v/v), and vortexed for an additional minute. The urine samples were then fortified with different concentrations of mitragynine (0, 0.01, 0.1, 0.5, 1, 5, and 10 μg/mL) and analyzed using ELISA. The variability of the assay was determined by calculating the standard deviation and average absorbance (A/A_0) value. The results were assessed based on the deviation from the readings and reported as the coefficient of variance (CV) obtained from multiple readings. The CV was calculated according to eq 4.

$$\text{Coefficient of variance, CV}\ (\%) = \frac{\text{Standard deviation}}{\text{Mean}} \times 100 \quad (4)$$

2.8. Correlation Study between CI-ELISA and LC−MS/MS. As part of the ELISA development, it is crucial to compare the reliability of the immunoassay with standard instrumental methods, such as liquid chromatography−mass spectrometry (LC−MS/MS). The concentration of mitragynine in urine samples was determined using both ELISA and LC−MS/MS. The results obtained from both methods were compared to determine the reliability of the developed immunoassay.

2.9. Preparation of Urine. Urine samples (n = 10) positive for mitragynine for LC−MS/MS analysis were prepared according to a previously reported method.[21] Metabolites in the urine were enzymatically hydrolyzed using β-glucuronidase prior to sample extraction and LC−MS/MS analysis. Briefly, urine sample aliquots (200 μL) were dissolved in 50 mM potassium phosphate hydrolysis buffer at pH 6 (200 μL). β-glucuronidase/arylsulfatase from *Helix pomatia* was added (200 μL) to achieve an enzyme activity of 20,000 units per sample. Samples were then mixed thoroughly using a gel rocker for 5 min and incubated at 37 °C for 3 h. Subsequently, samples were equilibrated for 10 min at room temperature and added to 0.1 M NaOH (500 μL). For the extraction step, methyl *tert*-butyl ether (MTBE) (3 mL) was added to each aliquot and vortexed for 10 min. The organic and aqueous layers were separated by centrifugation at 1000*g*. The samples were then kept in dry ice to freeze the aqueous layer. The organic layer was dried under nitrogen at 45 °C. The aliquots were reconstituted with methanol before LC−MS/MS analysis.

2.10. Liquid Chromatography−Mass Spectrometry (LC−MS/MS) Analysis. All analyses were performed using the Agilent Technologies 6460 LCMS Triple Quad LC/MS (Agilent Technologies, Agilent, USA) equipped with the Agilent Technologies 1260 Infinity High-Performance Liquid Chromatography (HPLC) LC-ESI-MS/MS system in the positive ionization mode. Chromatography was achieved using an XDB-C18 Eclipse column (4.6 × 150 mm, 5 μm) maintained at 40 °C, following a previous study,[22] with some modifications. The ESI was operated in the positive ionization mode. A total of 10 samples were analyzed using a mobile phase consisting of water/methanol (5:95, v/v). Mobile phase A contained 0.1% (v/v) of formic acid in water, and mobile phase B contained 0.1% (v/v) formic acid in methanol. The flow rate was set at 0.7 mL/min in the gradient mode, starting with an initial mobile phase composition at 5% B and increasing linearly to 95% B over 11.5 min. The ion source heater temperature was set to 350 °C, with a gas flow rate of 10.5 mL/min. Nebulizer pressure was maintained at 45 psi, and the capillary voltage was set at 2000 V. Multiple reaction monitoring (MRM) was used in the positive mode, with the transition of the precursor ion of mitragynine at m/z 399 to product ions at m/z 110, 174, and 238.

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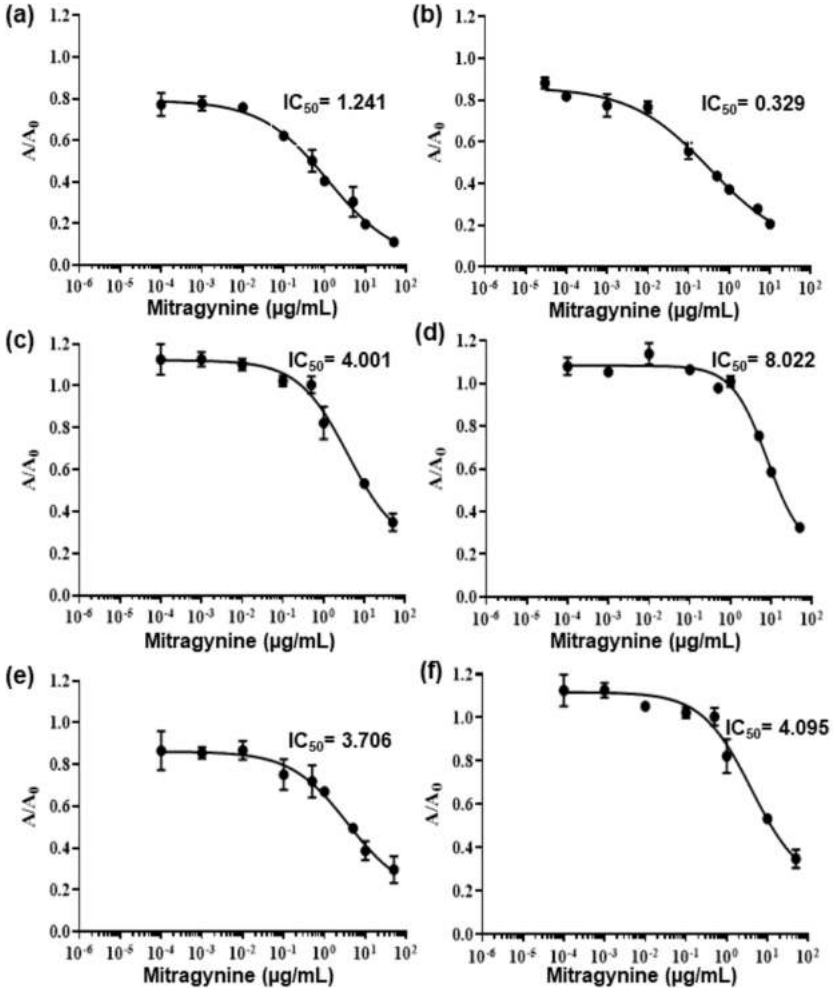

Figure 4. CI-ELISA using six combinations of different concentrations of the coating conjugate (MG-OVA) and antibody (anti-mitragynine of purified IgG) which immunized with C-22 MG-cBSA at (a) 5 μg/mL; 1/8000 (v/v), (b) 2.5 μg/mL; 1/1000 (v/v), (c) 1.25 μg/mL; 1/2000 (v/v), (d) 0.625 μg/mL; 1/4000 (v/v), (e) 1.25 μg/mL; 1/4000 (v/v), and (f) 1.25 μg/mL; 1/6000 (v/v values are mean ± SD of three replicates (n = 3)).

3. RESULTS AND DISCUSSION

3.1. Determination of Half-Maximal Inhibitory Concentration (IC_{50}). The checkerboard titration method was used to select a few combinations of coating conjugate concentrations and antibody dilutions for subsequent experiments to determine the assay sensitivity and achieve lower IC_{50} values. Based on the checkerboards (n = 3), six combinations of coating conjugates and antibodies concentrations were chosen, and three additional selections that showed promising

Table 2. Value of the CI-ELISA Curve Fitted with Four-Parameter Log Graphs (4 PL) Produces Lowest IC_{50} Values

parameters	value
coating conjugate (μg mL^{-1})	2.5
antibody dilution, (v/v)	1/1000
IC_{50} (μg mL^{-1})	0.329
slope	−0.449
top value of the y-axis, A	0.862
bottom value of the y axis determined from the 4PL graph, D	0.081
distance between the upper and lower asymptote (dynamic range), A/D	10.58
coefficient of determination, R^2	0.981

Table 3. ANOVA and Regression Coefficient for IC_{50} (Y_1) and A/D Value (Y_2) Responses[a]

factors	Y_1	p-Value	Y_2	P-value
regression		0.000		0.000
linear		0.000		0.000
squares		0.000		0.000
two-way interaction		0.000		0.000
constant	−1.375	0.000	6.383	0.000
$x1$	0.37405	0.448	−0.979	0.239
$x2$	0.01912	0.000	0.553	0.000
$x3$	−0.00233	0.003	−0.234	0.007
$x1 \times x1$	−0.00891	0.000	0.0037	0.838
$x2 \times x2$	0.000022	0.596	−0.0048	0.000
$x3 \times x3$	0.000051	0.234	0.00063	0.221
$x1 \times x2$	−0.00296	0.000	−0.00205	0.489
$x1 \times x3$	−0.00036	0.150	0.01838	0.000
$x2 \times x3$	0.000043	0.293	0.000075	0.879
R^2 (%)	92.63		90.01	
lack of fit		0.610		0.283
f-value	26.50		18.99	

[a]Significant ($p < 0.05$).

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Table 4. Polynomial Equation of Response Surface Model

responses	quadratic polynomial model	R^2 (%)	S
Y_1	$y_1 = -1.375 + 0.37405X_1 + 0.01912X_2 - 0.00233X_3 - 0.00891X_1X_1 + 0.000022X_2X_2 + 0.000051X_3X_3 - 0.00296X_1X_2 - 0.00036X_1X_3 + 0.000043X_2X_3$	92.63	0.103
Y_2	$y_2 = 6.383 - 0.979X_1 + 0.553X_2 - 0.234X_3 + 0.0037X_1X_1 - 0.0048X_2X_2 + 0.00063X_3X_3 - 0.00205X_1X_2 + 0.01838X_1X_3 + 0.000075X_2X_3$	90.01	1.235

signals were also included for further testing in the CI-ELISA format.

The sensitivity of the assay in the competitive format is highly dependent on the recognition of the antibody toward a free analyte rather than the bound antigen. Therefore, the selected optimum concentrations of the coating conjugate and antibody should be high enough to produce a strong signal through competition binding with the free analyte.[23] In the CI-ELISA, the mitragynine standard was introduced into the assay, competing with the bound antigen. The specific binding of the antibodies to mitragynine resulted in a decrease in enzyme activity, leading to a lower absorbance value. Figure 4 illustrates the six combinations of coating conjugate and antibody concentrations, which were determined by checkerboard ELISAs to obtain the IC_{50} value.

The lowest IC_{50} value was obtained at 0.329 μg/mL with a combination concentration of the coating conjugate at 0.25 μg/mL and the antibody at 1/1000 (v/v) dilution. The result obtained was in line with previous finding, which showed that lower coating conjugate and antibody concentrations can provide better sensitivity for the hapten immunoassay.[24] In this study, the IC_{50} response showed a favorable trend with a

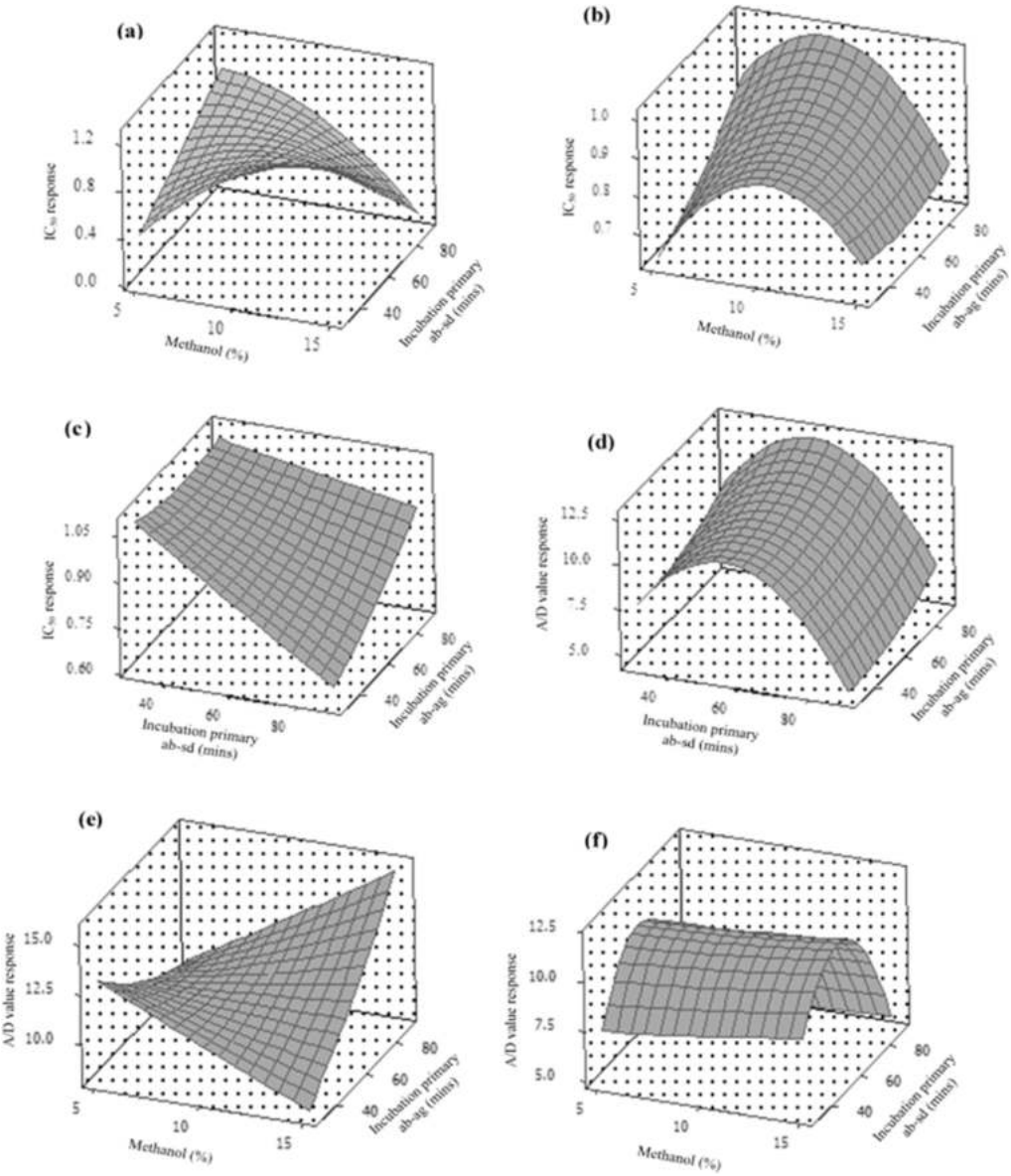

Figure 5. 3D surface plots for IC_{50} response as affected by (a) incubation of the primary ab-standard with methanol concentration, (b) incubation of the primary ab-antigen with methanol concentration, (c) incubation of the primary ab-antigen with the incubation of the primary ab-standard and for A/D value response as affected by (d) incubation of the primary ab-antigen with the incubation of the primary ab-standard, (e) incubation of the primary ab-antigen with methanol concentration, and (f) incubation of the primary Ab-standard with methanol concentration.

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ACS Omega 2023, 8, 47412−47426

Table 5. Optimized Parameter for IC_{50} and A/D Value Responses

no	MeOH (%)	incubation primary Ab-standard (min)	incubation primary Ab-antigen (min)	IC_{50} response		RSE (%)	A/D values		RSE (%)
				predicted	experiment		predicted	experiment	
1	10.01	60.13	60	0.190	0.187	1.58	18.49	18.6	0.59
2	10.06	57.10	60	1.411	1.436	1.77	10.72	10.51	1.95
3	10.60	61.13	60	1.55	1.57	1.29	5.752	5.838	1.49

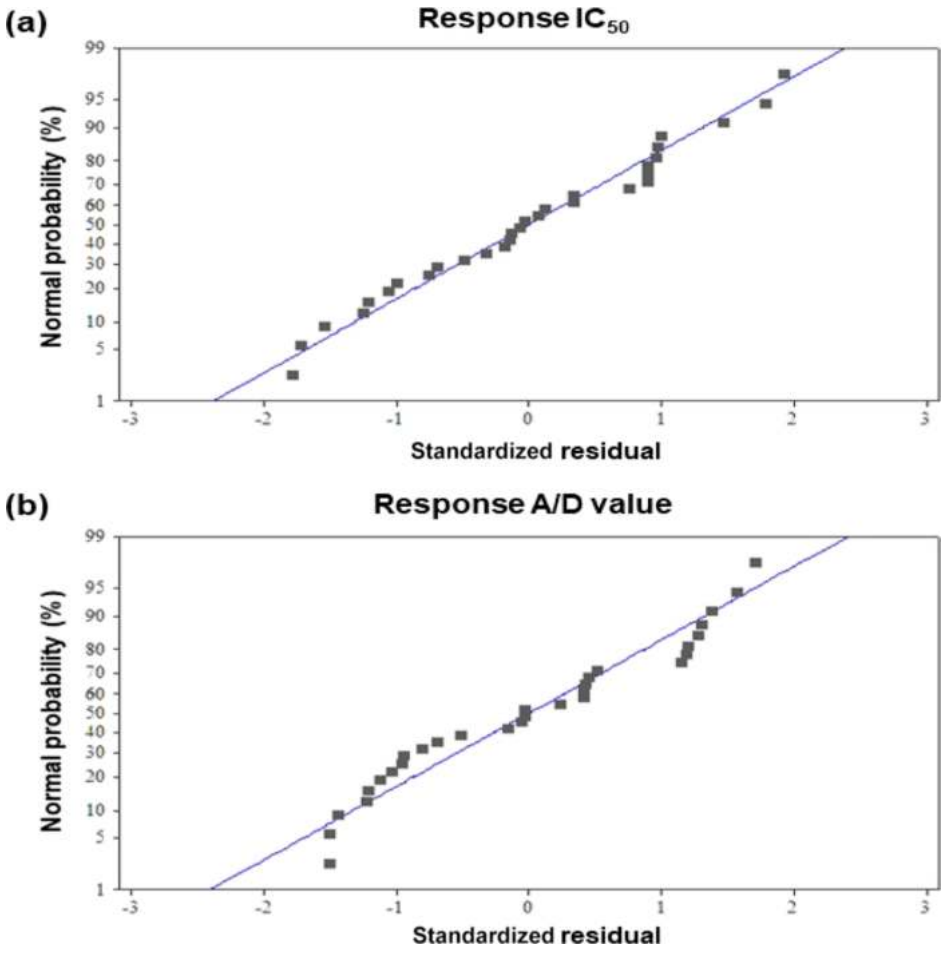

Figure 6. Fitted line plots of normal probability of the residual for predicted and experimental values for (a) response of IC_{50} values and (b) response of the A/D value.

coating conjugate concentration of 2.5 μg/mL compared to the highest concentration at 5.0 μg/mL and the lowest concentration at 0.625 μg/mL. This indicates that the highest and lowest concentrations of the coating conjugate reduced the assay sensitivity, resulting in higher IC_{50} values. For the antibody concentration, the result showed favorable condition with the 1/1000 dilution compared to the other dilutions, 1/2000 and 1/4000. The findings were fitted with four-parameter logarithmic graphs (4PL) and exhibited a best-fit value with r = 1. Table 2 represents the lowest IC_{50} values obtained from the optimum coating conjugate and antibody concentrations.

The IC_{50} value was used to determine the amount of analyte (inhibitor) required for the inhibition process, which indicates the effectiveness of an analyte in inhibiting specific biological function. This is because a smaller amount of antibody or limited binding sites will bind to the coating conjugate, resulting in higher competition with the free analyte. Achieving good assay sensitivity depends highly on optimizing the coating conjugate and antibody concentrations as well as ensuring the antibody recognizes the free analyte rather than the bound analyte. Additionally, a wider linear range is a criterion for a sensitive ELISA. Therefore, a combination of a coating conjugate concentration of 2.5 μg/mL and an antibody concentration of 1/1000 showed a good response in terms of IC_{50} values and was selected for assay optimization.

3.2. Optimization of Responses Using Response Surface Methodology. In order to achieve high assay sensitivity, the optimization of variable parameters in ELISA is crucial. Response surface methodology (RSM) is introduced as an alternative method to traditional optimization, allowing for the optimization of multiple parameters simultaneously. RSM offers several options for experimental design, including Doehlert design,[25] Box–Behnken design,[26] and central composite design (CCD).[27] This statistical analysis provides several advantages, including the ability to optimize one or more parameters, while considering the interaction between

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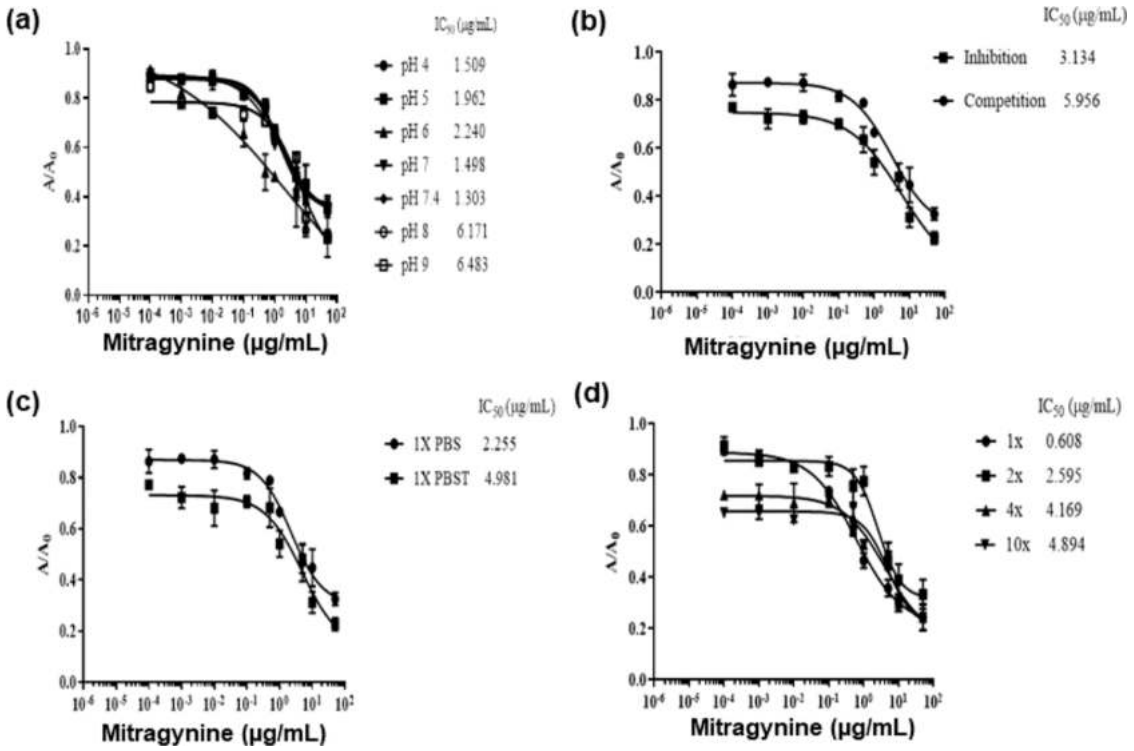

Figure 7. Effects of (a) pH, (b) pre-incubation of antibody and standard, (c) detergent (Tween-20), and (d) salt concentration in assay buffer on the IC_{50} response.

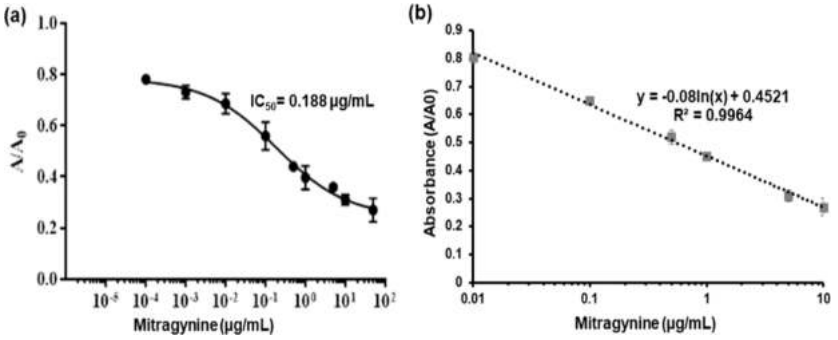

Figure 8. Calibration curve of (a) non-linear regression of the mitragynine standard (0.001−50.00 μg/mL) was fitted to a four-parameter logistic equation and (b) linear regression of the calibration curve using CI-ELISA. ELISA was performed using pre-coated wells of MG-OVA (0.25 μg/mL), competition between mitragynine standards at 0.001−50.00 μg/mL with anti-mitragynine of purified IgG (1:1000 v/v; 0.298 μg/mL), followed by addition of goat-anti rabbit-HRP (1/2500 v/v; 0.16 μg/mL). LOD = 0.412 μg/mL, LOQ = 1.25 μg/mL, error bar = standard deviation, n = 3.

them. Moreover, RSM enables the use of a small number of experiments, reducing the cost of reagents and the time required for experimentation.

In this study, optimization of IC_{50} (half-maximal inhibition) and A/D (top and bottom values from the 4PL graph) responses was performed using the Box−Behnken design. The objective was to determine the optimal conditions for methanol concentration (%), incubation time of the primary antibody with the standard, and incubation time of the primary antibody with the antigen. The Box−Behnken design was chosen over the CCD due to its cost-effectiveness and suitability for analytical data analysis.[28] The effects of these factors were studied and modeled. The optimized parameters were then analyzed using a 4 PL graph to obtain the IC_{50} (half-maximal inhibition) and A/D values, which determine the dynamic range of the assay.

3.3. Model Fitting and Statistical Analysis Data. The response surface modeling was constructed to obtain a good response in terms of the lowest IC_{50} and the highest A/D value by optimizing the variable parameters of the ELISA inhibition. The data were statically analyzed using Minitab 16, and a fitted equation model was constructed. A good regression model is capable of describing the experimental data and has a combination of high R^2 value, low p-values of model terms, and insignificant of lack of fit.[29] The significance of model terms with $p < 0.05$ determines the fitness of the statistical data in the regression equations, which is sufficient to describe the experimental data.

Based on the ANOVA data of the IC_{50} and A/D values of the response obtained, the p value from the lack of fit was found to be insignificant, greater than 0.1. An insignificant lack of fit value determines the acceptability of the model.[30] In this

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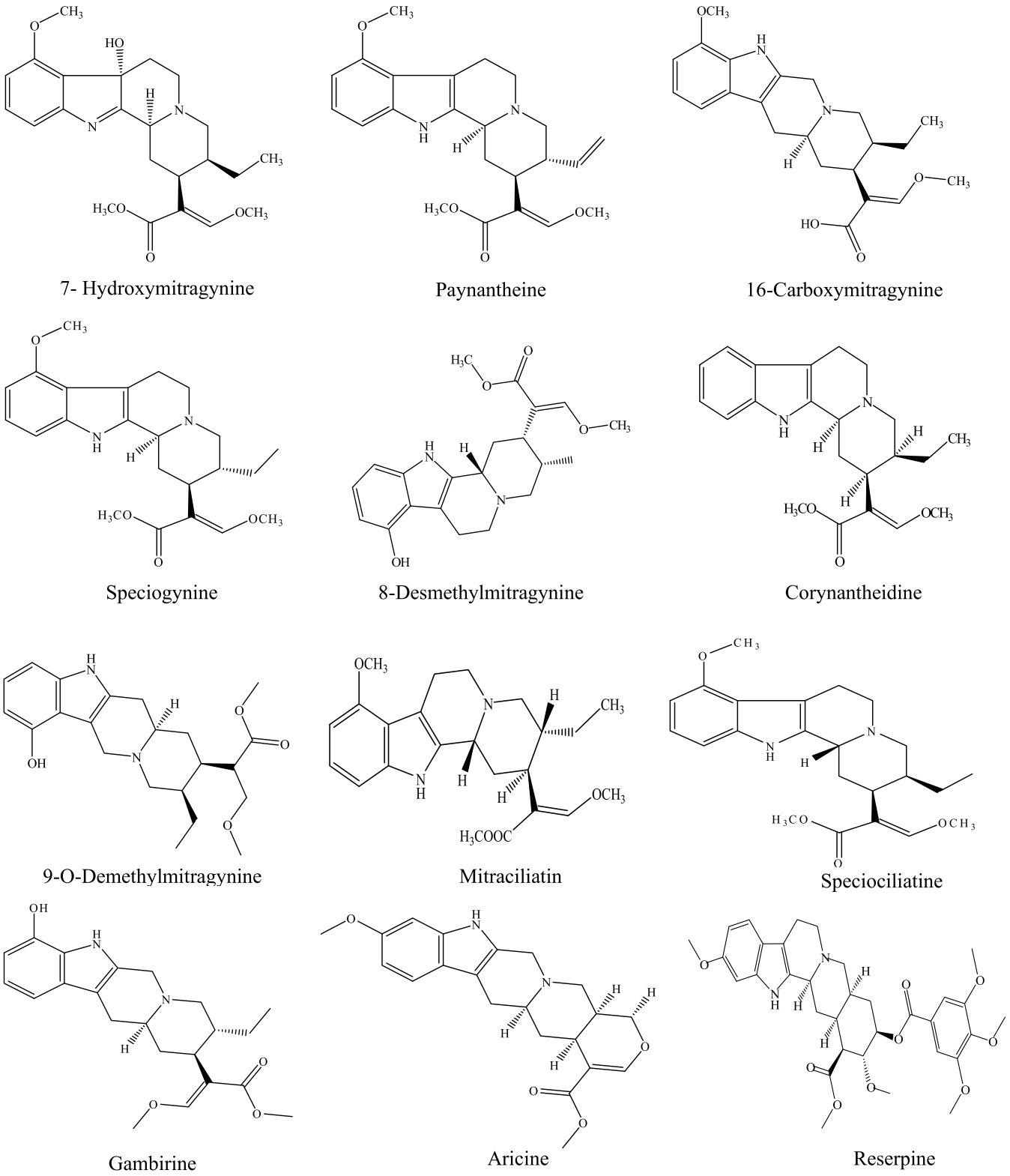

Figure 9. Chemical structures of mitragynine and its analogues in *kratom* and other various compounds.

analysis, the model shows good predictability with a p value of 0.533. The coefficient of determination (R^2) indicates the linearity of the model. The R^2 value obtained for this model is high at 0.90, which is closer to 1. This indicates that the model has a good regression and is closer to linearity.[31]

A total of 30 experimental runs with three factors were suggested based on the Box−Behnken design. The parameter for each factor, such as methanol concentrations, was chosen based on a previous study, with values of 5, 10, and 15%.[32] The findings from that study reported that higher concentrations of methanol above 10% would decrease assay sensitivity due to a decrease in antibody activity. This current study aims to evaluate the performance of organic solvent at lower and higher methanol concentrations above 10%. It has been

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ACS Omega 2023, 8, 47412−47426

Table 6. Cross-Reactivity Study of Anti-Mitragynine toward Mitragynine and Various Other Compounds

compounds	IC_{50} values	cross-reactivity (%)
mitragynine	0.187	100
7-hydroxymitragynine	0.971	19.26
16-carboxymitragynine	1.299	14.39
paynantheine	1.332	14.04
speciogynine	1.473	12.69
8-desmethylmitragynine	1.489	12.56
corynantheidine	1.854	10.08
9-*O*-demethylmitragynine	1.872	10.0
mitraciliatine	2.272	8.23
speciociliatine	2.285	8.18
gambirine	9.327	2.00
aricine	9.819	1.90
reserpine	9.889	1.89

identified that higher concentrations of methanol could improve assay sensitivity due to the better solubility of mitragynine in the assay system.

High concentrations of methanol could improve assay sensitivity, possibly due to the better solubility of mitragynine in the assay system. In terms of incubation period, a 60 min duration was determined to be the optimum condition for the ELISA incubation time. Longer incubation times have the potential to enhance reproducibility and sensitivity. Regarding the incubation of antibody with the standard and antigen, incubation times of 30, 60, and 90 min were selected for optimization. An experimental design was created based on this range, and the response variables in this model were IC_{50} and A/D values. For Box−Behnken experimental design, the experimental and predicted values of IC_{50} and A/D responses were analyzed with respect to the methanol concentration (X_1), incubation time (primary antibody standards) (X_2), and incubation time (primary antibody-antigen) (X_3). For more detailed information, please refer to the Supporting Information (Table S1).

3.4. Analysis of Variance. Determination of an individual and interaction effect from each factor was successfully analyzed. Table 3 presents the analysis of variance (ANOVA) of IC_{50} and A/D value responses for the significance of the model term, which was used to fit the predicted statistical model for the experimental data.

Significant responses for individual factors and interactions between the factors were obtained from the ANOVA ($p < 0.05$) and regression coefficient. The results indicate that the regression model for both responses was significant at $p = 0.000$, indicating the model's capability to fit the experimental data. The interaction effects observed were methanol concentration × methanol concentration and methanol concentration × incubation time of primary antibody with the standards for the IC_{50} response. Similarly, for the A/D value response, the interaction effects were identified as incubation of primary antibody with the standards × incubation of the primary antibody with the standards and methanol concentration × incubation of the primary antibody with the antigen.

However, insignificant terms can still be included in the model equation depending on their suitability. The insignificant terms contribute to the data, and the accuracy of the model design can be improved by up to 2% standard residual error (RSE) according to the validation of the model.[33] The high values of R^2 (IC_{50}: 92.63%; A/D value: 90.01%), the lack-of-fit insignificance (IC_{50}: 0.610; A/D value: 0.283), and the *f*-value (IC_{50}: 26.50; A/D value: 18.99) indicate a good fit of the statistical data to the predicted model, as shown in Table 4.

Table 4 presents the effect of the variables on the response using quadratic polynomial equations. The experimental data was analyzed and modeled using a polynomial equation. The model adequately fits the experimental data. The fitted quadratic polynomial model signifies the effect of each factor on the IC_{50} and A/D value responses. In the equation, a positive symbol indicates a synergistic effect from the factors, resulting in an increase in the variable value. On the other hand, a negative symbol represents an antagonistic effect, indicating a decrease in the variable value. Figure 5 illustrates the 3D surface plot depicting the interaction effects of methanol concentration (%), incubation time of the primary antibody with the standard (minutes), and the effect of the primary antibody with the antigen on the IC_{50} and A/D value responses.

Figure 5a−c depicts the interaction effects among the factors influencing the IC_{50} response, while Figure 5d−f represents the interaction effects on the A/D value response. Based on the findings in Figure 5b, the IC_{50} value exhibited an increase with the increasing methanol concentration, reaching a maximum point before decreasing at higher methanol concentrations.

Table 7. Intra-assay (Precision) and Inter-assay (Reproducibility) Variation of the CI-ELISA in Blank Urine[a]

	mitragynine (μg/mL)	intra-analyst			inter-analyst		
		mean A/A_0 ($n = 3$)	SD ($n = 3$)	CV (%)	mean A/A_0 ($n = 3$)	SD ($n = 3$)	CV (%)
intra-assay	0.01	0.648	0.060	9.25	0.708	0.053	7.48
	0.1	0.586	0.053	9.04	0.631	0.061	9.66
	0.5	0.469	0.041	8.74	0.575	0.013	2.26
	1	0.483	0.051	10.55	0.388	0.041	10.56
	5	0.343	0.021	6.122	0.278	0.020	7.19
	10	0.257	0.019	7.39	0.223	0.021	8.96
inter-assay	0.01	0.591	0.075	11.84	0.617	0.046	7.45
	0.1	0.433	0.026	6.00	0.543	0.031	5.70
	0.5	0.356	0.030	8.42	0.487	0.053	10.88
	1	0.335	0.023	6.86	0.356	0.014	3.93
	5	0.309	0.015	4.85	0.298	0.016	5.36
	10	0.242	0.011	4.54	0.234	0.01	4.27

[a]Data represents the mean ± SD of triplicates ($n = 3$).

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ACS Omega 2023, 8, 47412−47426

Table 8. Recovery of Mitragynine in Spiked Urine Samples as Measured by CI-ELISA and LC−MS/MS[a]

spiked concentration (μg/mL)	CI-ELISA			LCMS/MS		
	detected (μg/mL)	recovery (%)	CV (%)	detected (μg/mL)	recovery (%)	CV (%)
0.1	0.084	84.0	7.69	0.112	112.0	6.62
0.5	0.427	85.4	9.61	0.496	99.20	4.17
1	0.770	95.7	9.78	1.003	100.30	2.86

[a]Data represents the mean of triplicates.

Table 9. Quantification of Mitragynine in Positive Human Urine Using ELISA and LC−MS/MS Method[a]

urine samples (n = 10)	mitragynine concentration (μg/mL)	
	ELISA	LC−MS/MS
MGU 001	1.04 ± 0.09	1.068 ± 0.06
MGU 002	0.844 ± 0.11	0.836 ± 0.09
MGU 003	1.126 ± 0.20	1.178 ± 0.08
MGU 004	1.888 ± 0.52	1.762 ± 0.14
MGU 005	1.002 ± 0.37	0.672 ± 0.06
MGU 006	1.320 ± 0.50	1.368 ± 0.09
MGU 007	2.506 ± 1.17	2.706 ± 1.32
MGU 008	3.062 ± 1.20	3.158 ± 1.14
MGU 009	0.644 ± 0.06	0.632 ± 0.07
MGU 010	0.596 ± 0.13	0.614 ± 0.06

[a]Data represents the mean ± SD of triplicates (n = 3). MGU = mitragynine urine, represented by sample codes MGU 001 to 010.

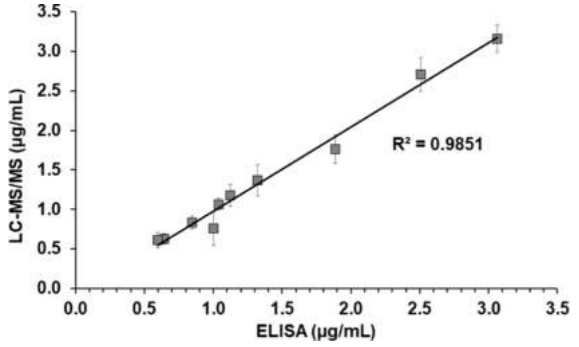

Figure 10. Correlation of mitragynine concentration in human urine as determined by ELISA and LCMS/MS.

This resulted in the formation of a quadratic surface plot. The increased methanol concentration negatively impacted the activity of the antibody, leading to a reduction in assay sensitivity.[18,34] In Figure 5d, the A/D values demonstrated an increase with prolong incubation time of the primary antibody with the mitragynine standard until reaching the maximum point. Subsequently, the response decreased with further increases in incubation time, forming a quadratic surface plot. A previous study has reported that the longer incubation times decrease the dynamic range of the assay system due to a less significant change in the reaction rate.[35] The incubation time did not significantly affect the assay.

3.5. Validation of the Model. Validation of the model and optimization of the IC_{50} and A/D value responses were performed using the optimized condition obtained from Minitab 16, as presented in Table 5.

The software suggested three sets of predicted optimized conditions, which were compared to the experimental data. The validation of the model was determined by comparing the experimental and predicted values.

The model's validity was assessed by calculating the percentage of standard residual error (RSE) between the experimental and predicted values, using eq 5.

$$\text{Residual standard error (\%)} = \frac{\text{experimental value} - \text{predicted value}}{\text{predicted value}} \times 100 \quad (5)$$

As shown in Figure 6, the normal probability plot of residuals obtained in this study showed a straight line, indicating that the errors followed a normal distribution.

Figure 6 shows that an adequate model has been obtained, as evidenced by the uniform distribution of the response variables' mean points and the normal probability plots. Based on the results, experiment 1 was selected as the optimum condition due to its lower IC_{50} value of 0.187 compared to the previous value of 0.329 μg/mL and the highest A/D value response of 18.6 compared to 10.58. The RSE for this study was less than 2%, indicating the accuracy of the model for prediction. Therefore, the optimum condition for ELISA sensitivity was further employed for the physical assay optimization.

3.6. Assay Optimization. The assay was further optimized to determine the optimal condition for ELISA sensitivity by considering various important parameters, including pH, pre-incubation, detergent (Tween-20), and ionic strength (salt concentration). The optimal concentrations of the coating antigen (0.25 μg/mL) and primary antibody (1/1000 v/v) as well as the incubation time of the reaction were determined by previous experiments using the checkerboard experiment and the RSM method. The effects of these important parameters on assay sensitivity were evaluated, ant the results are shown in Figure 7.

The pH value significantly affects the performance of ELISA. The IC_{50} values showed an increase with decreasing pH (acidic condition) and increasing pH (basic condition). However, there was no significant difference in absorbance values between pH 7, 7.4, and 8.0. Among them, the IC_{50} value at 7.4 (IC_{50} = 1.303 μg/mL) was the lowest. This pH condition is closer to physiological pH, which explains the better assay performance. Extreme pH conditions can denature proteins and alter the antibody structure, thereby affecting antigen−antibody binding.[36,37] Therefore, the optimal inhibition curve was obtained at pH 7.4 in the assay buffer.[38]

In the competitive direct ELISA, the effect of pre-incubation versus no pre-incubation was evaluated. Pre-incubation (inhibition method) significantly affected the assay performance. The IC_{50} value in the inhibition step of ELISA was significantly lower (IC_{50} = 3.134 μg/mL) compared to the competitive method (IC_{50} = 5.956 μg/mL). This result showed a similar trend with previous studies, which showed that pre-incubation (inhibition method) significantly increased ELISA sensitivity for the detection mitragynine (LOD = 32.47 μg/mL),[14] fenpropathrin (IC_{50} = 0.34 mg/L),[38] and

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ACS Omega 2023, 8, 47412−47426

chlorpromazine (IC_{50} = 0.58 ng/mL).[39] During the incubation step, allowing the antibody to bind with mitragynine before adding it to the plate reduces antibody binding to the plate, thus increasing the sensitivity level.

Detergent is another factor that influences ELISA sensitivity. The effect of using detergent as an antibody diluent was examined by comparing 1× PBST (Tween 20, 0.05%) with 1× PBS. The use of Tween 20 at 0.05% significantly decreased assay sensitivity. The IC_{50} value for the detergent-assisted assay was higher (IC_{50} = 4.981 μg/mL) compared to 1× PBS (IC_{50} = 2.255 μg/mL). Previous study has reported that the use of detergent can disrupt the antibody−antigen binding, thereby decreasing assay sensitivity.[40] However, another study reported that the use of detergent in the assay system can significantly enhance ELISA sensitivity.[41] This may be attributed to the hydrophobic interaction between the detergent and the non-polar hapten, which improves the antibody−antigen binding and subsequently increases ELISA sensitivity.

Moreover, the effect of ionic strength in the assay was determined by using different salt (NaCl) concentrations. The IC_{50} value decreased with the increase of ionic strength, resulting in IC_{50} values of 0.608, 2.595, 4.169, and 4.894 μg/mL for 1× (0.17 M), 2× (0.33 M), 4× (0.67 M), and 10× (1.67 M) salt concentrations, respectively. High salt concentrations increase the ionic strength, which may influence or disrupt the binding between the antibody and antigen. Similar finding has been reported in a previous study, where high ionic strength significantly decreased the assay sensitivity.[42] Although the 1× and 2× salt concentrations showed a similar absorbance value, the 1× salt concentration exhibited higher sensitivity with the lowest IC_{50} values. Therefore, 1× PBS was used as the assay buffer in this system. Thus, the optimized assay conditions included a pH of 7.4 with an IC_{50} value of 1.303 μg/mL, the addition of an inhibition step with an IC_{50} value of 3.134 μg/mL, the use of 1× PBS without detergent with an IC_{50} value of 2.255 μg/mL, and the use of 1× PBS with an IC_{50} of 0.608 μg/mL. Figure 8 depicts the IC_{50} value of mitragynine ELISA at 0.187 μg/mL under the optimized assay conditions.

3.7. Cross-Reactivity. A cross-reactivity study was conducted to determine the specificity of the anti-mitragynine antibody toward mitragynine. Cross-reactivity occurs when other compounds with structurally similar or related structures to mitragynine compete to bind with the anti-mitragynine antibody. The high specificity of the antibody refers to its ability and affinity to specifically recognize mitragynine, distinguishing it from structurally and non-structurally similar compounds. Figure 9 shows the chemical structures of mitragynine analogues and various other compounds used in the cross-reactivity studies.

The cross-reactivity study is essential to assess the specificity of the polyclonal antibodies and prevent false-positive results. The percentage of cross-reactivity (%) of the anti-mitragynine antibody toward mitragynine and other structurally related and non-related compounds was determined based on their IC_{50} values. Table 6 presents the cross-reactivity study of the anti-mitragynine toward mitragynine and various other compounds.

Based on the results, the anti-mitragynine antibody exhibited medium cross-reactivity with 7-hydroxymitragynine (19.26%), 16-carboxymitragynine (14.39%), paynantheine (14.04%), speciogynine (12.69%), 8-desmethylmitragynine (12.56%), corynantheidine (10.08%), 9-O-demethylmitragynine (10.0%), mitraciliatine (8.23%), and speciociliatine (8.18%). On the other hand, it showed low cross-reactivity toward gambirine (2.00%), aricine (1.90%), and reserpine (1.89%). The structure of mitragynine contains three stereocenters (3S, 15S, and 20S). 7-Hydroxymitragynine, which is terpenoid indole alkaloid, has a structure that is almost similar to mitragynine but differs in the position of C-7, where it contains a hydroxyl group. Previous study has reported that mitragynine is converted to 7-hydroxymitragynine in mouse and human liver (in vitro) by cytochrome P450 3A isoforms.[43] This metabolite of mitragynine explains the recognition of 7-hydroxymitragynine by the anti-mitragynine antibody. Paynantheine and speciogynine differ from mitragynine in the position of C-20, where it is replaced with a methylene group at C-18.[14] The slightly higher cross-reactivity percentages of mitraciliatine and speciociliatine are due to the different configuration at the position of C-3 and C-20. The lower cross-reactivity attributed to the difference in configuration compared to the structure of mitragynine. Compounds lacking a methyl group (epitope position) exhibited significantly lower cross-reactivity with the anti-mitragynine antibody. Additionally, more distant in configurations resulted in lower cross-reactivity percentages, as shown by the cross-reactivities of gambirine, aricine, and reserpine. Table 8 presents the cross-reactivity study of the anti-mitragynine antibody toward mitragynine and various other compounds.

3.8. Assay Precision and Reproducibility. The assay precision was examined using different concentrations of the mitragynine standard. Intra-day assay refers to the reproducibility of assay performance within a day across three different plates. On the other hand, inter-day assay measures the variation of data obtained from the assay over three consecutive days. Real samples, such as urine, typically contain various matrix components that can affect the results. Therefore, urine samples were spiked with respective concentration of mitragynine to assess the performance of the developed immunoassay in real sample, accounting for matrix effects. Table 7 depicts the precision and reproducibility of the intra-day and inter-day assay, as determined by two panelists.

The percentage of intra-assay and inter-assay CV (%) for intra-analyst ranged from 6.122 to 11.34% and 4.54 to 11.84%, respectively. Meanwhile, for inter-analysts, the CV (%) ranged from 2.26 to 10.56% and 3.93 to 10.88% for intra-assay and inter-assay, respectively. Both intra- and inter-assay CVs for intra- and inter analysts were within the acceptable range of <20%. In fact, the higher acceptance limit for coefficient variation in immunoassay is 30%.[1] These results indicate that the assay was reproducible and precise, demonstrating its robustness. For the inter-day assay, the results showed reproducibility in day-to-day variations. To assess the accuracy of the developed ELISA, mitragynine standards (0.1, 0.5, and 1.0 μg/mL) were spiked into urine samples and compared with the LC−MS/MS method.

3.9. Correlation Study between CI-ELISA and LC−MS/MS. ELISA is well known as a rapid method for biological sample. It offers high throughput, reliable results, and ease of performance. However, there is a possibility of interference and cross-reactivity with other compounds in biological samples. Therefore, confirmation using a gold standard method such as HPLC is necessary to validate the results of the developed immunoassay. Table 8 shows the percentage recovery of ELISA and LCMS/MS analysis, which ranged from 84.0 to 95.70% and 99.20 to 112.0%, respectively.

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ACS Omega 2023, 8, 47412−47426

The results demonstrate acceptable recovery values, with 70−130% considered as good recoveries.[44] The coefficient of variation (CV %) for ELISA and LCMS/MS analysis ranged from 7.69 to 9.78% and 2.86 to 6.62%, respectively. This indicates that the ELISA is an acceptable method for quantifying mitragynine and can complement LCMS/MS analysis. The quantitative analysis of mitragynine was compared with the LCMS/MS method, as shown in Table 9. The correlation between the detection methods of ELISA and LCMS/MS is presented in Figure 10, revealing a strong correlation with an R^2 value of 0.9928. These results indicate that the developed ELISA exhibits good sensitivity and can be used as a rapid method for quantifying mitragynine content in biological samples. Furthermore, the developed ELISA was employed in an electrochemical immunosensor to enhance assay sensitivity for similar detection purposes.

4. CONCLUSIONS

A CI-ELISA with high specificity and sensitivity toward mitragynine in human urine has been successfully developed. The ELISA system utilized MG-22-OCH_3 IgG as a detection antibody. The calculated LOD and LOQ were 0.412 and 1.25 μg/mL, respectively. The percentage recovery of the ELISA and LCMS/MS analysis was 84.0−95.70% and 99.20−112.0%, respectively. The coefficient of variation (CV %) for ELISA and LCMS/MS analysis ranged from 7.69 to 9.78% and 2.86 to 6.62%, respectively. The correlation between these two methods showed good agreement with an R^2 value of 0.9928. These results indicate that the developed ELISA exhibits excellent sensitivity and can serve as a rapid method for quantifying mitragynine content in biological samples. This will enable rapid toxicological screening for users of kratom abuse by enforcement and agency task forces. To the best of author's knowledge, there are several commercial rapid test kits for mitragynine available from Randox Toxicology (Ireland, UK), Premier Biotech (Minneapolis, USA), Safecare Biotech (Hangzhou, China), and CLIA Waived (San Diego, CA). However, these kits are costly and not commercially available in the local market. This work can be applied for future development of an on-site ELISA kit to enhance its robustness and accessibility.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c02734.

Box−Behnken experimental design (PDF)

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Radhiahtul Raehan Mustafa contributed to methodology, validation, formal investigation, analysis, data curation, writing original draft preparation, writing—review and editing, and visualization. **Rashidah Sukor** contributed to conceptualization, methodology, validation, formal investigation, analysis, data curation, visualization, supervision, project administration, and funding acquisition. **Siti Mariam Mohd Nor** contributed to supervision. **Nazamid Saari** contributed to supervision and funding acquisition. **Aliah Zannierah Mohsin** contributed to methodology, writing—review and editing, and visualization.

Notes

The authors declare no competing financial interest.

All procedures performed in studies involving animals were in accordance with the Institutional of Animal Care and Use Committee of Universiti Putra Malaysia (UPM/IACUC/AUP-R004).

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