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Highlights

- Novel sensing device based on the achievement of high-throughput by simultaneous preparation of up to 96 samples using multiwell plates for sulfonamides screening.
- Novel electrochemical Carbonic Anhydrase (CA) inhibition biosensor for sulfanilamide determination.
- CA inhibition mechanism was noncompetitive for sulfanilamide.
- Interference study demonstrated very good selectivity towards sulfanilamide.

INVESTIGATION OF SULFONAMIDES INHIBITION OF CARBONIC ANHYDRASE ENZYME USING MULTIPHOTOMETRIC AND ELECTROCHEMICAL TECHNIQUES

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ABSTRACT:

Two approaches for sulfonamides (SA's) determination, based on carbonic anhydrase enzyme inhibition have been investigated in this work. In the first method, nine different SA's have been screened using simultaneous multiphotometric measurement in multiwell plates. The sulfanilamide (SAD) showed significant inhibition compared to other sulfonamides. The carbonic anhydrase (CA) kinetic interactions reveal noncompetitive binding of SAD. Interferences from other inhibitors with enzyme were studied and the results showed very good selectivity toward SAD. In the second approach, an electrochemical enzyme inhibition biosensor, based on CA entrapped in a carbon paste electrode using carbon black nanoparticles and solid paraffin, was successfully applied to SAD measurements. Results from the quantitative analysis of SAD are discussed in terms of detection limit, linear range and sensitivity using multiphotometric and biosensor-based methods. The biosensor developed was successfully applied to the determination of SAD at submicromolar levels and it is recommended for application for in situ analysis.

KEYWORDS: Sulfonamides; Multiphotometric assay; Carbonic Anhydrase; Electrochemical Biosensor; Enzyme Inhibition.

1. Introduction

The presence of pharmaceutical drugs in food, water and soil currently represents one of the major issues for the environmental chemistry [1-10]. Frequently a high percentage of the ingested drugs is excreted from the dosed human and animals without metabolism or excreted in conjugated forms that can be easily converted back to the parent compounds [11]. Sulfonamides represent classes of synthetic antibiotic drugs that are widely used in human and veterinary medicine [12-15]. Among 10 000 sulfanilamide derivatives, 40 are applied as antibacterial drugs. The chemical structure of most of them contains heterocyclic radicals. The precursor of sulfonamides is sulfanilamide (p- aminobenzene sulfonamide) known as streptocid which was first synthesized in 1908. The hemotoxicity and the carcinogenic effects of some sulfonamides have been reported [16, 17]. It has been shown that certain decomposition products are more active and potentially more toxic than the parent compounds [18]. For that reasons, many methods have been used for sulfonamide detection including chromatographic, microbiology, immunoassays, electrochemical and biosensors Techniques [19-21].

High-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS) chromatographic methods are used as reference sensitive tools but present strong drawbacks such as complex and time-consuming treatments of the samples, extraction , clean-up and additional purification steps [21-24]. Moreover, the analysis usually has to be performed in a specialized laboratory by skilled personnel and is not suitable for in situ application. These issues turn out to be a major problem when rapid and sensitive measurements are required. On the other hand screening techniques can detect specific contaminant or a family of contaminants at the level of interest, and commonly provide semi-quantitative results [18, 21]. These methods enable reliable verification of several samples simultaneously and only samples indicating the presence of the contaminant will be selected for further analysis. The advantages of a screening strategies, high throughput, low sample rate, short analysis time, easy to use by unskilled persons, good selectivity, low cost, simple, can be used in the field and in routine laboratories.

Nowadays, the use of sulfonamides is widespread and often uncontrolled in veterinary practices which increase the potential presence of SA's in foodstuff and environment as reported in literature [25]. They are, in fact, among the new emergent environmental pollutants because of their increasing occurrence in different commodities [5, 26].

To respond to these issues, many electrochemical and antimicrobial screening tests have been adopted as alternatives to conventional reference methods for fast and simple detection

of some environmental pollutants [27-32]. However few studies have used enzymatic biosensor for sulfonamides determination [26]. Recently Lorena del Torno-de Román et al 2016 have been developed Tyrosinase amperometric biosensor sulfamethoxazole determination. The Tyrosinase has been cross-linked on screen-printed carbon electrodes previously modified by gold nanoparticles. The sulfamethoxazole has been evaluated in the concentration range from 20 µM to 0.2 mM [33], indicating low sensitivity of tyrosinase biosensor toward sulfamethoxazole.

Taking into account these reasons, our work aims to develop two new approaches applied to sulfonamides screening and analysis using carbonic anhydrase enzyme. Carbonic anhydrase is metalloenzyme that catalyses at least three reactions, the hydration of CO_2, the hydration of certain aldehydes and the hydrolysis of several esters [34-37].

In this work, colorimetric and electrochemical techniques have been used. The quantification of sulfonamides, considered as new emerging environmental pollutant is based on their action of inhibition on Carbonic Anhydrase enzyme (CA). In the first part, The CA residual activity has been determined after exposure to nine different sulfonamides using the colorimetric method. The chemical structures of the nine sulfonamides are shown in supplementary (Table 1). In this case our approach has been focused on the development of a new sensing device based on the achievement of high-throughput by simultaneous preparation of up to 96 samples using multi well plates. Nine different sulfonamides were tested for their inhibition of CA. The type of inhibition was determined as non-competitive. In the second part of this work, an electrochemical carbonic anhydrase based biosensor for inhibitive determination of sulfanilamide is proposed for the first time.

2. EXPERIMENTAL SECTION

2.1. Instrumentation

The CA activity assays and inhibition measurements were performed using photometric instrument and electrochemical analyzer.

The Multiphotometric assays were carried out using a BioTek ELx 800 photometric instrument (Micotitration plate reader, 96 wells).

Linear sweep Votammetry (LSV) measurements were carried out using a Palmsens Potentiostat-Galvanostat electrochemical analyzer, controlled by personal computer equipped with GPES for Windows software, provided by Eco Chemie B.V. (Utrecht, Netherlands).

2.2. Reagents

All chemicals used in this work were of analytical grade and were used without further purification. Sodium acetate buffer solution (50 mM, pH 4.5, 5.5, 6.5) prepared from sodium acetate and acetic acid from Sigma-Aldrich and Tris-HCl prepared from Tris powder and HCl (35%, v/v) were purchased from Carlo Herba.

4-nitrophenyl acetate (NPA), was from Sigma-Aldrich and used as carbonic anhydrase substrate.

Carbonic anhydrase lyophilized enzyme (3500 U/mg) was purchased from Sigma Aldrich. CA enzymatic aliquots of 64.4 U/mL were prepared in Tris-HCl buffer 50 mM, pH 7.8.

Acetonitrile (ACN) (99.9 %,W) from Sigma-Aldrich were used for preparing of freshly NPA substrate solutions. $HgCl_2$, NiCl, Na_2S are of analytical grade and were purchased from Sigma-Aldrich.

Carbon black (N 220) used in this study of industrial standard grade is obtained from Corporation (Ravenna, Italy). N 220 is characterized by having diameter of 32 nm and surface area of 117 m^2 /g [32]. Graphite having particle diameters <0.1 mm and 12 m^2/g as surface area, was purchased from Fluka.

All solutions were prepared using Millipore Milli-Q nanopure water. Experiments were all carried out at room temperature (25±1°C).

Sulfonamides including Sulfadiazine (SDZ), Sulfamethoxazole (SMX), Sulfacetamide (SCT), Sulfathiazole (SFT), Sulfamethiazole (SMT), Sulfamerazine (SMZ), Sulfadimethoxine (SDT), Sulfasalazine (SSZ) and Sulfanilamide (SAD) were from Sigma-Aldrich.

The sulfonamides stock solutions 10^{-2} M were prepared as follow: The sulfonamides powder were dissolved in few ml of acetone after that the water was added until 100 ml.

2.3. Biosensor preparation

Carbonic Anhydrase was immobilized into carbon paste electrode by entrapment as follow:

Carbon black (CB) nanoparticles or graphite (G) powder was mixed with solid paraffin. Solid paraffin was first liquefied by heating at around 45°C before mixing it with carbon material. After that carbon material was mixed by adding the adequate amount to liquefied solid paraffin using a pestle and mortar to form a homogeneous carbon paste. The homogeneous consistency was achieved at proportion of (50 carbon black: 50 solid paraffin) and (75 graphite: 25 solid paraffin) (w/w) [38]. CA was added to the obtained paste at the ratio of 5% (w/w) and well mixed. The electrode (3 mm diameter) was filled with CA-CB or CA-G prepared paste. The enzymatic electrodes were polished on print paper until a smooth surface

was obtained. The resulting entrapment enzyme electrodes were stored in Tris-HCl buffer 4°C when not in use.

3. Results and discussions

3.1. Multiphotometric assay measurements

3.1.1. Optimization of the measurement

The carbonic anhydrase activities were measured by monitoring the 4-nitrophenol, which is the enzymatic product of 4-nitrophenyl acetate (NPA) at 405 nm for 90 min in Tris-HCl buffer. Figure 1 shows the principle of CA and 4-nitrophenyl acetate reaction. ELISA microplates were used as a support for small volumes of enzymatic mixtures (200 µl total volume) and allowing multiassay photometric measurements.

Preferred position of Figure 1

3.1.1.a. Effect of pH on non-enzymatic degradation of substrate NPA

Since the NPA substrate is more soluble in acetonitrile than in buffer, the stock substrate solution (3 mM) was prepared in acetonitrile (CAN).

In order to monitor the spontaneous degradation of the 4-NPA in buffer solution, the stability was monitored at different pH values: pH 4.5, 5.5, 6.5 (acetate buffer), and 7.8, 8.5 (Tris-HCl buffer). In micotitration plate wells 50 µl of **NPA** stock substrate solution (3 mM) were mixed with 150 µl of different buffer solutions which corresponds to 750 µM of NPA.

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Preferred position of Figure 2.

Figure 2 shows the absorbance of non-enzymatic evolution (at 405 nm) versus time of NPA prepared solutions at different pH. The spontaneous hydrolysis of the 4-NPA in buffer solution is greater at basic pH than at low pH value. Even so, it should be noted that hydrolysis rates at basic pH is very low. Indeed, the variation of absorbance of 750 µM of 4-nitrophenyl acetate during 42 minutes is equal to 0.033 and 0.013 using 8.5 and 7.8 pH buffers respectively.

3.1.1.b. Substrate and CA concentration optimization

The CA enzymatic reaction using 4-nitrophenyl acetate has been followed at 405 nm for 90 min. Freshly substrate prepared solution in acetonitrile was chosen for further experiments to avoid the non-enzymatic degradation of 4-nitrophenyl acetate.

Since the non-enzymatic degradation of 4-nitrophenyl acetate (750 µM) is significantly higher when the buffer is alkaline we have studied in this part of work the CA enzymatic reaction at low NPA concentrations respectively 37.5 and 75 µM. Various enzyme concentrations were used for this aim 8.05 U/µl, 16.1 U/mL and 32.2 U/mL. The results obtained are reported in the figure below (Fig.3).

Preferred position of Figure 3.

These measurements were realized in order to choose optimal enzymatic conditions for further studies. For the concentration of 4-NPA equal to 37.5 µM, the velocity of the reaction of substrate hydrolysis is increasing with increasing CA concentration.

Since the absorbance change is not very important using 32.2 U/mL of CA and 37.5 µM of NPA comparing to 16.1 U/mL of enzyme and 75 µM of substrate, last conditions were chosen for further investigations.

3.1.1.c. Effect of pH on CA activity

As reported in literature [37, 39-41], most studies were performed in Tris-HCl buffer at pH 7.5 to 8.5. In this work we evaluate CA activity as a function of pH (acetate buffer: 4.5, 5.5, 6.5 and Tris-HCl buffer: 7.8, 8.5).

Preferred position of Figure 4.

In accordance with earlier studies, results obtained showed that CA anhydrase activity is low at pH from 4.5 to 6.5 and significant over pH 7 (Fig.4). Thus pH of 8.5 was selected in this work.

3.1.2. Sulfonamides determination based on CA inhibition using multiphotometric assay measurements

Nine different sulfonamides were tested for their inhibition of CA such as SDZ, SMX, SCT, SSZ, SFT, SDT, SMT, SMZ and SAD.

Measurement of enzymatic inhibition degree: In the usual procedure, buffer, enzyme and inhibitor were premixed in total volume of 195 µL, and the reaction was initiated by addition of 5 µL of NPA freshly prepared in ACN. The degree of inhibition (Abs (%)) due to the sulfonamide inhibitors was evaluated according to the equation:

$$\text{Abs (\%)} = \frac{\text{Abs}_0 - \text{Abs}_1}{\text{Abs}_0} \times 100$$

Where: Abs_0 and Abs_1 are the absorbance response of 4-nitrophenol which is the enzymatic product of 4-nitrophenyl acetate (NPA) at 405 nm for 90 min in Tris-HCl buffer recorded in absence and in presence of CA inhibitors.

The results for nine different sulfonamides tested showed that the activity of carbonic anhydrase was decreased by adding the SAD, SMX and SDZ at concentrations between 0.25 and 120 µM. As regards the other sulfonamides TBS, SSZ, SFT, SDT, SMT, SMZ no inhibition was observed at this concentration range.

The degree of CA inhibition by sulfanilamides SAD, SMX et SDZ, corresponding to 10 and 50 % has been determined and reported in table 1. The inhibition assays have been realized in Tris-HCl buffer at pH 8.5 using different concentration of SA's.

Table 1

The sulfanilamide SAD showed significant inhibition (Table 1) compared to other sulfonamides which allows us to study its influence on carbonic anhydrase activity at various concentration as shown in the following paragraphs.

The CA inhibition profile of SAD has been registered in the figure 5. The inhibition assays have been realized in Tris-HCl buffer at pH 8.5 using 25 µM of SAD. Under these conditions 70.5 % CA inhibition degree was obtained.

Preferred position of Figure 5.

The degree of CA inhibition by sulfanilamide was studied using different concentrations of inhibitor (**0.25-10 µM)** and SAD calibration curve was plotted (Figure 6).

Preferred position of Figure 6.

The figure 6 shows the relation between the SAD concentration and the inhibition rate of 4-NPA enzymatic hydrolysis. The CA activity decreases exponentially with increasing SAD concentration. The calibration curve shows a linear range from 0.25 to 1 µM. The equation of the curve is y = 58.48 x + 4.14 and 0.25 µM was equal to limit of detection (LOD) assuming that 10% of inhibition corresponding to LOD [42].

3.1.3. Determining the type of inhibition of CA by SAD

Each enzyme mechanism is reflected by specific kinetic characteristics that allow us to determine the type of inhibition of the enzyme studied. Sulfonamides are known to be potent, specific inhibitors of hydrase as well as esterase activities of carbonic anhydrase. SAD inhibition illustrated by Lineweaver and Burk representation was plotted in figure 7. The CA inhibition has been studied using different SAD concentrations at different concentrations of substrate as shown in Figure 7.

Preferred position of Figure 7.

The results obtained from the **Lineweaver and Burk representation (**figure 7) shows that the K_m is not changed with increasing SAD concentration. On the other hand the V_{max} lowered with increasing the SAD concentration. These results confirm that the inhibition by sulfanilamide is noncompetitive for 4-nitophenyl acetate hydrolysis. Recall that noncompetitive inhibition lowers the V_{max}. The obtained results are in accordance with previous works [37, 40] where sulfonamides inhibition was studied using CA type B and CO_2 as substrate. It is reported in these works that bovine CA in situ prepared is strongly inhibited by sulfonamides and these inhibitions appear to be noncompetitive.

3.1.3. Interferences

The activity of CA was followed using different anionic and cationic elements and benzoic acid. The concentration of these chemical compounds used for this study is equal to 10 µM.

Preferred position of Figure 8.

At 10 µM interfering concentration, figure 8 shows that CA is not inhibited by $HgCl_2$, Ni Cl, Na_2S, and benzoic acid but inhibited by 10 µM KCN. The percentage of inhibition corresponds to 48.3% of the initial CA activity.

3.2. CA based biosensor design and electrochemical measurements

3.2.1. CA based electrochemical biosensor

The carbon paste biosensor has been successfully used for enzyme immobilization in our previous work [43]. The Tyrosinase enzyme has been immobilized on the surface of carbon paste electrode using the cross-linking method in presence of glutaraldehyde. In the present work the CA enzyme was immobilized into carbon paste electrode by entrapment using carbon black nanoparticles or graphite powder and solid paraffin. The electrochemical measurements of 4-nitrophenol produced by CA based biosensor in presence of NPA was monitored by Linear Sweep Votammetry (LSV).

Because of the adsorption process of the oxidized products of phenol derivatives, on the electrode surface, the active area of the biosensors is reduced and thus signal decrease is observed as reported in literature [44, 45]. Both designed biosensors (CA-Carbon black and CA-Graphite) are concerned by these phenomena. This decrease of the electrochemical signal is more important in the case of graphite based biosensors than the other biosensors prepared with carbon black nanoparticles. After 5 measurements without changing bio-electrode surface, the signal decreases to 30.9 % and 13.1 % for CA-Carbon black and CA-Graphite based biosensors respectively. Therefore, the carbon black nanoparticles was chosen for CA based biosensor design and used for further inhibition studies. Furthermore to avoid the adsorption process of the oxidized products, the biosensor surface was generated after each use by polishing.

3.2.2. Inhibition study on CA based biosensor

According to spectrophotometric study reported in this work, SAD showed a significant inhibition of the carbonic anhydrase activity. Consequently, this sulfonamide compound was used for electrochemical investigations. Electrochemical measurements were done in 5 mL Tris-HCl 50 mM, pH 8.5 using Ag/AgCl (3 M KCl) reference electrode, platinum as counter electrode and CA-Carbon black as working electrode.

The experiments were realized in the presence of constant concentration of 4-nitrophenyl acetate (3 mM). In order to avoid the adsorption phenomenon, the biosensor surface was generated after each use. The inhibition profile of SAD has been registered in figures 9. The Linear Sweep Votammetry (LSV) has been made in 50 mM Tris-HCl buffer at 25 mV/s.

In this method the CA activity is monitored by measuring the oxidation current of the enzymatic product (4-nitrophenol). The extent of CA activity is inversely correlated with the amount of SAD inhibitor present in the system.

The degree of inhibition (I (%)) due to the SAD inhibitor was evaluated according to the equation:

$$I(\%) = \frac{I_0 - I_1}{I_0} \times 100$$

Where I_0 and I_1 are the currents recorded before and after CA inhibition, respectively.

The Inhibition degree equal to 75% of CA initial activity were registered in Tris-HCl buffer pH 8.5 using 25 µM of SAD as indicated in Figure 9. The relative standard deviation (RSD) for three repetitions is equal to 5%.

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Preferred position of Figure 9.

Using the developed CA-CB biosensor, the degree of inhibition was evaluated in presence of SAD at various concentrations. The degree of inhibition calculated with respect to the biosensor response without SAD addition (I_0). The concentration of SAD has been varied between 0.5 and 20 µM. The inhibition increases with increasing SAD concentration and the

corresponding degree of inhibition range was between 13.3% and 66.7% as shown in figure 10.

Preferred position of Figure 10.

A linear range, from 0.5 to 5 µM SAD concentration, was obtained from inhibition data recorded and summarized the equation y = 7.55 x + 10.42. Data reported correspond to 3 measurements for each concentration of CA-CB biosensor inhibitor (Figure 10). The estimated detection limit of SAD by CA based biosensor equal to 0.4 µM

3.2.3. Stability of the CA-CB based biosensor

Temperature is an important parameter that influences the enzyme stability. Storage stability, or shelf life, refers to an enzyme's maintaining its catalytic properties in the period between manufacture and eventual use. The polypeptide's native tertiary structure of the enzyme is commonly known as affected by the temperature changes. These changes can undergo some further change leading to permanent inactivation of the enzyme in special case. For these raisons in this work, CA-CB based biosensor stability was followed at 4°C storage temperature. The results obtained were reported in the figure 11.

Preferred position of Figure 11.

The CA-CB based biosensor storage at 4°C for 18 days does not compromise its usability, since the residual activity of CA-CB based biosensor remains over 76%.

4. Conclusion

In the first part of this work, the photometric assay with multiple analyses was successfully employed for sulfonamides screening. The sulfanilamide SAD showed significant inhibition compared to other sulfonamides. The calibration curve presented a linear range from 0.25 to 1 µM and LOD equal to 0.25 µM (10% of inhibition). Under the optimal experimental conditions, the inhibition mechanism was determined from Lineweaver and Burk plots and was found to be noncompetitive for SAD.

In the second part, an electrochemical carbonic anhydrase based biosensor for inhibitive determination of sulfanilamide was elaborated for the first time. The calibration curve showed a linear range from 0.5 to 5 µM SAD concentration, and estimated LOD equal to 0.4 µM. The activity of CA-CB biosensor over 18 days remains at 76 % of initial activity. The CA-CB biosensor developed is an easy and sensitive technique for in situ and routine analysis for detection of SAD which is considered as drug but also as emergent environmental pollutant.

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Figure Captions:

Fig. 1. Principle of the reaction of CA and 4-nitrophenyl acetate.

Fig. 2. Non enzymatic degradation of 4-nitrophenyl acetate (750 µM) at different pH conditions, a: pH 4.5; b: pH 5.5; c: pH 6.5; d: pH 7.8 and d: pH 8.5.

Fig. 3. Enzymatic activity monitoring using various concentrations of CA and 4-NPA (a: CA 8,05 U/ml vs 37,5 µM NPA) (b: CA 16,1 U/ml vs 37,5 µM NPA), (c: CA 32,2 U/ml vs 37,5 µM NPA) and (d: CA 16,1 U/ml vs 75 µM NPA) at pH equal to 8.5.

Fig. 4. PH changes effect on hydrolysis of 4-NPA by carbonic anhydrase, (e: pH 8.5), (d: pH 7.5), (c: pH 6.5), (b: pH 5.5) and (a: pH 4.5).

Fig. 5.Typical response of enzyme inhibition, carbonic anhydrase activity at pH 8.5 without (b) and with 25 µM of SAD (a). The inset is the chemical structure of SAD.

Fig. 6. Calibration curve of CA inhibition by SAD in Tris-HCl buffer 50 mM, pH 8.5. For each concentration of SAD the experiment was repeated three times.

Fig. 7. Lineweaver-Burk plots of CA catalysis hydrolysis of different concentration of 4-NPA in absence (a) and in presence of different concentration of SAD (b) 0. 25, (c) 10 and (d) 50 µM at Tris-HCl 50 mM, pH 8.5.

Fig. 8. Effect of different chemical compounds on carbonic anhydrase activity. (Black line NPA + CA), (Brown line CA+Benzoic acid+NPA) , (Blue line $HgCl_2$), (green line CA+NiCL+NPA) , (Purple line CA+Na_2S+NPA) and (Red line CA+KCN+NPA).

Fig. 9. The inhibition profile of SAD using Linear Sweep Votammetry (LSV). d: baseline, a: CA+ 3mM of NPA, b: CA+ 25µM SAD+ 3mM of NPA and c: 3 mM of NPA at Tris HCl 50 mM + KCl 50mM, pH 8,5 and 25 mV/s.

Fig. 10. Effect of SAD on CA-CB based electrochemical biosensor response. For each concentration of SAD the experiment was repeated three times.

Fig. 11. Storage stability of CA-CB biosensor at 4°C.

Table 1: SAD, SMX and SDZ concentration corresponding to 10% and 50% of CA inhibition

SA's μM	**10 % of inhibition**	**50 % of inhibition**
SAD	0.25	1
SMX	1	100
SDZ	20	120

Figures

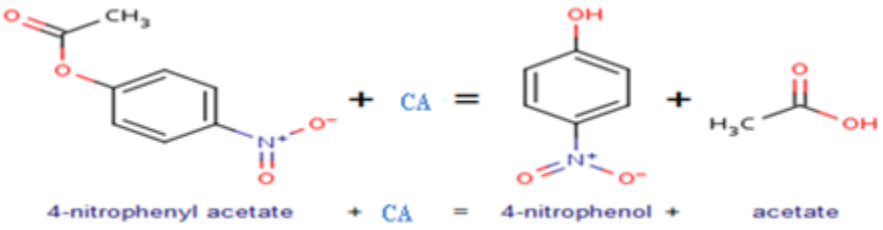


Fig. 1

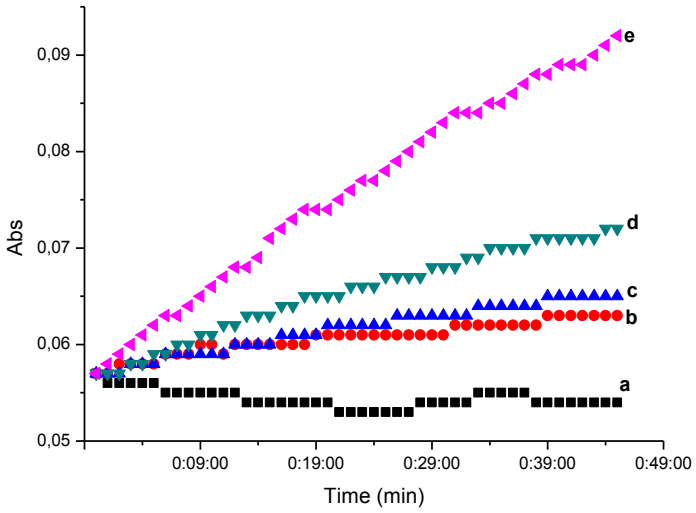
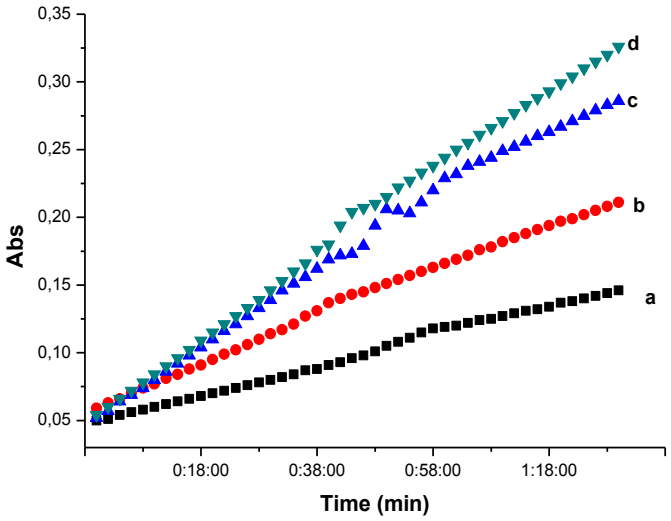


Fig.2



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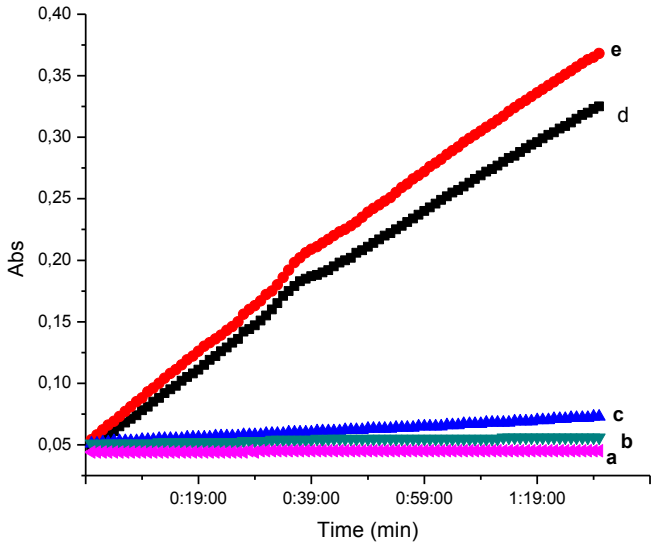

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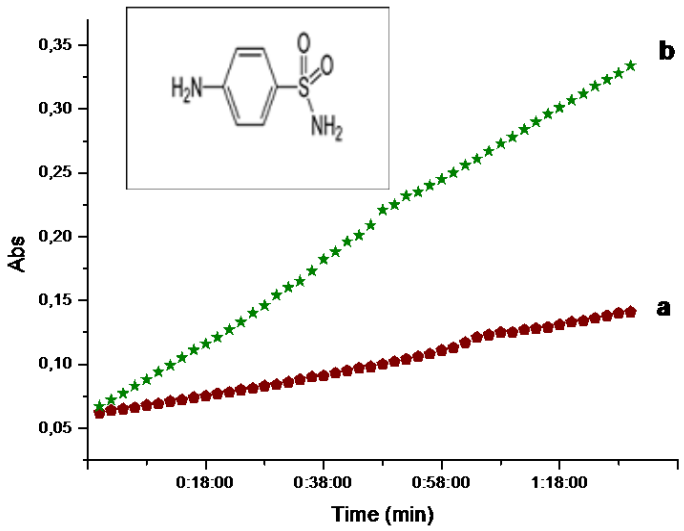

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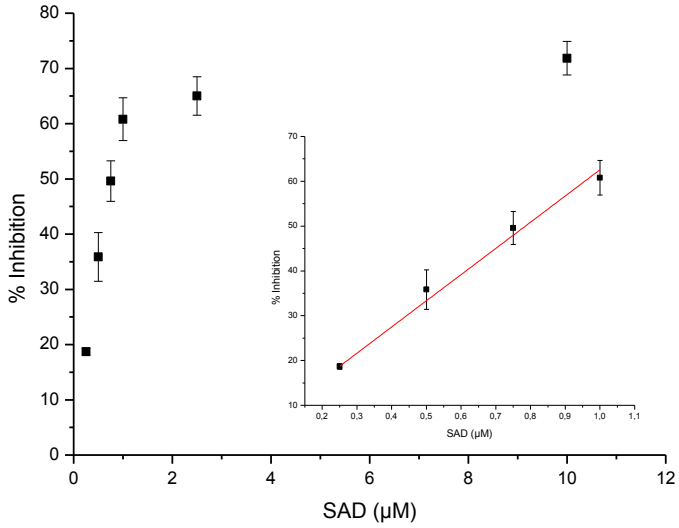

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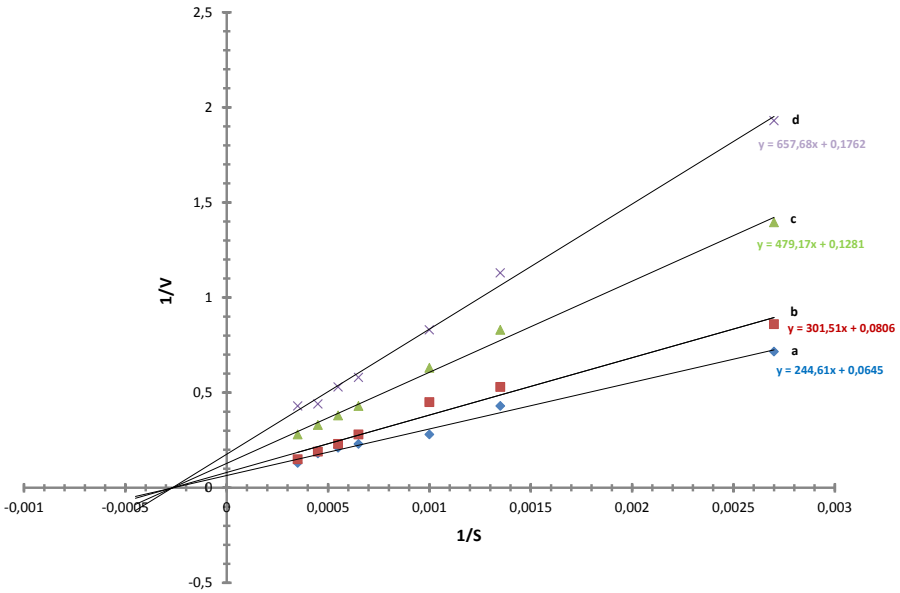

Fig.7

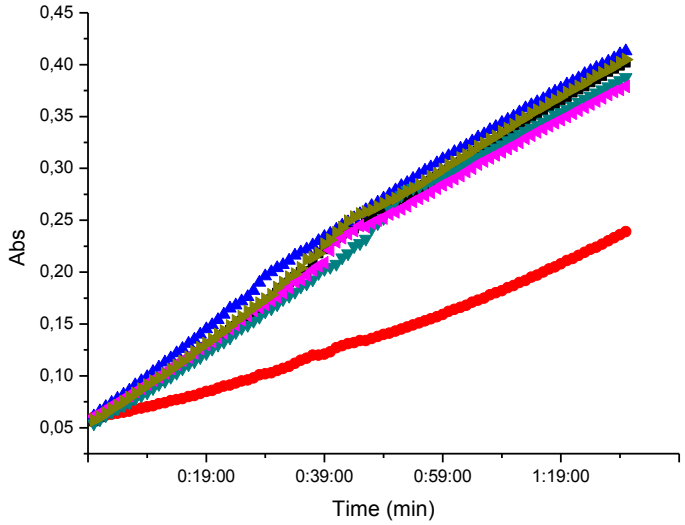

Fig.8

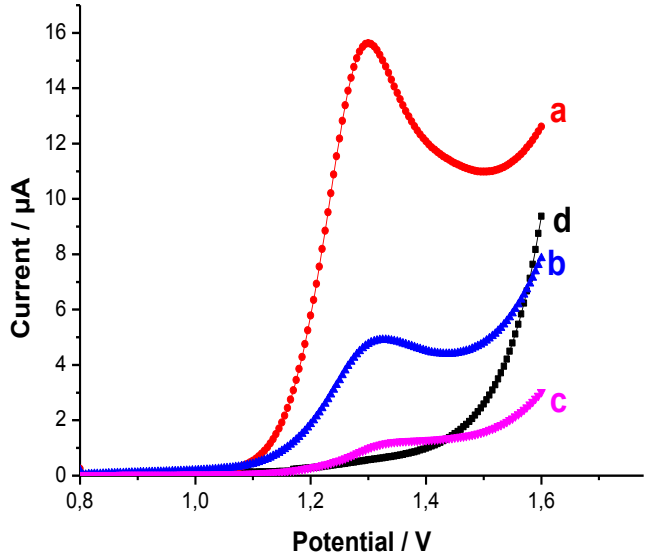

Fig.9

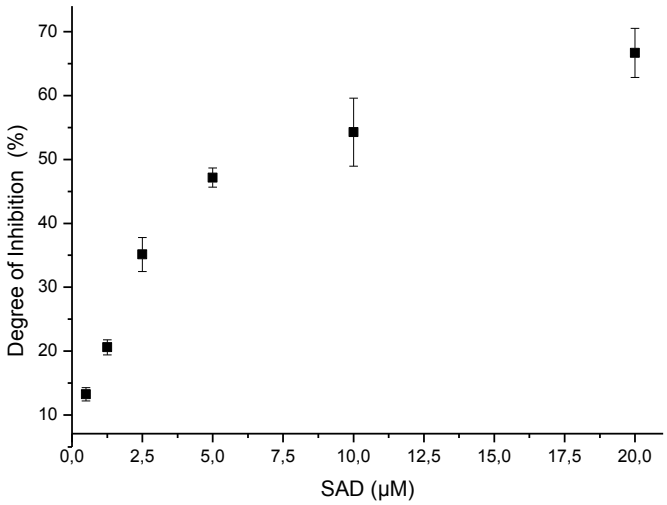

Fig.10

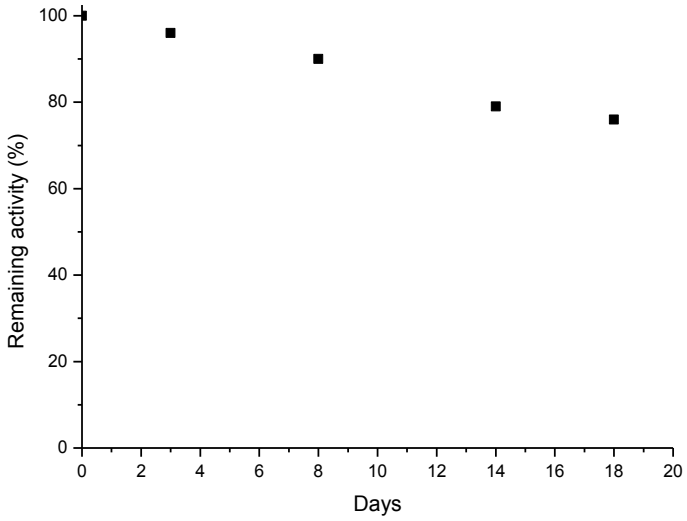

Fig.11