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Bi-enzymatic optode detection system for oxalate determination based on a natural source of enzyme

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Abstract

This work describes a simple and low cost methodology for oxalate determination in food samples, which employs a bromothymol blue-based pH optode for the determination of CO_2 generated in the enzymatic reaction between oxalic acid and oxalate oxidase. The enzyme was immobilised on barley seeds, together with catalase enzyme, and placed in a stirring bar type enzymatic reactor. The system showed a linear response range from 0.0080 up to 0.100 mol l^{-1}, when the measurements were carried out in 0.050 mol l^{-1} succinate buffer at pH 4.0 and 25°C. In these conditions, the lifetime of the system was about 120 h, with a relative standard deviation <2% (four measurements of a 0.020 mol l^{-1} oxalate solution). A value of 0.075 mol l^{-1} was obtained for the apparent Michaelis–Menten constant, with a maximum velocity of 1908 μmol min^{-1} for oxalic acid oxidation. No significant differences were found at a confidence level of 95%, when the results were compared with those obtained with the AOAC official standard method (974.24) for oxalate determination in spinach.

Keywords: Oxalate oxidase; Barley seeds; Catalase; pH optode

1. Introduction

The determination of oxalate is important in various fields, such as clinical analysis, food analysis and even cattle breeding. An excess of oxalate in the urine of a patient can indicate renal failure, kidney lesions and pancreatic insufficiency [1,2]. For some susceptible persons, the ingestion of a large quantity of food which contains a concentration of oxalate can cause loss of calcium in the blood and injury in the kidneys. Furthermore, the determination of oxalate in plants is important in the case of pasture for feeding cattle [3,4]. A high concentration of oxalate in the pasture can produce needle-shaped crystals of calcium oxalate [5,6], which can cut the throat of the animal and kill it by asphyxia. The same problem happens with trees and grass [3,6], ornamental [5] and comestible [7] plants.

Different methods have been described in the literature for oxalate determination by employing chromatography [8–12], spectrophotometry [2,13,14] and permanganometric titration [15]. However, these methods are expensive, time consuming, have poor selectivity and require laborious sample pre-treatment. In order to overcome these disadvantages, enzymatic systems and biosensors have frequently been described in the literature, allowing for simple, selective

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and fast determination of many analytes. Enzymatic systems based either on the pure oxalate oxidase enzyme [16–20] or on the naturally immobilised enzyme [21], employing conductimetric [22], spectrophotometric [23–26], biothermochip [27], potentiometric [16] and amperometric detection [17–21,28] have been described for the determination of oxalate in different matrices, including biological samples.

Optical fibre chemical sensors (optodes) and biosensors have been widely described in the literature, showing some advantages over electrochemical sensors, such as electrical safety and lack of need for a reference signal [29,30]. Optodes are very appropriate for the development of biosensors, since many enzymatic reactions produce carbon dioxide or ammonia [31,32], which can be selectively detected by means of a pH optode.

Optode designs for determination of carbon dioxide have been frequently based on the principle of the Severinghaus CO_2 electrode. In this sensor, the CO_2 molecules diffuse through an hydrophobic layer (silicone permeable membrane), which separates the sodium bicarbonate buffer internal solution from the sample. The carbon dioxide is converted in HCO_3^- in the internal solution, changing its pH. In case of optodes, the pH change is followed by using colorimetric or fluorescent pH indicators. This idea was firstly reported by Optiz and Lübbers [33] and Zhujun and Seitz [34], for the determination of gaseous and dissolved CO_2, respectively, by employing a pH optode based on the hydroxypyrenetrisulphonic acid (HPTS) fluorescent indicator dissolved in a bicarbonate buffer solution, isolated from the environment by a silicone rubber membrane. Afterwards, contributions based on a similar principle have been found in the literature [35–38]. Some modifications in the above mentioned approach have also been described, which eliminate the internal solution reservoir, by entrapping the buffer solution as a fine emulsion in silicone rubber [39,40]. Another approaches also eliminate the use of the internal buffer solution, by employing ion pairs formed by a pH indicator anion and a quaternary ammonium cation associated to a few molecules of water, which allow the pH-sensitive dye to function as an indirect indicator for CO_2 [41].

The optodes above described have been employed as sensors for indirect determinations of enzymatic substrates which produce CO_2 in the respective reaction and the determination of L-glutamate [42] and pyruvate [43] by means of the CO_2 optode proposed by Optiz and Lübbers [33] and Zhujun and Seitz [34] have been carried out.

The oxalate oxidase enzyme (OxO) produces CO_2 in the reaction with oxalate substrate, in the presence of oxygen, according to

$$(COOH)_2 + O_2 \overset{OxO}{\rightarrow} 2CO_2 + H_2O_2 \quad (1)$$

The reaction represented by Eq. (1) is relatively slow and some authors [44] suggest the use of a catalase or a peroxidase enzyme together with the OxO enzyme (Eq. (2)), which reacts with the hydrogen peroxide produced in reaction (1), increasing the production of carbon dioxide:

$$2H_2O_2 \overset{catalase}{\rightarrow} 2H_2O + O_2 \quad (2)$$

This work describes the use of a pH optode based on bromothymol blue as sensor for the indirect determination of oxalate, by measuring the carbon dioxide produced in the reaction of this analyte with oxalate oxidase. This enzyme was immobilised in barley seeds, which preserve its natural environment, improving the performance of the system. This performance was further improved by immobilising catalase together with oxalate oxidase and putting them into a stirring bar type enzymatic reactor.

2. Experimental

2.1. Instrumentation

The instrumentation employed in this work consisted of a radiometric source (Oriel 6883), with a quartz-halogen lamp (12 V, 100 W), whose radiation was modulated at 200 ± 2 Hz by means of an optical chopper (Oriel 75152) and focused onto one arm of an optical fibre bundle. The modulated light source was guided to the distal end of the optical fibre, where the CO_2/pH sensing reagent was immobilised. The reflected radiation was focused onto the entrance slit of a monochromator (Oriel 77250) and detected by a photomultiplier tube (Oriel 77340), connected to a lock-in amplifier (Stanford Research System SR 510), provided with a pre-amplifier (Stanford Research System SR 552). The lock-in amplifier was synchronised to the same frequency as the optical chopper.

A PC-AT 486 microcomputer was employed to control the monochromator and the lock-in amplifier, through an electronic interface (ADLink ACL-8111). Software was written in Microsoft VisualBasic 3.0 to perform these tasks.

2.2. Reagents and solutions

All chemical reagents were of analytical grade. Buffer solutions were prepared using deionised water and the actual pH of the solutions were determined by employing a pH electrode connected to a pH-meter (Micronal B374).

For the construction of the pH optode, nitric acid, ethanol, *o*-xylene (Mallinckrodt), 3-aminopropyl-triethoxysilane (Aldrich) and bromothymol blue (Grupo Química) were employed. *o*-Xylene was previously dried overnight with anhydrous Na_2SO_4 before using in the silanisation.

Oxalate oxidase (E.C. 1.2.3.4), catalase (E.C. 1.11.1.6) and sodium citrate were purchased from Sigma. Sodium oxalate, sodium salicylate, ascorbic acid, hydrochloric acid, 1-octanol (Merck), ammonium hydroxide, sodium acetate, potassium permanganate (Nuclear), sodium succinate (Aldrich), glutaraldehyde (Fluka) and sodium hydroxide (Synth) were employed as purchased.

2.3. Construction of the CO_2/pH optode

The optode was constructed as previously described [45], by employing a borosilicate bifurcated optical fibre bundle (5 mm diameter), constituted by fibres of 70 μm i.d. The surface of the common end of the optical fibre bundle was initially treated with ethanol, nitric acid and ethanol, in this sequence, and allowed to dry at ambient temperature. Then, the silanisation was carried out by introducing the common end of the bundle into a 5.0% (v/v) 3-aminopropyl-triethoxysilane in *o*-xylene. After this process, the silanised common end was rinsed with *o*-xylene, in order to eliminate any excess of silane, then with ethanol, to clean off the solvent, and allowed to dry at ambient temperature. The immobilisation of the pH sensing reagent was done by introducing the optical fibre tip into a 0.04% (w/v) bromothymol blue (BB) aqueous solution, containing 6.4×10^{-4} mol l^{-1} NaOH, and rinsed with deionised water. After all these steps, the common end of the optical fibre bundle showed an intense blue colour, indicating that the immobilisation was successful.

2.4. Immobilisation of the enzymes

The oxalate oxidase (OxO) and catalase enzymes were immobilised on a barley seed (*Hordeum vulgare*) support, previously ground in a mortar and sized in the range of 20–35 mesh, by means of proper sieves. To 0.5 g of support material, 2500 U of catalase, 8 U of OxO and 5.0 ml of 1% (v/v) glutaraldehyde prepared in 0.050 mol l^{-1} succinate buffer solution (pH 4.0) were added. The mixture was left to react for 12 h at 5°C in order to effect the immobilisation.

Barley seeds were chosen as support material because they naturally contain oxalate oxidase in their composition, offering to the enzyme an environment very close to the natural one, which can improve its stability and, therefore, the lifetime of the enzymatic system. The catalase enzyme was chosen to be co-immobilised with OxO because it shows higher selectivity for hydrogen peroxide than peroxidase enzyme.

The enzymes were immobilised onto the support material by employing glutaraldehyde, a widely used homo-bifunctional reagent. This reagent possesses two reactive carbonyl groups, allowing the formation of inter-molecular cross-linked bonding between the support and the enzyme, through its carbonyl group, which reacts with the amine groups of the enzymes and the support [44].

2.5. Assembly of the system

The system used in this work was based on those described by Fernandes et al. [46], by replacing the CO_2 selective electrode by the optode covered by a PTFE membrane (Black Swan MFG Co.) permeable to this gaseous species. Fig. 1 shows a schematic representation of the system. The stirring bar reactor was filled with 0.5 g of the support material, containing the immobilised enzymes, which was hold in the interior of the reactor with the use of a 390 mesh Nylon® grid. Measurements were carried out in a cell maintained at 25°C with a thermostatic bath (Quimis Q.214.D2).

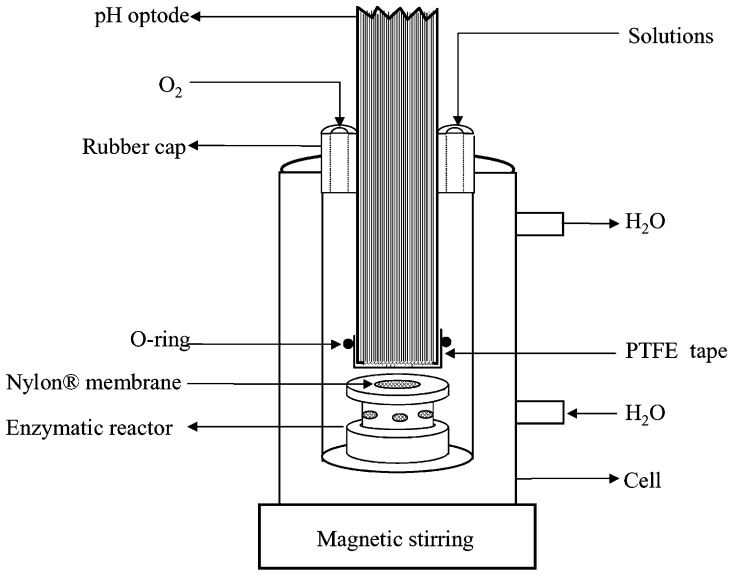

Fig. 1. Schematic diagram of the system for oxalate determination.

2.6. Analytical procedure

Each set of measurements was carried out by pipetting 10.00 ml of succinate buffer solution (in adequate pH) into the cell shown in Fig. 1. A preliminary spectrum was initially run, in order to determine the wavelength of maximum sensitivity (530 nm at pH 4.0). After the proper wavelength had been set, air was bubbled into the solution for 10 min, in order to saturate it with oxygen, and the reflectance was continuously monitored until a steady state was obtained. Then, aliquots of an oxalic acid reference solution were added to the buffer solution and the variation in the diffuse reflectance was monitored. The additions of the solutions were made every 3 min, as soon as a steady state was reached, due to the permeation of carbon dioxide through the membrane.

After a set of measurements, the basic form of the BB indicator was regenerated by immersing the optode in ammonia vapours for 5 min. During the same interval of time, the bi-enzymatic reactor was vigorously shaken inside a cleaning buffer solution, in order to eliminate any residue of analyte from its interior.

2.7. Determination of the kinetic parameters

Curves of reflectance versus time for oxalate concentrations between 0.01 and 0.15 mol l^{-1} were initially plotted. The tangents to these curves were determined, showing an increase as the concentration of oxalate increases. The values of slope of these tangents (reflectance min^{-1}) were divided by the slope of the analytical curve (reflectance μmol^{-1} oxalate), in order to obtain the initial rates of the enzymatic reaction in the proper unit (μmol oxalate min^{-1}). The reciprocal of velocity values correspondent to each oxalate concentration were employed to plot a double reciprocal graph [47], for obtaining the values of the Michaelis–Menten apparent constant and the maximum rate.

2.8. Determination of oxalate in food samples

The performance of the system was evaluated through the determination of oxalate in spinach, comparing the results with those obtained with the AOAC 974.24 official method [48], which is based on permanganometric titration.

Spinach leaves were collected in three different grocery stores of Campinas (Brazil). The old yellow

leaves were discharged and an amount of 2.0 kg of green (new and adult) leaves were used.

Each sample was pre-treated as recommended by the official method. They were liquefied in a commercial liquefier, with 250 ml of deionised water, for 15 min. After cooling down to ambient temperature, the paste was homogenised with a Teflon stirring bar and two portions of 100 g from each sample were transferred to 600 ml beakers. Then, 55 ml of 6.0 mol l^{-1} HCl solution and five drops of octanol (Merck), acting as antifoam agent [15], were added. Each mixture was heated to boiling for 15 min, cooled to ambient temperature and transferred to a 500 ml volumetric flask, which was completed with deionised water. After being vigorously shaken, the mixture was left to stand overnight and then filtered through quantitative filter paper. The first 100 ml of the filtered solution were discharged and the remaining solution (solution 1) was collected in appropriate glass flasks for further determination of oxalate by the standard and proposed methods.

The determination of oxalate by the official method was carried out with 25.00 ml of the solution 1, in which 5 ml of a tungstophosphoric acid solution (prepared by mixing 2.5 g of $Na_2WO_4{\cdot}H_2O$ to 4 ml of H_3PO_4 with concentration 1.2 mol l^{-1}, and diluting to 100 ml) were added and left to stand for at least 5 h. After this period of time, the mixture was filtered, an aliquot of 20.00 ml was taken and the pH was adjusted to 4.0–4.5 with concentrated ammonia. To this solution, 5 ml of a pH 4.5 acetate buffer solution, containing 0.45 mol l^{-1} $CaCl_2$, was added and left overnight in order to precipitate oxalate ions. Afterwards, the precipitate was centrifuged at 1700 rpm and washed with a 0.9 mol l^{-1} acetic acid solution, saturated with $CaCl_2$. Finally, the precipitate was dissolved in 5 ml 1:1 sulphuric acid and titrated with a 0.00200 mol l^{-1} $KMnO_4$ standard solution.

In the proposed method, 10.00 ml of a 0.050 mol l^{-1} succinate buffer solution (pH 4.0) were transferred to the cell and three aliquots of known volume of solution 1 were sequentially added and the variations in the diffuse reflectance were measured. This procedure was carried out in triplicate for each sample. The concentration of oxalate in the sample was determined by interpolation, employing the analytical curve obtained with an oxalate reference solution.

3. Results and discussion

3.1. Effect of the temperature and pH

Table 1 shows the reflectance intensity signals for a 0.030 mol l^{-1} oxalate solution as a function of the temperature (20–35°C) and pH (3.0–5.0), in experiments carried out in a 0.050 mol l^{-1} succinate buffer. As can be noted, the highest sensitivity was obtained at 25°C and pH 4.0. Therefore, the subsequent experiments were carried out in these conditions.

3.2. Interference studies

The interference of ascorbic, citric, acetic and salicylic acids on the determination of oxalate was evaluated. Ascorbic acid was studied mainly because it reacts easily with oxygen and is frequently found in several kinds of samples. Citrate ion was also investigated because it has been employed in the immobilisation of the OxO and oxalate determination, by using an oxalate oxidase-based biosensor [49]. Finally, acetic and salicylic acids were employed as representative types of linear and aromatic monocarboxylic acids.

Table 2 summarises the recovered signals obtained in the determination of a 0.035 mol l^{-1} oxalate solution in the presence of the concomitant species at two concentration levels. Ascorbate ion did not present any interference, probably because the solution was

Table 1
Effect of the temperature and pH on the reflectance intensity (mV), in a solution of 0.050 mol l^{-1} succinate buffer containing 0.030 mol l^{-1} oxalate

Temperature (°C)	Reflectance (mV)		
	pH = 3.0	pH = 4.0	pH = 5.0
20	0.20	1.02	0.78
25	0.25	1.26	0.96
30	0.22	1.12	0.70
35	0.18	1.00	0.80

Table 2
Recovery (%) obtained for the determination of a 0.035 mol l^{-1} oxalate solution in the presence of interfering species

[Interferent] (mol l^{-1})	Acetate	Salicylate	Citrate	Ascorbate
0.030	98	100	95	101
0.050	106	96	61	100

saturated with oxygen before the measurements, which is in accordance with the results obtained by Petrarulo et al. [14], who also employed OxO enzyme from barley seeds for the colorimetric determination of oxalate in blood plasma. The interferences of acetate and salicylate were not significant, indicating that acetate buffer can be used in this determination, instead of succinate buffer. The most significant interference was obtained for citrate ion, mainly when its concentration was higher than the oxalate concentration. This result shows that citrate buffer is not to be recommended for the immobilisation of the enzymes onto barley seeds or for use as a buffer solution for pH adjustment in the proposed system.

3.3. Figures of merit

The analytical performance of the proposed system was evaluated regarding the linear response, lifetime and repeatability. Experiments were carried out in the pH 4.0 succinate buffer solution at 25°C. It is worth remembering that the overall performance of the system depends on the enzymatic reactor as well as the optical fibre sensor. The optode performance itself has been described previously by Sotomayor et al. [45] and is in accordance with the following results.

The bi-enzymatic system showed a linear response range from 0.0080 to 0.100 mol l^{-1}, which can be expressed according to the following equation:

$$\Delta\text{reflectance} = -0.037(\pm 0.045) + 68.70(\pm 0.77)[\text{oxalate}], \quad r^2 = 0.9992 \tag{3}$$

The linear response range shown by the proposed system is broader than those of similar bi-enzymatic systems described in the literature, which employ spectrophotometric [44] and amperometric [50] detection.

The repeatability of the system was evaluated by successively adding four aliquots of a 20 mmol l^{-1} oxalate solution to 10.00 ml of succinate buffer solution, yielding a relative standard deviation of 1.9%. This result shows good agreement those described by Fernandes et al. [44], who have employed OxO and peroxidase immobilised on sorghum seeds.

The lifetime of the system was estimated as being at least 120 h, encompassing the enzyme activity and optical sensor performance. This value was obtained considering the sensitivity of the sensor, measured as the slope of the analytical curve, as a function of time. As can be seen in Fig. 2, the sensor sensitivity decreases abruptly after 120 h, although it can be still used after this period. The lifetime of the present sensor has been found to be equivalent to the lifetime of a similar biosensor [44].

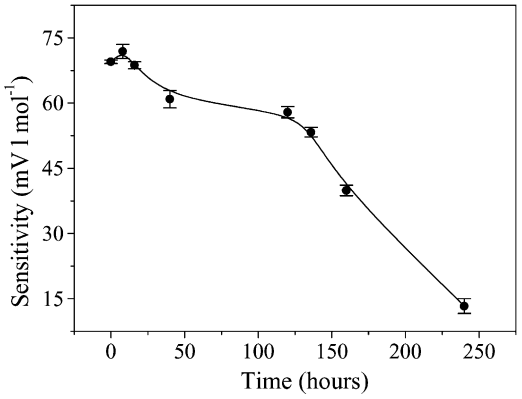

Fig. 2. Sensitivity of the enzymatic system (as the slope of the analytical curves) as a function of time (0.050 mol l^{-1} succinate buffer pH 4.0, 25°C, in the oxalate concentration range from 0.0080 to 0.100 mol l^{-1}).

3.4. Kinetic parameters

In order to verify the behaviour of the system, kinetic parameters such as apparent Michaelis–Menten constant (K_{MM}^{app}) and enzyme activity were also evaluated by employing the Lineweaver–Burk graph [47], in order to verify the behaviour of the system. A value of 0.075 mol l^{-1} for the K_{MM}^{app} was obtained, with maximum velocity of 1908 μmol oxalate min^{-1}.

Although the apparent constant value obtained is higher than those for OxO in solution (1.1×10^{-4} mol l^{-1}) [51], Michaelis–Menten constant values for OxO in the range from 1.3×10^{-1} to 1.6×10^{-4} mol l^{-1} have been described in the literature [17,19,23,24,52], for different procedures of immobilisation. The increases in K_{MM}^{app} for the OxO may be attributed to the mass transfer limitations of the substrate to access the immobilised enzyme [53] and also to the modification of the quaternary structure of the actives sites of the enzyme [13]. Although

Table 3
Results obtained in the determination of oxalate in spinach by the official AOAC method (measured in duplicate) and the proposed method (measured in triplicate)

Sample	AOAC method	Bi-enzymatic method	Relative error
1	735 ± 45	670 ± 4	8.8
2	615 ± 1	580 ± 21	5.7
3	610 ± 40	602 ± 22	1.3

the value of K_{MM}^{app} is high, the production of CO_2 is fast enough to allow a sensitive detection of oxalate using the proposed system.

3.5. Determination of oxalate in spinach

The proposed system was applied to determine oxalate in spinach, which contains ca. 800–1000 mg of oxalate in 1000 g of the plant [54]. The results were compared to those obtained with the AOAC 974.24 official method [48]. Table 3 shows the results obtained, which do not show any significant difference at a 95% confidence level. It is important to note that a determination can be performed in 15–20 min with the proposed method, while it takes 3 days with the official method, indicating the good performance of the bi-enzymatic system.

4. Conclusions

The bi-enzymatic system showed good performance for the determination of oxalate in real samples. The natural-like environment provided by barley seeds for oxalate oxidase improves the lifetime of the enzyme, providing a fast, simple and inexpensive methodology for oxalate determination. Furthermore, the use of an optical fibre chemical sensor as detector seems to be a feasible alternative to the well-established electrochemical sensors. Finally, the proposed methodology for oxalate determination in foods was less laborious and faster than the official method.

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