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Automated flow-through amperometric immunosensor for highly sensitive and on-line detection of okadaic acid in mussel sample

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ABSTRACT

An electrochemical immunosensor for okadaic acid (OA) detection has been developed, and used in an indirect competitive immunoassay format under automated flow conditions. The biosensor was fabricated by injecting OA modified magnetic beads onto screen printed carbon electrode (SPCE) in the flow system. The OA present in the sample competed with the immobilized OA to bind with anti-okadaic acid monoclonal antibody (anti-OA-MAb). The secondary alkaline phosphatase labeled antibody was used to perform electrochemical detection. The current response obtained from the labeled alkaline phosphatase to 1–naphthyl phosphate decreased proportionally to the concentration of free OA in the sample. The calculated limit of detection (LOD) was 0.15 μg/L with a linear range of 0.19–25 μg/L. The good recoveries percentages validated the immunosensor application for real mussel samples. The developed system automatically controlled the incubation, washing and current measurement steps, showing its potential use for OA determination in field analysis.

1. Introduction

The detection of okadaic acid (OA) is a challenging and important issue for shellfish industries worldwide. Okadaic acid (OA) is a lipophilic marine biotoxin produced by *Dinophysis* and *Prorocentrum Dinoflagellates* [1]. OA intoxication is considered as the most of concern diarrhetic shellfish poisoning (DSP) for human health. Studies carried out on animals have also proved the cancerogenic, mutagenic and immunotoxic effects of OA [2]. The European commission (EC) has implemented regulation on the concentration of OA, and the maximum permitted level is 160 μg/Kg of mussels (EC No.853/2004 15). The European Food Safety Authority (EFSA) has suggested to decrease the maximum permitted level from 160 μg/Kg to 45 μg/Kg of mussels [3]. Mouse bioassay was recommended as a reference method by legislation [4]. However, its ethical problems in addition to poor selectivity and accuracy led the European Commission to look for alternative methods such as conventional chromatographic method, enzymatic biosensor and immunoassay [5–8]. Nevertheless, there is still need of simple, sensitive and consistent methods to perform rapid monitoring of real samples for field analysis.

An alternative and interesting approach is the use of flow injection analysis system (FIA) for rapid, sensitive and on-line detection of the target molecules. The potential advantages of the FIA are rapidity, precision, and accuracy due to the high degree of control and constancy of analytical parameters. Furthermore automation makes routine tasks easier and less cumbersome [9,10]. There are numerous examples of immunoassay automation with colorimetric, fluorescent, chemiluminescent and electrochemical signal transduction steps [11,12]. Among all these, on-line monitoring by electrochemical means has been emerged as a powerful tool both in research and industrial settings [13,14]. There are reports of flow systems which combine an immunoassay with an electrochemical detection step using screen printed carbon electrode (SPCE) [15,16]. The preparation of SPCEs is simple, inexpensive, versatile, mass produced with the possibility of miniaturization. The SPCE based biosensor offers the advantages over the conventional electrochemical biosensors for disposability and portability, and have been extensively used in the fabrication of electrochemical biosensors [17–19]. The flow immunoassay systems have been reported with micro-plate, bead, membrane and capillary immunoassay formats. However, micro-particles are the most commonly used solid surface for the immobilization of antibody, antigen or relevant reagent. The system based on micro-particle is also known as bead based immunoassay [20]. One of the benefits of the bead based immunoassay is the high surface area per volume of the

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micro-particles and the possibility to increase the immobilization surface as compared to the restricted area of the fixed size micro-well. The ability to immobilize higher numbers of binding molecules helps to improve the sensitivity and detection limit of the assay [21,22]. Beads made of various materials are commercially available. However, magnetic beads (MBs) have gained much attraction due to their ease in being retained in the flow system during different steps of biosensor fabrication as compared to non MBs [23]. The effectiveness of online magnetic trapping system in some application of flow injection bead based immunoassay using chemiluminescence and electrochemical detection has been reviewed. However, the development of bead-based immunoassay with electrochemical transduction step has been increased [24,25].

In previous study, we have shown that a SPCE could be tailored with OA modified streptavidine MBs to produce an indirect competitive immunosensor. This device was successfully used in the batch mode for low level determination of OA in mussel samples [26]. However, the incorporation of the MB based immunosensor into an automated flow system could offer attractive advantages over batch system [27]. To our knowledge, this is the first approach using electrochemical automated continuous flow system for the analysis of OA in mussel. The ultimate goal of this work is the development of a biosensor for OA analysis, which can be incorporated into field analysis to provide online monitoring of the OA in mussel samples. The fully automated flow system is based on the incorporation of OA modified MBs into the central flow cell, which were retained there by the application of an external magnet. The device was connected with a flow injection system and amperometric detection based on an indirect competitive immunoassay was performed for the sensitive and on-line detection of OA.

2. Experimental

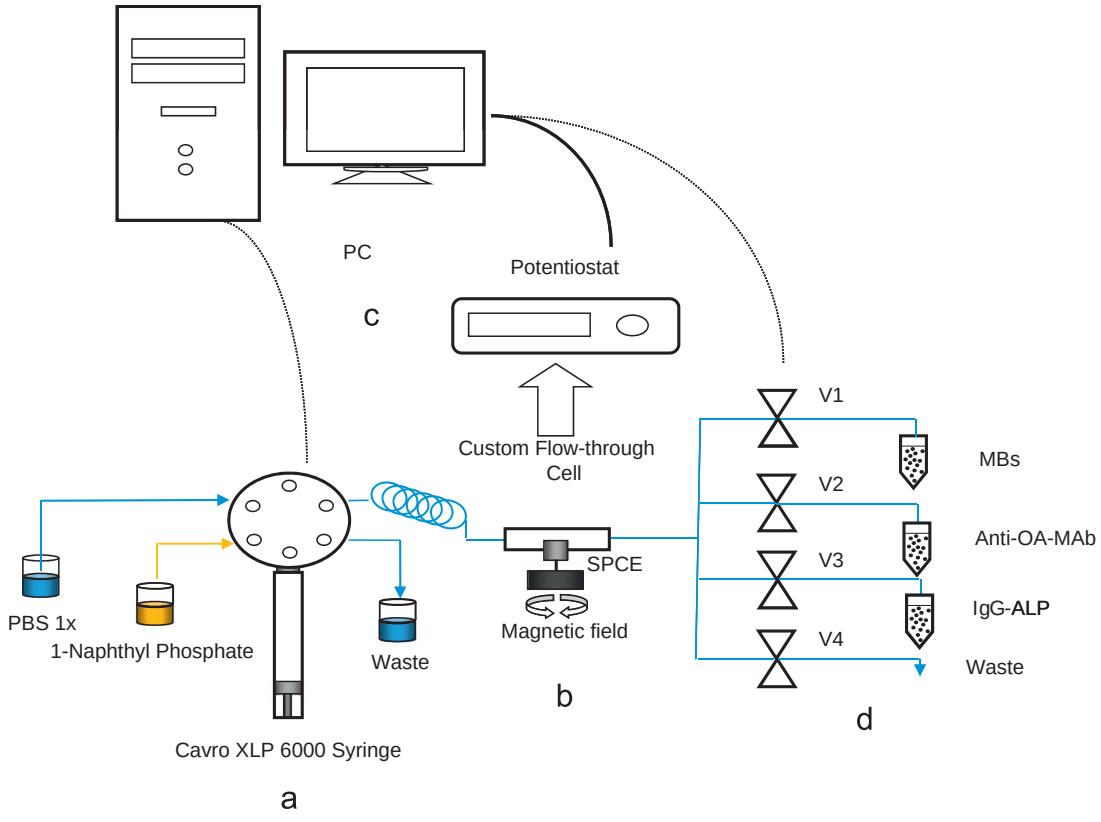
2.1. Reagents

OA potassium salt from Sigma was dissolved in ethanol (0.1 g/L) and subsequently diluted in phosphate buffer saline (PBS 1x). Buffer components, tween 20, bovine serum albumin (BSA), diethanolamine (DEA), 1-naphthyl phosphate (1-NP), alkaline phosphatase (ALP)-labeled goat anti-mouse IgG antibody, *N*- hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyle)-*N*′-ethylecarbodiimide hydrochloride (EDC) were purchased from sigma (France). EZ-link amine-PEO_3-biotin was from Pierce (France). Monoclonal antibody (anti OA-MAb, developed in mouse) against OA was obtained from Novus Biologicals (UK) showing up to 50% cross reactivity with some OA analogs. Streptavidin-coated magnetic beads Dynabeads MyOne C1 were from Invitrogen (USA). Adem-MagSV (single magnet position adapted for 1.5 mL micro tubes) was from Ademtech S.A (France).

2.2. Instrumentation

SPCE systems, with graphite as working and counter electrode and Ag/AgCl as a reference electrode, were manfactured using a DEK 248 screen-printing system. A custom flow through-cell with 4 mm-diameter magnet on the central chamber was used to immobilize the MBs over the electrode surface.

The automatic flow system is shown in the Scheme 1. All the parameters such as flow rate, incubation time, washing step, injection volumes and signal measurements were completely controlled by a graphical custom interface developed in LabVIEW 8.5. The reagents were injected or aspirated by



Scheme 1. Schematic diagram of apparatus for continuous flow automated system for OA analysis, (a) Syringe pump, (b) Electromagnetic device, (c) recorder device, and (d) Selection valves.

controlling a bidirectional syringe pump (Cavro XLP 6000, Tecan, Switzerland) and four solenoid valves (NResearch, West Caldwell, NJ, USA). The flow stream delivered the reagents into the custom magnetic flow cell, which is connected to the syringe with 200 μL holding coil to avoid contamination. Finally, chrono-amperometric measurements based on SPCE as transducer surface were performed using a potentiostat (Tacussel, France), connected to the PMD1208FS data acquisition card (Measuring Computing, USA).

2.3. Immobilization of OA on streptavidin magnetic beads

The immobilization of the OA on MBs was performed according to our previously described procedure [26]. Briefly, 50 μL of beads (10 mg/mL) were taken in 1.5 mL microtube and subsequently washed six times with 50 μL of PBS (1x). The MBs were then incubated with 50 μL of biotinylated OA for 1 h at room temperature; then washed three times with 50 μL of PBS (1x). The biotinylated magnetic beads (OA-MBs) were resuspended in 50 μL of PBS (1x) and stored at 4 °C. The MBs were collected using the Adem-Mag SV during all the modification process.

2.4. Amperometric analysis of OA

Firstly, the SPCE was incubated with 100 μL of 1%BSA for 1 h at room temperature to prevent non specific binding. Afterward, the SPCE was disposed into the flow cell and connected to the potentiostat. The stock solution of modified MBs (3 μL) was diluted in 300 μL of working buffer (PBS 1x pH 7.4) and injected to the flow cell, where the magnetic field trapped the modified MBs over the immunosensor surface.

After deposition of modified MBs on the SPCE surface, the competition step was performed by aspiring OA standard solutions at different concentrations or spiked samples and anti OA-MAb solution. Then ALP labeled goat anti-mouse IgG secondary antibody solution was also aspired. Both, the competition mixture and the secondary antibody solution were incubated for 30 min at room temperature. Finally, a mixture containing 10% DEA buffer (pH 9.5) and 5 mg/mL 1-NP was continuously passed over the immunosensor surface for 5 minutes. Washing steps were performed by injecting washing buffer (PBS 1x, 0.5% tween) between each step. Amperometric detection was performed by applying a potential of 200 mV vs Ag/AgCl. The height of the resulting electrochemical signal was recorded and plotted against OA concentration to give a calibration curve. During all the experiments, the magnetic field was applied to attach the modified MBs specifically to the immunosensor surface and they were not carried away by the continuous flowing.

2.5. Sample preparation

Mussels (Normandy, France) were blended with hand held homogenizer and extraction was carried out with methanol:water (80:20; 0.6 g/mL) for 5 min at 4000 rpm. Crude extract was centrifuged for 5 min at 2000 rpm, 1 mL of extract was evaporated in a speed VAC concentrator (Organomation Association, Inc; Berlin, USA) and the residue was resuspended in 1 mL of PBS (1x). The samples were spiked with the two standard concentration of OA (20, 10 μg/L or 40, 10 μg/kg of mussels) after filtration, to study matrix effect and OA recovery

3. Results and discussion

3.1. Optimization of parameters for automated flow detection system

Several factors that affect the biochemical reaction must be considered because the reaction conditions in automated flow immunosensors are different than those of conventional batch immunosensors. Optimization and validation of an analytical system are key factors in the development of an automated flow system. Before using the system for OA detection, it was necessary to optimize the system. Since our flow system is able to perform continuous and stop flow, both techniques were applied to the flow immunoassay. The flow rates of the sample and the reagents have an effect on efficiencies of the antigen–antibody reaction. A high flow rate would remove the immobilized modified MBs on the SPCE surface and could produce unstable signal, while a slow flow rate could suffer from signal tailing. Continuous flow was used for washing step and substrate injection and stopped flow was selected for antibody reactions in order to get a better interaction between antibody and the immobilized OA. Many experiments were carried out and optimized conditions are provided in the Supporting Table 1, while the effect of different flow rates for MBs on the immunosensor performance has been shown in the Fig. 1.

3.2. Optimization of immunoassay procedure

Immunosensors based on the competition strategy were performed, as sandwich assay are not possible for OA (MW 805) due to its small size. Secondary antibody labeled with ALP was used to perform electrochemical detection, as anti-OA MAb is not labeled (Scheme 2). Under optimal detection conditions, value of the electrochemical signal depends on the immunocomplex reaction in the flow cell. The possible affecting factors are the working dilution and the incubation time of antibodies. In order to gain optimal dilution, experiments with different dilutions of anti OA-MAb and secondary labeled antibodies were performed. As reported in our previous work, high antibody concentration increases the signal intensity but decreases the sensitivity of immunoassays, and causes background signal. Best working dilutions found were 1/1000 and 1/2500 for anti-OA-MAb and second antibody, respectively. Secondly, the incubation time for both anti-OA-MAb and second antibody should be set up to guarantee the optimal contact time between the sample mixture and the immunosensor surface. Different incubation times with

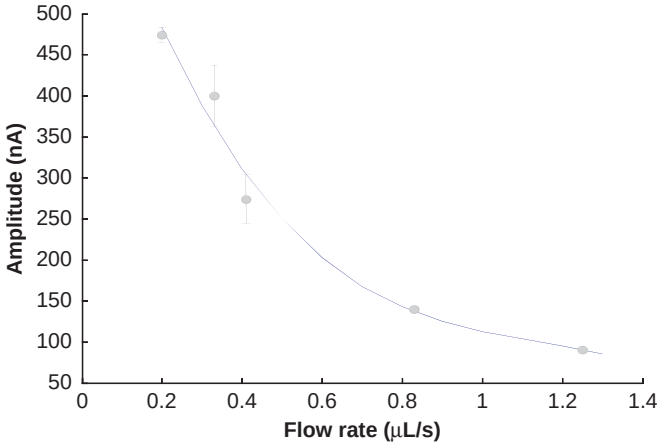
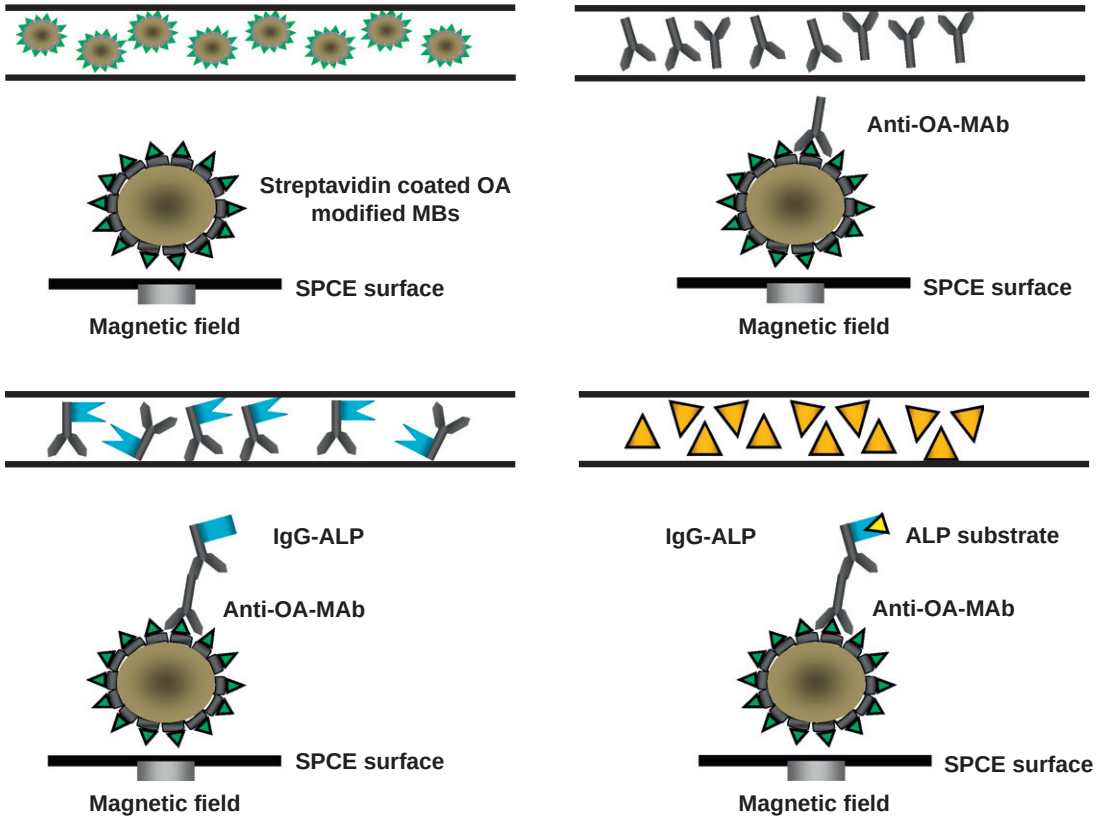


Fig. 1. Dependence of amperometric response of immunosensor on flow rate at an applied potential of 200 mV vs Ag/AgCl under optimal conditions.



Scheme 2. Schematic representation of the working principal for the flow-based immunoassay.

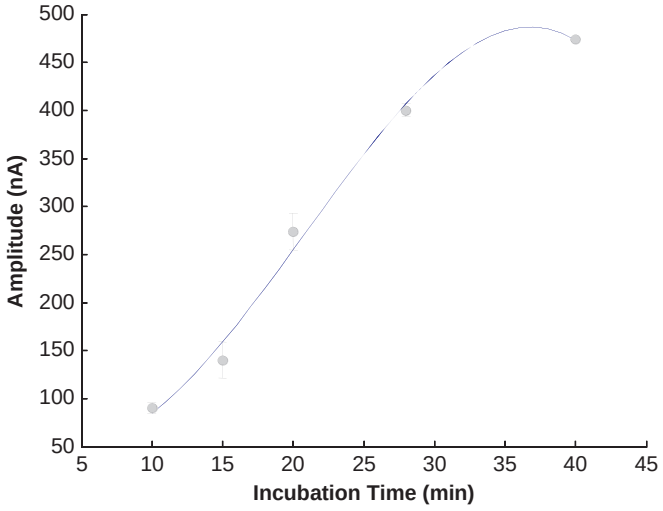


Fig. 2. Dependence of amperometric response of immunosensor on anti-OA-MAb and secondary labeled antibody incubation time at an applied potential of 200 mV vs Ag/AgCl under optimal conditions.

stop flow technique were performed. Although, the maximum signal amplitude was observed at 35 min, but an increase of non specific signal was also noticed. Therefore an optimal incubation time of 30 min was selected for the next experiments to avoid the non specific signal. Fig. 2 showed the effect of different incubation times on the signal amplitude of the immunosensor.

Under the selected conditions described above, the electrochemical response of the enzymatic product is directly proportional to the concentration of immobilized OA on the electrode surface. Assays were performed by injecting OA modified MBs with and without free OA, and non modified MBs into the flow channel, followed by anti-OA-MAb and secondary enzyme labeled antibody injection. The signal value was higher in case of modified MBs without OA as compared to the modified MBs with free OA, and non-modified MBs, demonstrating flow system to be applied for OA detection (Supporting Fig. 1).

3.3. Detection of OA in automated flow system

Under the optimal conditions, an indirect competitive immunoassay was performed with typical automated flow system. Anti-OA-MAb solutions containing different concentrations of OA were aspired into the flow cell and incubated over the immunsensor surface with stop flow technique. The immobilized OA and the free OA in solution competed to bind the limited binding sites of the anti OA-MAb in solution. The amperometric response obtained from the enzymatic catalysis of secondary alkaline phosphates labeled antibody to 1-NP decreases proportionally to the OA concentration. Fig. 3A showed the calibration curve obtained with the developed system. Anti-OA-MAb binding was expressed as percentage of the control without OA. The % binding decreased proportionally to the OA concentration in the range of 0.19–25 μg/L with a correlation coefficient of 0.995 (Fig. 3B). The LOD was considered as the toxin concentration corresponds to the 80% of anti OA-MAb binding depending upon the maximum value of standard deviation (5%). The detection limit was 0.15 μg/L lower than the result of previously developed batch system (0.38 μg/L) [26]. The decreased LOD could be attributed to decreased diffusion distance and increased reactive surface in our flow system. The present work has extended the previous studies from our group into a fully automated continuous/stop flow system. The results demonstrated the advantages displayed by the use of flow based immunoassay as compared to

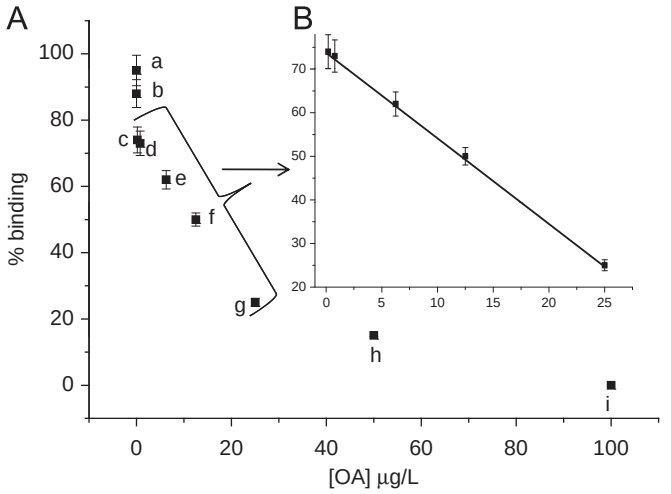


Fig. 3. (A) calibration plot of different OA concentrations (µg/L) against % binding (a) 0.001, (b) 0.01, (c) 0.19, (d) 0.78, (e) 6.25, (f) 12.5, (g) 25, (h) 50, (i) 100; (B) Linear relationship between % binding and OA concentration.

Table 1
Recovery percentages obtained by spiking the mussel samples with known amount of OA.

Spiked OA (µg Kg^{-1})	Spiked OA (µg L^{-1})	[OA]^b (µg L^{-1})	R.S.D %	R.E %	R^a %
40	20	19.25	5	3.75	96.25
10	10	9.6	5	4	96

[OA]^b=found OA concentration, R.S.D %=relative standard deviation percentage; R.E %=relative error percentage; R^a%=recovery percentage.

the conventional batch-wise system. The incorporation of the OA modified SPCE into the automated flow system led to decrease in the complexity of the system, in addition to increasing the sensitivity of the method.

Recently, an electrochemical immunosensor based on protein-G-magnetic beads for OA detection was developed by our group [28]. Although, the method has the advantage of label free detection, but the developed flow system exhibited LOD (0.15 µg/L) almost 3.3 folds lower than the LOD of label free method (0.5 µg/L), in addition to low matrix effect. Similarly, Campas et al. [29] and our group [7] have developed strategies to amplify the signals and to reduce the LOD value. This, however, has complicated the system and the sensors required longer analysis time. Furthermore, Prieto-Simon et al. [30] have reported a KinExA-based immunosensors, which required 24 h coating time. Our developed system showed LOD comparable to the above described systems, however, the purpose of this work was to develop a simple and fully automated immunosensor for on-line detection of OA to overcome the complexity involved in the previously described methods.

3.4. Application to real sample analysis

The method was applied to determine OA in real samples. The mussel samples were spiked after filtration to study the matrix effect, apart from evaluating the OA recovery. Mussel extracts were spiked with two different known concentrations of the OA (40, 10 µg/kg). No significant difference in spiked and obtained values was observed (Table 1). As already demonstrated in our previous study, the low matrix effect could be attributed to the specificity of the antibody–antigen reaction and the high number of washing steps. This great advantage demonstrated that our fully automated system can be used to quantify OA in real mussel samples, compared to the enzymatic biosensor that suffered a high matrix effect. The recovery results for the developed flow system validated the suitability of the method even at extremely lower concentration of OA than the highest level allowed by the European Commission (160 µg/kg) (Comission Regulation (EC) NO. 853/2004). The precision of the immunoassays was evaluated by the relative standard deviation (%RSD) for the triplicate measurements. Relative standard deviation with this method was (5%) indicating acceptable and precision and fabrication reproducibility. The accuracy (%RE) of OA determination was examined by comparing the real and the measured values. The spiked OA concentrations were 20 and 10 µg/L, while the values obtained with this method were 19.25 and 9.6 µg/L with a RSD of 5%. These results indicated that the measured values are in good agreement with the spiked values. Thus, the present method could satisfy the need of automated immunoassay for OA determination in real mussel samples.

4. Conclusion

In this work, we have extended our previous studies into the use of automated flow system for OA detection. Modified super paramagnetic beads were injected into the flow cell as solid support to immobilize OA and subsequently an indirect competitive immunoassay format was performed for electrochemical detection. This paper presented a novel simple fully automated continuous/stop flow immunoassay method for OA based on a disposable immunosensor integrated to a flow injection system. The flexible automated flow system provided a robust environment for the rapid, sensitive and online detection of OA in mussel samples. The incorporation of the modified magnetic beads into flow device with SPCE electrode to measure an electrochemical signal has increased the ability of the system to determine OA at low concentration (0.15 µg/L) as compared with previous studies using immunosensor under batch conditions (0.38 µg/L) [26]. Owing to the wider applications in many fields, the automated continuous system can be employed for online detection of different target analytes in many domains such as clinical, environmental and food determinations. Our developed system provided a basis for integrated process monitoring and control employing immunosensors.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2012.05.045.

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