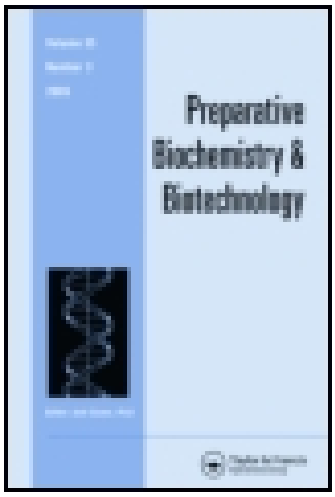

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A New Multi Enzyme Type Biosensor for Triglyceride Determination

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Abstract

An amperometric multi-enzyme biosensor for determination of triglycerides (TGs) was constructed by mounting gelatine membrane bound three enzymes on a glassy carbon electrode (working electrode), then connecting it to electrometer along with Ag/AgCl reference electrode and Pt auxiliary electrode. Characterization and optimization of the multi-enzyme biosensor which is prepared with glycerol kinase (GK) (E.C.2.7.1.30), glycerol-3-phosphate oxidase (GPO) (E.C.1.1.3.21) and lipase (E.C.3.1.1.3) were studied. In the optimization studies for the bioactive layer components of the prepared biosensor, the optimum amounts of gelatin, BSA and glutaraldehyde was calculated as 1 mg/cm^2, 1 mg/cm^2 and % 2.5 respectively. Optimum pH and temperature of the reaction of biosensor was determined as 7.0 and 40 ^{o}C, respectively. Linear range of triolein for the biosensor was found from the calibration curve between several substrate concentration and Δ Current. After optimization and characterization of the biosensor, its operationability in triglycerides was also tested.

KEYWORDS: Multi-enzyme biosensor, TG, amperometry, glycerol kinase, glycerol-3-phosphate oxidase, lipase

1. INTRODUCTİON

Triglycerides (TG) are transesterification products of fatty acids and glycerol which plays a very important role in the energy metabolism and the transportation of fats in human body. Normal levels of TG in human blood and serum is between the range of 150–190 mg/dl. Increased levels of TG in blood is a major indicator of nephritic syndrome, chronichepatitis, pancreatitis, liver disease, hypothyroidism, hyperlipidemia, diabetes mellitus and alcoholism [1].

The most common methods for glycerol determination, such as liquid chromatography and spectrophotometric analysis based on chemical and enzymatic reactions, in particular, oxidase–peroxidase method [2-6], are disadvantageous since it requires highly qualified operator and expensive devices, besides, complex sample pretreatment is necessary.

Biosensors are comparatively more simple, sensitive, easy to operate, cheap, rapid and also they don't require any experienced personnel to operate [7]. Some scholars have reported biosensors for determination of TG [8-11]. Biosensors which are based on dissolved oxygen meters require expensive chemicals like peroxidase and NADH and also not rapid and very sensitive [12]. Thermal biosensors are also not sensitive as they measure the heat occurs during the breakdown of triglycerides by lipase. Porous silicon

based potentiometric biosensors are also not a sensitive method for the determination of TG as they measure the slight changes in pH due to hydrolysis of TGs to fatty acids. [9,13] Amperometric biosensors are mostly preferred as they do not require any expensive materials and chemicals and also not time consuming [14].

Some amperometric enzyme biosensors prepared with test kits for determination of TG were reported. [15-17]. But these test kits for enzymatic analysis of glycerol provided by known firms like Mannheim and Sigma are very expensive. Also they are not suitable for everyday use. Development of a novel multi enzyme based biosensors for TG determination can be a way to solve these problems and meet the requirements mentioned. Their basic advantages are high specificity, facility of data processing, fast results and low analysis prime cost.

In this study, we proposed a new amperometric multi-enzyme based biosensor constructed with three enzymes which were individually co-immobilized onto the surface of a glassy carbon electrode with the help of cheap and easy to find chemicals like gelatine and glutaraldehyde.

2. MATERİALS AND METHODS

All reagents were of analytical grade unless stated otherwise and were purchased from Sigma-Aldrich (St.Louis, MO, USA). Glycerol kinase (*Cellulomonas sp*.) (E.C.2.7.1.30), Glycerol-3-phosphate oxidase (*Aerococcus viridans*) (E.C.1.1.3.21), *C. Rugosa* Lipase (E.C.3.1.1.3) were purchased from Sigma-Aldrich (St.Louis, MO, USA). Enzyme

portions were prepared at certain concentrations and were stored at -20 ^{0}C until use. Artificial serum solution was prepared with 4.5 mM KCl, 5 mM $CaCl_2$, 1.6 mM MgCl, 4.7 mM D(+) glucose, 2.5 mM urea, 0.1% human serum albumin, and 145 mM NaCl.

Palmsense Potentiostat/Galvanostat (Netherlands), a triple-electrodes system contains glassy carbon working electrode, Ag/AgCl reference electrode and platinum wire counter electrode (Basi, W. Lefayette,USA) were used for amperometric detection for TG. To prepare buffer solution, magnetic stirrer (RCA, UK) and pH meter with an electrode (Schott handlylab, Spain) were used. All measurements were carried out at a constant temperature using a circulating thermostat (Nüve BM 302, UK). In all electrochemical experiments a faraday cage (from iBAS, Warmickshire, UK) was used to block out external static electric fields.

2.1. Preparation Of Substrate Solution

Triolein solution was used as a substrate for lipase, glycerol kinase and glycerol-3-phosphate oxidase. Triolein solution was prepared as described [18]. Substrate solutions with varying concentrations were prepared weekly with triolein in 0.1 M sodium phosphate buffer (pH 7.0) and stored at 4 ^{o}C until used.

2.2. Preparation Of Enzyme Mixture

The enzyme mixture containing lipase, GK and GPO were prepared by mixing these enzymes individually. All enzymes were portioned in 0.1 M sodium phosphate buffer (pH 7.0). Each of these portions was 10µL. ATP and $MgCl_2$ portions were also prepared

the same way and all of these portions were mixed together to obtain enzyme mixture which contains lipase, GK and GPO along with ATP and $MgCl_2$ having an enzyme ratio of 10:5:2 respectively.

2.3. Preparation Of The Gelatin Membrane

Gelatin (45 mg) and BSA (30 mg) was dissolved in 300 µL 0.1 M sodium phosphate buffer (pH 7.0) which contains 31.5 µM $MgCl_2$ and kept in 40 ^{o}C for 15 minutes for homogenisation. It is important to keep the prepared solution over the room temperature before the construction of the biosensor and co-immobilization of the enzymes.

2.4. Construction Of The Biosensor And Measurements

Glassy carbon electrode was used as a working electrode. Prior to immobilization of enzymes working electrode (glassy carbon electrode) surface was polished on microfiber cloth until a shining surface was obtained. Then it was thoroughly rinsed with distilled water by sonication.

10 µL gelatin membrane was mixed with 40 µL of enzyme mixture that contains lipase, GK and GPO then spreaded evenly onto the top layer of the glassy carbon electrode and kept in +4 ^{o}C for 30 minutes. Afterwards it was dipped into % 2.5 glutaraldehyde solution for crosslinking for 5 minutes and then washed with distilled water. The constructed biosensor was taken into the reaction cell that contains 0.1 M sodium phosphate buffer (pH 7.0) along with Ag/AgCl reference electrode, Pt auxillary electrode and connected through potentiostat.

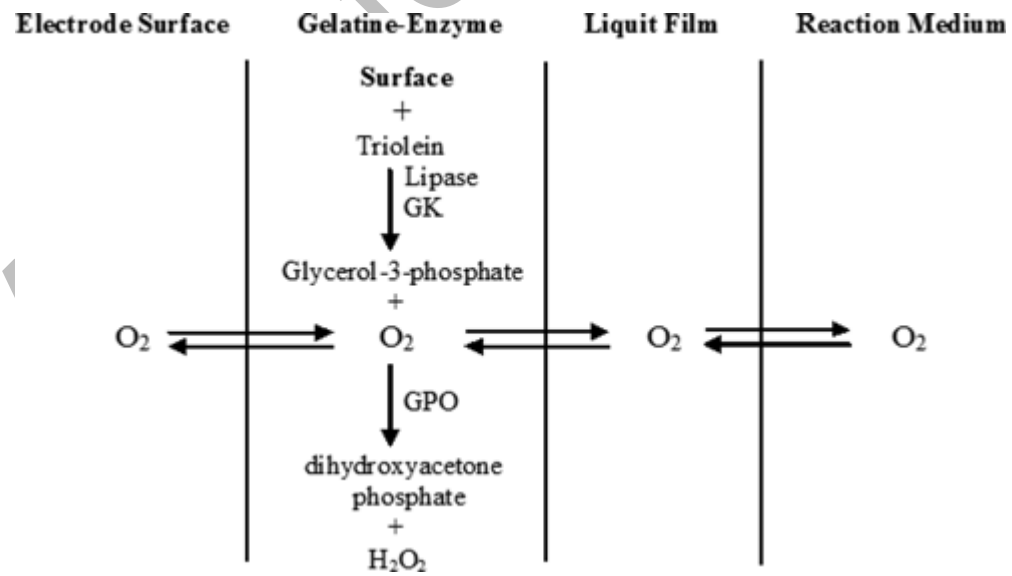
All the measurements were carried out at 40 ^{o}C in the reaction cell with varying concentration of triolein. All electrodes were polarized at -0.7 V potential and current was measured amperometrically. The principal of this biosensor is as follows. As soon as dissolved triolein was added into the reaction media, it was converted to dihydroxyacetone phosphate and H_2O_2 by the co-immobilized lipase, glycerol kinase and glycerol-3-phosphate oxidase enzymes. The O_2 need during the formation of dihydroxyacetone phosphate was taken from the reaction media thus it was determined according to the applied potential.

The reactions involved in this biosensor are:

$$\text{Triglyceride} \xrightarrow{\text{lipase}} \text{glycerol} + \text{fatty acids}$$

$$\text{glycerol+ATP} \xrightarrow[\text{MgCl2}]{\text{GK}} \text{glycerol}-3-\text{phosphate} + \text{ADP}$$

$$\text{glycerol}-3-\text{phosphate} + \text{O2} \xrightarrow{\text{GPO}} \text{dihydroxyacetone phosphate} + H_2O_2$$



3. RESULTS AND DİSCUSSİON

3.1. Co-İmmobilization Of Lipase, Glycerol Kinase, Glycerol-3-Phosphate Oxidase İnside Gelatin Membrane

Lipase, GK and GPO enzyme mixture have been co-immobilized into gelatin membrane through adsorption. Glutaraldehyde solution was used as a cross-linking agent between lipase, glycerol kinase and glycerol-3-phosphate oxidase. Bovine serum albumin was also used to reinforce the structure of enzymes leading to increased size in order to prevent leakage of enzymes from the pores of the gelatin membrane. One –CHO group of glutaraldehyde was linked to $–NH_2$ groups of enzyme while another –CHO group was bound to $–NH_2$ groups of BSA.

3.2. Effect Of The Gelatin Amount On Biosensor Response

Measurements were carried out with the constant amount of enzymes and glutaraldehyde concentration, in order to investigate the effect of differing gelatin amounts on the GCE surface. Results obtained were given in Fig.1

As it is seen in Fig.1, According to the biosensor response, linearity and R^2 values, the gelatin amount of 45 mg (0.98 mg/cm^2 on electrode surface) was chosen as optimum. In low gelatin amounts (30 mg), substrate may easily leak out from the bioactive layer because of the low concentration of gelatin. Thus, deviation from linearity was seen and a lower R^2 value was obtained for low gelatin amounts. In high gelatin amounts (60 mg), the concentration of the bioactive layer also increases and makes it harder for substrate to

diffuse through the membrane. Thus, a lower biosensor response and R^2 value was obtained.

3.3. Effect Of Glutaraldehyde Concentration On Biosensor Response

For the determination of the effect of glutaraldehyde concentration on the biosensor response, enzyme amounts and gelatin amount were kept constant and varying concentrations of glutaraldehyde solutions were used in the experiments. As it is seen in Fig.2, optimum glutaraldehyde concentration was 2.5%. Lower biosensor response was obtained when using low glutaraldehyde concentration which made the substrate leak out easier from the gelatin membrane because of fewer crosslinks with gelatin. When using higher concentrations of glutaraldehyde (%3.5), more crosslink numbers prevent the substrate from diffusing into the membrane.

3.4. Effect Of Ph On Biosensor Response

The effect of pH of the reaction buffer on the response of prepared biosensor was shown in Fig.3. All experiments has been carried out at 40 ^{o}C in 10 mL final reaction volume with phosphate buffer pH 7.0. Citric acid buffer was used for pH 4.0, phosphate buffer was used for the pH ranging between 5.0 – 8.0 and glycine buffer was used for pH 9.0. The biosensor response was increased as the pH increased up to 7.0, then it was decreased. During the reaction time, applied current changes according to the pH of the media using the same substrate concentration. These increase and decreases were calculated and put on a graphic (current vs pH). The maximum current difference has been accepted as maximum activity and calculations were made according to this. The

optimum pH of this biosensor was similar to the amperometric biosensor based on PVA membrane (pH 7.0) [1] and potentiometer sensor (pH 7.0) [13] and lower than other biosensors based on oxygen meter based sensor (pH 8.0 and 7.5) [8,12], and amperometric sensors (pH 8.0) [7,11].

3.5. Optimum Temperature

The effect of temperature on the response of biosensor was evaluated at varying incubation temperatures from 20 to 50 ^{o}C. All experiments has been carried out in 10 mL final reaction volume and 0.1 M phosphate buffer pH 7.0. The response of the biosensor was increased as temperature was increased up to 40 ^{o}C, thereafter it decreased as it is shown in Fig.4. It is known that the potimum temparatures of enzymes may vary than their free forms after their immobilization. Optimum temparatures of free lipase, GK and GPO enzymes are 37 ^{o}C, 50 ^{o}C and 37 ^{o}C, respectively. It is clear that there is no common optimum temparature for these ezymes. In thıs study biosensor was prepared by co-immobilizing three enzymes together and temperature (40 ^{o}C) that the maximum biosensor response was obtained has been accepted as optimum temperature for all following experiments. This optimum temperature is higher than other biosensors based on oxygen meter based sensor (33 and 39.5 ^{o}C) [8,12], and amperometric sensor (37 ^{o}C) [7]. It was seen that this new immobilization technique had also increased the thermal stability of the co-immobilized enzymes.

3.6. The Effect Of The Buffer Concentration On Biosensor Response

Ionic strength of the media may differ according to the changes in buffer concentrations. These changes also effect the biosensor response. Therefore, effect of different buffer concentrations (33, 66, 100, 133 and 166 mM) on biosensor responses were studied and maximum response was obtained with 100 mM phosphate buffer as it seen in Fig.5. All tests has been carried out at 40 ^{o}C in 10 mL final reaction volume with phosphate buffer pH 7.0.

3.7. Effect Of Substrate Concentration (Linearity)

The effect of substrate concentration on biosensor response was studied with different triolein concentrations ranging from 0.14 mM to 18 mM. All experiments has been carried out at 40 ^{o}C in 10 mL final reaction volume in 0.1 M phosphate buffer pH 7.0. Fig. 6 shows the calibration curve of the prepared biosensor for triolein. Linear graph, defined by the equation y = 0,054x + 0,154 (R^2 = 0,9940) was obtained. Linearity was found in the range of 0.14-1.125 mM. There is a deviation from linearity as it is seen from the standard curve at higher concentrations. The deviation was due to the limited amount of enzymes in the bioactive material or insufficient dissolved oxygen which is also a co-substrate of the enzymes.

There was a linear relationship between applied current and triolein concentration ranging from 0.14 mM to 1.125 mM in reaction mixture, which is comparable to other biosensor studies based on PVC membrane (0.5-2.1 mM) [19], enzyme based sensor (0.3-2.5 mM) [20], porous silicon based biosensor (0.2-2.1 mM) [13], CA membrane bound

enzyme sensor (0.2-3.5 mM) [13], but lower than dissolved oxygen metric biosensor (5-20 mM) [8].

The minimum detection limit of the biosensor was 0.14 mM, which is lower than oxygen meter based sensor (0.35 mM) [8], potenciometric sensor (0.42 mM) [13], amperometric biosensor (0.32 mM) [7]

3.8. Reproducibility

The reproducibility of the biosensor was tested by 6 average standard solutions that contain same amount of triolein (0.40 mM). The standard deviation (SD), variation coefficient (cv) and average value (X) were calculated as 0.051×10^{-3} mM, 2.21 % (n=6) and 0.42 mM respectively.

3.9. Stabilities Of The Biosensor

First, the thermal stability of the biosensor was studied. Prepared biosensor was incubated at 40^{o}C for 1, 2 and 3 hours. The biosensor showed 91%, 85% and 72% activity after these incubations, respectively. It may be possible to say that our immobilization material (gelatin) was effected from high temperatures and showed less activity.

pH stability of the biosensor was also studied. Prepared biosensor was incubated in 100 mM phosphate buffer pH 6, pH 7 and pH 8 for 3 hours. After incubation the biosensor showed 60%, 72%, 63% activity, respectively.

In operational stability studies of the biosensor, 12 consecutive measurements were made in optimized conditions. It was determined that biosensor showed 98% activity after 6, and 82% activity after 12 measurements.

For storage stability studies, the long term performance of the biosensor was evaluated intermittently over a period of 30 days by monitoring its response against triolein. The biosensor was stored in a dark environment with wet cotton 4 ^{o}C. Although this condition provide a moisturizing media for the biosensor, biosensor's bioactive layer may get dry after a few weeks. In this conditions after 4 days storage period, the biosensor didn't lose any activity at the end of the 5th, 10th, 15th, 20th and 30th days, the final activity of the biosensor were 98.1, 83.3, 74.4, 68.9 and 61.1%, respectively.

3.10. Serum Aplications

The biosensor was also applied to the artificial serum samples for TG detection. Average results from 5 different measurements were given in Table 1. The results in the table shows that TG levels of the artificial serum samples analyzed by the fabricated biosensor matches the results of spiked artificial serum samples.

4. CONCLUSİONS

An amperometric sensor was constucted by immobilizing lipase, GK, GPO enzymes in gelatin membrane and mounting it onto the surface of a glassy carbon working electrode. The current measured was directly proportional to the triolein concentration.

There are many TG biosensors fabricated similar to the one in this study. But this new immobilization method also made it possible to co-immobilize the enzymes individually without using an enzyme kit. Characterization and optimization parameters were also studied. The developed TG biosensor is characterized by linear range of 0.14-1.125 mM and have a wider linear range and lower detection limit according to the literature and showed %78.4 activity on two weeks after immobilization into the gelatin membrane. Also the comparison between the present biosensor and various TG biosensors were given in table 2.

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Table 1. TG detections in artificial serum samples

Added TG (mM)	Found by biosensor(mM)	Recovery (%)	Relative differences (%)
0.2	0.2050	102.5	2.5
0.3	0.3012	100.4	0.40
0.4	0.4051	101.2	1.20
0.5	0.4962	99.2	0.80
0.6	0.5911	98.5	1.50

Table 2. A comparison of the present biosensor with various TG biosensors

Technique	Support of Immobilization	Type of Electrode	Optimum pH	Potential for Max. Current	Detection Limit	Lineer Range	Storage Stability	Ref
Potentiometric	Porous Silicon	NR	NR	NR	522 mg/dl	522-1860 mg/dl	6 months	[1]
Potentiometric	Silicon nitride layer of the EISCAP	EISCAP	NR	NR	500 uM	1-7 mM	NR	[23]
Electrochemical	Polyaniline/single walled carbon nanotubes	ITO	7.4	NR	50 mg/dl	50-400 mg/dl	13 weeks	[21]
Electrochemical	Gold polypyrole	Au	6.5	0.1	20 mg/dl	50-700 mg/dl	Half-life of over 7 months	[22]
Electrochemical	Zinc oxide nanoparticles	Pt	7.5	0.4	20 mg/dl	50-650 mg/dl	Half-life of 7 months	[15]

Amperometric	Egg Shell Membrane	Pt	7.0	0.4	0.28 mM	0.56 to 2.25 mM	50% loss in 70 days	[17]
Amperometric	PVA	Pt	7.0	0.4	19 mg/dl	50-200 mg/dl	50% loss in 70 days	[19]
Amperometric	Gelatin membrane	GCE	7.0	0.7	12 mg/dl (0.14 mM)	12-100 mg/dl (0.14 - 1.125 mM)	40% loss in 30 days	present

NR – not reported

Figure 1. The effect of gelatine amount on the biosensor response (T= 40 ^{o}C, 0.1 M phosphate buffer pH 7.0)

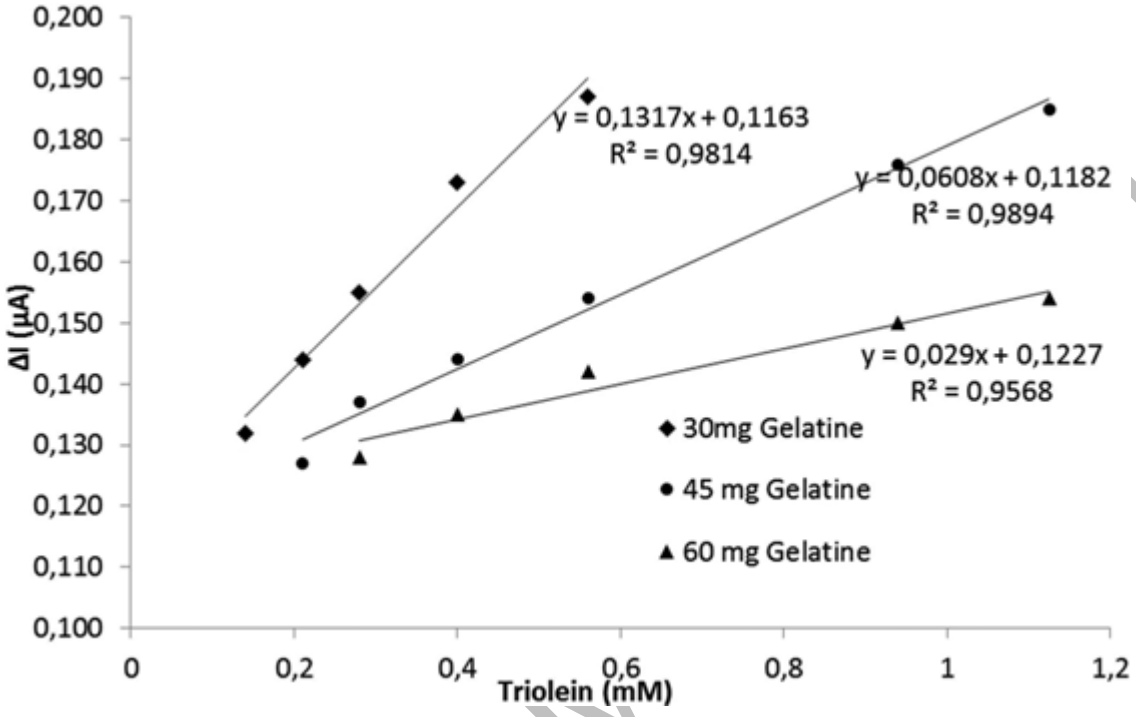

Figure 2. The effect of glutheraldehide percentage on the biosensor response (T= 40 ^{o}C, 0.1 M phosphate buffer pH 7.0)

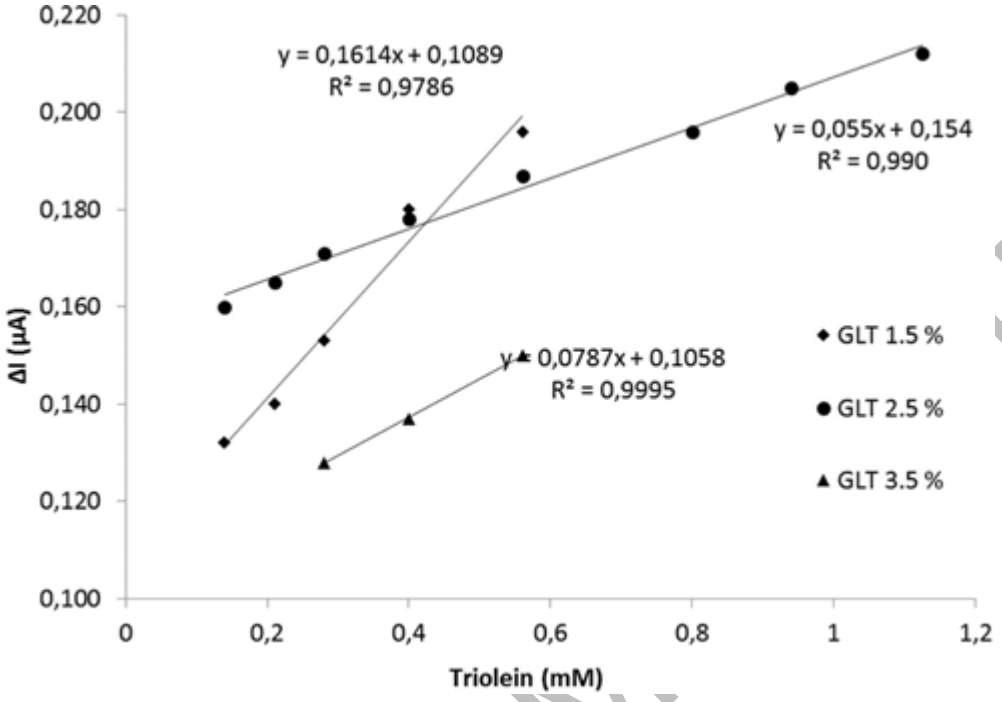

Figure 3. pH effect on the biosensor response (T= 40 ^{o}C)

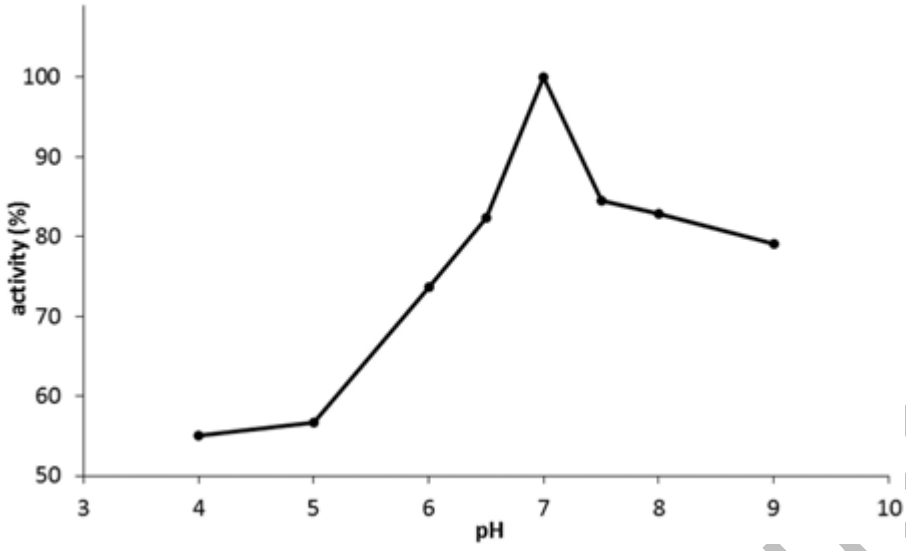

Figure 4. Temparature effect on the biosensor response (0.1 M phosphate buffer pH 7.0)

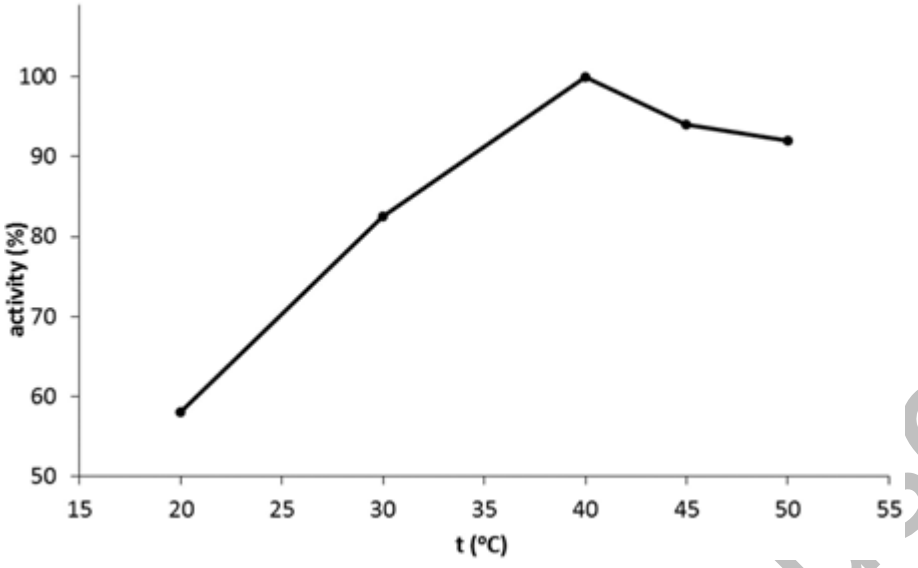

Figure 5. The effect of buffer concentration on the biosensor response (0.1 M phosphate buffer pH 7.0, T= 40 ^{o}C)

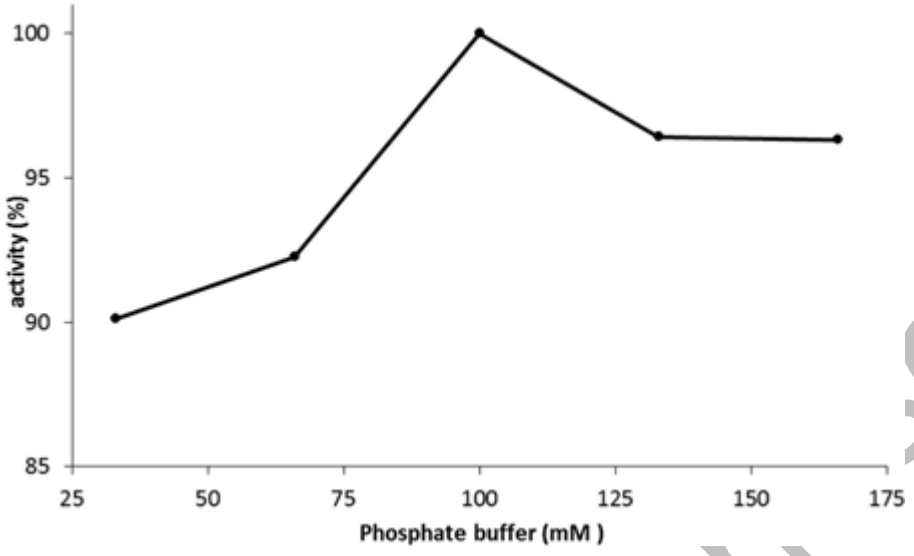

Figure 6. Calibration curve of the multi enzyme biosensor for triolein (0.1 M phosphate buffer pH 7.0, T= 40 ^{o}C)