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Ascorbic Acid Determination in Biological Fluids Using Ascorbate Oxidase Immobilized on Alkylamine Glass Beads in a Flow Injection Potentiometric System

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ABSTRACT

A flow injection method was developed aimed at the determination of ascorbic acid in biological fluids, particularly fruit juices. The enzyme ascorbic oxidoreductase (EC 1.10.3.3), extracted from *Cucurbita maxima,* was immobilized onto alkylamine glass beads using glutaraldehyde as a bifunctional agent. The ascorbic acid concentration was related to oxygen saturation. Fall in oxygen concentration, as a result of ascorbic acid oxidation, was detected by a low cost, homemade oxygen electrode. The calibration graph was linear over the range 0.05 to 3.00 m*M* (RSD 1%), the maximum number of samples that could be analysed was 90/h. The immobilized enzyme retained its initial activity for 2 mo with more than 600 assays.

Index Entries: Flow Injection Analysis (FIA); ascorbic acid; oxygen electrode; glass beads; *Cucurbita maxima.*

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INTRODUCTION

Ascorbic acid (vitamin C) can not be synthetized by humans and certain mammals, but it is present in many fruits and vegetables. It is a relatively strong reductant, and much loss may occur during processing or storage (*1*). Ascorbic acid levels can be used as an indicator of freshness. Frequently, it is used as an antioxidant in industrial processed food, also the determination of ascorbic acid concentration in blood and urine is important as a clinical indicator (*1,2*).

Many classical chemical methods have been developed for determination of vitamin C, such as, reduction of 2,6-dichlorophenolindophenol, derivatization with 2,4-dinitrophenylhydrazine, fluorimetric determination, and dipyridyl reaction (*3*). However, these methods suffered from a lack of specificity, owing to interference from other reducing agents such as cysteine, glutathione, acetol, and iron (II), which are often present in biological fluids and plant extracts or with sulfite, which is commonly added to soft drinks as a preservative (*3–6*). Other more specific analytical procedures have been applied, requiring expensive manipulation and separation techniques, such as High Performance Liquid Chromatography (HPLC) (*7*).

However, since the 1980s some inexpensive and simple analytical procedures or devices have been proposed for determination of ascorbic acid, most of them using Flow Injection Analysis (FIA), that can provide a fast procedure compared to batch systems. Lazaro et al. (*5*) developed a flow injection spectrophotometric system for simultaneous determination of ascorbic acid and sulfite in fruit juices based on the reaction of these species with chloramine T. Uchiyama et al. (*8*), described a flow injection method for vitamin C using cucumber juice as the carrier, which has a high ascorbate oxidase activity, using amperometric detection. Schaffer et al. (*9*) proposed a new method using a fibre optic ascorbic acid biosensor as a transducer in a flow injection configuration, which had ascorbate oxidase immobilized onto the surface of a oxygen optrode, these systems also had internal automatic dilution and buffering. Greenway and Ongomo (*4*), built up a flow injection system with ascorbate oxidase immobilized in cyanogen bromide activated-sepharose 4B, with three amperometric electrode detectors, including a Clark oxygen electrode, with two Sepharose columns, one of them being a reactive column and other a blank column. Verma et al. (*6*) described a flow injection spectrophotometric determination of ascorbic acid in pharmaceuticals by background correction. Uchiyama and Umetsu (*10*) developed a batch system for determination of ascorbic acid based on a concentration-step amperometric sensor using Cucumber juice in a thin porous carbon felt and a Clark oxygen electrode as a transducer. Korel and Lennox (*2*), developed a organic conducting salt electrode to determine ascorbate levels in aqueous samples. Fung and Mo (*11*) applied square-wave voltametry for

the determination of ascorbic acid using a flow injection system. Last year (1992), we reported a biosensor using ascorbate oxidase immobilized on alkylamine glass beads using a low cost homemade potentiometric oxygen electrode in a batch system (*12*). This paper describes the application of this simple technique for the determination of ascorbic acid in soft drinks and fruit juices samples using a flow injection configuration.

MATERIALS AND METHODS

Chemicals were purchased from Sigma Chemical Co (St. Louis, MO) and VETEC (Rio de Janeiro, Brazil) and were reagent grade or better. The alkylamine glass beads were obtained from H. H. Weetall (Ciba-Corning Diagnostics, Medfield, MA).

The enzyme (ascorbic oxidoreductase EC 1.10.3.3.) was extracted from *Cucurbita maxima* and partially purified by ammonium sulphate precipitation (40–60% (w/v) cut) following the procedure of Carvalho et al. (*13*). Protein concentrations were determined by the Lowry method using bovine serum albumin as the standard (*14*).

Commercial fruit pulps were purchased from COMIMSO Ltda. (Frutotal) and Sorvetes e Produtos Alimenticios do Nordeste S.A. (Kibon).

IMMOBILIZATION

The enzyme was immobilized using glutaraldehyde as the bifunctional agent through Shiff base formation on alkylamine glass beads (500 Å pore size 80–120 mesh) according to Weetall (*15*).

FLOW INJECTION SYSTEM

A schematic diagram of the flow system is shown in Fig. 1. A plastic column (8 cm in length, 0.5 cm in id) was filled with the immobilized enzyme. A solution of ascorbic acid (0.05–10 m*M*, 180 μL) was injected into the system and carried by a citrate–phosphate buffer (pH 6.0, 0.1*M*) at a flowrate of 2.9 mL/min upward throughout the column containing the immobilized enzyme. The electrode was located immediately above the packed bed of immobilized enzyme. The electrode chamber had a volume of 0.75 mL and the electrode one of 0.42 mL leaving a sample volume (for flowthrough) of 0.33 mL. At a flowrate of 2.9 mL/min the mean residence time in the chamber can be calculated to be of the order of 7 s. The experiments were performed at room temperature (23°C) and the column was stored with the same buffer at 4°C. Fall in oxygen concentration, as a result

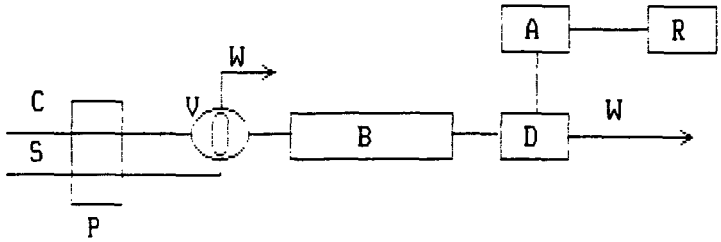

Fig. 1. Flow injection manifold for the determination of ascorbic acid in fruit juices. (S) sample; (C) buffer flow, 2.9 mL/min; (W) waste; (P) peristaltic pump; (V) injection valve, 180 μL; (B) column (8 cm in length, 0.5 cm i.d.) containing ascorbate oxidase immobilized on alkylamine glass beads; (D) detection flow cell with oxygen electrode; (A) amplifier; (R) recorder.

of ascorbic acid oxidation, was detected by a potentiometric oxygen electrode, described by Marques Jr. and Lima Filho (*16*). The oxygen consumed by the enzyme reaction is proportional to the ascorbic acid content of the sample.

The system was calibrated with ascorbic acid solution (50 m*M*) and appropriate dilutions, which were prepared freshly each day. The results were calculated as an average of threefold or more repeated measurements. This system was used to determine ascorbic acid concentrations in commercially available fruit pulp. The fruit sample pulp were diluted to the linear range of the calibration curve with citrate–phosphate buffer solution (pH 7.0, 0.1*M*) to protect the biosensor from the low pH present in these juices, then filtered.

CARRIER STREAM pH CURVE

The pH curve was determined using a citrate–phosphate buffer (0.1*M*, pH 5.0, 6.0, 7.0) and boric acid–borax buffer (0.1*M*, pH 8.0) as carrier stream.

RESULTS AND DISCUSSION

Crude ascorbate oxidase from *Cucurbita maxima* had a specific activity of 0.24 m*M* ascorbic acid/min/mg protein. After purification this increased to 2.86 m*M* ascorbic acid/min/mg protein (a purification factor of approx 12). The specific activity of the immobilized preparation was 1.91 m*M* ascorbic acid/min/mg protein indicating a 33% loss of specific activity on immobilization.

The effect of pH on the carrier stream on the activity of the immobilized enzyme was studied from pH 5.0 to 8.0 (Fig. 2). The results have shown that the maximum response was obtained at pH 6.0.

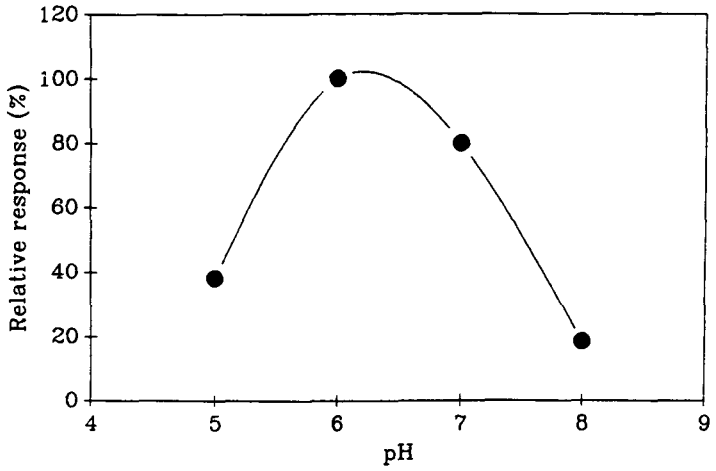

Fig. 2. Effect of carrier stream pH on the activity of immobilized ascorbate oxidase. Citrate-phosphate buffer (0.1M, pH 5.0, 6.0, 7.0) and boric acid-borax buffer (0.1M, pH 8.0); 0.8 m*M* of ascorbic acid.

Typical responses to ascorbic acid, obtained by the flow injection method are shown in Fig. 3. This output shows that continuous determinations are easily possible.

The linear range of the system for different column lengths was also examined (Fig. 4). The linear range increased with increasing column length. This could be explained based on the increase of sample dispersion into the column that increased dilution. The choice of column length needs to be related to the desired concentration range. A column with 8.0 cm length (0.5 cm i.d.), was suitable for the linear response in the range between 0.05–3.0 m*M*. However, it is still possible to determine ascorbic acid until concentrations of 6 m*M* (Fig. 5). This range is compared to other FIA systems with ranges such as: 0.23–0.96 m*M* (*5*), 0–0.11 (*6*), 0.5–7 m*M* (*8*), 0.05–6 m*M* (*9*), 0.002–6 m*M* (*11*).

The effect of sample volume on the peak height and sampling rate was studied from the range 100–220 μL. A loop with 180 μL was found to be the best volume for the relation between sensitivity and sample rate (Fig. 6). The response with a sample volume of 220 μL was 200% greater then a sample of 100 μL. The sample rate for a 100 μL volume was 40% faster than that for 220 μL.

The effect of flowrate on the response and sampling rate was studied from 0.75 to 5.3 mL/min (Fig. 7). The choice of flowrate involves a compromise between sensitivity and sampling rate. A flowrate of 2.9 mL/min was then chosen. The sample rate throughput was 50/h. With a flowrate of 5.3 mL/min, 90 samples/h could be analyzed, but at a loss of 80% of maximum column conversion rate. This is the sample rate that we chose, is according to those obtained by other FIA systems (Table 1).

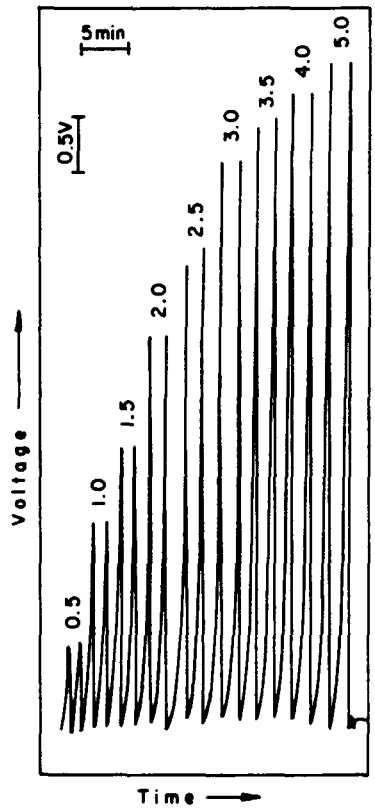

Fig. 3. Recorder output for the calibration graph. The peak corresponds to the concentration of ascorbic acid (m*M*) in duplicate. Column length, 8 cm; flow-rate, 2.9 mL/min; sample volume, 180 μL; pH 6.0.

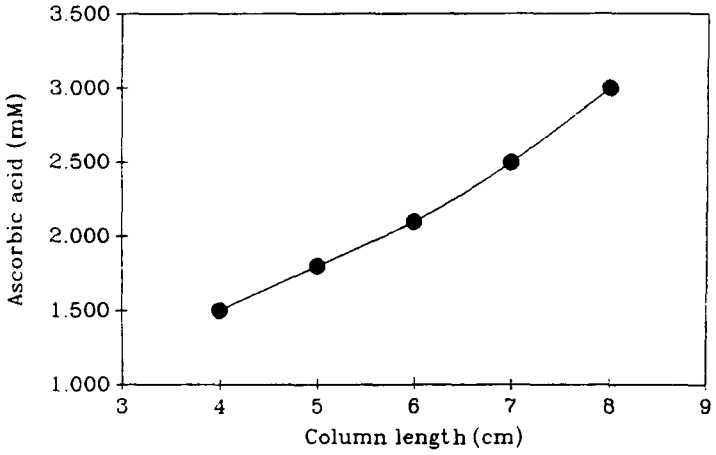

Fig. 4. Relationship between column length (0.5 cm i.d.) and maximum concentration of ascorbic acid measureable with a linear response. Flowrate, 2.9 mL/min; sample volume, 180 μL; pH 6.0.

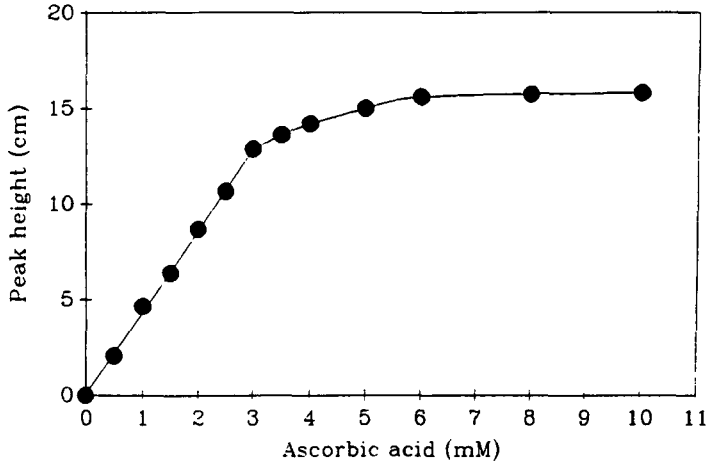

Fig. 5. Calibration curve for ascorbic acid obtained by FIA. Column length, 8 cm; flowrate 2.9 mL/min; sample volume, 180 μL; pH 6.0.

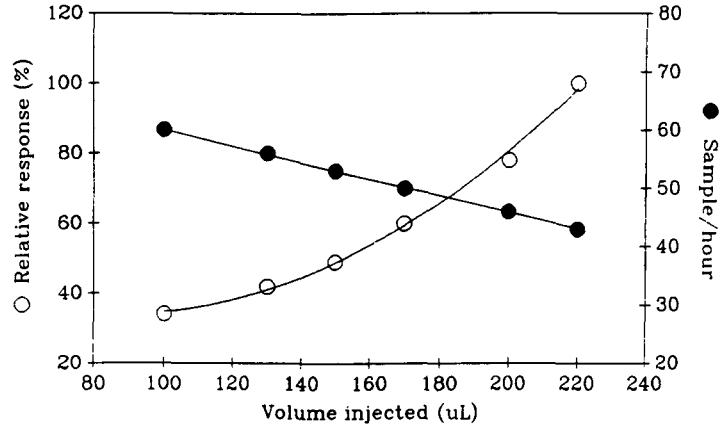

Fig. 6. Effect of injected sample volume on the relative response for ascorbic acid. Column length, 8 cm; flowrate, 2.9 mL/min; ascorbic acid concentration, 0.5 m*M*; pH 6.0.

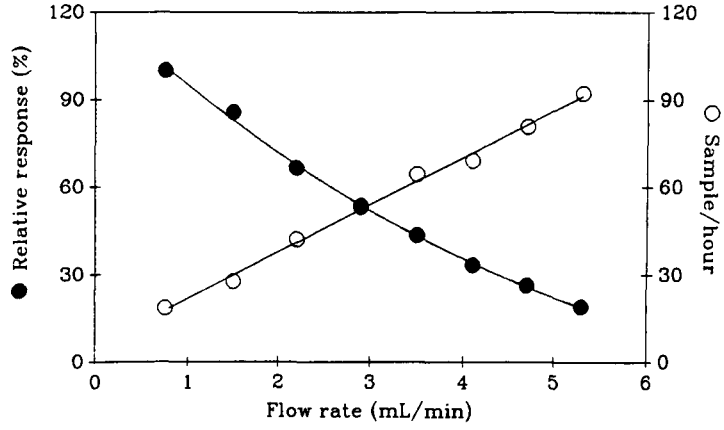

Fig. 7. Effect of flowrate of the carrier stream on the relative response for ascorbic acid. Column length, 8 cm; sample volume, 180 μL; ascorbic acid concentration, 1.5 m*M*; pH 6.0.

Table 1
Relationship Between Sampling Rate and Flowrate

Reference	Flowrate, mL/min	Samples/h
4	2.8	30
5	1.5	45
8	4.8	60
9	—	40
11	0.5	60
(This work)	2.9	50

Table 2
Results of Ascorbic Acid Determination
(Average of a Fivefold Repeated Measurement)
in Fruit Juices Obtained Using FIA Oxygen Electrode System

Sample	Source	Ascorbic acid in pulp, m*M*
Acerola (*Malpighia coccifera L.*)	Frutotal	41.20
Caju (*Anacardium occidentale L.*)	Frutotal	13.40
Graviola (*Anona cearensis Barb. Rodrig.*)	Kibon	0.90
Pitanga (*Myrcia ramulosa D.C.*)	Kibon	0.66
Caja (*Spondias Lutea brasiliensis*)	Kibon	0.60

The effect of buffer ionic strength on the biosensor response was investigated over the range 0.025–0.4*M* and no variation in response was detectable.

The enzymatic column has been operated satisfactorily for 2 mo with more than 600 assays, without loss of activity. In comparison with the lifetime, without loss of activity, obtained with other enzymatic methods, e.g., 8 d (*8*), 3 wk (*4*), these results are much better than expected and than those described in the literature.

The FIA system showed a sensitivity of 195 mV/m*M* ascorbic acid tested over the range 0.05–3.0 m*M*. (This response was prior to any voltage amplification for recording purposes.)

The relative standard deviation (RSD) obtained with 10 injections of ascorbic acid (1.5 m*M*) was 1%. The RSD obtained with other FIA systems are reported between 0.6–4.0% (*4–6,8,9,11*).

Ascorbic acid determination in fruit juices could be performed more rapidly with this technique than with the classical analytical procedures (*9*). Table 2 shows the results of the ascorbic acid determination (average

of fivefold repeated measurement) in fruit juices prepared from commercial pulps.

CONCLUSION

The results obtained in this work show that the flow injection system described here with an immobilized ascorbate oxidase reactor, and low cost homemade oxygen electrode, offers a fast, precise, and cheap procedure for the determination of ascorbic acid.

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