TRENDS IN SCIENCES 2025; 22(1): 8849 RESEARCH ARTICLE
https://doi.org/10.48048/tis.2025.8849

Optimization of Acetylcholinesterase and Sucrose Concentration using Response Surface Methodology (RSM) Approach in the Development of Paper-Based Biosensor for Pesticides Detection

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Received: 15 August 2024, Revised: 28 September 2024, Accepted: 5 October 2024, Published: 15 November 2024

Abstract

Excessive use of pesticides, particularly organophosphate pesticides (OPs), to increase agricultural productivity may give a risk to consumers if consumed as these pesticides are neurotoxic substances. To increase agricultural productivity, fruits and vegetables are frequently treated with excessive pesticides, particularly organophosphate pesticides (OPs). Several methods have been studied and developed to identify pesticide residue in crops, however the standard laboratory procedures for OPs analysis involves the use of HPLC or GC-MS. These methods are complex in sample preparation and require expert labor to operate. Paper-based biosensors are low-cost platforms for fast detection in the field, offering an easy and reusable option. In this study, a colorimetric paper-based biosensor based on immobilizing acetylcholinesterase (AChE) using sol-gel silica matrices and indoxyl acetate is developed. The Response Surface Methodological (RSM) approach was used to optimize AChE concentration and sucrose as a stabilizer, allowing simultaneous optimization of multiple variables using smaller-size datasets. This study used the central composite design (CCD) fractional factorial design using Design Expert 7.0 software. A quadratic model was selected to represent both immobilization yield and relative AChE activity responses. The model was evaluated using ANOVA values and Lack of Fit test which confirmed that the quadratic models for both responses are suitable for experimental data. Validation of the model demonstrates that the predictive model accurately represents of the validation research. The Limit of Detection (LoD) of the biosensor for pure profenofos pesticide after a 20-minute incubation time is 1 ppm, as indicated by a 32.55 % decrease in color intensity. After a week of storage at 4 °C, the biosensor showed a loss of 5.92 % on immobilization yield and 2.55 % on relative activity indicating a good storage stability. The mean value of lettuce samples was 200.60 ± 1.2 a.u, with pesticide concentration of 2.57 ± 1.2 ppm (n=3).

Keywords: Biosensor, Paper-based, Pesticide, Optimization, RSM, Organophosphate, Colorimetry

Introduction

Pesticides are neurotoxic substances, frequently employed to eradicate pests and plant diseases in order to boost crop yields, notably in vegetables. Pesticides, on the other hand, constitute a concern because they are poisonous and, if taken [1], may lead to illness or even death. Organophosphate pesticides (OPs) are a commonly used form of pesticide. Detection of OPs is required to assure the quality of crops particularly for vegetables, from pesticide residue contamination.

The standard method for identifying OPs is either High Performance Liquid Chromatography (HPLC) or Gas Chromatography - Mass Spectrometry (GC-MS) [2]. Both methods possess comparable limitations, such as being time-consuming, expensive, complex, requiring many chemicals, and requiring specialized skills to operate. As a result, development of a simpler, faster, and less expensive method is demanding so that people especially farmers and consumers can use it easily. In recent years, several studies have been undertaken to determine organophosphate and carbamate insecticides by inhibiting acetylcholinesterase (AChE) enzyme activity, a key enzyme for normal operation of the central nervous system in both humans and insects [3]. Some detection approaches primarily rely on electrochemical enzyme-based biosensor [4-6]. Although these methods have simplified the testing process, they still require electronic devices and correlated equipment, leading to a complex detection.

Paper-based biosensors offer ease of production, affordability, and impressive reusability characteristics. It provides low-cost platform for fast and easy detection especially when it is employed in field. The colorimetric-based detection is based on the inhibition by pesticides against the AChE enzyme which catalyzes the hydrolysis reaction of a chromogenic reagent [7]. In this study, we developed a colorimetric paper-based biosensor using entrapment approach for immobilizing AChE within sol-gel silica matrices. This method has been deemed a superior way for immobilization because it may protect the enzyme from any negative environmental impacts and may increase the enzyme's bioactivity due to diffusion or mass transfer through the pores [8]. Silica sol-gels can be prepared from siloxane precursors via hydrolysis and polymerization [9]. Tetraethyl orthosilicate (TEOS) is commonly used as the major network building agent in sol-gel methods. The fundamental rationale for utilizing TEOS is that it allows the development of resilient networks with moderate reactivity and a high level of control through simple adjustments in the synthesis parameters such as pH, temperature, and additives [10-11].

Sucrose has the ability to influence medium viscosity and acts as an activator in enzymatic reactions [12]. Additionally, a study found that a combination of sucrose and proteins, such as Bovine Serum Albumin, has a beneficial effect in maintaining the stability of alkaline phosphatase, nucleoside phosphorylase, and xanthine oxidase enzymes [13]. Therefore, the application of sucrose at an optimal concentration is expected to help maintain the stability of AChE enzymes immobilized on the pesticide detection biosensor. The optimization process was carried out using Response Surface Methodology (RSM) which is a set of mathematical and statistical tool for designing experiment, modelling and analyzing problems. This method is considered as useful for optimizing response level of process, influenced by several variables. This study aims to find the optimized condition for immobilization using RSM towards immobilization yield and relative AChE activity. The variables employed in the experimental design were the concentration of AChE and sucrose.

Materials and methods

Chemicals

Acetylcholinesterase (AChE) from *Electrophorus electricus* type VI-S (C3389-500UN, enzyme activity of 200 - 1,000 U/mg) and all other chemicals, TEOS (tetraethyl orthosilicate, $(C_2H_5O)_4Si$), indoxyl acetate, Tris-base, H_3PO_4 HCl and sucrose were purchased from Sigma-Aldrich (St. Louis, MO, USA) at the highest purity available. All solutions were prepared with ultrapure water (Millipore, Germany). Profenofos pesticide (Callicron 500 EC) was purchased from Faculty of Agriculture, Brawijaya University.

Preparation of Silica-based Sol-gel mixture

An alkoxy precursor, 66.83 µl of TEOS 98 %, was added into 43.16 µl of ultrapure water which was previously mixed with 570 µl of ethanol 96 % in stirring condition at 400 rpm for 3 min. 10 µl of 5 mM NaOH was then subsequently added into the mixture and keep stirring until it was well mixed. The sol-gel was then transferred to a 5 mL vial and freshly made before further analysis.

Fabrication of paper-based biosensor

4 µl of AChE enzyme was dropped into aluminum foil and mixed with 4 µl of sol-gel mixture. The mixture of enzyme-sol gel was then dropped on the Whatman filter paper as the platform of the biosensor which has dimension of 0.8×3 cm with reaction zone area of 0.8×0.8 cm. The modified-paper was dried in air for 15 min in room temperature. Afterwards, 4 µl of sucrose was added into the platform area and allowed to dry for another 15 min. Subsequently, the immobilization yield and relative enzyme activity were determined afterwards.

Determination of immobilization yield

The modified paper was immersed in a 225 µl of 20 mM Tris-HCl buffer, pH 7.5, in a microtube and allowed to stand for 15 min. Then, 196 µl of the remaining soaking solution was transferred to a microplate well. Subsequently, absorbance was measured at a wavelength of 630 nm using a microplate reader. Immobilization yield defines as the percentage of the total immobilized enzyme from the free enzyme in solution [14-15] and was calculated using the following formula:

$$Immobilization\ yield\ (\%) = \frac{\Delta A0 - \Delta A1}{\Delta A0} \times 100\%$$

where ΔA0 represents the absorbance of the free enzyme and ΔA1 represents the absorbance of the enzyme lysate in the soaking solution.

Determination of relative immobilized enzyme activity

The relative enzyme activity was determined as the percentage of the immobilized enzyme activity compared to the activity of the free enzyme in solution. For the determination of free enzyme activity, in a microwell plate, 190 µl of 20 mM Tris-HCl buffer pH 7.5 was mixed with 5 µl of AChE enzyme and 5 µl of 5 mg/mL indoxyl acetate sequentially. The absorbance was measured shortly after mixing at a wavelength of 630 nm every 2 min for a total measurement of 20 min. The enzyme activity was determined as reported by [15] using extinction coefficient of $\varepsilon = 3{,}900\ M^{-1}cm^{-1}$. The immobilized enzyme activity was determined by previously calculated the lysed enzyme activity which was the activity of the remaining soaking solution after immersing the modified paper in 20 mM Tris-HCl buffer pH 7.5. A 5 µl of 5 mg/mL indoxyl acetate was then immediately added into the remaining solution and the absorbance was measured. The relative activity of the immobilized enzyme is calculated using the following formula:

$$Relative\ activity\ (\%) = \frac{\text{V free} - \text{V lysed}}{\text{V free}} \times 100\%$$

where V free represents the activity of the free enzyme, while V lysed represents the activity of the lysed enzyme or the enzyme that was not immobilized in the sol-gel.

Optimization of sol-gel conditions

RSM experimental design

The Response Surface Methodology (RSM) approach was used to optimize 2 independent process variables: Concentration of AChE (U/mL) and concentration of sucrose (%, m/v) on immobilization yield (%) and relative AChE activity (%) as the dependent variables. Central composite design (CCD) is a fractional factorial design in RSM, used to study the quadratic response surface and construct second-order polynomial models in RSM [16]. In this study, CCD was constructed using Design Expert 7.0 software (DX, 10.0.07; Stat Ease Inc., Minneapolis, USA). Table 1 represents the experimental design of Response Surface Methodology (RSM) which yields 13 CCD model designs.

Table 1 Design of experiment using RSM method.

Design	Independent variables in coded form		Independent variables in actual form	
	X1	X2	X1 (Concentration of AChE, U/mL)	X2 (Concentration of Sucrose,% m/v)
1	1	1	60	20
2	−1	−1	20	5
3	0	1.414	40	23
4	1	−1	60	5
5	1.414	0	68	12.50
6	-1.414	0	11.72	12.50
7	−1	1	20	20
8	0	0	40	12.50
9	0	−1.414	40	1.89

Design	Independent variables in coded form		Independent variables in actual form	
	X1	X2	X1 (Concentration of AChE, U/mL)	X2 (Concentration of Sucrose,% m/v)
10	0	0	40	12.50
11	0	0	40	12.50
12	0	0	40	12.50
13		0	40	12.50

The ranges and levels of independent variables in **Table 1** were selected based on the preliminary experiments and literature reports to ensure feasibility. Three sequential key stages were conducted for optimization using RSM method: First, the design of statistically planned experiments; second, the estimation of coefficients in a mathematical model; and third, the prediction of the response and validation of the model within the experimental framework [17-18].

Determination of paper-based biosensor performance

Steady states measurement

Determination of apparent steady-state kinetic constants were conducted using indoxyl acetate as substrate at varied concentrations. Catalytic constants were measured at pH 7.5, and calculated using nonlinear least-square regression by fitting the observed data to the Michaelis-Menten equation, $v = v_{max}$ [S]/K_m+[S] (Sigma Plot 12, Systat, Chicago, IL, USA). All measurements were conducted in triplicates and the results are given as mean value $\pm$ standard deviation (SD) with $p < 0.05$.

Determination of limit of detection (LoD), response time and stability

The performance of biosensor was determined by calculating the limit of detection (LoD), response time and its stability with time. LoD was defined as the lowest concentration of pesticide at which consistent color change could be observed. Pure pesticide solutions containing profenofos with varying concentrations was used as the analyte. The selected LoD is where the color intensity decreases from indigo blue color to white, which can persist for up to 20 min. The color changes were observed using an scanner (EPSON TX121), and the quantification was inspired by a report published previously [19]. The blue color intensity value was calculated using Adobe Photoshop CS 5 software through the average RGB (Red Green Blue) values or "Mean RGB". A mean value of 0 indicates black color, while a mean value of 255 indicates white color [20]. Therefore, the intensity of blue fading to white can be calculated using the following formula.

$$Intensity\ (\%) = \frac{Mean\ \text{a} - Mean\ \text{b}}{Mean\ \text{a} - Mean\ \text{control}} \times 100\%$$

where Mean a represents the mean color value of the paper before anything is dropped. Mean b represents the mean value of the biosensor after adding the pesticide, while Mean control represents the mean value of the biosensor without adding the pesticide.

The response time is the time it takes for the biosensor to respond, by the decrease in the intensity of the indigo blue color after the pesticide is added. The stability of biosensor was evaluated by storing the biosensor at varying times and temperatures in a closed container under constant humidity.

Results and discussion

Evaluation of response analysis method

Analysis of statistic model selection was developed using both Design Expert software package and ANOVA. The experiment factors in coded and actual form as well as the experimental responses is shown in **Table 2**. Design expert was used to select the model and to draw response surface plot. The analysis of model selection was based on the description of sequential model sum of squares, the lack of fit test, and the summary of statistical models. The types of model that can be used based on the software include: Linear, 2-factor interaction, quadratic and cubic model.

Following software's recommendation quadratic model was selected for both responses as they were not aliased. The final empirical model, represented in coded factors for immobilization yield (Y_1) and relative enzyme activity (Y_2) are shown in Eq. (1) and (2);

$$Y_1 = 95,69 + 1,85X_1 - 0,11X_2 - 2,34X_1X_2 - 3,25X_1^2 - 1,92X_2^2 \quad (1)$$

$$Y_2 = 90,34 + 4,20X_1 + 0,10X_2 - 0,44X_1X_2 - 4,22X_1^2 - 3,05X_2^2 \quad (2)$$

Table 2 Experiment factors in coded and actual form and experimental responses.

Design	Independent variables in coded form		Independent variables in actual form		Responses	
	X_1	X_2	Concentrations of AChE (U/mL)	Concentrations of Sucrose (%, m/v)	Immobilization yield (%)	Relative AChE activity (%)
1	1	1	60.00	20.00	88.42	87.22
2	−1	−1	20.00	5.00	86.75	78.12
3	0	0	40.00	23.11	92.11	83.33
4	1	1	60.00	5.00	93.05	86.49
5	1.414	1.414	68.28	12.50	93.88	88.44
6	-1.414	−1.414	11.72	12.50	85.73	75.24
7	−1	−1	20.00	20.00	91.48	80.62
8	0	0	40.00	12.50	95.69	92.30
9	0	0	40.00	1.89	92.82	85.04
10	0	0	40.00	12.50	94.25	88.46
11	0	0	40.00	12.50	93.54	86.75
12	0	0	40.00	12.50	97.84	93.16
13		0	40.00	12.50	97.12	91.02

Analysis of variance (ANOVA) was then conducted to assess the accuracy level of the selected quadratic model based on the ANOVA values and Lack of Fit. Additionally, both relationship between the variables X_1 (concentration of AChE) and X_2 (concentration of sucrose) with response Y_1 (immobilization yield) and Y_2 (relative AChE activity) can be elucidated. **Table 3** shows that the quadratic models for both responses are suitable for experimental data which were confirmed by a high correlation coefficient and the absence of a lack-of-fit of the model equation of the data. The model F-values for immobilization yield and relative AChE activity are 7.73 and 12.63, respectively, indicating that the model for both responses is statistically significant. Furthermore, the lack of fit is not statistically significant (p-value > 0.05) for the model. The lack of fit values of 0.4271 and 0.8480 for immobilization yield and relative AChE activity, respectively, indicates that they are insignificant when compared to the pure error. Model terms A, AB, A^2 and B^2 are found to be significant in the immobilization yield model, however model terms A, A^2 and B^2 are not significant in the relative AChE activity model.

Table 3 ANOVA for response quadratic model.

Source	Sum of Squares	df	Mean Square	F Value	*p*-Value Prob > F	Remarks
Response: Immobilization yield						
Model	138.42	5	27.68	7.73	0.0091	significant
A	27.26	1	27.26	7.61	0.0282	significant
B	0.10	1	0.10	0.029	0.8689	not significant
AB	21.93	1	21.93	6.12	0.0426	significant
A^2	73.34	1	73.34	20.47	0.0027	significant
B^2	25.57	1	25.57	7.14	0.0319	significant
Residual	25.08	7	3.58			
Lack of Fit	11.69	3	3.90	1.16	0.4271	not significant
Pure Error	13.39	4	3.35			
Cor Total	163.50	12				
Response: Relative AChE activity						
Model	310.58	5	62.12	12.63	0.0022	significant
A	141.38	1	141.38	28.76	0.0010	significant
B	0.083	1	0.083	0.017	0.9006	not significant
AB	0.78	1	0.78	0.16	0.7017	not significant
A^2	124.08	1	124.08	25.24	0.0015	significant
B^2	64.77	1	64.77	13.17	0.0084	significant
Residual	34.41	7	4.92			
Lack of Fit	5.71	3	1.90	0.26	0.8480	not significant
Pure Error	28.71	4	7.18			
Cor Total	344.99	12				

A = X_1 variable (concentration of AChE)
B = X_2 variable (concentration of sucrose)
AB, A^2, B^2 = interaction between variables

Interaction of AChE and sucrose concentration

Analysis of the combined effect of the factors on immobilization yields was carried out by employing 2D contour graphs and 3D surface plots as shown in **Figure 1**.

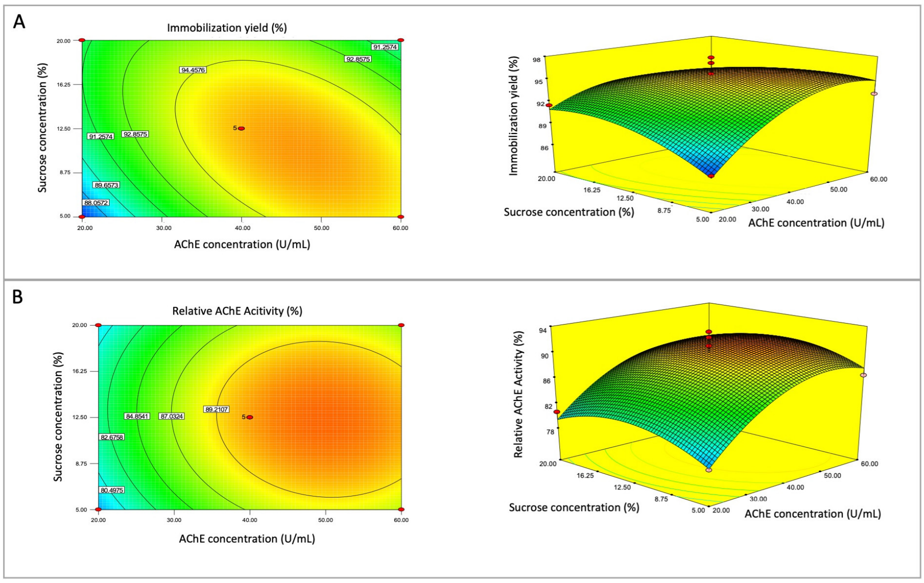

Figure 1 2D contour and 3D surface plots of (A) Immobilization yield and (B) Relative AChE activity

The immobilization yield grows exponentially with increasing AChE and sucrose concentrations, reaching a maximum of 97.84 % (**Figure 1(A)**). A further rise in AChE and sucrose concentration reduces immobilization yield implying that the enzyme loss rate increases and the diffusional substrate into the enzyme is limited [21-22]. Sucrose acts as a stabilizer by inducing the structural stabilization. During air-drying, enzyme will experience a decrease in water content. Sucrose can stabilize enzymes through a mechanism known as water replacement by replacing water and forming hydrogen bonds with the enzyme to retain its conformation [13-23]. **Figure 1(B)** indicates that as the AChE concentration increases, so does the sucrose concentration. The maximum relative activity of 93.16 % was achieved at AChE concentration of 40 U/mL and sucrose concentration of 12.5 %. However, the decrement in relative activity can be observed when the concentration of AChE and sucrose above the optimal threshold. By increasing the enzyme concentration, more enzymes will be immobilized. This is related to the amount of enzyme protein bound and trapped in the material used for immobilization, which increases the relative enzyme activity [24-25]. Furthermore, increasing the sucrose concentration may result in increased activity as well. Sucrose can affect viscosity and act as an activator in enzymatic reactions [12]. Some disaccharides such as sucrose and trehalose exhibit cryoprotective and lipoprotective properties that improve the stability of protein molecules such as enzymes [26].

In this study, the optimized combination of concentrations of AChE enzyme and sucrose on the responses of immobilization yield and relative AChE activity was identified using Design Expert 7.1.5 software. Desirability is a method used to assess how well the optimal solution fits the aims of the response.

A desirability value of 1 represents the ideal condition, while a desirability value of 0 suggests that the response should be rejected [27-29]. The optimized condition of the model presented was AChE enzyme concentration of 48.27 U/mL and a sucrose concentration of 11.39 %, yielding in a predicted response of 96.0197 % and a desirability value of 0.872.

Validation of models

Model validation was conducted by performing the experiments in order to verify the optimization results. The findings were then compared to the optimization calculation results based on the prediction model as shown in **Table 4**. The experimental results were closely matched to those obtained by RSM, confirming the findings of response surface optimization. Based on these conditions, the developed prediction model can closely depict the conditions of the validation research.

Table 4 Model validation result.

Responses	Mathematical model	Experiment
Immobilization Yield (%)	96.0197	95.55 ± 0.02
Relative AChE Activity (%)	91.3039	90.55 ± 0.21

Evaluation of paper-based pesticide biosensor

Table 5 depicts the color changes produced by the paper-based biosensor as the pesticide concentration increases. The intensity of the blue color reduces with the addition of pesticides and as the pesticide concentration increases. The fading of the blue color is due to the competitive inhibition response in which the pesticide attaches to the enzyme's active site. As a result, the blue color generated by the AChE enzyme's breakdown of the indoxyl acetate substrate is reduced [30-31]. The mean value represents the average pixel value corresponds to the intensity of the blue color visible on the biosensor. A mean value of 0 indicates black, while a mean value of 255 indicates white [20]. The color intensity decreases by 32.55 % when applying 1 ppm of pesticide, meaning that the higher the concentration of added pesticide, the higher the mean value, which indicates a decrease in the intensity of the blue color. The limit of detection of the biosensor for pure profenofos pesticide with a 20-minute incubation time is 1 ppm.

Table 5 Evaluation of paper-based biosensor detection on pesticides.

Parameter	Pesticide (ppm)					
	0	1	5	10	25	100
Colorimetric visualization						
Mean (a.u)	217.94 ± 0.18	229.23 ± 0.29	230.17 ± 0.28	236.99 ± 0.04	239.78 ± 0.36	244.53 ± 0.06
Intensity (%)	100.00	67.45	64.73	45.07	37.02	23.33
Limit of detection (ppm)	1					
Response time (minutes)	20					
Km (mM)	2.01					
Vmax (mM/min)	0.00074					
R^2	0.995					

a.u (*arbitrary unit*), ppm (*parts per million*).

A paper-based biosensors have been developed based on the enzymatic reaction between acetylcholine and indoxyl acetate, triggering a color change from white to blue on the biosensor as previously reported [31]. Our study used entrapment using TEOS pre-cursor for sol-gel medium, offer a low concentration of approximately 48.27 U/mL to produce an optimal response. Storage stability was evaluated by storing the paper-based biosensor at 4 °C in a closed container under constant humidity for 7 days. After a week of storage, the loss percentage of 5.92 and 2.55 % were observed on the immobilization yield and relative activity, respectively, indicating favorable storage stability of the biosensor.

Determination of pesticide residue in vegetable

The paper-based biosensor was applied on lettuce sold in local market. The lettuce was chopped and soaked in 5 mL Tris-Cl buffer 20 mM pH 7.5 for 15 min. Subsequently, the biosensor was dipped into the solution and the color change was observed after 20 min. The mean value of lettuce samples was 200.60 ± 1.2 a.u with pesticide concentration of 2.57 ± 1.2 ppm (n=3) which is still below the maximum residue limit for profenofos pesticides regulated by the Indonesian National Standard (SNI) at 5 ppm.

Conclusions

In this study, we successfully developed a colorimetric paper-based biosensor using sol-gel silica matrices for the immobilization of AChE using Response Surface Methodology (RSM) approach. Central Composite Design (CCD) allowed us to effectively model and optimize the immobilization yield and relative enzyme activity, confirmed by ANOVA and Lack of Fit tests. The quadratic models for immobilization yield and relative AChE activity were validated suitable for measuring pesticide concentration in lettuce sample, proving its practical applicability. The optimized biosensor demonstrated a limit detection of 1 ppm for profenofos pesticide and exhibited good storage stability with minimal loss in performance over a week at 4 °C. These findings suggest that the developed biosensor is a promising low-cost, reusable tool for the rapid detection of organophosphate pesticide residues in agricultural products, with potential applications in ensuring food safety.

Acknowledgements

The research was supported by the funding from the Ministry of Education and Culture of Republic of Indonesia.

References

[1] MG Lionetto, R Caricato, A Calisi, ME Giordano and T Schettino. Acetylcholinesterase as a biomarker in environmental and occupational medicine: New insights and future perspectives. *BioMed Research International* 2013; **2013**, 321213.

[2] D Harshit, K Charmy and P Nrupesh. Organophosphorus pesticides determination by novel HPLC and spectrophotometric method. *Food Chemistry* 2017; **230**, 448-453.

[3] SD Wijayanti, L Tsvik and D Haltrich. Recent advances in electrochemical enzyme-based biosensors for food and beverage analysis. *Foods* 2023; **12(18)**, 3355.

[4] B Jiang, M Lu and M Xu. Amperometric sensing of organophosphorus pesticides based on covalently attached multilayer assemblies of diazo-resin, prussian blue single-walled carbon nanotubes, and acetylcholinesterase. *Revue Roumaine de Chimie* 2019; **64(9)**, 763-774.

[5] HF Cui, TT Zhang, QY Lv, X Song, XJ Zhai and GG Wang. An acetylcholinesterase biosensor based on doping Au nanorod@SiO2 nanoparticles into TiO2-chitosan hydrogel for detection of organophosphate pesticides. *Biosensors and Bioelectronics* 2019; **141**, 111452.

[6] H Hu, B Wang, Y Li, P Wang and L Yang. Acetylcholinesterase sensor with patterned structure for detecting organophosphorus pesticides based on titanium dioxide sol-gel carrier. *Electroanalysis* 2020; **32(8)**, 1834-1842.

[7] Z Sun, L Tian, M Guo, X Xu, Q Li and H Weng. A double-film screening card for rapid detection of organophosphate and carbamate pesticide residues by one step in vegetables and fruits. *Food Control* 2017; **81**, 23-29.

[8] NA Mohidem, M Mohamad, MU Rashid, MN Norizan, F Hamzah and HB Mat. Recent advances in enzyme immobilisation strategies: An overview of techniques and composite carriers. *Journal of Composites Science* 2023; **7(12)**, 488.

[9] DL Jürgen-Lohmann and RL Legge. Immobilization of bovine catalase in sol-gels. *Enzyme and Microbial Technology* 2006; **39(4)**, 626-633.

[10] GJ Owens, RK Singh, F Foroutan, M Alqaysi, H Cheol-Mim, Chinmaya Mahapatra, K Hae-Won and JC Knowles. Sol-gel based materials for biomedical applications. *Progress in Materials Science* 2016; **77**, 1-79.

[11] MC Gonçalves. Sol-gel silica nanoparticles in medicine: A natural choice. Design, synthesis and products. *Molecules* 2018; **23(8)**, 2021.

[12] R Todorova and K Nedev. Effect of high concentrations of sucrose on the enzymatic activity of a-chymotrypsin. *IUBMB Life* 2000; **49(6)**, 491-496.

[13] P Srirangsan, K Kawai, N Hamada-Sato, M Watanabe and T Suzuki. Stabilizing effects of sucrose-polymer formulations on the activities of freeze-dried enzyme mixtures of alkaline

phosphatase, nucleoside phosphorylase and xanthine oxidase. *Food Chemistry* 2011; **49(1)**, 271-280.

[14] RA Sheldon and SV Pelt. Enzyme immobilisation in biocatalysis: Why, what and how. *Chemical Society Reviews* 2013; **42(15)**, 6223-6235.

[15] M Pohanka, M Hrabinova, K Kuca and J P Simonato. Assessment of acetylcholinesterase activity using indoxylacetate and comparison with the standard Ellman's method. *International Journal of Molecular Sciences* 2011; **12(4)**, 2631-2640.

[16] M Kumari and SK Gupta. Response surface methodological (RSM) approach for optimizing the removal of trihalomethanes (THMs) and its precursor's by surfactant modified magnetic nanoadsorbents (sMNP) - An endeavor to diminish probable cancer risk. *Scientific Reports* 2019; **9**, 18339.

[17] SK Behera, H Meena, S Chakraborty and BC Meikap. Application of response surface methodology (RSM) for optimization of leaching parameters for ash reduction from low-grade coal. *International Journal of Mining Science and Technology* 2018; **28(4)**, 621-629.

[18] K Mahalik, JN Sahu, AV Patwardhan and BC Meikap. Statistical modelling and optimization of hydrolysis of urea to generate ammonia for flue gas conditioning. *Journal of Hazardous Materials* 2010; **182(1-3)**, 603-610.

[19] TJ Bergman and JC Beehner. A simple method for measuring colour in wild animals: Validation and use on chest patch colour in geladas (*Theropithecus gelada*). *Biological Journal of the Linnean Society* 2008; **94(2)**, 231-240.

[20] ML Gulrajani. *Colour measurement principles, advances and industrial applications*. Woodhead Publishing, Cambridgeshire, 2010.

[21] S Arana-Peña, NS Rios, D Carballares, C Mendez-Sanchez, Y Lokha, LRB Gonçalves and R Fernandez-Lafuente. Effects of enzyme loading and immobilization conditions on the catalytic features of lipase from *Pseudomonas fluorescens* immobilized on octyl-agarose beads. *Frontiers in Bioengineering and Biotechnology* 2020; **8**, 36.

[22] LJ Li, WJ Xia, GP Ma, YL Chen and YY Ma. A study on the enzymatic properties and reuse of cellulase immobilized with carbon nanotubes and sodium alginate. *AMB Express* 2019; **9**, 112.

[23] L Momeni, S Mahmodian, B Shareghi, AA Saboury and S Farhadian. The functional and structural stabilization of trypsin by sucrose. *International Journal of Biological Macromolecules* 2017; **99**, 343-349.

[24] V Minovska, E Winkelhausen and S Kuzmanova. Lipase immobilized by different techniques on various support materials applied in oil hydrolysis. *Journal of the Serbian Chemical Society* 2005; **70(4)**, 609-624.

[25] I Alemzadeh, SSS Kavardi and A Kazemi. Optimization of lipase immobilization. *International Journal of Engineering* 2012; **25(1)**, 1-10.

[26] SN Timasheff. Control of protein stability and reactions by weakly interacting cosolvents: The simplicity of the complicated. *Advances in Protein Chemistry* 1998; **51**, 355-432.

[27] G Derringer and R Suich. Simultaneous optimization of several response variables. *Journal of Quality Technology* 1980; **12(4)**, 214-219.

[28] J Li, C Ma, Y Ma, Y Li, W Zhou and P Xu. Medium optimization by combination of response surface methodology and desirability function: An application in glutamine production. *Applied Microbiology and Biotechnology* 2007; **74**, 563-571.

[29] IJ Jeong and KJ Kim. An interactive desirability function method to multiresponse optimization. *European Journal of Operational Research* 2009; **195(2)**, 412-426.

[30] M Kavruk, VC Özalp and HA Öktem. Portable bioactive paper-based sensor for quantification of pesticides. *Journal of Analytical Methods in Chemistry* 2013; **2013**, 932946.

[31] X Guo, X Zhang, Q Cai, T Shen and S Zhu. Developing a novel sensitive visual screening card for rapid detection of pesticide residues in food. *Food Control* 2013; **30(1)**, 15-23.