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Short communication

Development of cholesterol biosensor based on immobilized cholesterol esterase and cholesterol oxidase on oxygen electrode for the determination of total cholesterol in food samples

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Abstract

The development of a cholesterol biosensor by co-immobilization of cholesterol esterase (ChEt) and cholesterol oxidase (ChOX) on oxygen electrode is described. The electrode consists of gold cathode and Ag/AgCl anode. The enzymes were immobilized by cross-linking with glutaraldehyde and Bovine Serum Albumin (BSA). The immobilized enzymatic membrane was attached to the tip of the electrode by a push cap system. The optimum pH and temperature of the sensor was determined, these are 6 and 25 °C respectively. The developed sensor was calibrated from 1–75 mg/dl of cholesterol palmiate and found linear in the range of 2–50 mg/dL. The calibration curve was drawn with V_i (ppm/min)(initial velocity) vs different concentrations of cholesterol palmiate (mg/dL). The application of the sensor to determine the total cholesterol in different real food samples such as egg, meat was investigated. The immobilized enzymatic layer can be reused over 30 times and the stability of the enzymatic layer was studied up to 9 weeks.

Keywords: ChEt; Cholesterol biosensor; ChOX; Food sample analysis; Stability

1. Introduction

Cardiovascular diseases in people are increasing day by day and cardiac arrest is a major cause of death world over. There are several causes for this but one of the most important reasons is hypercholesterolemia i.e. the increased concentration of cholesterol in the blood [1,2]. Cholesterol belongs to the sterol group of fats. It is present in egg yolk, dairy products, goat meat etc. Therefore, cholesterol is one of the most frequently determined analytes in clinical as well as in analysis of food samples. The development of efficient rapid analytical methods for their estimation in food and clinical samples is important. HPLC [3], gas–liquid chromatography [4,5] methods used for the determination of total cholesterol offer sensitivity and selectivity but are neither suitable for rapid nor cost effective detection. Enzymatic procedures have practically replaced the chemical methods based on the classical Libermann–Burchard reaction, used traditionally for free and total cholesterol determination [6]. Owing to the advantages of simplicity, rapidness and cost effectivity, a few cholesterol biosensors have been developed which are based on cholesterol oxidase (ChOX) and cholesterol esterase (ChEt) [7,8], cholesterol oxidase [9–14], Cytochrome P450scc [15], fiber-optic biosensor [16,6], acoustic wave [17].

Most of the above reported cholesterol biosensors were applied for clinical analysis. Application of this sensor in the field of food sample analysis is very limited. In this paper our aim is to develop a fast, economic, simple cholesterol biosensor based on immobilized ChEt and ChOX on a polycarbonate membrane attached to the tip of the oxygen electrode and use it for determination of cholesterol in food. For quality control, determination of cholesterol in food is important as food high in cholesterol may increase its level in the blood [18].

The current enzymatic methods are based on ChEt and ChOX, which catalyze the following reactions [19,20].

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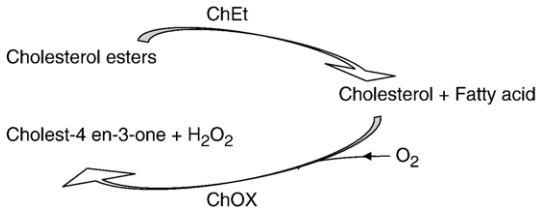

2. Materials and methods

2.1. Materials

Cholesterol esterase (EC 3.1.1.1.3,3.6 U mg^{-1}), cholesterol palmiate, nonaethylene glycol monododecyl ether used as a surfactant were purchased from Sigma, USA. Cholesterol oxidase (EC 1.1.3.6, 15 Umg^{-1}) was procured from SRL, India. Mono and di-sodium phosphate, glutaraldehyde, Bovine Serum Albumin (BSA), sodium chloride were obtained from Merck, Germany. Polycarbonate membrane (pore size 0.4 μm) was purchased from Sartorius, Germany. All the food and meat samples were purchased from local market of Jadavpur, Kolkata, India.

2.2. Apparatus

Oxygen consumption during the reactions was measured polarographically at −0.7 V using an oxygen electrode. The probe consists of a silver anode and a gold cathode (with a sensitive end of 0.5 mm diameter). This probe is connected to the DO meter (Orion, 850 plus). When a polarizing voltage (−0.7 V) is imposed across the cathode/anode, by the principles of electrode chemistry, it reduces oxygen at the cathode, causing a measurable current to flow. This current is in proportional to the difference in partial pressure of oxygen (pO_2) across the membrane, which is in turn proportional to the absolute pressure out side the membrane, assuming pO_2 inside the membrane is zero. The higher the oxygen concentration in the reaction medium, and the higher the external pO_2 the more is the current flow. The DO meter used this signal to calculate the dissolved oxygen concentration in the reaction medium in parts per million (ppm). For constant stirring of the sample a magnetic stirrer (REMI India, model 1 MLH) was used.

2.3. Preparation of cholesterol palmiate solution

Cholesterol palmiate is insoluble in water. So, it is important to make the homogeneous solution using either propanol/triton-X or nonaethylene glycol monododecyl ether. Standard solution of cholesterol palmiate was prepared by dissolving 400 mg of cholesterol palmiate followed by addition of nonaethylene glycol monododecyl ether by stirring till the solution was clear and colorless at 65 °C. Then hot phosphate buffer saline (PBS) was added and volume made up to 100 ml. The solution was allowed to reach its normal temperature before being used and was stored at 4 °C.

2.4. Immobilization of enzymes

A 10 μl (10×4) aliquot from a 40 μl mixture prepared by mixing 25 μl of BSA (10%), 10 μl of a bifunctional cross-linker, glurataldehyde (2.5%) and 5 μl of PBS containing different amount of cholesterol oxidase (2.5 μl) and 150 U ml^{-1} of cholesterol esterase (2.5 μl) was spread over the polycarbonate membrane and dried at room temperature (25 °C) for 1 h [21]. The membrane was washed thoroughly with PBS (pH 7) to remove any excess glutaraldehyde and stored in PBS at 4 °C when not used.

2.5. Development of the sensor

The immobilized enzyme membrane was mounted on the tip of the oxygen electrode with a push cap system. A rubber washer was used between the membrane layer and push cap to make it leak proof. The enzyme electrode was connected to the DO meter with a socket. The buffer saturated with air had an oxygen concentration 7–8 ppm at 25 °C. Zero oxygen concentration was obtained by an oxygen scavenging solution of sodium sulfite.

2.6. Assay procedure

The enzyme electrode was polarized for about 30–40 min every time before use. The DO meter was calibrated with the help of the manufacturer's instruction manual. Assay was started by adding different concentrations of cholesterol plamiate. In order to restore 100% oxygen saturation, the enzymatic membrane was washed several times with PBS before the following assay. Oxygen diffusion from air decreases the efficiency of the assay when carried out with enzyme in the solution [22,23]. To overcome this problem, an air sealed reaction chamber of glass (capacity 12 ml) was used. But with enzyme immobilized on an electrode, environmental oxygen does not interfere as the sensor measures oxygen at the level of the film [23]. For sample measurement, the background O_2 concentration was subtracted every time. All the experimental data given are average of 5 times repeats.

Buffer and all other solutions were prepared with double distilled water, immediately before use.

2.7. Sample preparation

Sample extraction was performed by using a modification of the method of Folch et al. [24] and Ishikawa et al. [25]. Fresh goat meat and chicken meat were gutted and the muscle tissue was cut into small pieces. About 5 g of each sample was homogenized in a mixer–grinder (REMI, India) with 50 ml of chloroform–methanol mixture. The homogenized tissue was then filtered. For sensor measurement the mixture solution was evaporated at 80 °C in water bath. After that the sample residue was dissolved in nonaethylene glycol monododecyl ether following the same procedure as the standard cholesterol palmiate preparation.

2.8. Cholesterol estimation by spectrophotometric method

By the colorimetric enzymatic kit (CHOD-PAP) method (Human ®, Germany) the desired colour of cholesterol in the

samples was reached and then analyzed in a spectrophotometer (Hitachi U-2000) at 500 nm.

3. Results and discussion

3.1. Optimization of pH in sensor responses

The effect of the pH on the response of the cholesterol sensor, containing immobilized ChEt (150 U ml^{-1}) and ChOX (60 U ml^{-1}) was examined over the range of 4–9 at a fixed concentration of cholesterol palmiate (33.33 mg/dl).

The effect of pH on the activity of DO/ChEt/ChOX probe is shown in Fig. 1. It can be seen in figure that the peak signals were obtained at pH 6.0, whereas the mixture of ChEt and ChOX in free solution showed an optimum pH of 6.5 at 30 °C [26]. This shift of optimum pH to the acidic region may be due to change in microenvironment for immobilization. Other reported values show that the optimum pH of these co-immobilized enzymes as 7.5 [26] and 7.0 [8]. This anomaly may be due to the difference in sensor fabrication and process for enzyme immobilization. Further experiments were carried out in buffer of pH 6.0.

3.2. Optimization of temperature on sensor responses

Temperature exerts a marked influence on the enzymatic activity as well as the performance of the oxygen electrode because all the enzymes have a narrow temperature range for their functioning. The effect of temperature was studied by exposing the cholesterol palmiate solution at a fixed concentration 33.33 mg/dl over the temperature range of 10–50 °C (Fig. 2).

O_2 consumption (Vi ppm/min, initial velocity) by the cholesterol sensor increased with increase in temperature till it reached 20 °C and further increased slightly at 25 °C (Vi=0.6405 ppm/min). Beyond 25 °C, the consumption of O_2 gradually decreased. A bell-shaped temperature profile is obtained in the figure with a distinct optimum temperature at 25 °C. This value is lower than that of the earlier reported values [26,8]. This difference may be due to the difference in electrode preparation and immobilization of the enzymes. Further experiments were carried out at 25 °C.

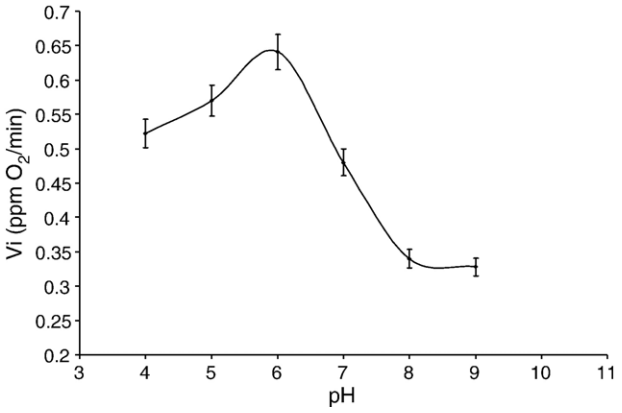

Fig. 1. Optimization of pH performed at 25±2 °C by making use of 0.2 M citrate–phosphate buffer (pH 4, 5, 6), sodium phosphate buffer (pH 7 and 8), glycine–NaOH buffer (pH 9), containing 33.33 mg/dl of cholesterol palmiate.

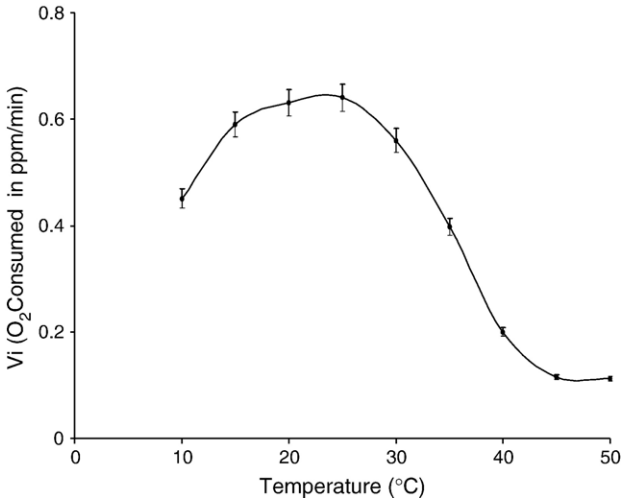

Fig. 2. Temperature profile determined at pH 6.0 (at varying temperatures of 10±2 to 50±2 °C), containing 33.33 mg/dl of cholesterol palmiate.

3.3. Enzyme loading

In these sets of experiments, the immobilized enzyme membranes were prepared by co-immobilizing of ChEt and ChOX. The cholesterol biosensor was standardized with four different ChOX concentration (15, 30, 60 100 U ml^{-1}) keeping the loading concentration of ChEt fixed at 150 U ml^{-1} and each biosensor was evaluated based on the characteristics of its oxygen consumption (Vi ppm/min) to the concentration of cholesterol palmiate at pH 6.0 and temperature 25 °C. It was found that the loading concentration of ChOX at 60 U ml^{-1} and ChEt at 150 U ml^{-1} has exhibited superior performance over the other three loading concentrations (Fig. 3). So, for subsequent experiments the loading concentration of ChOX at 60 Uml^{-1} and ChEt at 150 Uml^{-1} were taken as the optimum loading concentration.

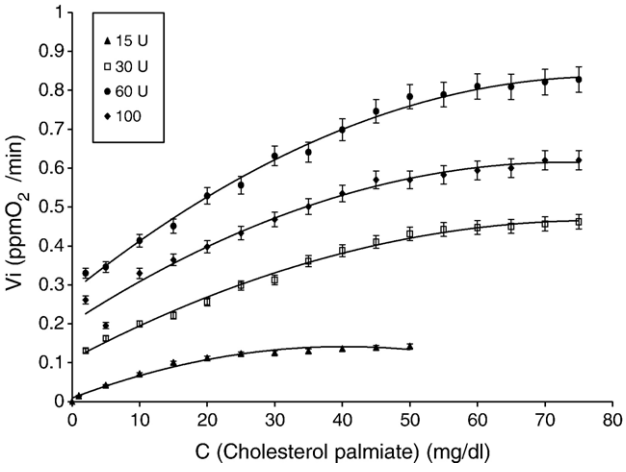

Fig. 3. Effect of enzyme loading concentration on the sensor responses varying the loading concentration of cholesterol oxidase (▲ 15 U ml^{-1}, □ 30 U ml^{-1}, ◆ 100 U ml^{-1}, ● 60 U ml^{-1}), keeping the concentration of cholesterol esterase fixed at 150 U ml^{-1}.

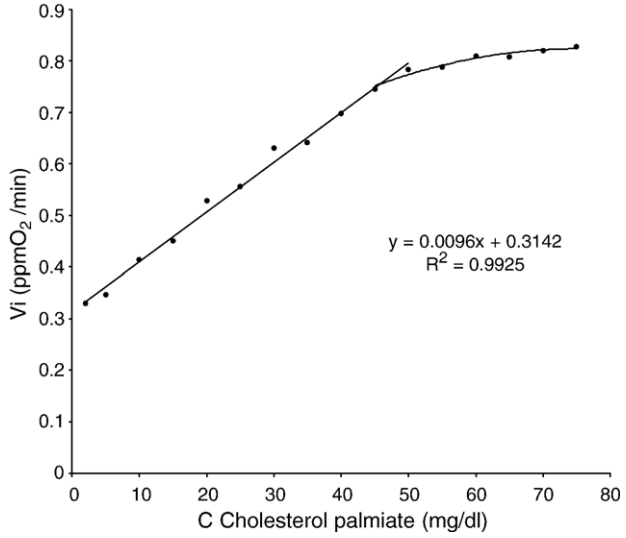

Fig. 4. Calibration curve of the coupled cholesterol esterase and cholesterol oxidase system at varying substrate concentrations at 25±2 °C and pH 6.0.

3.4. Calibration of the sensor

An oxygen consumption in ppm vs substrate concentration (mg/dl) curve were drawn. Normally the time required to reach maximum O_2 consumption value i.e. the response time of the sensor was 90 s (data not shown). The optimum concentration of substrate, which can be estimated by enzyme sensor can be determined by measuring O_2 consumption at varying concentrations of cholesterol palmiate (1–75 ml/dl) in the assay mixture at pH 6.0 and temperature 25 °C. The effect of cholesterol palmiate concentration on the sensor is shown in Fig. 4. A linear relationship was found between substrate concentration and O_2 consumption (Vi ppm/min) from 2–50 mg/dl of cholesterol palmiate. The following regression equation with a $R^2=0.9925$ was obtained.

$$Vi(\text{ppm/min}) = 0.0096[\text{ Cholesterol palmiate}] + 0.3142$$

At this stage, the biochemical reaction reaches saturation point with the substrate. Above this substrate concentration (50 mg/dl), the ratio of increase in O_2 consumption vs increase of substrate concentration gradually decreased.

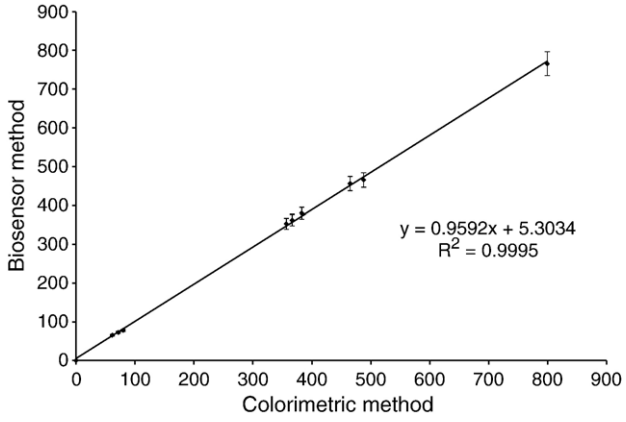

Fig. 5. Correlation between the total cholesterol values determined by the Libermann–Burchard method and the present method using coupled cholesterol esterase and cholesterol oxidase system.

Table 1
Determination of cholesterol in different real food samples (10 times diluted except duck egg) by the developed cholesterol biosensor

Sample	Vi (ppm O_2/min)	Cholesterol mg/100g
Poultry egg yolk	0.752	456.04
Poultry egg yolk (boiled)	0.761	465.4
Poultry egg (white)	−0.01	0
Deshi egg yolk	0.653	352.92
Deshi egg yolk (boiled)	0.662	362.3
Duck egg yolk (40 times diluted)	0.498	765.8
Goat meat	0.384	72.7
Goat meat (cooked)	0.389	77.91
Goat liver	0.679	380
Chicken meat	0.377	65.4

*Values reported as the average of 5 repeats.

3.5. Estimation of cholesterol in real samples

In order to check the validity of our sensor, we have analyzed the total cholesterol in real food samples. These values obtained by sensory method were also compared with enzymatic kit (CHOD-PAP) method. The results are compared graphically in Fig. 5 with $R^2=0.9995$. The data given are average of five measurements. The values of the total cholesterol of different food samples is represented in the Table 1. It was found by both of these methods that the cholesterol content increased after cooking. It may be due to the retention and tissue moisture losses [27].

3.6. Storage stability of the membrane

The long term stability and repeatability of the immobilized membrane was investigated in identical condition for each time. The membrane was kept for 9 weeks at 4 °C in sealed polypropelene bags containing PBS (pH 7.0). The enzymatic membrane can be reused over 30 times with 10–12% loss of its initial activity (data not shown).

For long-term storage, the enzyme activities were almost the same as the initial activity up to 2 weeks (Fig. 6). After that the enzyme activity started to decrease with storage time and reached almost 50% of its initial activity after 8 weeks. The activity further decreased below 50% after 9 weeks.

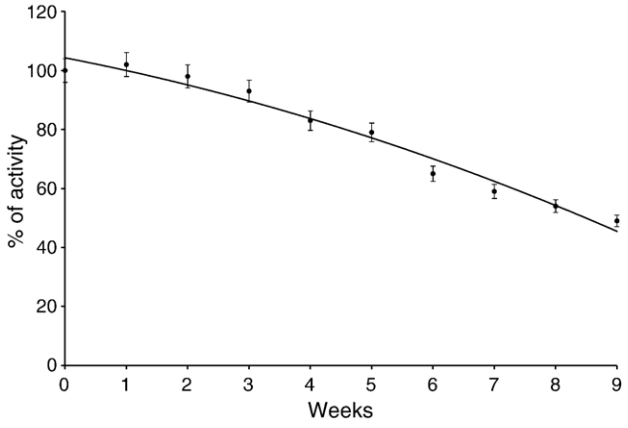

Fig. 6. Storage stability of the immobilized membrane.

4. Conclusion

A rapid, economic and simple DO probe based cholesterol biosensor was developed for the estimation of the total cholesterol in food substances. The biosensor can be prepared by co-immobilizing ChEt and ChOX. This sensor exhibits linearity with the concentration range from 2–50 mg/dl of cholesterol palmiate at pH 6.0 and temperature 25 °C. In this study, it has been demonstrated that the developed sensor can be applied in food samples for the estimation of the total cholesterol. This sensor also shows close results as with the colorimetric method.

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References

[1] J. Zicha, J. Kunes, M.A. Devynek, Abnormalities of membrane function and lipid metabolism in hypertension, Am. J. Hypertens. 12 (1999) 315–331.
[2] R. Foster, J. Cassidy, E. O'Donoghue, Electrochemical diagnostic strip device for total cholesterol and its subfraction, Electroanalysis 12 (2000) 716–721.
[3] W.W Wong, D.L. Hachey, L.L. Clarke, S. Zhang, M. Llaurador, W.G. Piond, An improved HPLC method to purify erythrocyte cholesterol for estimation of *in vivo* cholesterol synthesis using the deuterium method, Appl. Radiat. Isotopes 45 (1994) 529–533.
[4] J.P. Blomhoff, Serum cholesterol determination by gas–liquid chromatography, Clin. Chim. Acta 43 (1973) 257–265.
[5] E. Agullo, B. Susna, Gas–liquid chromatographic determination of total free cholesterol in egg pastas, Food Res. Int. 29 (1996) 77–80.
[6] M.D. Marazuela, B. Cuesta, M.C. Moreno-Bondi, A. Quejido, Free cholesterol fiber-optic biosensor for serum samples with simplex optimization, Biosens. Bioelectron. 12 (1997) 233–240.
[7] G. Li, J.M Lia, G.Q. Hu, N.Z. Ma, P.J. Wu, Study of carbon nanotubes modified biosensor for monitoring total cholesterol in blood, Biosens. Bioelectron. 20 (2005) 2140–2144.
[8] S. Singh, A. Chaubey, B.D. Malhotra, Amperometric cholesterol biosenor based on immobilized cholesterol esteresa and cholesterol oxidase on conducting polypyrrole film, Anal. Chim. Acta 502 (2004) 229–234.
[9] X. Tan, M. Li, P. Cai, L. Luo, X. Zou, An amperometric cholesterol biosensor based on multiwalled carbon nanotubes and organically modified sol–gel/chitosan hybrid composite film, Anal. Biochem. 337 (2005) 111–120.
[10] J.-C. Vidal, J. Espuelas, E. Garcia_Ruiz, J.-R. Castillo, Amperometric cholesterol biosensors based on the electropolymerization of pyrrole and the electrocatalytic effect of Prussian-blue layers helps with self-assembled monolayers, Talanta 64 (2004) 655–664.
[11] S. Brahim, D. Nariresingh, A. Guiseppi_Elie, Amperometric determination of cholesterol in serum using a biosensor of cholesterol oxidase contained within a polypyrrole–hydrogel membrane, Anal. Chim. Acta 448 (2001) 27–36.
[12] J.-C. Vidal, J. Espuelas, J.-R. Castillo, Amperometric cholesterol biosensor based on an immobilized monolayer of flavin adenine dinucleotide cofactor, Anal. Biochem. 333 (2004) 88–98.
[13] M.K. Ram, P. Bertoncello, H. Ding, S. Paddeu, C. Nicolini, Cholesterol biosensors prepared by layer-by-layer technique, Biosens. Bioelectron. 16 (2001) 849–856.
[14] M. Situmorang, P.W. Alexander, D.B. Hibbert, Flow injection potentiometry for enzymatic assay of cholesterol with a tungsten electrode sensor, Talanta 49 (1999) 639–649.
[15] V. Shumyantseva, G. Deluca, T. Bulko, S. Carrara, C. Nicolini, S.A. Usanov, A. Archakov, Cholesterol amperometric biosensor based on cytochrome P450scc, Biosens. Bioelectron. 19 (2004) 971–976.
[16] S.-H. Kim, S.M. Nam, G.S. Byun, S.Y. Yun, S. Hong, Determination of cholesterol by a diode laser/fiber optic colorometric spectometer, Bull. Korean Chem. Soc. 21 (2000) 389–392.
[17] S.P. Martin, D.J. Lamb, J.M. Lynch, S.M. Reddy, Enzyme-based determination of cholesterol using quartz crystal acoustic wave sensor, Anal. Chim. Acta 487 (2003) 91–100.
[18] D. Das, Biochemistry, 10th edition, Academic Publishers, Kolkata, India, (2000), 73.
[19] Ch.C. Allain, L.S. Poon, C.S.G. Chan, W. Richmond, P.C. Fu, Enzymatic determination of total serum cholesterol, Clin. Chem. 20 (1974) 470–474.
[20] J.M. Fernandez_Romero, M.D. Luque de Castro, M. Valcareal, Determination of total cholesterol in serum by flow injection with immobilized enzymes, Clin. Chim. Acta 167 (1987) 97–104.
[21] A.K. Basu, P. Chattopadhyay, U. Roychoudhury, R. Chakraborty, Development of an amperometric hypoxanthine biosensor for determination of hypoxanthine biosensor for determination of hypoxanthine in fish and meat tissue, Indian J. Exp. Biol. 43 (2005) 646–653.
[22] A.F. Albantov, Reducing the influence of sample oxygen in biosensor design, Biosens. Bioelectron. 7 (1992) 575–585.
[23] A. Haouz, A. Geloso-Meyer, C. Burstein, Assay of dehydrogenase with an O_2-consuming biosensor, Enzyme Microb. Technol. 16 (1994) 292–297.
[24] J. Folch, M. Less, G.H.S. Stanley, A simple method for the isolation and purification of total lipids from animal tissue, J. Biol. Chem. 226 (1957) 497–509.
[25] J.J. Ishikawa, J. MacGee, J.A. Morrison, G.J. Glueck, Quantitative analysis of cholesterol in 5 to 20 ml of plasma, J. Lipid Res. 15 (1974) 286–291.
[26] V. Malik, C.S. Pundir, Determination of total cholesterol in serum by cholesterol esterase and cholesterol oxidase immobilized and co-immobilized on to arylamine glass, Biotechnol. Appl. Biochem. 35 (2002) 191–197.
[27] A.M. Pearson, T.R. Dutson (Eds.), Edible Meat By-Products, vol. 5, Elsevier Sci. Publisher Ltd., Linton Road, England, 1988, p. 38.