Determination of Oxalate in Grass by FIA with a Spectrophotometric Detection System Using a Two Enzyme Reactor

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A new methodology for soluble oxalic acid determination in grass samples was developed using a two enzyme reactor in an FIA system. The reactor consisted of 3 U of oxalate oxidase and 100 U of peroxidase immobilized on *Sorghum vulgare* seeds activated with glutaraldehyde. The carbon dioxide was monitored spectrophotometrically, after reacting with an acid-base indicator (Bromocresol Purple) after it permeated through a PTFE membrane. A linear response range was observed between 0.25 and 1.00 mmol l^{-1} of oxalic acid; the data was fit by the equation $A=-0.8(\pm1.5)+57.2(\pm2.5)$[oxalate], with a correlation coefficient of 0.9971 and a relative standard deviation of 2% for $n=5$. The variance for a 0.25 mmol l^{-1} oxalic acid standard solution was lower than 4% for 11 measurements. The FIA system allows analysis of 20 samples per hour without prior treatment. The proposed method showed a good correlation with that of the Sigma Kit.

Keywords Oxalate determination, oxalate oxidase, grass, *Sorghum vulgare* seeds

The oxalic acid determination in urine and plasma has great importance in clinical diagnostics of renal calculus and hyperoxalury, a disease caused by a genetic disorder.[1] In the same way, the amount of this substance in foods such as orange juice, beer, grass, *etc.*, is related with food quality. The oxalic acid present in grass may be prejudicial for neurological systems, bone, demineralization, hypocalcemic and magnesia tetanus, leading to the death of the animals.[2] The levels of this compound in vegetables change according to growth stage, season, soil manuring and species[3,4], sometimes reaching toxic levels.

Several determination methods are described in the literature, such as chromatography[5-8], amperometry[9-12] and spectrophotometry.[13-17] However, the majority present high cost and/or need previous sample treatment to avoid interferences. The use of the oxalate oxidase enzyme [E.C.1.2.3.4] from some vegetable sources, such as barley[18], sorghum[19], spiny pigweed[20] and banana[21], can be an interesting alternative to increase the speed and selectivity, at low cost. Naturally immobilized enzymes may be used several times, allowing automation of the analytical system[22], in contrast to their soluble form. Although the enzyme reacts selectively with substrate, several substances, such as L-ascorbic acid, have presented interferences in all available procedures. The oxalate oxidase catalyzes the oxidation of oxalic acid to CO_2 and H_2O_2 in the presence of oxygen, but the reaction is relatively slow.[23] The immobilization of peroxidase together with oxalate oxidase to improve the reaction speed, increases the carbon dioxide produced.[23] The CO_2 can be monitored after permeation through a PTFE membrane in a permeation cell, without interference, since only CO_2 passes through the membrane. The evaluation of the performance of the new procedure and the application of this to seven different grass samples is described. Comparisons are made with results obtained with the Sigma Kit.

Experimental

Solutions and reactants

Oxalic acid: The solution of this acid was prepared by dissolving a quantity of the solid in 0.05 mol l^{-1} (pH=4.0) succinate buffer containing 1.0×10^{-3} mol l^{-1} edta (dissodium salt, Merck) to obtain a 5.0×10^{-2} mol l^{-1} solution.

Bromocresol Purple (BCP): A quantity of 0.27 g of the indicator was dissolved in 20 ml of ethanol and the volume was completed to 500 ml with doubly distilled water, previously deaerated by heating, obtaining a stock solution of 1.0×10^{-3} mol l^{-1}. This solution was always prepared on the same day as the experiment, eliminating carbon dioxide. The solution pH was adjusted to 7.0 with NaOH and stored in a flask protected against ambient CO_2.

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Enzyme immobilization

Ten grams of *Sorghum vulgare* seeds (variety BR303) were ground in a mortar and sieved through a 20 mesh Granutest® Sieve. The pieces were washed with distilled water and dried at room temperature, and then these were immersed in a 25% (w/v) glutaraldehyde solution for 12 h at 5°C. After this time the supernatant was rejected and the sorghum seeds were washed 10 times with water, 4 times with a 0.10 mol l^{-1} glycine (Carlo Erba) solution and 4 times with water. About 1 g of this material was immersed in 1 ml of a solution of succinate buffer containing 3U of oxalate oxidase [E.C.1.2.3.4] and 100U of peroxidase [E.C.1.11.1.7]. This mixture was suspended for a week at 5°C and, after this time, the material was washed with succinate buffer. The obtained material was used to make a two enzyme reactor.

FIA system

The FIA system consisted of a peristaltic pump (Ismatech Model mp13GJ4), a spectrophotometer (Zeiss Model PM2D), an automatic injector/comutator (Micronal Model B352) and a chart recorder (Cole Parmer 9375). Tygon tubes from Technicon of different internal diameters and a polyethylene tube of 0.7 mm of internal diameter were used in the system. The permeation cell consisted of two acrylic pieces with a groove 0.5 mm in depth and 100 mm of length in each, separated by a Teflon membrane.

Grass sample preparation

From about 10 m^2 of pasture, young grass blades were collected cutting at 10 cm from apical. Seven different species of grass were collected and conditioned in paper bags. After sampling, the grasses were weighted and put in a ventilated oven at 60°C, for 48 h. After this, the samples were cooled to room temperature, weighted again, ground in a mill and stored in plastic bags. The loss of weight during this procedure is shown in Table 1. Amounts of 200 mg of these samples were introduced into flasks containing 10.0 ml of succinate buffer containing 1 mmol l^{-1} of edta (disodium salt) and the flasks were closed. These flasks were put in a water bath with the temperature controlled to 60°C for 12 h. The oxalic acid extracted into the supernatant was analyzed.

Procedure

A polyethylene tube of 2 mm of internal diameter and 100 mm of length was filled with 273 mg of sorghum seeds containing the two immobilized enzymes, which was used as a two enzyme reactor. The reactor (ER) was inserted into the flow system as illustrated in Fig. 1. Quantities (100 µl) of standard solutions of oxalic acid were injected, as well as the grass samples, which, upon passing through the reactor, produced carbon dioxide. The segment of the sample containing this gas in contact with an acid solution aided its permeation through the membrane to the Bromocresol Purple indicator solution (pH=7.0) in the other stream. The changes of absorbances due to the alteration in the acid base equilibrium of the indicator, monitored at 540 nm, were recorded. These changes were proportional to the oxalic acid concentration. The samples were also analyzed by a commercial Sigma Kit (procedure number 591C).

Table 1 Weight of grass for pasture samples collected

Grass	m_v	m_{aps}
Quicuio	217	64.0
Tifton-85	162	50.0
Tangola	280	87.0
Brachiaria decumbens	207	64.0
Setaria splendida	314	73.0
Setaria anseps	216	53.0
Tanner glass	163	51.0

m_v=weight in gram of the vegetal "in natura" and m_{aps}= weight in gram of the dried samples.

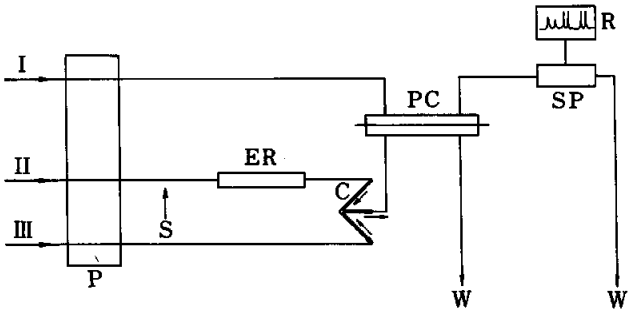
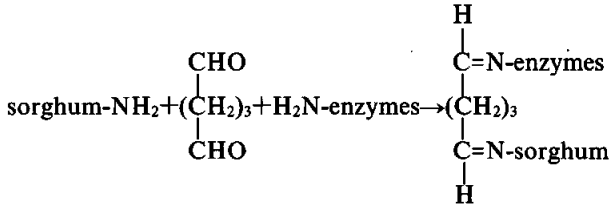


Fig. 1 FIA system with spectrophotometric detection. I: solution of Bromocresol Purple (5.0×10^{-5} mol l^{-1}); II: 0.05 mol l^{-1} succinate buffer solution at pH 4.0, containing 0.001 mol l^{-1} of Na_2H_2edta; III: 1.0 mol l^{-1} phosphoric acid solution; P: peristaltic pump; ER: enzymatic reactor; S: sample; C: confluence; PC: permeation cell; SP: spectrophotometer; R: chart recorder and W: waste.

Results and Discussion

Several enzyme immobilization procedures have been used to increase the enzyme stability by make the possible reutilization the enzyme, thus decreasing the cost per analysis.[23] Among several immobilization procedures, the use of a bifunctional reagent such as glutaraldehyde, which promotes immobilization by crosslinked bonding, has been employed extensively.[24] The immobilization procedure of the enzymes by glutaraldehyde on sorghum seeds can be represented as:



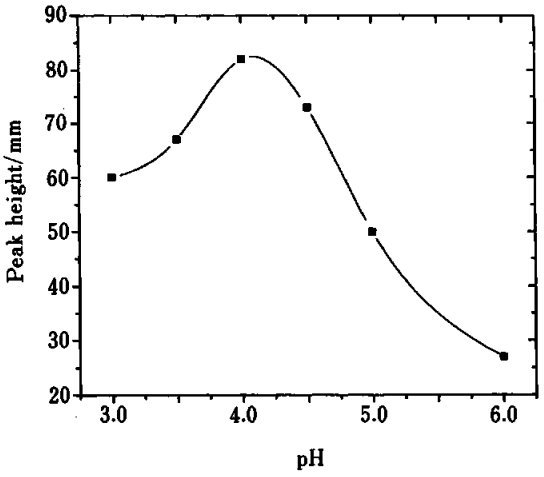


Fig. 2 Influence of pH on activity of the immobilized enzymes. Signal obtained for 0.50 mmol l^{-1} oxalic acid prepared in succinate buffer. The temperature was 25°C and the dimension of enzyme reactor was 100 mm (length) and 2 mm (inner diameter). The sample volume injected was always 100 µl.

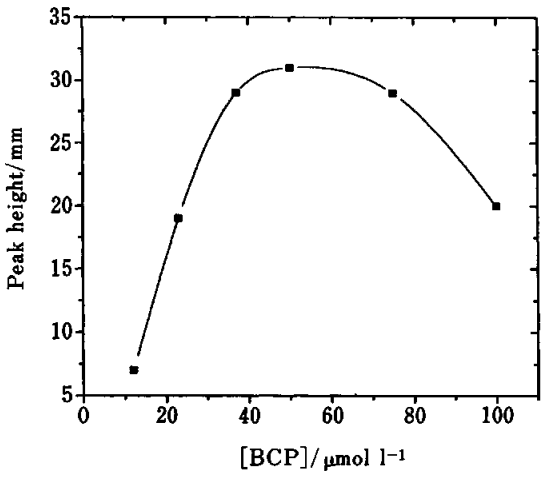


Fig. 3 Effect of the concentration of indicator (BCP) on analytical signal. Oxalic acid concentration was 0.5 mmol l^{-1}; sample volume was 100 µl and the temperature was 25°C.

The immobilization should occur by the reaction between amino groups of the sorghum seeds and aldehyde groups of the glutaraldehyde molecule, while the other aldehyde group can react with amino groups of the enzymes. Additional interactions are possible, such as those of hydrophilic or hydrophobic regions of the matrix with those of the enzyme. The stability of the immobilized enzyme is very good, showing the same activity for at least three months when stored in a refrigerator.

Several arrangements of the FIA system were tested to reach high sensibility, speed of analysis and reproducibility. The temperature was fixed at 25.0±0.5°C, although a temperature change does not cause significant influence on the response of the system. Figure 2 shows the behavior of the response as a function of pH. For pH<4 the response decreased, probably due to a significant decrease in peroxidase activity, while for pH>4 the activity of oxalate oxidase is decreased.

The indicator concentration was tested to verify the best response for spectrophotometric detection. Its behavior is shown in Fig. 3. Thus the concentration of the indicator was fixed as 5.0×10^{-5} mol l^{-1}, for all measurements. This behavior may be explained as being the best linear range for absorbance decrease due to deprotonation of the Bromocresol Purple.

The best conditions were established as being a sample loop of 100 µl; reactor of 100 mm length and 2 mm internal diameter and a flow of 1.0 ml min^{-1} (carrier and indicator) as can be observed from the results presented in Table 2. This condition allows an analyses' frequency of 20 samples per hour. A great advantage of this methodology is that it does not need the use of a coil after gas permeation for stabilization of the absorbent species. This is presumably due to the phosphoric acid confluence system that dislocates the equilibrium towards carbon dioxide liberation.

Table 2 Operational parameters of FIA system: carrier of 0.05 mol l^{-1} succinate buffer at pH 4.0, containing 1.0 mmol l^{-1} of Na_2H_2edta; temperature of 25.0±0.5°C and oxalic acid solution of 0.50 mmol l^{-1} prepared in same buffer as the carrier

Parameter		Performance	
Flow (ml min^{-1})	0.5	Signal/time[a]	2.6
	0.7		3.1
	1.0		3.3
	1.3		3.0
	1.5		1.7
Sample loop (µl)	10	Signal/time[a]	1.5
	25		2.6
	50		2.8
	100		4.7
	200		4.2
L/d ratio of enzymatic reactor	5.0(10)	Signal/time[a]	10.0
	3.3(10)		7.5
	2.5(5)		8.0
	1.7(5)		6.7
	10 (20)		6.3
	6.7(20)		6.0

a. Time of washing necessary until the signal reaches the base line.
L=length; d=inner diameter of the reactor.
The values between brackets represent the length of reactor in mm.

Using these pre-established conditions, successive injections of oxalic acid standard solutions and grass samples were performed. A diagram of these injections is shown in Fig. 4. A linear response range between

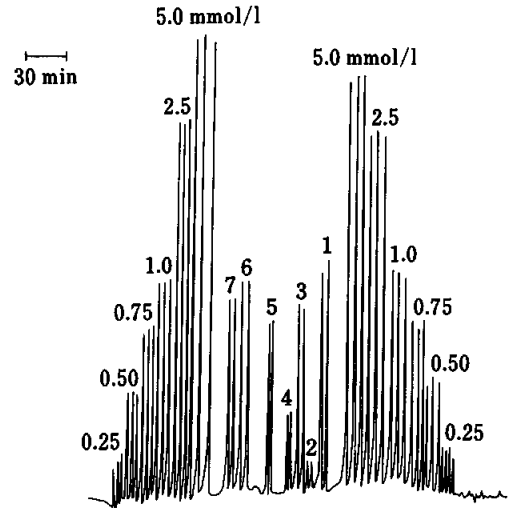


Fig. 4 Diagram obtained for standard oxalic acid solution and grass samples, using a 0.05 mol l^{-1} succinate buffer at pH 4.0 containing 1.0 mmol l^{-1} of edta as carrier. From right to left ascendent sweeping for standard oxalic acid solutions, samples and descendent sweeping for standard solutions. The samples were diluted to linear range before the injection. The temperature was 25°C and the dimension of enzyme reactor was 100 mm (length) and 2 mm (inner diameter). The sample volume injected was always 100 µl.

0.25 and 1.00 mmol l^{-1} was observed; the data were fit by the equation $y=-0.8(\pm1.5)+57.2(\pm2.5)$[oxalate], with a correlation coefficient of 0.9971 and a relative standard deviation of 2% for $n=5$. The samples were diluted to linear range. The repeatability of the system during the samples analysis was verified by injecting a 0.25 mmol l^{-1} oxalic acid solution for 11 times, after different samples and the relative standard deviation was about 3.8%.

The system was applied to determine soluble oxalic acid from grass of 7 different samples (represented by seven different numbers in Fig. 4) and the method proposed was compared with one that is commercially available.[2] The results calculated by the equation of the standard solutions considering the dilutions' factors are presented in Table 3 and show a good correlation between the methods. The correlation between the methods was adjusted by the equation $y=-0.44(\pm10.5)+1.004(\pm0.011)$ with a correlation coefficient of 0.9997.

The main advantage of the proposed method is the high stability of the system, allowing at least 200 determinations without significant change in sensitivity, lowering the cost per analysis. After three months the activity of the two enzyme reactor had decreased to about 70%, of its initial activity while in continuous use.

The enzyme immobilization procedure on sorghum seeds presented excellent stability and performance, allowing a high sensitivity of the system for oxalate detection in real samples. The developed methodology shows good repeatability (about 4%) and excellent correlation with a commercially available method from Sigma, even without prior treatment. The great number of analysis using the same system makes the cost per analysis cheap enough to apply in real samples, principally in grass samples used for animal pasture.

Table 3 Quantities of soluble oxalic acid present in samples of pasture grass determined by the proposed method and by the Sigma Kit

Sample	m_{dm}/mg	Proposed method[a]	Sigma Kit[a]
1 Quicuio	210	595	624
2 Tifton-85	233	56	56
3 Tangola	275	596	589
4 *Bracharia decumb.*	257	132	130
5 *Setaria splendida*	217	1456	1430
6 *Setaria anseps*	204	1912	1914
7 Tanner glass	216	394	380

a. The values are expressed in mg of oxalic acid per 100 g of the dried sample. m_{dm}=dried weight of the sample analyzed.

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