PERIODICA POLYTECHNICA SER. CHEM. ENG. VOL. 40, NO. 1-2, PP. 93-100 (1996)

PREPARATION OF A CHOLESTEROL OXIDASE ENZYME REACTOR AND ITS APPLICATION FOR THE DETERMINATION OF TOTAL CHOLESTEROL IN DIFFERENT FOOD PRODUCTS WITH A FIA SYSTEM[1]

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Abstract

An immobilized enzyme reactor was prepared for determination of cholesterol with amperometric flow injection (FIA) technique. The immobilization of cholesterol oxidase was studied on glass, Acrylex C-100 polymer and on alkylamino-bonded silica beads. The activated loads were packed into a glass column. The hydrogen peroxide formed in the enzyme reaction was determined by electrochemical detection.

Glutaraldehyde-activated alkylamino silica seems to be the best means for enzyme immobilization. The mode of immobilization, the effects of linked enzyme units on activity, pH, temperature and stability of the enzyme reactor were also investigated. Total cholesterol in different food materials was measured by the optimized FIA system. The accuracy and reproducibility of the FIA method developed was compared to the conventional GC measurement. The results show that the described FIA method is also suitable for cholesterol determination in food products.

Keywords: cholesterol determination, enzyme reactor, FIA, multicomponent foods.

Introduction

There is a growing need for routine measurement of cholesterol in foodstuffs, due to the well-known effects of exaggerated cholesterol intake on the pathomechanism of cardiovascular disease. There are many published methods for cholesterol analysis including colorimetry [1], high performance liquid chromatography [2, 3], gas liquid chromatography [4, 5] and gas chromatography [6, 7]. For clinical analysis these methods usually are replaced by enzymatic tests, which are based on the following reactions [8]:

$$\text{cholesterol esters} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{free cholesterol} + \text{acids}$$

$$\text{cholesterol} + O_2 + H_2O \xrightarrow{\text{cholesterol oxidase}} \text{cholest-4-en-3-on} + H_2O_2$$

[1]This research work was supported by OTKA (National Foundation of Science and Research, Hungary). Project number: T-014420.

The formed H_2O_2 can be determined by different analytical procedures such as colorimetry [1, 9], fluorometry [8], chemiluminescence [10] and amperometry [11].

Enzymatic determinations are reproducible, sensitive, rapid and easy to automate. The usual solution for automation is flow injection analysis abbreviated as FIA [9, 10, 11, 12, 13]. The economy of such FIA measurements can be increased by the application of immobilized enzyme reactor(s).

In this paper we report on the optimization of parameters for immobilization of cholesterol oxidase enzyme. A flow injection system is described for cholesterol determination in different food extracts based on the enzyme reaction and electrochemical detection. The results are compared with those obtained by conventional GC method.

Materials and Methods

Reagents and standards: Chloroform, methanol, ethanol, petroleum ether, hexane and TRITON X-100 were purchased from Reanal Ltd., Hungary. All solvents were of analytical grade. Cholesterol oxidase enzyme (EC. 1.1.3.6. from Nocardia erytropolis) was obtained from Merck, Germany.

A standard solution was prepared with cholesterol from Reanal Ltd., Hungary. For the FIA method 300 mg cholesterol was dissolved in 100 ml water containing 10% Triton X-100. This stock solution was diluted in the range of 10 – 100 mg/100 ml concentration. For the GC procedure cholesterol was dissolved in petroleum ether in the range of 0.05 – 2 mg/ml, and 2 mg/ml squalene (Sigma Chemical Co.) was used as internal standard. All reagents used were of analytical grade.

Sample preparation: The cholesterol content of different commercial food products, such as whole egg powder, egg yolk, pig liver, liver pâté, pasta (8 eggs/kg), and Hungarian cheese ('Anikó') was determined. The samples were prepared by direct saponification followed by solvent (hexane) extraction. The organic phases were evaporated on a rotary evaporator. The nonsaponificable residues were dissolved in petroleum ether for GC and in 10% Triton X-100/water solution for FIA.

Flow Injection System

Immobilization of cholesterol oxidase enzyme:

1. 1 g of cleaned and dried controlled porosity glass beads (Sigma Chem. Co.) was activated with 5 ml acetone solution containing 1% of

3-aminopropyl-tritoxy-silane at 70°C for 15 h. 50 units of the enzyme were immobilized with glutaraldehyde (2% aldehyde in 0.1 mol/l phosphate buffer, pH = 7.5) [14].

2. 50 units of cholesterol oxidase enzyme were linked to the surface of 0.1 g Acrylex C-100 polymer with 20 mg N-Cyclohexyl-N'-(β-(N-methylmorpholino)-ethyl)carbodiimide-p-toluenesulfonate salt (CMC, Sigma Chem. Comp., C1011) in 5 ml phosphate buffer (0.01 mol/l, pH = 6.5) for 16 hours at 4°C [15].
3. Cleaned and dried Lichroprep-NH_2 granules were activated by glutaraldehyde solution (5% aldehyde in 0.05 mol/l $NaHCO_3$ solution), then 3 ml of different enzyme solutions [5, 10, 15 and 20 enzyme units in 3 ml 0.01 mol/l phosphate buffer at pH = 6.5) were circulated through the filled column. After the immobilization process the column was washed with 0.1 mol/l glycerol buffer at pH = 8 [12].

In every case a narrow glass tube (ø: 0.4 cm, $V = 0.7$ cm^3) was used as reactor.

The optimum amount of enzyme was studied by the following procedure: 40 mg of activated Lichroprep-NH_2 granules were treated with 5, 10, 15 and 20 IU of cholesterol oxidase enzyme.

Flow injection system: The applied flow system (ANASYSCON) contained a peristaltic pump, injection valve, enzyme reactor and voltametric detector (oxygen electrode YSI 5331) and a computer with CAFCA software as shown in *Fig. 1.*

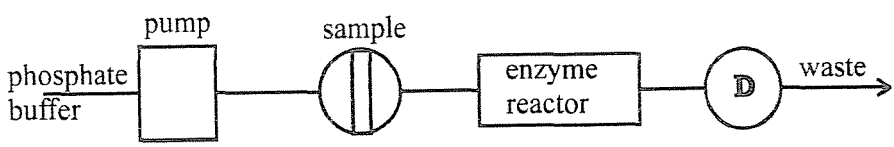

Fig. 1. Flow injection system with immobilized enzyme reactor and electrochemical detection for determination of total cholesterol

Gas chromatography: GC was performed on a Packard apparatus equipped with flame ionization detector and HP 19094 capillary column filled with methyl-silicone oil (Id: 0.25 mm, film: 0.1 μm. Column temperature was 265°C, injector temperature was 320°C and detector was 300°C. 1 μl sample (in petroleum ether with internal standard) was injected [7].

All the results shown are the averages of three parallel measurements. The reproducibility of measurements was characterized by relative standard deviation (r.s.d.) values.

Results and Discussion

Enzyme immobilization studies: The enzyme immobilization on controlled porosity glass beads was successful (as was shown in manual activity tests), but due to some practical difficulties – including many preparation steps, utilization of acetone solution, etc. – this process was excluded from further experiments.

The ACRYLEX C-100 type granules seem to be suitable for enzyme-fixation. The only problem is, however, that the beads swell during the immobilization process causing a big resistance against continuous flow. This results in extremely long cycle time for FIA ($\approx 8 - 10$ min/injection) and the pressure is too high in the system. It seems that the Acrylex-type polymer is suitable for producing an enzyme membrane for a selective oxygen electrode [15], but it is not suitable for preparing an enzyme reactor.

In the case of Lichroprep-NH_2 beads, the procedure of enzyme immobilization is simple, there is no swelling, and due to this fact the cycle time is much shorter than with Acrylex polymer. In further experiments the Lichroprep-NH_2 beads were used for filling the enzyme reactor.

The results of measurements with the FIA system for optimizing the amount of enzyme in the reactor are shown in *Fig. 2.* In agreement with previous results [12], the optimum can be seen at the rate of 10 IU/40 mg granules. Below this ratio the amount of enzyme is not sufficient for a higher reaction rate, while above it probably the large enzyme molecules retard substrates to reach the active centre.

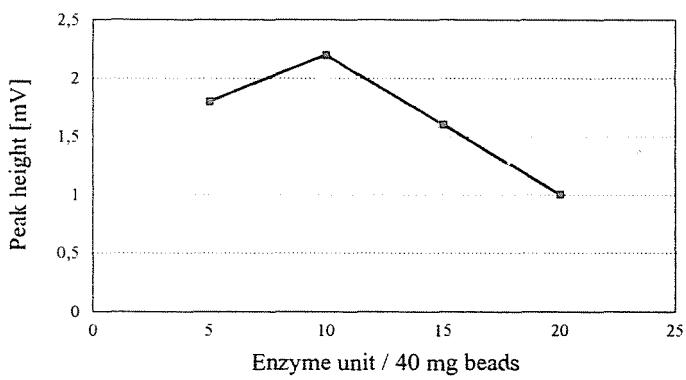

Fig. 2. Effect of enzyme concentration on the sensitivity of reactor (Cholesterol standard: 50 mg/100 ml)

The loss of activity of the described enzyme reactor during storage was also studied. The cholesterol oxidase reactor was stored in 0.01 mol/l phosphate buffer (pH = 7.4, $T = 4°C$) for 10 weeks. The results obtained with FIA measurements show that the enzyme reactor was very stable, the activity loss was only about 5% (*Fig. 3*).

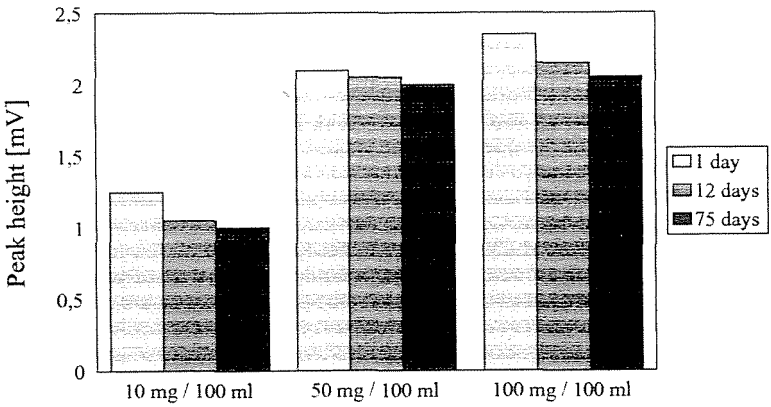

Fig. 3. Effect of enzyme reactor storage on peak height (storage temperature: 4°C, pH: 7.4)

Soluble enzymes are often very sensitive to temperature and pH. Usually the unfavourable environmental parameters lead to lower enzyme activity because of denaturation. *Fig. 4* shows the effects of pH and temperature of carrier solution on peak height.

The peak height for 50 mg/100 ml cholesterol concentration was slightly increased from 30°C to 50°C, while above 50°C the activity decreased drastically. Since temperature has only a slight effect on the enzyme activity, 30°C is satisfactory for the reaction. At this temperature the lifetime of the enzyme is also longer. The optimum pH range for the enzyme-processing is between 6.0 and 6.5.

In a FIA system, the flow rate of carrier solutions (and reagents) also influences the sensitivity of the method, because it affects the reaction rate. *Fig. 5* shows that the optimum flow rate of carrier is about 0.7 ml/min – when 30 μl sample is injected.

According to our results, we suggest the following parameters for the determination of cholesterol by FIA procedure (see also on *Fig. 1*):

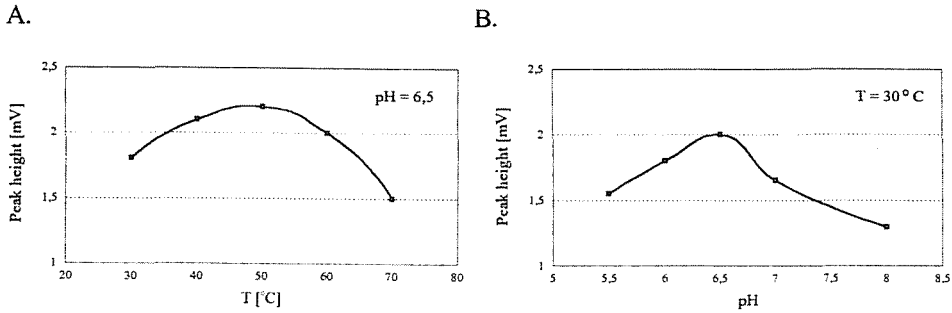

Fig. 4. Effects of temperature (A) and pH (B) on peak height in FIA system. (Cholesterol standard: 50 mg/100 ml, carrier: 1 ml Triton X-100 in 100 ml 0.1 mol/l phosphate buffer)

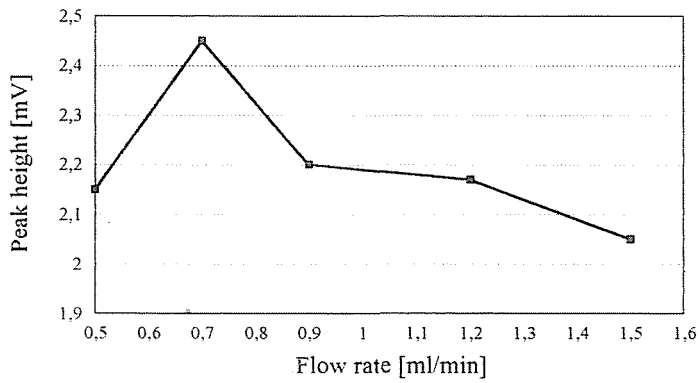

Fig. 5. Effect of flow rate on peak height (Injection: 30 μl, pH = 6.5, t = 30°C, cholesterol standard = 100 mg/100 ml)

Carrier:	1% Triton X-100 in 0.1 mol/l phosphate buffer
Enzyme reactor:	Lichroprep-NH_2 activated with glutaraldehyde
pH of carrier:	6.5
Temperature:	30°C
Sample injection:	30 μl
Flow rate:	0.7 ml/min
Concentration range:	10 – 100 mg cholesterol/100 ml

Fig. 6 shows a typical detector signal and calibration curve for cholesterol determination with the optimized FIA system. In this concentration range the calibration curve is non-linear. The cycle time is a little longer compared to other FIA methods [11, 12]. This fact can be explained by the lower flow rate and probably by the higher resistance of the packed reactor.

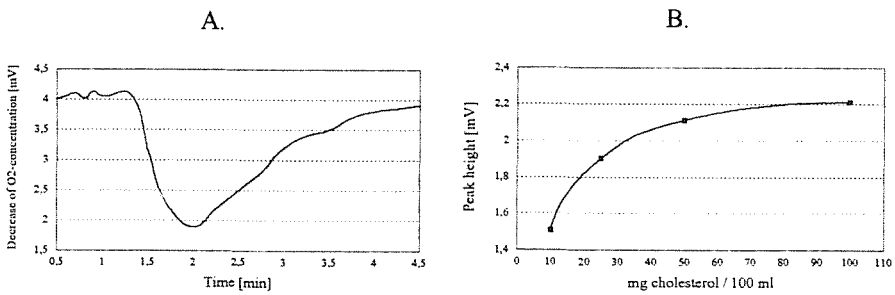

Fig. 6. Typical detector signal (A) and calibration curve (B) of the optimized FIA system for cholesterol determination

The reproducibility of the assays was tested by measurements of repeated injections of standard solutions ($n = 5$). The highest relative standard deviation (r.s.d.) was 1.8 indicating that the reproducibility of FIA method is acceptable.

Finally, the cholesterol content of six different products was determined by the above described FIA procedure and by conventional GC method. The results are shown in *Table 1.*

Table 1
Cholesterol content of some food products

Food products	Cholesterol content by GC (mg/100 g)	Cholesterol content by FIA (mg/100 g)
Whole egg powder	1186	1427
Egg yolk	1411	1686
Pig liver	363	308
Liver paté	185	181
Pasta	106	121
Cheese 'Aniko'	57	48
r.s.d.	< 2%	< 4%
Corr. coef. with GC	–	0.99

The good correlation coefficient ($R = 0.99$) shows that the described FIA method is suitable for cholesterol determination. The slightly higher r.s.d. value – compared to the same value in the case of standards – is due to the errors originating from sample preparation. Moreover, our results clearly show that the detergent containing solution (10% Triton X-100 in distilled water) is applicable for sample resolving after saponification and extraction. The obtained aqueous solution is more suitable for enzymatic cholesterol determination than the conventional organic solvents.

Acknowledgement

The authors owe special thanks to their student Ildikó Berecz, for her conscientious experimental work. The authors acknowledge the technical help of Ágnes Miskolczi and Rita Dóczi.

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