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Amperometric biosensor for ascorbic acid

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Abstract: A L-ascorbic acid biosensor based on ascorbate oxidase has been developed. The enzyme was extracted from the mesocarp of cucumber (*Cucumis sativus*) by using 0.05 mol L^{-1} phosphate buffer, pH 5.8 containing 0.5 mol L^{-1} NaCl. After the dialysis *versus* phosphate buffer

0.05 mol L^{-1} pH 5.8, the enzyme was immobilized onto nylon net through glutaraldehyde covalent bond. The membrane was coupled to an O_2 electrode and the yielding reaction monitored by oxygen depletion at -600 mV using flow injection analysis optimized to 0.1 mol L^{-1} phosphate buffer pH 5.8, as the carrier solution and flow-rate of 0.5 mL min^{-1}. The ascorbic acid calibration curve was linear from $1.2x10^{-4}$ to $1.0x10^{-3}$ mol L^{-1}. The evaluation of biosensor lifetime leads to 500 injections. Commercial pharmaceutical samples were analyzed with the proposed method and the results were compared with those obtained by high-performance liquid chromatography (HPLC).

Keywords: biosensor; ascorbic acid; flow injection analysis.

Introduction

Ascorbic acid or vitamin C is a soluble vitamin present in many biological systems and multivitamin preparations, which are commonly used as supplement to inadequate dietary intake. In addition, the concentration of ascorbic acid in foodstuffs, beverages and pharmaceuticals can be an index of quality, since it varies during production and storage stages [1, 2]. Ascorbic acid has an important role in body health. It is necessary source for formation of collagen, assists the absorption of iron via reduction to the ferrous valence and lack of it provokes increased susceptibility to many kinds of infections and slows down the rate at which wounds and fractures heal [1]. It acts in amino acids metabolism and in biosynthesis of suprarenal hormone, minimizing the stress effect. Nevertheless, the excess of ascorbic acid can cause gastric irritation and diarrhea, giving as metabolic product the oxalic acid, which can cause renal problems [3]. Thus, vitamin C determination is very important for biological and food industry.

The ascorbic acid has been determined by many analytical methodologies. Oliveira and Watson [4] reviewed the chromatographic techniques for the determination of supposed dietary anticancer compounds including ascorbic acid in biological fluids. High performance liquid chromatography (HPLC) with electrochemical detection has been described as useful method for selective and sensitive determination of ascorbic acid in foods and biological fluids [5-7]. Zeng *et al*. have proposed a spectrophotometric method [8].

The amperometric determination of ascorbic acid based on its electrochemical oxidation can be done at bare platinum or glassy carbon electrodes, operating at potential above +500 mV. However, this overpotential is lowered substantially by using chemically modified electrodes (CME). CMEs based on ferrocene [9], on ferricyanide [10] or on 7,7,8,8-tetracyanoquinodimethane (TCNQ) [11] in

conjunction with tetrathiafulvalene or ferrocene [12] have been proposed as efficient methods to reduce the oxidation potential of ascorbic acid.

Enzymes have been used to improve the selectivity of many reactions using the amperometric detection. In this case, biosensors constructed using commercial ascorbate oxidase [13] or raw materials based on extracts from cucumber [14-17] and zucchini [18] have been proposed in the literature as alternative methods to determine ascorbic acid.

Ascorbate oxidase from cucumber have been used to eliminate ascorbic acid interference from malate determination in analysis of citrus juice [19] and also to determine glutathione [20], organophosphorous pesticida ethyl paraoxon [21] based on enzymatic inhibition method.

This work describes the construction of a biosensor using crude ascorbate oxidase extracted from cucumber, immobilized onto nylon net and subsequent coupling to oxygen electrode. Ascorbate oxidase is used to catalyze the oxidation of L-ascorbic acid in the presence of oxygen, as follow:

$$2\,\text{L-ascorbate} + O_2 + 2H^+ \rightarrow 2\,\text{dehydro-L-ascorbate} + 2H_2O$$

The method monitor the oxygen depletion, which is related to the ascorbate concentration, and it is successful applied to analysis of ascorbic acid in pharmaceutical formulations.

Experimental

Reagents

L-Ascorbic acid and albumin bovine were purchased from Sigma Chemical Co. (St. Louis, USA); the nylon membrane from A. Bozzone (Appiano Gentile, Italy) was kindly supplied by Prof. M. Mascini (University of Firenze, Italy); dialysis membrane was purchased from Viskase Co. (USA); cucumbers (*Cucumis sativus*) were obtained from commercial sources; three medicine samples (Redoxon, Cewin and Vitamina C, containing 200 mg mL^{-1} of ascorbic acid) were purchased from a local drugstore. All the other chemicals were of analytical grade. The ascorbic acid solutions were freshly prepared in 0.1 mol L^{-1} phosphate buffer pH 6.2.

Equipments

Lyophilization was performed with Labconco mod. LYH-Lock 1L.

The FIA system was constituted by a peristaltic pump (Ismatec, model IPC), using an injection valve (Reodyne Inc.) with a 50 mL loop and a flow-through cell, where it was positioned the oxygen electrode (Universal Sensor Inc., Model 4001). The amperometric measurements were performed with an amperometric detector (Universal Sensor, model 3001), connected to a chart recorder (Kipp and Nonen, Model BD 111). The chromatographic measurements were carried out with a HPLC system, utilizing a Waters (Model 501) pump, a Tunable Absorbance Detector (Waters, Model 486) and a Data Module (Waters Model 746).

Ascorbate oxidase extraction

The ascorbate oxidase was extracted from cucumbers according to procedure described by Machólan [14]: 97 g vegetal tissue was homogenized in a blender and distributed in 5 equal parts. Each part was homogenized with phosphate 0.05 mol L^{-1} buffer solution pH 5.8, containing 0, 1 10^{-3}, 2 10^{-2}, 5 10^{-1}, 1 10^{-3} and 1 mol L^{-1} NaCl, respedtively. The extracts were sucked through a Buchner funnel and centrifuged at 9000 rpm for 30 min at 4ºC. The supernatants were dialyzed *versus* 0.05 mol L^{-1} potassium phosphate buffer solution pH 5.8, under constant stirring during 7 h at 4ºC. Aliquots of 1 mL of those extracts were distributed in flasks and lyophilized under vacuum (-50ºC) during 10 h. Afterwards, it was stored at 4ºC in dessecator and used as ascorbate oxidase source.

Enzyme assay

The free enzyme activity (DA/min) was carried out by adding into a cuvette 2.6 mL phosphate buffer pH 6.2, 0.3mL of enzymatic extract and the reaction was started by addition of 100 mL of 1.5 10^{-4} mol L^{-1} ascorbic acid. The absorbance decreasing was monitored at 265 nm (25^{0}C) against a blank, in which no substrate was added. The protein concentration was measured by microbiuret method [22], taking albumin bovine as standard. The catalytic activity was calculated according to [23]:

$$z = \frac{\Delta A.V.1000}{\Delta t \varepsilon.d.v.C_p}$$

where A: absorbance decreasing in 60 s at 265 nm, V: volume of assay (3 10^{-3}L), t: time (60s), e: molar

absorptivity of ascorbic acid (1.0 $mmol^{-1}$ mm^{-1}, according to [24]), d: light path (10mm), v: volume of sample used in assay (3 10^{-4} L), C_p: protein concentration (11g L^{-1}).

Biosensor construction

10 μL of the enzymatic extract containing the ascorbate oxidase was mixed with 20 μL of glutaraldehyde solution 0.5% (v/v) and covalently immobilized onto nylon net membrane previously activated according to Mascini [13]. The membrane was stored overnight at 4ºC, washed with phosphate buffer and fixed over the polypropylene membrane of the O_2 electrode by an o-ring.

Analytical curve of ascorbic acid using the biosensor

The analyses of ascorbic acid were carried out by using the typical setup used for flow injection system. The best flowing out of the measuring system was determined by injecting 50 μL of 3 10^{-3} mol L^{-1} ascorbic acid solution in the 0.1 mol L^{-1} phosphate buffer solution pH 5.8 and flow rate ranging from 0.3 to 1.0 mL min^{-1}. The oxygen decreasing was monitored following the oxygen electrode response at a fixed potential of -600 mV that was recorded with a chart speed of 2 cm min^{-1}. Once the flow rate was optimized, the pH influence on the biosensor response was investigated and the analytical curves was constructed using the amperometric measurements performed by injecting 50 μL of ascorbic acid solution from concentration of 1.3 10^{-4} to 3.0 10^{-2} mol L^{-1}, respectively.

Measurement of ascorbic acid in pharmaceutical products

The ascorbic acid was determined in three pharmaceutical products. Considering the nominal value displayed by manufacturers, the samples were diluted in phosphate buffer 0.1 mol L^{-1} pH 5.8, in order to obtain solutions of 2.5 10^{-4} and 5.0 10^{-4} mol L^{-1} concentrations for each pharmaceutical sample. The analytical procedure was similar to the one described to obtain the ascorbic acid curve. In order to compare the results, the samples were also analyzed by HPLC according to Nicholson and Macrae [25]. The analyses were carried out on a Waters Model 486 detector sensitive to absorption at 265 nm. A C-18 column was used and a solution of H_2O:methanol:acetic acid (93:5:2) as the mobile phase. Chromatograms were obtained using aliquots of 10 mL of the sample under flow rate of 0.50 mL min^{-1}.

Results and Discussion

Ascorbate oxidase extraction and activity

The supernatants containing the ascorbate oxidase obtained from the cucumber mesocarp in different concentration of NaCl in phosphate buffer are colorless and present pH values 5-6.

Figure 1 exhibits the influence of NaCl concentration on the free enzyme activity (DA /min). In the absence of the salt, the ascorbate oxidase enzyme presented value of DA /min = 0.132. Nevertheless, the concentration of 1.0 10^{-3} mol L^{-1} NaCl is enough to double the respective activity. Then, the value increases up to 0.5 mol L^{-1}, where the maximum activity is obtained. This value is slightly different from that reported in the literature that selected 0.1 mol L^{-1} NaCl concentration for ascorbate oxidase extracted from mango *(Mangifera indica)* [26]. Further experiments were carried out by using 0.2 mol L^{-1} NaCl concentration; the enzymatic extract presented 11mg of protein per ml.

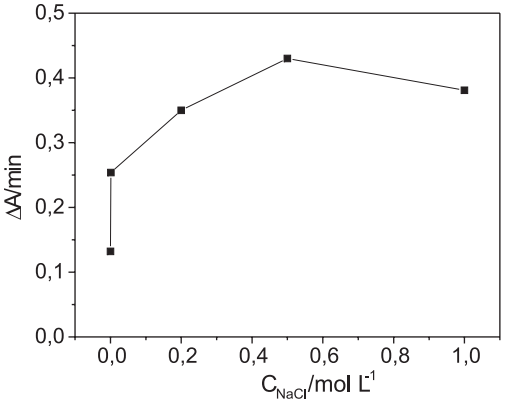

Figure 1. Influence of NaCl concentration in phosphate buffer solution as ascorbate oxidase extractor.

The apparent specific catalytic activity of enzymatic extract obtained was $9.5x10^4$ U mg^{-1}. This value is in agreement with the value ($1.70\ 10^3$ U mg^{-1}) related to the purified ascorbate oxidase [24].

Biosensor performance

Table 1 compare the influence of flow rate on the response time (t_r) and the time to establish base line (t_w) for detection of $1\ 10^{-5}$ mol L^{-1} ascorbic acid solution. Best current intensity is obtained with 0.3 mL min^{-1}, but the response time (time to reach the maximum intensity) is longer than in the other flow-rates investigated. No influence is observed on the time required to establish the baseline. Taking into account the sampling rate and the current intensity, a flow rate of 0.5 mL min^{-1} was selected for further measurements.

The effect of pH on the activity of the immobilized enzyme was investigated evaluating the signal for: 0.1 mol L^{-1} HCl/KCl, pH 2.2; 0.1 mol L^{-1} potassium hydrogenphthalate/KCl, pH 3.0; 0.1 mol L^{-1} potassium hydrogenphthalate/NaOH, pH 4.5; 0.1 mol L^{-1} formic acid/NaOH, pH 3.8 and 4.8; 0.1 mol L^{-1} phosphate buffer at pH 6.2 and 7.4. As shown in Figure 2, the maximum activity occurred around pH=4. Nevertheless more stable baseline is observed at pH5.8, which was chosen as best pH value.

Table 1. Influence of flow rate on the dispersion of analyte. Ascorbic acid: $1.0\ 10^{-5}$ mol L^{-1}.

Flow rate/mL min^{-1}	i/nA	t_{resp}/s	t_w/min
0.3	0.348	60	1
0.5	0.293	15	1
0.7	0.277	15	1
1.0	0.165	15	1

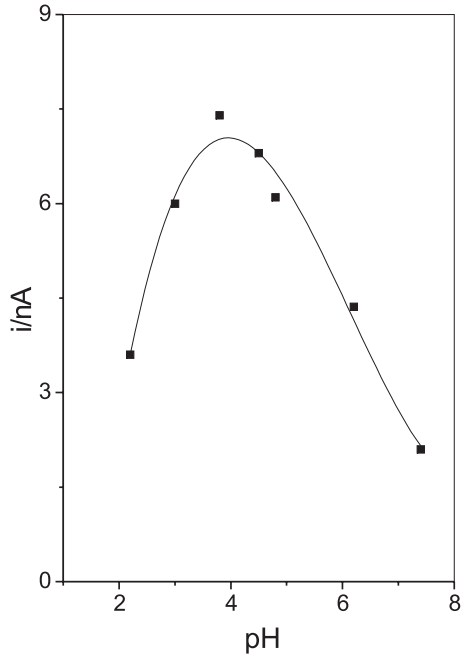

Figure 2. Effect of pH on the biosensor response. Ascorbic acid: $3.0x10^{-4}$ mol L^{-1}, flow rate: 0.5 mL min^{-1}.

The reproducibility of the analytical readout was investigated recording the FIA signal using injection volume of 50 μL, under a flow rate of 0.5 mL min^{-1}. For each ascorbic acid solution injected in triplicate, is observed firstly a concomitant increasing followed by the decreasing side. As shown in Fig. 3, the proposed system presents a good reproducibility, a constant baseline and sampling time of 1 minute.

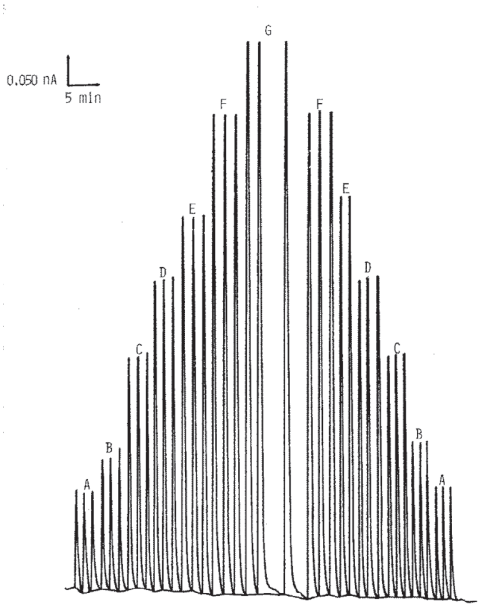

Figure 3. Flow diagram for amperometric determination of ascorbic acid. Carrier solution: 0.1 mol L^{-1} phosphate buffer, pH 5.8. Flow rate: 0.5 mL min^{-1}. A: $5.0x10^{-4}$; B: $1.0x10^{-3}$; C: $2.0x10^{-3}$; D: $3.0x10^{-3}$; E: $5.0x10^{-3}$; F: $1.0x10^{-2}$; G: $3.0x10^{-2}$ mol L^{-1}.

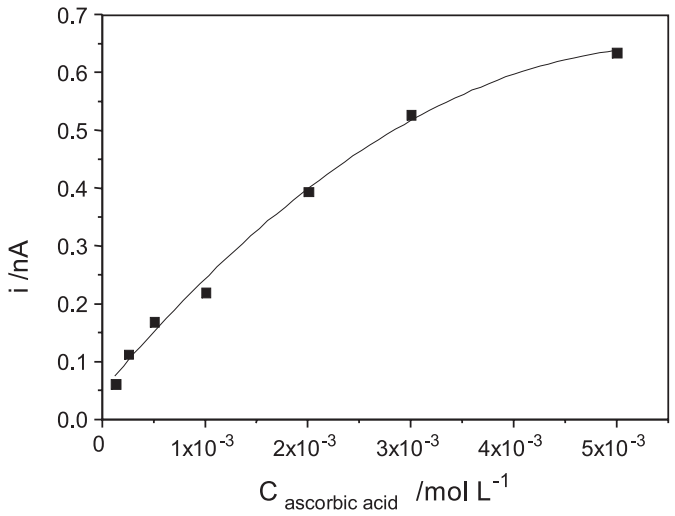

Figure 4. Analytical curve of ascorbic acid. Carrier solution: 0.1 mol L^{-1} phosphate buffer, pH 5.8. Flow-rate: 0.5 mL min^{-1}

The relationship between current intensity and ascorbic acid concentrations are represented in the Figure 4. The analytical curve shows a linear range ($y=6.27\ 10^{-2} + 166.2x$) for ascorbic acid concentration from $1.2\ 10^{-4}$ to $1.0\ 10^{-3}$ mol L^{-1}; following a mathematical equation that fits whole the curve (from $1.2\ 10^{-4}$ to $5.0\ 10^{-3}$ mol L^{-1}) assigned as $y=0.0483 + 213x - 19032\ x^2$. Due to good data reproducibility, the whole curve could be useful for the ascorbic acid determination.

In order to test the lifetime of the amperometric biosensor measurements were carried out using it almost daily during 25 days leading to 500 injections. No significant loss in enzymatic activity was observed. The results indicate one decade in linear response (10^{-4} to 10^{-3} mol L^{-1}), as well as described in reference [15], where the enzyme was supplied by the cucumber juice. Other references using an enzymatic extract partially purified [16], a slice of cucumber [14] or a commercial enzyme [13], also present one-decade linear response from 10^{-5} to 10^{-4} mol L^{-1}. The sampling rate is 75s more convenient than the other references [13,14,16].

The method was applied for determination of ascorbic acid in commercial samples and the data were compared with analysis based on HPLC method as described in the literature [25]. Analytical results obtained with both the biosensor and HPL are displayed on Table 2. A neglected deviation from both methods is observed. The correlations values (% correlation) that were calculated taking into account the HPLC values as reference, present values from 104 to 11% indicating good results for three samples investigated.

Table 2. Ascorbic acid concentrations in medicine obtained with both biosensor and HPLC methodologies.

Samples	C /mg mL^{-1}			% Correlation
	Nominal	Biosensor (n=3)	HPLC (n=2)	
Vitamin C	200	203±2	183±8	111
Cewin	200	252±2	230±20	109
Redoxon	200	217±2	207±10	104

Conclusion

The ascorbate oxidase extracted from cucumbers with 0.1 mol L^{-1} phosphate buffer solution containing 0.5 mol L^{-1} NaCl presented a high enzymatic activity. It can be used without previous purification when the biosensor is constructed by the immobilization onto nylon net and coupled to oxygen electrode.

The simplicity of the biosensor construction, the low cost of analysis, the sampling rate and the agreement between the results obtained with both proposed method and HPLC indicate viability of the proposed methodology.

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Resumo: Desenvolveu-se um biossensor para ácido L-ascórbico empregando ascorbato oxidase. A enzima foi extraída do mesocarpo de pepino com solução tampão fosfato 0,05 mol L^{-1}, pH 5,8 contendo NaCl 0,5 mol L^{-1}. Após diálise versus solução tampão fosfato 0,05 mol L^{-1}, pH 5,8 a enzima foi imobilizada em rede de nylon através de ligação covalente com glutaraldeído. A membrana foi acoplada em eletrodo de O_2 e a reação monitorada pelo consumo de oxigênio a -600 mV em análise em fluxo (solução tampão fosfato 0,05 mol L^{-1}, pH 5,8 como carregador e vazão 0,5 mL min^{-1}). A curva analítica apresentou-se linear entre $1,2x10^{-4}$ a $1,0x10^{-3}$ mol L^{-1}. O tempo de vida do biossensor foi de 500 análises. Amostras de medicamentos foram analisadas com a metodologia proposta e os resultados comparados com os obtidos com HPLC.

Palavras-chave: biossensor; ácido ascórbico; análise por injeção em fluxo.

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