2nd WORKSHOP ON BIOSENSORS

FOR WATER MONITORING

April 08-10, 2015, University of Rome Tor Vergata, Italy

Aptamer-based biosensing platform for the *Saxitoxin* detection

Principle of the method

Aptamers are single-stranded DNA, RNA, or peptide molecules, selected in vitro, capable of binding to its target antigen with high affinity and specificity. Aptamers have been developed against a wide variety of targets ranging from small organics to large proteins. The high affinity and specificity is due to the tertiary structure formed. Unlike antibodies, some aptamers exhibit stereoselectivity. Aptamers are discovered via an iterative process known as systematic evolution of ligands by exponential enrichment (SELEX), that begins with the synthesis of a very large oligonucleotide library consisting of randomly generated sequences of fixed length flanked by constant 5' and 3' ends that serve as primer. Separation of target bound and unbound oligonucleotides has been performed using strategies such as affinity chromatography, filtration, SPR sensors, and magnetic bead immobilization. Selected aptamers have been demonstrated for use in assays and platforms that have traditionally employed antibodies as analytical recognition elements.

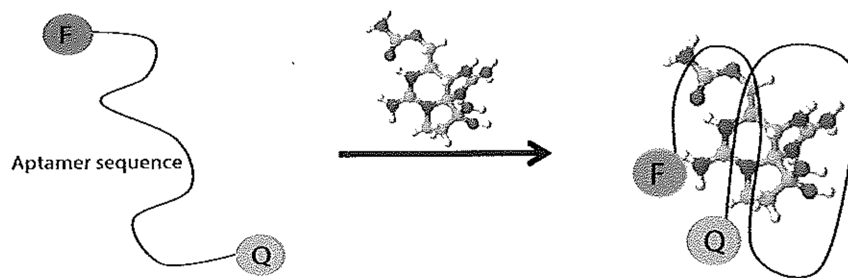
Compared to antibodies, aptamers have several advantages:

- Manufacturing costs and time are all lower compared to that of monoclonal antibody production.
- Once the aptamer (nucleic acid) sequence is known, the aptamer can be produced on the fly using an oligo synthesizer to meet ones immediate needs.
- Easy to label with reporters, enzymes, or fluorescent tags.

In this demonstration, we will use an aptamer that targets Saxitoxin (STX), a low molecular weight neurotoxin (299.29 g/mol) mainly produced by certain marine dinoflagellates that, along with its

family of similarly related paralytic shellfish toxins, may cause the potentially fatal intoxication known as paralytic shellfish poisoning.

The aptamer is modified with a fluorophore and a quencher at the two extremities. In absence of the target the fuorophore is distantiated from the quencher (left). In presence of the target, the aptamer will undergo a binding-induced conformational change, allowing a decrease of the fluorescence signal, due to the proximity of the fluorophore and the quencher (right).



Procedure

Fluorescence measurements will be obtained using a Cary Eclipse Fluorimeter (Varian) at 25° C. Being the aptamer sequence modified with Alexa488 an excitation at 488 nM (± 5 nm) and acquisition between 510 and 520 nm with 5 nm bandwidths will be used. Binding curves will be obtained by sequentially adding increasing concentration of saxitoxin in a solution containing the aptamer receptor (50 nM of saxitoxin-binding aptamer in 100 µl HEPES 50 mM NaCl 150 mM pH 7.4). All the signals will be recorded at the equilibrium time (15 minutes). The observed K_D will be obtained using the following pseudo-Langmuir Equation:

$$F(T) = F(0) + \left(\frac{[T]*(F_B - F_0)}{K_D^{obs} + [T]} \right)$$

where [T] = target concentration; F_B= fluorescence in the presence of saturating concentration of target and F_0 = background fluorescence.

Selected literature

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Jayasena, S.D., 1999. Aptamers: an emerging class of molecules that rival antibodies in diagnostics. Clin. Chem. 45 (9), 1628–1650.
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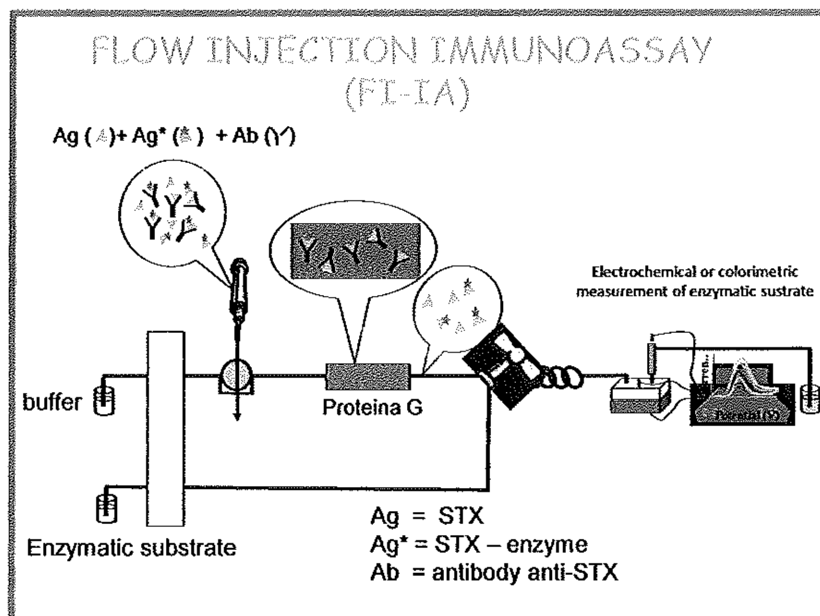
An flow-injection immunoassay system for detection of *Saxitoxin*

Principle of the method

Flow-injection immunoassay (FI-IA) is a method of conducting immunoassays using a small, reusable immunoreactor and a continuously flowing buffer stream into which samples and necessary reagents are introduced by injection. The sample containing the analyte is first equilibrated with given amounts of enzyme-analyte conjugate (Ag*) and a fixed amount of primary antibody (Ab). After a brief equilibrium incubation period, the reaction mixture is injected through the immunosorbent reactor containing excess Protein G (high affinity for the constant region -Fc region- of immunoglobulins, in this case it binds the primary antibody). The antigen–antibody complexes (AgAb and Ag*Ab) are trapped on the Protein G column. All unbound species are washed away by the carrier stream buffer. The free fraction of the tracer (Ag*) is monitored. The enzyme activity of unbound tracer can be detected by diverting a flowing solution of substrate through the channel 2, and monitoring the product downstream with an appropriate flow-through electrochemical or colorimetric detector. The Protein G column does not need to be regenerated between assays.

For Saxitoxin (STX) determination, a FI-IA system based on the amperometric/colorometric detection of horseradish peroxidase (HRP), which serves as the enzymatic label for tracer Ag in the eluate is developed.

Schematic representation of the flow injection immunoassay (FI-IA)



Materials

a) Four peristaltic pomp MINIPLUS®3 (Gilson, USA)
b) 100 µL injection loop
c) Six-way Rheodyne injection valve, model 7125 (Cotati, CA)
d) Borosilicate glass chromatography column (30 mm length and 0.07 mL bed volume) equipped with nylon membrane and filled with Protein G agarose beads
e) Plastic microplate (96 microwells)

REAGENTS

1. Saxitoxin (STX) as target analyte
2. Rabbit polyclonal antibody anti-STX
3. Saxitoxin-HRP
4. 3,3',5,5'-tetramethylbenzidine + H_2O_2 substrate supersensitive solution.

BUFFERS

Buffer solution: 50 mmol l^{-1} phosphate buffer

Carrier buffer (channel 1): 50 mmol l^{-1} phosphate buffer, also containing 100 mmol l^{-1} NaCl, 10 mmol l^{-1} $MgCl_2$ and 0.03% (v/v) Tween 20

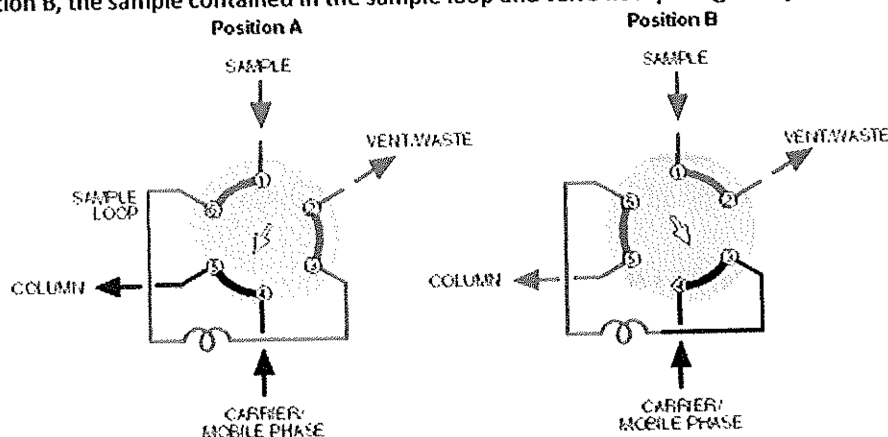
Substrate solution (channel 2): 0.5 mmol l^{-1}TMB and 10 mmol l^{-1} H2O2 prepared in 100 mmol l^{-1} phosphate-citrate buffer, also containing 100 mmol l^{-1} NaCl.

Stop solution: 0.16M sulfuric acid (50 µL/well)

PROCEDURE

1. Add the Ab* solution in each vial, containing the sample/standard(400 µL)
2. Inject 200 µL of mixed solution in flow system
3.Open the injection valve (position B)
4. Collect the eluate solution after 1 min and for 4 min
5. Close the injection valve (position A)
6. Waiting 2 min before the subsequent injection
5. Transfer the collected solution (100 µL) in the wells of microplate
6. Measure the enzymatic product at 405 nm using the microplate reader

Schematic representation of the injection valve with 6 channels: with the valve in Position A, sample flows through the external loop while the carrier flows directly through to the chromatographic column. When the valve is switched to Position B, the sample contained in the sample loop and valve flow passage is injected onto the column.



Schematic representation of the FI-IA immunoassay set-up

Selected literature

-M. Badea, L. Micheli, M.C. Messia, T. Candigliota, E. Marconi, T. Mottram, M. Velasco-Garcia, D. Moscone, G. Palleschi (2004). "Aflatoxin M1 determination in raw milk using a flow-injection immunoassay system". Analytica Chimica Acta, vol. 520 (pp. 141-148)

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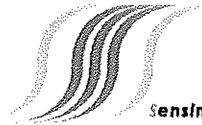

2nd WORKSHOP ON BIOSENSORS

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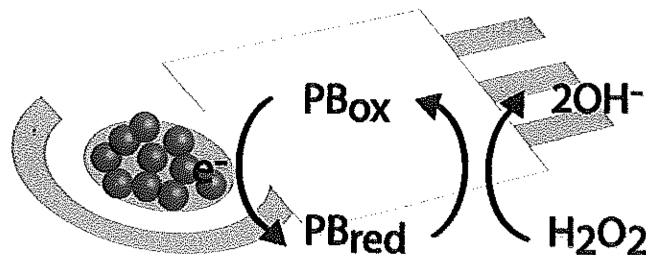
Prussian Blue: "artificial peroxidase" to detect H_2O_2

Principle of the method

Ferric hexacyanoferrate or Prussian blue (PB)is an electrochemical mediator and it has been referred to as an "artificial peroxidase" [1,2]. It is electrochemically reduced to form Prussian white (PW), which is capable of catalyzing the reduction of hydrogen peroxide at low potentials (around 0 V vs. Ag/AgCl), which is an excellent characteristic as it allows to work in the presence of a wide range of interferences.



Materials

a) Screen-Printed Electrodes;
b) Oven;
c) Portable potentiostat PalmSens (Palm Instruments, The Netherlands);
d) Magnetic stirrer.

Reagents

1. Potassium ferricyanide ($K_3[Fe(CN)_6]$);
2. Ferric chloride ($FeCl_3$);
3. Hydrochloric acid (HCl);
4. Hydrogen peroxide (H_2O_2).

Procedures

SPEs fabrication: a briefly (theoretically) explanation about the screen-printing technique of the electrodes will be provided to groups.

PB chemical deposition onto SPEs:

1. Prepare a solution of 0.1 M $K_3[Fe(CN)_6]$ in 0.01 M HCl (Solution A);
2. Prepare a solution of 0.1 M $FeCl_3$ in 0.01 M HCl (Solution B);
3. Cast 5 µL of Solution A onto the working electrode surface;
4. Cast 5 µL of Solution B onto the working electrode surface and gently mix solutions A and B;
5. Let the mix reacts for 10 minutes, then wash with a solution of 0.01 M of HCl;
6. Cure the modified SPEs in the oven at 100°C for 90 min.

Cyclic voltammetry experiments:

1. Fill a beaker with 10 mL of phosphate buffer (0.05 M phosphate buffer (pH 7) containing 0.1 M KCl);
2. Perform a cyclic voltammetry experiment in the range from -0.4 to 0.4 V at a scan rate of 50 mV/s;
3. Add H_2O_2 into the beaker to obtain a concentration equal to 1 mM;
4. Perform a cyclic voltammetry experiment in the same conditions at point 2.

Amperometric calibration curve of H_2O_2:

1. Fill a beaker with 10 mL of phosphate buffer (0.05 M phosphate buffer (pH 7) containing 0.1 M KCl), add a magnetic bar inside and put the beaker over a magnetic stirrer;
2. Start the amperometric detection applying -0.05 V (vs. Ag/AgCl) and allow the current line to stabilize;
3. Add amount of H_2O_2 equal to 10 µM in the beaker;
4. Repeat point 3 until H_2O_2 reaches 50 µM.

References

[1] F. Ricci, G. Palleschi, Sensor and biosensor preparation, optimisation and applications of Prussian Blue modified electrodes. Biosens. Bioelectron. 21 (2005), 389-407.

[2] S. Cinti, F. Arduini, D. Moscone, G. Palleschi, A.J. Killard, Development of a hydrogen peroxide sensor based on screen-printed electrodes modified with inkjet-printed Prussian blue nanoparticles, Sensors 14 (2014), 14222-14234.

Sensing toxicants in Marine waters makes Sense using biosensors

2nd WORKSHOP ON BIOSENSORS

FOR WATER MONITORING

April 08-10, 2015, University of Rome Tor Vergata, Italy

Electrochemical sensor for detec on of sulfonamides

Principle of the method

Over the last few decades, the occurrence of micropollutants in the aqua c environment has become a worldwide issue of increasing environmental concern. Pharmaceu cals and other micropollutants have been the focus of recent research due their occurrence in di erent environmental compartments worldwide. Among of these micropollutants, sulfonamides are a class of widely used human and veterinary an bio cs but pose a risk as environmental pollutants. Several methods have been reported for the determina on of sulfonamides in water, soil and pharmaceu cal and food products including high-performance liquid chromatography (HPLC), capillary electrophoresis, gas chromatography and spectrophotometric method, and electroanly cal methods [1-3].

The principle of the method is the determina on of sulfonamides using two kind of electrodes based on carbon nanomaterials. Linear Sweep Voltammetry (LSV) and Square Wave Voltammetry are used as electroanaly cal techniques.

Materials

a) Graphite and Carbon Black N220 paste electrodes;
b) Portable poten ostat PalmSens (Palm Instruments, The Netherlands);
c) Magne c s rrer.

Reagents

1. Sulfamethoxazole, Sulfacetamide,Sulfadiazine;
2. Methanol, Sodium hydroxide (NaOH);
3. Potassium phosphate monobasic (KH_2PO_4) ,Potassium phosphate dibasic (K_2HPO_4);

Procedures

Prepara on of carbon paste electrode:

1. The carbon paste was prepared by hand mixing an adequate amount of carbon materials with liquid para n using a pestle and mortar to form a homogeneous carbon paste.

2. The resul ng pastes were packed into the well of the working electrode to a depth of 2 mm with 3 mm of diameter.

3. The surface exposed to the solu on was polished on a print paper to give a smooth nish before use. The body of the working electrode was a Te on tube.

Linear Sweep Voltammetry experiments:

1. Fill a beaker with 10 mL of phosphate bu er (0.05 M phosphate bu er, pH 6);
2. Perform a Linear Sweep voltammetry experiment in the range from 0.3 to 1.3 V at a scan rate of 50 mV/s;
3. Add Sulfonamide into the beaker to obtain a concentration equal to 100μM;

Square Wave Voltammetry experiments:

1. Fill a beaker with 10 mL of phosphate bu er (0.05 M phosphate bu er (pH 6) ;
2. Start the Square Wave test in the range from 0.6 to 1.3V at a frequency of 10Hz.
3. Add amount of sulfonamide equal to 1 μM in the beaker;
4. Make a calibra on curve in the range from 1 to 10 μM.

References

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[2] Hana Dejmkova, Miroslav Mikes, Jiri Barek, Jiri Zima. Determina on of Sulfamethizole Using Voltammetry and Amperometry on Carbon Paste Electrode. Electroanalysis 2012 ; (24) 1 – 6.

[3] SusanSadeghi ,AliMotaharian, AliZeraatkarMoghaddam, Electroanaly cal determina on of Sulfasalazine in pharmaceu cal biological samples using moleculary imprinted polymer modi ed carbon paste electrode; Sensors and Actuators B 168 (2012) 336–344.

UNIVERSITA' degli STUDI di ROMA
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2nd WORKSHOP ON BIOSENSORS

FOR WATER MONITORING

April 08-10, 2015, University of Rome Tor Vergata, Italy

Spectrophotometric method for detection of sulfonamides

Introduction

Lately, the drugs are receiving more attention for their power consumption and pollutants in the environment. Since then, a wide range of pharmaceuticals has been detected in fresh and marine waters. For this reason, the work of scientific research has been conducted to study their fate, transport via natural aqueous systems and their ecological impact. Pharmaceuticals are increasingly produced and used in large quantities each year, which raises concerns about their presence and side effects that increase over time.

Studies in several countries have shown the presence of more than 80 pharmaceuticals in municipal wastewater at concentrations ranging from ng/L to μg/L (Kümmerer, 2001; Heberer 2002). The increasing use and improper disposal of these drugs leads to continuous inputs leading to their presence and "persistence" in ecological environments.
Antimicrobial agents are one of the largest classes of pharmaceuticals with general application in human and veterinary medicine. These compounds are of particular importance in the environment because of their ubiquity and their potential to disrupt the bacterial populations in natural systems. Among these agents, sulfonamides have recently been subject to increasing environmental concern because of their impact on the environment.Survey of the literature reveals various methods available for the determination of sulfonamide derivatives. The methods include the gas chromatography (GC), high performance liquid chromatography (HPLC), electroanalytical methods and spectrophotometric methods[1-4].

The objective of this work is to develop spectrophotometric method for the determination of sulfonamides present as residues in seawater.

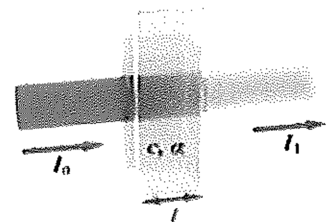
1. Spectrophotometric analysis

Spectrophotometry is a versatile analytical tool. The underlying principle of spectrophotometry is to shine light on a sample and to analyze how the sample affects the light. Advantages of spectrophotometry are:

- it is usually non-destructive (can measure and recover sample),
- it is selective (often a particular compound in a mixture can be measured without separation techniques),
- it has a short time interval of measurement.

1.1. Principle of spectrophotometric method

- Interaction between a compound of interest and a monochromatic radiation
- a part of the radiation is absorbed by the compound and a rest of the radiation is detected by a detector
- Quantity of the absorbed radiation is directly proportional to the quantity of the compound.



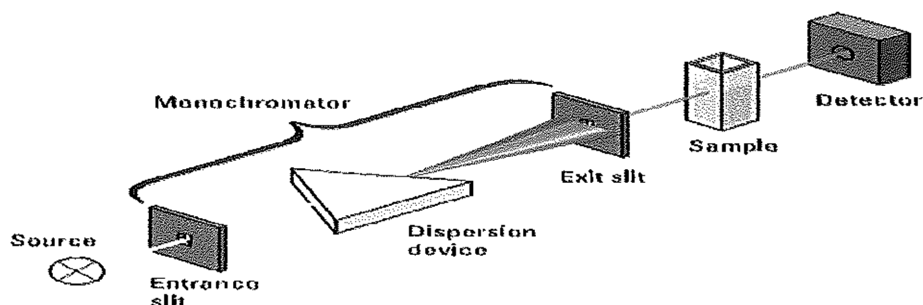
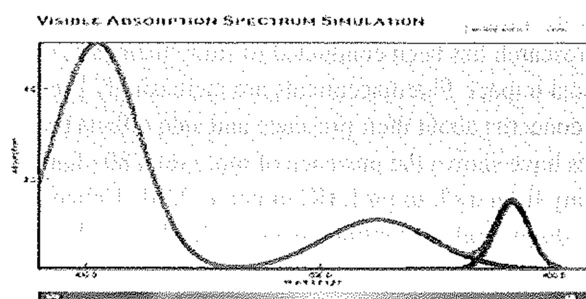


Fig.1: Compounds of spectrophotometer

1.2. Plotting absorption spectrum :

The absorption curve is obtained by continuous measurement of absorbance at various wavelengths and plotting these data to a graph, where the x axis contains the wavelength (independent variable) and the y axis is used for the absorbance (dependent variable). Regions with the highest absorbance values are called the absorption maxima.



A / λ

Fig.2: Absorption spectrum

2. Principle of the proposed method

The method is based on the diazotization of sulfonamides such as sulfanilamide (SA), sulfadiazine (SDZ), sulfacetamide (SFA), sulfamethoxazole (SFMx), sulfamerazine (SFMr), sulfadimethoxine (SDm), and sulfamethiazole (SFMt), Sulfathiazole (ST) with sodium nitrite, sulfamic acidand hydrochloric acid followed by coupling with N-(1-naphthyl) ethylenediaminedihydrochloride (NED) to form a pink product.

3. Proposed chemical reaction for the method

The proposed chemical reaction for the method is shown in the figure below:

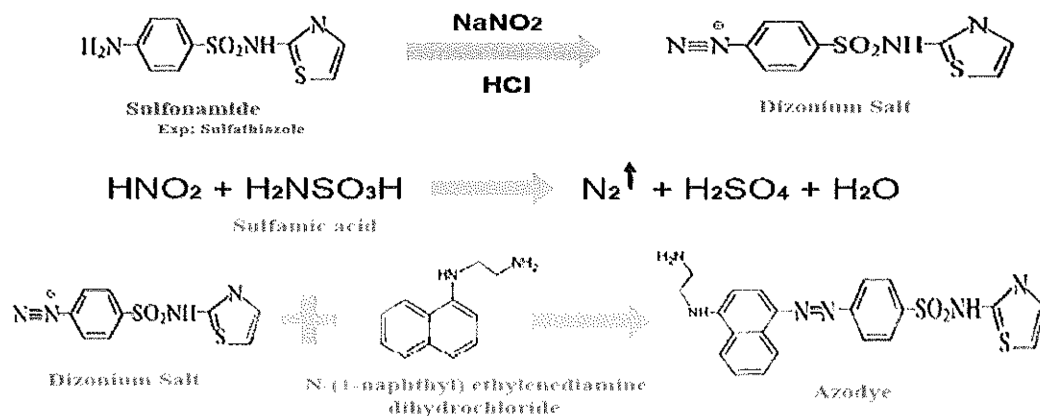


Fig.3: Reaction sequence for the formation of the pink coloured products

4. **Materials**
   a) Spectrophotometer UV, Visible ;
   b) Cuvettes 1cm;
   c) Volumetric flasks 100mL;
   d) Volumetric flasks 10mL
   e) Magnetic stirrer.
   f) Pipettes 0.2, 1 and 10 mL
5. **Reagents**
   a) Sulfonamide derivatives (RSO_2NH_2): sulfadiazine, sulfamerazine;
   b) Hydrochloric acid (HCl);
   c) Sodium Nitrite ($NaN0_2$);
   d) Sulfamic acid (H_3NSO_3);
   e) N-(1-naphthyl) ethylenediaminedihydrochloride ($C_{12}H_{14}N_2$).
6. **Procedure**

a) Preparation of standard solution

About 100 mg of sulfonamide (sulfadiazine, sulfamerazine) weighed accurately, dissolved in 40 mL of 1mol Hydrochloric acid in a 100 mL volumetric flask, and diluted up to the mark with water (1000 μg/mL).

b) Preparation of working standard solution

Working standard solution of sulfonamide containing 10μg/mLwas prepared by further dilution.

c) Preparation of solutions

- Hydrochloride 1M;
- Sodium nitrite (1%w/v): dissolve 1g of $NaN0_2$ in 100ml of distilled water;
- Sulfamic acid (2% w/v): dissolve 2g of H_3NSO_3 in 100ml of distilled water;
- N-(1-naphthyl)ethylenediamine dihydrochloride (1% w/v): dissolve 1g of $C_{12}H_{14}N_2$ in100ml of distilled water.

d) Spectrophotometric measurement

1. Transfer aliquots of Sulfonamide derivative solutions (Sulfadiazine, Sulfamerazine) ranging from 0.2-1mL (10 μg/mL) into each of the series of 10 ml standard flasks;
2. Add 1ml of cold sodium nitrite (1% w/v), and 1 mL of 1 mol hydrochloric acid with swirling at room temperature;
3. After 5 min, add 1 mL of sulphamic acid (2% w/v) and 1 mL of N-(1-naphthyl)ethylenediamine dihydrochloride (1% w/v) with swirling;
4. Adjust the volumes to the mark with distilled water, then mix the solutions thoroughly;
5. Measure the absorbance of the pink coloured at 536 nm against reagent blank;
6. Make a calibration curve in the range of concentration of 0.2μg/mL to 1μg/mL.

References:

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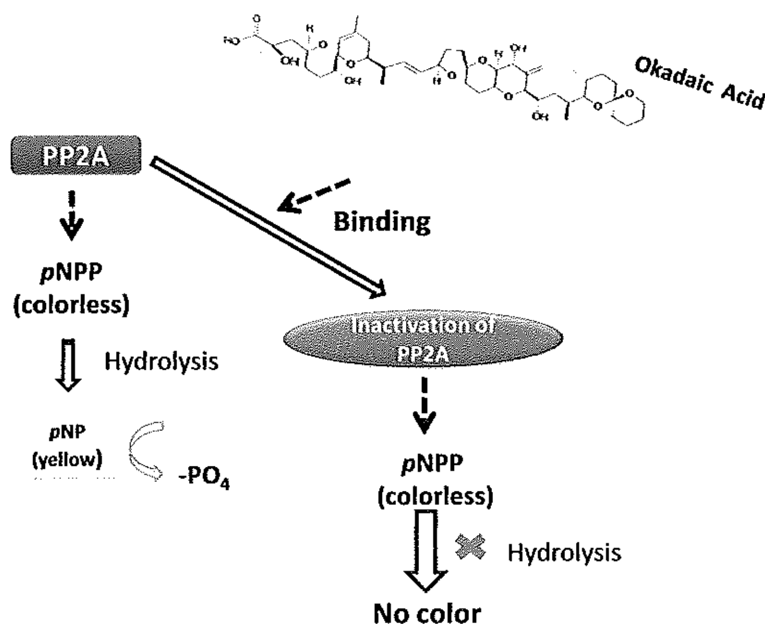
Colorimetric assay based on Protein Phosphatase-2A inhibition for Okadaic Acid detection

Principle of the method

The proposed method is a colorimetric assay in which the activity of protein phospharase-2A (PP2A) is determined by measuring the rate of color production from the release of yellow *p*-nitrophenol using *p*-nitrophenyl phosphate as the substrate. In the presence of OA enzyme inhibition occurs and consequently the rate of color production decreases proportionally to the concentration of the toxin.



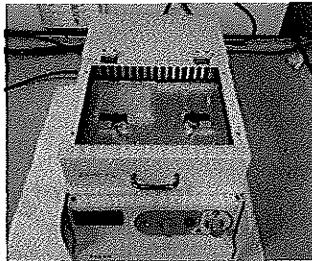
Materials and Apparatus

a) Microtitre well plate and multichannel pipette

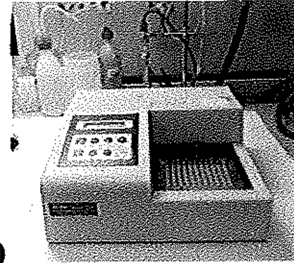
b) Microtitre stirrer

c) Microtitre plate reader

(a)



(b)



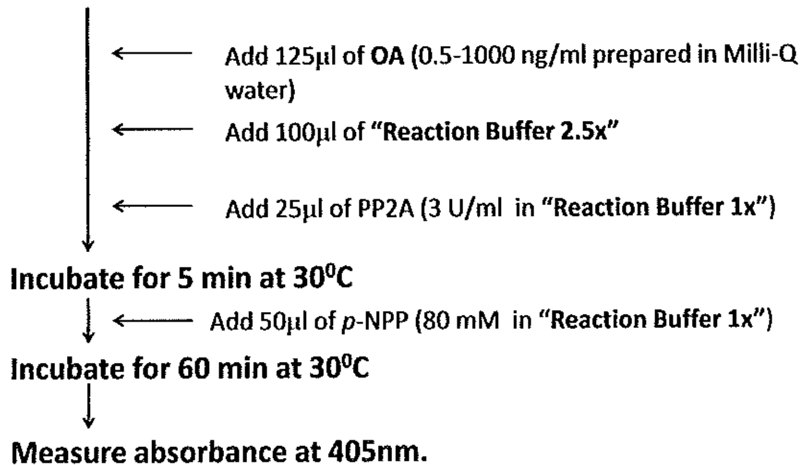
(c)

Reagents

1. Microcystin-LR (MC-LR) from *Microcystis aeruginosa*
2. 4-Nitrophenyl phosphate disodium salt hexahydrate (*p*NPP)
3. PP2A (purified enzyme) from human red blood cells in Dilution Buffer
4. Reaction Buffer 2.5 x and 1 x
5. Milli-Q water

Procedure

96 Well plate



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An ELIME assay for detection of *Salmonella enterica*

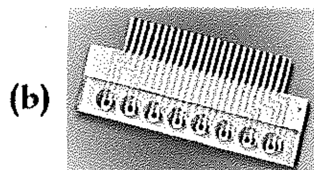
Principle of the method

The proposed ELIME (Enzyme-Linked-Immuno-Magnetic-Electrochemical) assay is based on the use of magnetic beads (MBs), as support of a sandwich immunological chain, coupled with a strip of 8-magnetized screen-printed electrodes (SPEs). The product of the enzymatic reaction is quickly measured by chronoamperometry at an applied potential of -100 mV for 60 seconds.

Materials

a) Autolab with GPES software
b) Strip of 8-SPEs localized at the bottom of 8 wells
c) magnetic particle concentrator
d) rotation device

(a)

(b) 

(c)

(d)

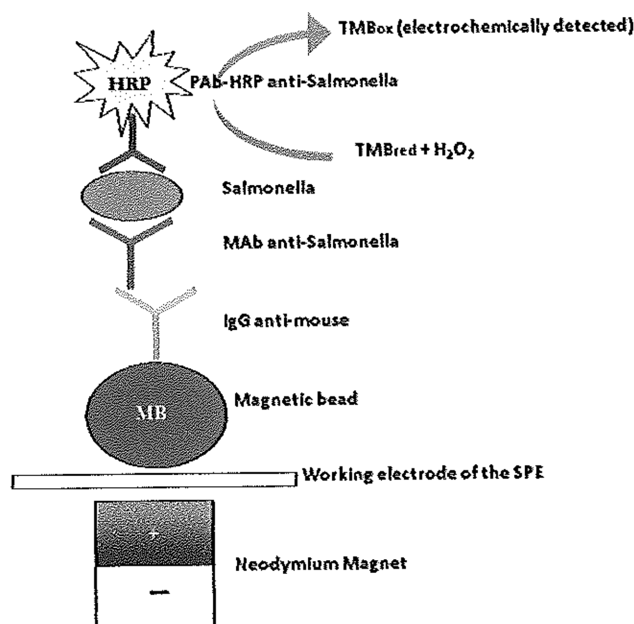
Reagents

1. Pan Mouse IgG MBs precoated with IgG anti-mouse and previously coated with a broad reactivity MAb anti-Salmonella (produced in mouse), blocked with dry milk (3% w/v) and stored in PBS +0.02% NaN_3.
2. Rabbit polyclonal antibody-HRP (2 mg/mL) anti-*Salmonella*.
3. 3,3',5,5'-tetramethylbenzidine + H_2O_2 substrate supersensitive solution.
4. *Salmonella* Typhymurium as target analyte.

Procedure

- 1. Shake and transfer 10 µl of the coated and blocked beads suspension (stored at 4°C) into 2 ml Eppendorf tube (in number required for the calibrators and/or saliva samples to analyze);
- 2. wash 3 times with 1 ml PBS + 0.2% dry milk, every time shaking and discarding the supernatant;
- 3. add 200 µl of Salmonella standard solutions + 200 µl of PAb-HRP (diluted 1:50 in PBS+0.2% dry milk) and incubate for 30 min;
- wash 2 times with 1 ml of PBS + 0.05% Tween 20, and one time with PBS;
- resuspend the MBs in 100 ml of PBS;
- transfer 20 µl of each suspension (two replicates for each calibrator and/or sample) into separate wells of the electrochemical strip;
- localize the MBs onto the surface of the 8-screen-printed working electrodes by use of a specially designed support that includes eight neodymium magnets, positioned underneath the wells;
- add 70 µl of substrate (TMB + H_2O_2) into each well;
- Measure, after 2 min of incubation, the electroactive enzymatic product by chronoamperometry.

Schematic representation of the sandwich immunological chain concentrated onto the WE surface



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E. Delibato, G. Volpe, D. Romanazzo, D. De Medici, L. Toti, D. Moscone, G. Palleschi. Development and Application of an Electrochemical Plate Coupled with Immunomagnetic Beads (ELIME) Array for *Salmonella enterica* detection in meat samples. *Journal of Agricultural and Food Chemistry* **2009**, 57:7200-7204.