Enzyme Inhibition Sensor for Malachite Green and Leuco- Malachite Green Detection in Tilapia Fish

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Abstract

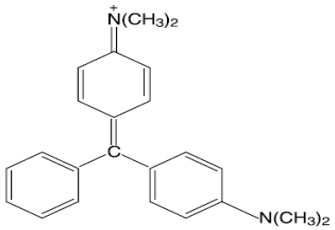
Malachite Green (MG) and Leuco-Malachite Green (LMG) are chemicals extensively used as anti-fungal and anti-parasite agents in the aquaculture industry. Over dosage of these chemicals is hazardous to human health and environment. Detection of MG and LMG is essential for quality and safety monitoring of aquaculture products to meet international standards and to ensure acceptance by foreign markets. An electrochemical enzyme inhibition sensor on screen-printed carbon working electrode with onboard carbon counter and silver–silver chloride pseudo-reference electrode MG and LMG detection is described in this paper. The detection was based on the inhibition of MG and LMG towards butyryl-cholinesterase enzyme (BuChE) in the presence of butyryl- thiocholine substrate on the sensor surface. The mixtures of BuChE enzyme (4 UmL^{-1}) and polypyrrole matrix (75 mM) were electro- polymerized on the sensor surface with a constant potential of 1.0V for 20 minutes. The sensor was soaked for 5 minutes with MG and LMG standard or sample containing MG and LMG before the electrochemical measurement was taken by adding butyryl- thiocholine substrate in KCl (0.08M, pH 7.5) which acts as the enzyme mediator/substrate system. The current measurement was conducted using chronoamperometry at 400mV vs. on board screen-printed Ag-AgCl pseudo- reference electrode. MG and LMG lowest detection limit of 0.25 ppb was achieved. The optimal sensor configuration was then compared to a conventional LC-MS/MS method for analyzing fish samples. The sensor showed good selectivity for MG and LMG with higher recovery percentage (60 - 80%) in the presence of other compounds in the sample matrix. It also showed very high sensitivity using the amplification strategy applied with the use of gold nanoparticles and conductive poly- pyrrole polymer. This makes the developed sensor a potential tool for the rapid, simple and sensitive detection of MG and LMG residues in the aquaculture industry.

Keywords: Gold nanoparticles, enzyme inhibition sensor, screen-printed carbon electrode, malachite green, rapid detection, aquaculture industry.

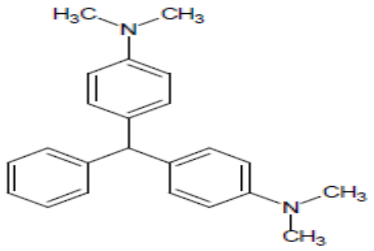
Introduction

Malachite green (MG) is a basic triphenylmethane dye ($C_{23}H_{26}N_2O$) (Figure 1) and was used in the aquaculture industry as anti-fungal, anti-bacterial and anti-protozoan. It is effective against white spot disease and mycoses and also used in controlling skin and gill flukes in fish. MG sometimes is used for ulcerative dermal necrosis treatment in fish and fish eggs (Culp and Beland, 1996). Moreover, MG is used as a medical disinfectant, anthelminthic, food additive and colouring agent as well as dye in textile and paper industries (Analytix Issues 5, 2011). Recently, many countries are concerned about the toxicity and the effects of MG and LMG towards aquatic environment and human health. A previous study has reported that MG and LMG residues are being carcinogenic, mutagenic, teratogenic and also can cause chromosomal fractures and respiratory toxicity (Culp and Beland, 1996). Hence, it has been banned for use in food fish in many countries including the USA, Canada and the European Union (EU) and the usage of MG and LMG in fishery industry is not approved by the US Food and Drug Administration (Srivastava *et al.*, 2004; 1998).

Due to the toxicity effects of MG and LMG, various methods have been developed for the determination of this dye, especially for use in fishery industry. Current detection methods for MG and LMG in water and in different matrices were used - HPLC, GC-MS and LC-MS/MS with different detectors such as fluorescence, visible (VIS), mass spectrometry and electrochemical detection. Many of the currently used methods for MG and LMG detection are time consuming (having tedious extraction procedure) and high instrumentation cost.



MalachiteGreen (MG)



Leuco-Malachite Green (LMG)

Figure 1: Molecular structure of MG and LMG (Sudova *et al.*, 2007)

Therefore, rapid detection of MG and LMG is the key to the prevention of problems related to health and safety. Rapid detection of chemical contamination, pathogens, spoilage microorganisms and other microbial contaminants in food is critical to ensure food safety and quality (Tothill, 2011). Currently, biosensor devices can offer a very attractive alternative technology for contaminant detection since they can be rapid, sensitive and simple to perform (Alocilja and Radke, 2003) as well as can provide real-time and on-site analysis (Tothill, 2011). Real-time detection of chemical contaminants is important since it provides immediate interactive information regarding the sample being tested and enables food manufacturers to take corrective measures before the product is released for consumption. This method

has widely been used in many applications such as pollution control and monitoring of mining, industrial and toxic gases, environmental monitoring, quality control, drug development, agricultural and veterinary analysis and crime detection. Electrochemical detection of MG and LMG using a flow cell of Boron-doped diamond thin-film electrodes was reported by Ngamukot *et al*. (2006).

The work presented in this paper reports the construction of an enzyme inhibition sensor using screen-printed thick film electrodes. As biological recognition elements, butyrylcholinesterase (BuChE) enzyme that specifically reacted with MG and LMG is applied. Screen-printed technology provides a low cost thick film technology and suitable for mass production. They allow both real time and in situ monitoring that requires a low sample or reagent volume, less sample wastage, rapid analysis time, cost effectiveness and possibility for development of disposable devices.

Materials and Methods

Source of chemicals

Malachite green oxalate (free zinc) (MG), leuco-malachite green (LMG), butyrylthiocholine substrate (BTC), butyrylcholinesterase enzyme (BuChE – 221U/mg), pyrrole, sodium hydroxide (NaOH), sodium acetate and hydroxylamine hydrochloride (HAH) used in the present study were obtained from Sigma-Aldrich (Malaysia). Potassium hexacyanoferate (II) trihydrate ($K_4Fe(CN)_6$), disodium hydrogen phosphate dihydrate, natrium dihydrogen phosphate monohydrate, potassium chloride (KCl), acetonitrile (ACN) (HPLC grade), *p*-toluene sulphonic acid (*p*-tsa), methanol (HPLC grade), formic acid (FA), acetic acid and hydrochloric acid (HCl) were purchased from Merck. Ascorbic acid (AA) was purchased from FLUKA. Dichloromethane (DCM) used for sample extraction was purchased from Fisher Scientific. The dry milk was purchased from Santa Cruise Biotechnology and bovine serum albumin (BSA) from Bio-RAD. Both reagents A bicinchoninic protein assay and reagent B cuprum sulfate were obtained from Thermo Scientific and were used in protein assay analysis.

Screen-printed carbon electrode fabrication and electrochemical assays

Screen-printed carbon electrodes (SPCE) consisting of carbon working electrode, carbon counter electrode and silver–silver chloride pseudo-reference electrode were fabricated by Screen Print Technology Sdn Bhd. Sungai Petani, Kedah, Malaysia. The SPCE used in this work consisted of a carbon working electrode with a 5 mm diameter giving a 19.6 mm^2 planar area. All electrodes were then tested using a multimeter before use. The sensor edge connector was purchased from DropSense Ltd. UK through Metrohm Sdn Bhd, its product distributor in Malaysia.

Electrochemical measurements were carried out by placing a 50 µL solution onto the electrode, covering the 3-electrode area. Each measurement was carried out in triplicates using a new strip in a non-deaerated and unstirred solution. Measurements were performed using the Autolab Type II (Eco Chemie, The Netherlands) with NOVA 1.6 software. Cyclic voltammetric measurements were carried out by scanning at 50 mV s^{-1} between -1.0 and 1.0 V relative to Ag/AgCl reference electrode. Stock solutions of 50 mM potassium ferrocyanide were prepared in 0.1 M KCl.

The assay was carried out by electrodeposited of 2U BuChE in 0.08M

polypyrrole on carbon working electrode. The electrode was washed with Phosphate Buffer (PB) before adding 10 µL of standard Malachite Green or Leuco-Malachite Green (~99% purity, used as standard reference material) (0 – 10 ppb) and incubated for 5 min at room temperature (25 ℃). The butyrylthiocholine substrate was added to the sensor surface and the changes in current were observed versus time for different Malachite Green and Leuco-Malachite Green concentrations at a constant current of +400 mV.

For the percentage of inhibited BuChE enzyme activity (% Inhibition), it was measured after exposure to MG and LMG standard which act as an inhibitor to the enzyme. The % value was calculated following Equation 1 (Skladal *et al*., 1997).

$$\% \text{ Inhibition} = (I_0 - I_i / I_0) \text{ X } 100 \qquad \text{Equation 1}$$

where the % Inhibition represents the percentage of BuChE enzyme inhibition, I_0 is the activity in the absence of MG and LMG inhibitor; and I_i is the activity in the presence of MG and LMG inhibitor based on currents (µA). The working temperature of BuChE enzyme was in a range of 25 to 37℃, therefore all inhibition study of the enzyme was performed at room temperature in the laboratory.

Specificity of enzyme inhibition sensor

Three different dyes in triphenylmethane group (pararosaniline, methylene blue and nile blue A) were used to examine the specificity of this enzyme inhibition sensor. The assays were conducted using various dilutions of the three dyes replacing MG and LMG. Chronoamperometric assays were conducted with a fix potential setting at 0.4V and measurement duration about 120 sec with KCl solution (0.1M, pH8) as a working electrolyte. The current signal was based on the activity of BuChE enzyme in the presence of butyrylthiocholine substrate.

Recovery study

A fish tissue was spiked with MG and LMG standard at various concentrations (0, 0.5, 1, 2, 6 and 10 ppb) for 20-30 minutes before sample extraction. MG and LMG were extracted following Faridah Salam *et al*. (2011) procedure. Similar chronoamperometric assay as specificity experiment was conducted. Percentage recovery (% Recovery) of each spiked MG / LMG concentration was calculated based on inhibition enzyme activity.

Comparative study between enzyme inhibition sensor and LC-MS/MS technique

The performance of the developed enzyme inhibition sensor was tested with the analysis of MG in tilapia fish collected from local markets. A set of triplicates of 10 fish samples weighing 5 g each from various sources was used. The overall procedure is summarized in Figure 2.

Figure 2: Extraction procedure for MG and LMG in fish samples (following Faridah Salam *et al*., 2011)

Selected tilapia fishes from the local market were also used to test the performance of the developed enzyme inhibition sensor. A 5 g fresh tissue was extracted similar to the study described previously.

MG analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Treated and selected samples from the local outlets were analysed using liquid chromatography tandem mass spectrometry (LC-MS/MS). MG and LMG were analysed using hypersil GOLD CN column (packing with 5µm particle size and column size about 50mm diameter X 2.1mm height). MG and LMG standard solution was prepared in tilapia fish tissue. Methanol and 0.1% (v/v) formic acid were used as a mobile phase. Electro-spray ionization method with positive mode detector was used to detect MG and LMG residues. The result was analysed using X-Calibur software.

Results and Discussion

Optimum set potential for chronoamperometric assay of Malachite Green and Leuco-Malachite Green

Optimum fixed potential for chronoamperometric assay of Malachite Green and Leuco-Malachite Green on the enzyme inhibition sensor was determined by scanning the positive current (0-1.2V) in the presence of BTC substrate. The signal current (zero – 1.2 V) for two MG and LMG concentration (2ppb and 6ppb) over background current was plotted. The highest signal/background ratio for both MG and LMG concentrations (2 and 6 ppb) was at 0.4 V (Figure 3). Therefore, the applied potential use for chronoamperometric assay was 0.4V. Ngamukot *et al*. (2006) also reported similar applied potential for the amperometric detection of total malachite green (MG and LMG) using bio-analytical system and 1.1 V for homemade flow cell system (Ngamukot *et al.*, 2006).

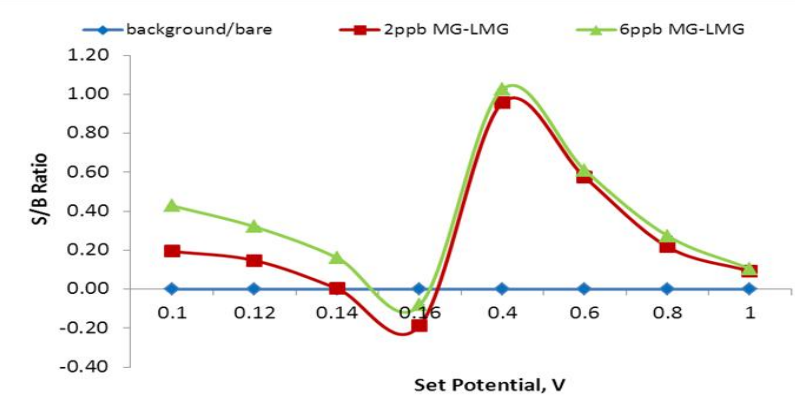
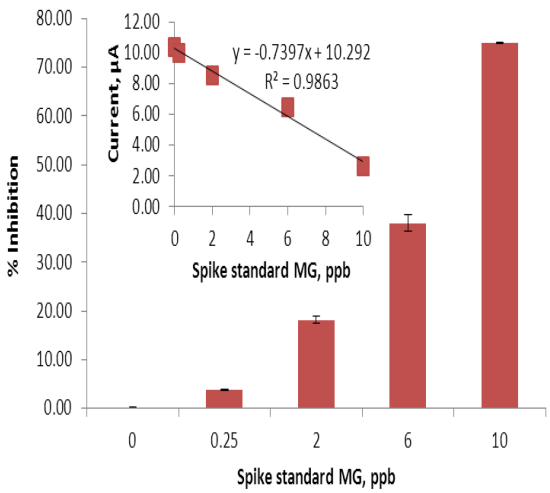


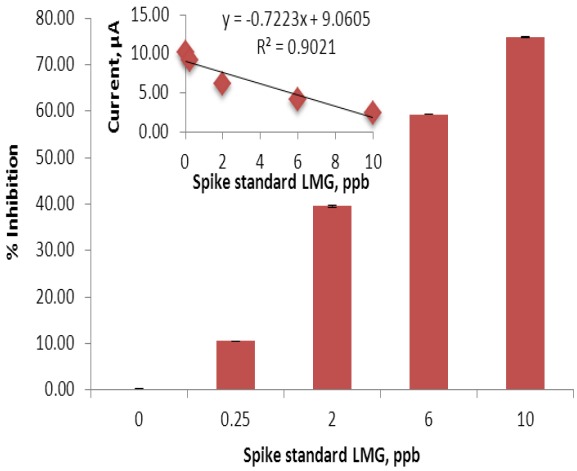
Figure 3: Optimum fixed potential selection for chronoamperometric assay of Malachite Green and Leuco-Malachite Green on the enzyme inhibition sensor at 0-1V

Malachite Green and Leuco-Malachite Green calibration curve using electro-deposited polypyrrole nano gold- enzyme inhibition sensor

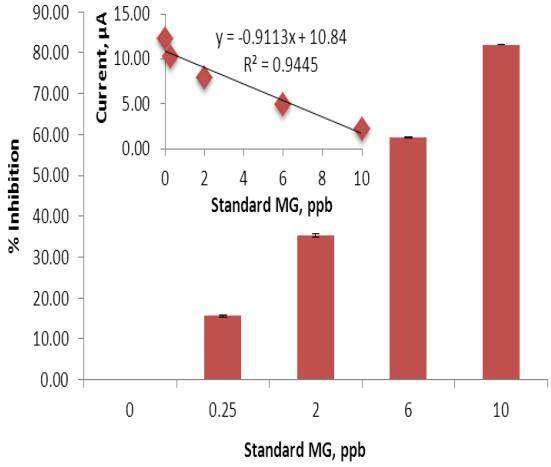
Standard calibration curve for Malachite Green and Leuco Malachite Green was constructed based on enzyme inhibition format. In this format, nano-gold coated with BuChE enzyme in pyrrole was electro- deposited on the carbon working electrode surface using cyclic voltametric method.

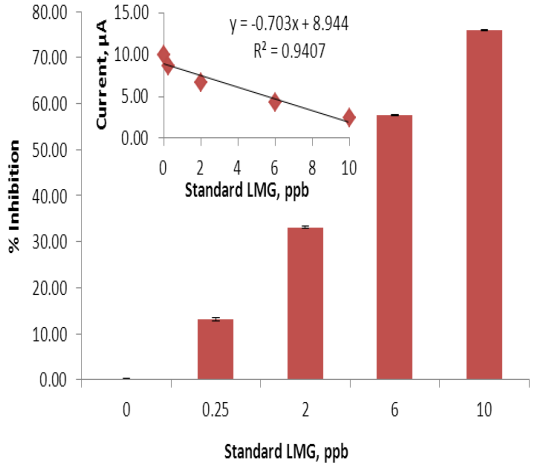
Figure 4 (a, b) shows that the percentage inhibition of enzyme sensor was increased when Malachite Green or Leuco-Malachite Green concentration increased. In other word, the signal current was decreased (I, µA) which indicated that the current was inversely proportional to the Malachite Green or Leuco-Malachite Green concentration. The current decreased linearly with the increase of Malachite Green or Leuco-Malachite Green concentration in the range of 0- 10 ppb**.** The calibration curve obtained showed that the calculated limit of detection of this enzyme inhibition sensor based on screen printed SPCE coated enzyme BuChE was found to be at ~ 0.25 ppb.





(a) Standard curve of MG and LMG spike sample, ppb respectively





(b) Standard curve of MG and LMG standard solution, ppb respectively

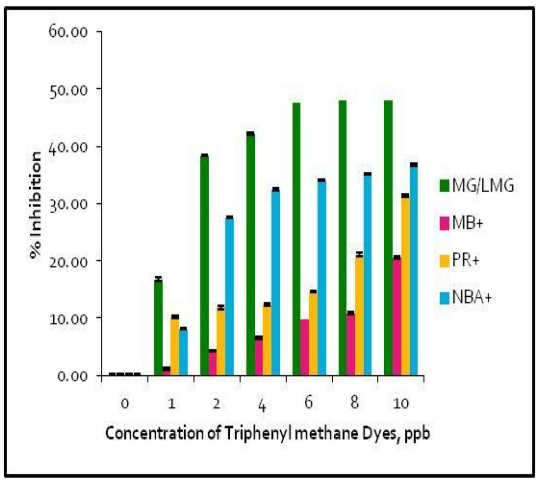
Figure 4: Calibration curve of MG and LMG with BuChE enzyme at different concentration of MG and LMG (a) Standard curve of MG and LMG in spike sample (b) Standard curve of MG and LMG in standard solution

Specificity of enzyme inhibition sensor

BuChE enzyme (E.C.1.1.8) used for enzyme inhibition sensor development was selected from equine serum and known as pseudo-cholinesterase enzyme. This enzyme has two binding sites and contains serine residue at its active site. The active site of BuChE enzyme is specific to a certain substrate such as butyrylcholine and

butyrylthiocholine. Malachite Green and Leuco-Malachite Green residues have the ability to inhibit the BuChE enzyme activity. The cross reactivity analysis was conducted similar as Malachite Green and Leuco-Malachite Green assays. Three types of dye in triphenylmethane group such as methylene blue (MB^+), pararosanaline (PR^+) and Nile Blue A (NB^A) were used for specificity test replacing Malachite Green and Leuco-Malachite Green at different concentrations (0-10ppb).

Figure 5 shows that Malachite Green and Leuco-Malachite Green were strong inhibitors for BuChE enzyme which showed about 50% inhibition. Methylene blue, pararosanaline and Nile Blue A exhibited lower than MG and LMG (10-20% inhibition).



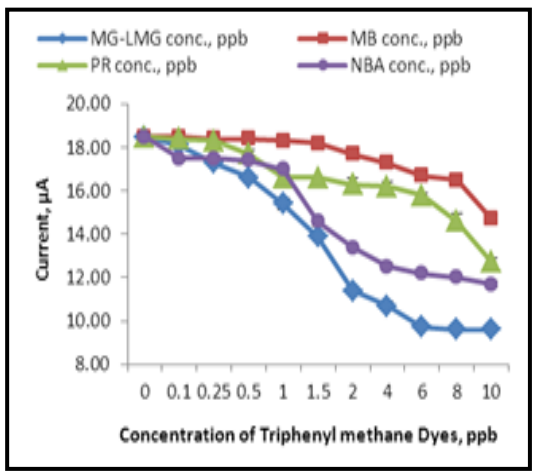


Figure 5: The plot of current, µA and percent inhibition (% Inhibition) of the triphenylmethane dyes at different concentrations (0-10ppb)

Recovery study

The results from Table 1 show that the % recovery obtained was about 60-85%. Table 2 shows that the % recovery of MG analysing using LC/MS was about 56-84%.

The MG recoveries obtained was mostly relayed to the fish matrix effects and MG losses during the extraction process. Scherpenisse and Bergwerff (2005) also found very low MG recovery when analyzing using LC-MS/MS.

Table 1: The percentage recovery of spiked tilapia tissue with MG standard analysed by enzyme sensor

Total MG spike, ppb	Found value, ppb	% RSD[1]	% Recovery
0.25	0.20	3.32	81.11
2	1.68	2.95	83.82
6	3.85	6.05	64.17
10	8.07	10.00	80.70

[1]RSD = Relative standard deviation

Table 2: The percentage recovery of spiked tilapia tissue with MG standard analysed by LC/MS

Total MG spiked, ppb	Found value, ppb	% RSD[1]	% Recovery
0	0.00	0.39	
0.25	0.21	0.20	84.26
2	1.44	0.05	71.77
6	3.38	0.12	56.38
10	4.60	0.09	45.98

[1]RSD = Relative standard deviation

Validation of sample analysis using enzyme sensor and LC-MS/MS

The performance of developed MG enzyme sensor was validated with LC-MS/MS techniques by analyzing actual sample collected from local outlets. Sample extraction was conducted following Faridah Salam *et al.* (2011) procedure (Figure 2). The total MG detected and relative standard deviation percentage (RSD) in tilapia fish samples was higher using MG enzyme sensor compared with LC-MS/MS techniques (Table 3).

Table 3: Comparative study of total MG determination using developed MG biosensor and LC-MS/MS method (Mean values ± % RSD, n =3)

Unknown samples	Total MG detected, ppb		% RSD	
	Enzyme sensor	LC-MS/MS	Enzyme sensor	LC-MS/MS
S1	1.48	0.84	1.15	0.67
S2	1.40	0.90	0.88	0.68
S3	1.57	1.08	2.96	1.55
S4	1.54	1.08	2.56	1.24
S5	0.66	0.52	2.90	1.51
S6	0.68	0.57	2.56	1.47
S7	0.69	0.66	3.15	0.97
S8	0.53	0.42	2.52	1.24
S9	0.59	0.48	3.00	1.11
S10	0.55	0.47	2.41	1.18

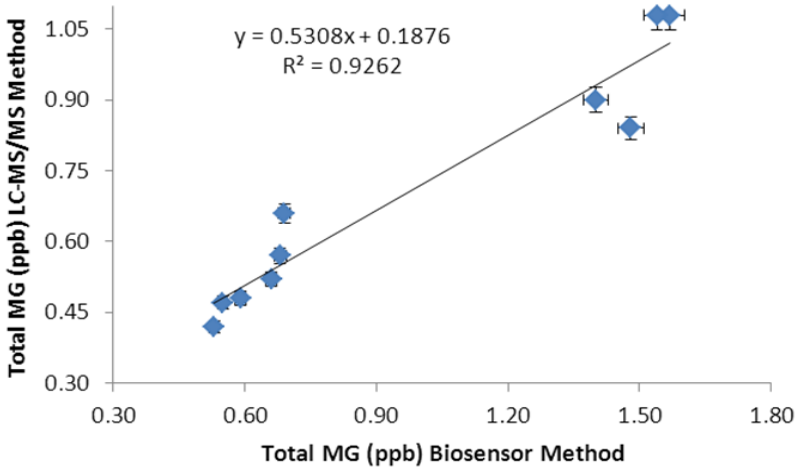


Figure 6**:** Correlation plot of MG analysis using Enzyme Sensor and LC-MS/MS techniques.

However, the correlation plot of samples analyzed using enzyme sensor and LC-MS/MS showed R^2=0.92, which was acceptable and significant correlation of both methods for analyzing MG residues in fish samples (Figure 6).

Conclusions

The BuChE enzyme was successfully immobilized using electro-polymerization of polypyrrole (Ppy) and reliable to be used as an enzyme inhibition sensor platform. The parameters of chrono amperometric analysis

and sensor probe were optimized which gave reliable results as standard method LC-MS/MS. Therefore, the developed enzyme inhibition sensor is a significant tool for monitoring and screening the contaminants and toxic substances in the aquaculture industry. This sensor prototype also is beneficial for human health and is able to be used in real time and on site application.

Acknowledgement

The authors would like to thank the Ministry of Agriculture and Agro-based Industries Malaysia for funding this research project (MOA Science Fund RB3199SF10).

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