SCREEN-PRINTED ELECTRODE MODIFIED WITH SILVER HEXACYANOFERRATE-NAFION® FOR ASCORBIC ACID DETERMINATION

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ABSTRACT

A flow injection system using a new and/or re-used graphite screen-printed electrode modified with silver hexacyanoferrate and a Nafion® polymer layer (AgHCF/GSPE) was employed for the determination of ascorbic acid in orange juice and drugs samples. Both modified electrodes showed an initial sensitivity of $0.015 \text{ A.cm}^{-2}.\text{M}^{-1}$, with a linear response over the range from 50 to 300 mg L^{-1} , relative standard deviations smaller than 2%, detection and quantification limits of *c.a.* 5.0 and 25.0 mg.L^{-1} . The response of the electrodes was stable, with no variation of base line after 6-8 hours of continuous operation. With this system it is possible to measure 65-70 samples ($20 \mu\text{L}$) per hour. By using 1.06 mg of AgNO_3 , 4.12 mg of $\text{K}_3\text{Fe}(\text{CN})_6$, 50.5 mg of KNO_3 and $800 \mu\text{L}$ of HNO_3 , it is possible to obtain between 25 and 30 modified electrodes. The proposed system showed to be inexpensive, versatile, robust and suitable for industrial application. The surface morphology of the bare and/or modified graphite screen-printed electrode was characterized by scanning electron microscopy.

Keywords: Silver hexacyanoferrate; Ascorbic acid; Food and pharmaceutical sample; Re-used screen printed electrodes; Flow amperometry

INTRODUCTION

The theory, practical considerations, and importance of ascorbic acid determination have been discussed before¹⁻³. Numerous methods based on flow injection analysis⁴⁻¹¹, spectrophotometry¹²⁻¹⁴, chromatography¹⁵⁻¹⁷ and others are available for the determination of ascorbic acid in different matrices. However, when a rapid determination is needed^{18,19}, procedures involving modified electrodes are more convenient²⁰⁻²¹. The use of screen-printed electrodes (SPE) in analytical applications has many advantages compared to other systems due to their unique properties such as small size, fast response time, low cost, good reproducibility etc.

Many papers have been published using these electrodes with very promising results²²⁻²⁶. For example, the use of metal hexacyanoferrates (MHCF) such as Prussian Blue (PB) features interesting electrochemical, electrochromic, photophysical and magnetic properties^{22,27}. Despite the success of sensors and biosensors²⁸ developed based on thin films of PB, the analogous - Mn, Cu, Ni, Co and so forth., and also mixed-metal hexacyanoferrates (Ni-Tl or Fe-Cu among others) have also received increasing attention recently²⁹.

The use of Prussian Blue to chemically and electrochemically modify SPE's was only reported in 2001³⁰ and 2003²² respectively, despite their versatility, simplicity and suitability of modifying surfaces with metal hexacyanoferrates. Nowadays, SPE's are being used for an increasing number of applications, demonstrating their potentiality in electroanalysis^{28,29,31}.

Silver hexacyanoferrate film or silver nanoparticles has been used for determination of silver, butylated hydroxyanisole (BHA), dopamine, uric acid and so forth³²⁻³⁴. We have previously reported the preparation and characterization of conducting polymer/silver hexacyanoferrate nanocomposite³⁵, although we did not use that material for any determination.

In this paper, a new or re-used graphite SPE was modified with silver hexacyanoferrate (coated with Nafion) for ascorbic acid determination in orange juices and medicaments. To the best of our knowledge, the use of this thin film electrochemically deposited onto SPE to determine ascorbic acid or other species has not yet been reported. The synthesis of the AgHCF film presented was realized under galvanostatic condition in the presence of AgNO_3 , $\text{K}_3\text{Fe}(\text{CN})_6$, KNO_3 and HNO_3 , and the operational stability of the film was evaluated by injecting a standard solution (ascorbic acid in phosphate buffer) over several hours in a flow-injection system with the electrode polarized at -50 mV versus Ag/AgCl. The method is based on the electrocatalytic oxidation of ascorbic acid by silver hexacyanoferrate, and the use of the (re-used) screen-printed electrode and flow injection system contribute to a versatile and low-cost approach, well suited for industrial applications and environmental. The use of Nafion® polymeric membrane improves versatility and applicability since foreign species are readily repelled.

EXPERIMENTAL

Reagents, standards and samples

All solutions were prepared with distilled water with analytical-grade chemicals without additional purification. Solutions used for deposition and activation of the AgHCF film onto the graphite SPE were prepared by mixing (i) 1.25 mM AgNO_3 + 2.5 mM $\text{K}_3\text{Fe}(\text{CN})_6$ + 0.1 M HNO_3 + 0.1 M of KNO_3 (solution defined as S_1); (ii) 0.1 M of HNO_3 plus 0.1 M KNO_3 (solution S_2) and (iii) 0.25 M of KH_2PO_4 plus 0.13 M K_2HPO_4 (solution S_3 or phosphate buffer solution, PBS, pH 5.5). The working standard solution of ascorbic acid ($50 - 300 \text{ mg.L}^{-1}$) was prepared by diluting a fresh stock solution (1000 mg.L^{-1}) with solution S_3 .

The Nafion® solution was diluted to 1% (v/v) with ethanol and the pH of the solution was adjusted to 5.5 by adding a concentrated solution of NH_4OH .

Samples of orange juice and drugs were obtained from local stores. All orange juices were produced in Chile. Drug samples were: #1 and #6 from China; #2, #3 and #4 from Chile and #5 from Colombia. Before introduction into the flow-injection system, the samples (orange or drugs) were suitably prepared and diluted with PBS; aliquots of the natural orange juice were diluted between 1000 and $8000 \mu\text{L}$ in 100 mL of buffer solution; equivalent mass of the tablet was diluted in 100 mL distilled water and after $1000 \mu\text{L}$ of this solution was diluted in 250 mL of buffer solution.

For comparative studies, the HPLC method³⁶ was used employing a 100-RP-18 250x4.6 mm column and $5 \mu\text{m}$ LiChrospher (USA). Before introduction (20 μL) the sample solution was filtered using a standard filter paper (Whatman, qualitative).

Instruments and apparatus

Cyclic voltammetry was conducted using a PS potentiostat system (Palm Instruments BV, The Netherlands) connected to a PC. A three-compartment electrochemical cell suited with electrodes in the form of an electrochemical sensor type AC1.W.R (BVT Technologies, Czech Republic) was used. Hydrodynamic amperometric detection experiments were performed with a PS potentiostat using a constant potential of $E_{\text{appl}} = -50 \text{ mV}$, an interval of 0.05 s and time_{run} between 1000 and 3000 s. Flow-injection experiments were carried out with an ALITEA eight-channel peristaltic pump (Sweden) equipped with Tygon tubing, a home-made injector and tubing (0.8-mm i.d., wall thickness < 0.2 mm). The flow-cell (Fig. 1), which ensured the wall-jet flow around the working electrode (model FC₁) was fabricated by BVT Technologies (Czech Republic). The surface morphology of the graphite SPE was characterized by scanning electron microscopy (SEM) with a Shimadzu Superscan SS-550 microscope (Japan). Before material characterization, the electrodes were coated with a 20-25 nm Au layer.

Figure 1. FI amperometry system employed for ascorbic acid determination. (A) Profile view of the electrochemical cell with the screen-printed electrode during the AgHCF thin film preparation. (B) General view of the FI system used (peristaltic pump, Tygon tubing, injector, flow-cell and potentiostat connected to a computer). (C) Detail of the wall-jet flow-cell used (showing the in/out channel) with the SPE (BVT Technologies).

Procedure for AgHCF/GSPE modified electrode

New graphite screen-printed electrodes were used as received. The reutilization of the electrode was done after 6-8 hours of continuous using. This last was treated in alkaline media (100 mM) and distilled water before new electrodeposition (below, steps i-iv). The figure 2 show the CV profile of the film deposited onto the new and re-used electrode.

Figure 2. Cyclic voltammogram of the AgHCF film obtained in 0.1 M of KNO_3 plus 0.1 M HNO_3 (solution S_2) with new (red) and re-used SPE electrode (blue). Scan-rate = 50 mV s^{-1} . The potential scan was initiated at 350 mV vs. Ag/AgCl.

Unless specified, for cyclic voltammetry experiments the scan rate was 50 mV s^{-1} . The silver hexacyanoferrate films were deposited on the graphite SPE using the cell showed in figure 1A.

The method used was: (i) Using the solution S_1 (around 20 mL), the SPE electrode was submitted to an anodic current of 1 mA during 45 s. After keeping the system under standby conditions for 20 s, the potential was cycled between 800 and 100 mV versus Ag/AgCl (15 cycles); (ii) Using the solution S_2 , it was done the activation of the film. After careful washing with water, the electrode was cycled three times between 800 and 100 mV versus Ag/AgCl; (iii) After washing with water, the modified electrode was left to dry for 10-20 minutes at room temperature; (iv) The modified electrode was conditioned in solution S_3 ; the dried AgHCF graphite SPE was kept at -50 mV versus Ag/AgCl for 10 min.

The ready AgHCF-graphite SPE was immersed in solution S_3 for 5-10 minutes to attain equilibrium conditions. In order to minimize the interfering species, the AgHCF modified electrode was covered with Nafion® polymer layer. This solution was diluted and neutralized to pH 5.5 before using; it was dropped 5 to 10 μL onto the working electrode.

The FI system was used for verifying the operational stability of the modified electrode with successive injections of standard solution (100 mg L^{-1} of ascorbic acid in PBS).

Flow-injection system

The flow-injection system is illustrated in Fig. 1B and was defined after investigation of the hydrodynamic, chemical and electrochemical parameters (Tab. 3). The sample was injected into the FIA system as 10, 20 or 50 μL sample loop and pushed by its carrier stream (S_3 solution) at 0.4, 1.0 or 1.6 mL min^{-1} . This flowed through a 10, 25 or 50 cm towards the detector with a fixed applied potential at the working electrode of -100, -50 or 100 mV vs. Ag/AgCl. The passage of the sample trough the wall-jet cell (Fig. 1C) produced a transient signal with a peak current proportional to the ascorbic acid content in the sample. Precision was expressed as relative standard deviation calculated after successive measurements.

RESULTS AND DISCUSSION

Preparation of AgHCF film

Silver hexacyanoferrate can be deposited onto the surface of a solid electrode to form an electroactive monolayer and be used for amperometric ascorbic acid determination. This film was deposited onto new or re-used graphite screen printed electrodes. The functionality of the AgHCF film depends on the confectioning procedure and the pH value; in the formation of the film the pH must be between 1.0-1.5. At $\text{pH} > 7.5$ the film start to decompose and at alkaline media there is a destruction of the film caused by decomposition of hexacyanoferrate(II) in iron hydroxide and by-products³⁷. Moreover, the conditioning of the modified electrode in acidic medium (0.1 M HNO_3 plus 0.1 M KNO_3) is important for keeping the operational stability.

For using in FI system, we have fixed the pH as 5.5 because, if necessary to immobilize enzyme onto the film, denaturation of the protein will not be

observed. At this pH the response is 90-95% of the obtained at acidic medium.

So, for continuing the experiments with FI system, the Nafion/AgHCF/graphite SPE had the pH kept at 5.5. This pH value was fixed as phosphate buffer solution (solution S₃), and was used as carrier stream in the flow injection system.

The decomposition of the film in alkaline medium is the basis of the procedure for re-using the SPE's after continuous use (6-8 hours). By figure 2 it is possible to note similarity between the deposition on new and re-used electrode. The electrochemical response of the re-used electrode was nearly unchanged (deviation of less than 2% in the recorded current). This procedure opens a new possibility for lowering the costs of analysis.

Electrochemical catalysis of ascorbic acid oxidation at the graphite SPE/AgHCF film

Figure 3-A illustrates a series of cyclic voltammograms of the AgHCF film on the graphite SPE in the absence and presence of increasing concentrations of ascorbic acid. The peak potential of ascorbic acid oxidation at the AgHCF film-modified electrode appears at potentials less positive (75 mV) than that at a bare SPE electrode (not shown). Also, an improvement in the current response is observed when the electrode is modified. The response of the modified electrode is proportional to the concentration of ascorbic acid in the range from 1.0 to 3.0 mM (Tab. 1 and Fig. 3-B).

Table 1. Values of anodic current at different concentrations of ascorbic acid (0, 1.0, 1.5, 2.0, 2.5 and 3.0 mg.L⁻¹)

Conc. AA, mM	Current, μ A
0	0.642
1.0	2.523
1.5	4.408
2.0	5.766
2.5	8.062
3.0	9.623

Figure 3. (A) Cyclic voltammogram of the graphite SPE/AgHCF electrode in 0.25 M (solution S₃) in crescent concentration of ascorbic acid (up to 3.0 mM): blue (0.0), red (1.0), green (1.5), yellow (2.0), violet (2.5) and dark blue (3.0). (B) Linear calibration plot of AA ($y = 0.032 + 3.083x$ with $R^2 = 0.9729$)

In the presence of hexacyanoferrate(III), ascorbic acid is oxidized to dehydroascorbic acid (ADHA, the nominal oxidized form of V-C):

The corresponding half-reactions can be represented as: $\text{AA} \rightarrow \text{ADHA} + \text{H}^+ + \text{e}^-$ and $[\text{Fe}^{\text{III}}(\text{CN})_6] + \text{e}^- \rightarrow [\text{Fe}^{\text{II}}(\text{CN})_6]$, for the oxidation and the reduction process, respectively. Finally, with silver hexacyanoferrate, the following can represent the electrochemical oxidation of ascorbic acid occurring as a one-electron process and the resulting current corresponding to the oxidation of the pair $\text{Fe}(\text{CN})_6^{4-/3-}$:

Figure 4. Characterization of the graphite screen-printed electrodes (SPE) by scanning electron microscopy (SEM). A, bare SPE at magnitude 10,000; B, C or D, modified SPE with AgHCF at magnitudes 10,000, 20,000 or 45,000.

The cyclic voltammogram scanned between 800 and 100 mV vs. Ag/AgCl in the absence and presence of 1.0 mM ascorbic acid presented clear anodic and cathodic peaks. In the absence of AA, the response is due to the $\text{Fe}^{\text{III}}(\text{CN})_6^{3-}/\text{Fe}^{\text{II}}(\text{CN})_6^{4-}$ redox process. In the absence of AA the peak separation is 70 mV

whereas in the presence of AA is 71 mV, which indicates that, in terms of reversibility, the redox response attributed to the Fe(III)/(II) process is not affected much by the presence of AA. The deviation from the theoretical value of 59 mV for an ideal reversible process can be due to the polymeric binder present in the film which can affect the conductivity of the film³⁸. It is possible that the binder could cover some of the active sites of the electrode surface as well. The formal potential of the the process in the absence and presence of AA are 421 and 420 mV respectively, so essentially it does not change. The number of electrons (n) transferred in the process can be calculated as 0.8440 and 0.8342. Both values approach unity, confirming that the transfer of one electrode is involved in the process^{39,40}.

The cyclic voltammograms shown in figure 3 essentially show that the intrinsic redox response of the film due to the Fe(III)/(II) couple is not affected by the presence of AA and the reversibility does not change. Both anodic and cathodic peaks are still present, indicating that the response of the film is not altered by the presence of AA and that its oxidation probably takes place in the outer layer of the film. These results are in agreement with those obtained with Prussian Blue films modifying glassy carbon electrodes⁸.

Scanning electron microscopy

Figure 4 shows the SEM analysis for the SPE samples prepared by the electrochemical deposition of silver hexacyanoferrate on a new graphite screen-printed electrode. We can see that the bare electrode presents a surface with low quality and homogeneity; this explains the partial deposition of AgHCF onto the surface. Despite this fact, the modified electrode was used to analyze ascorbic acid in real samples and presented satisfactory results (Fig. 5). The resulting material (film) consists of agglomerates with several segments, some of which with linear shapes about 500 nm to 1 μ m long and 150 to 250 nm thick. This solid morphology presented by the agglomerates may be responsible for the great operational stability of the film with regard to oxidation of ascorbic acid.

Performance of the AgHCF/SPE sensor in FIA system

Optimization of the variables involved in the system design was performed by the univariate method. Table 2 gives the range over which each variable was studied and also the selected values. For these investigations, the flow system shown in figure 1C was employed with a 50.0 or 100.0 mg.L⁻¹ of AA standard solution. The potential of the working electrode was selected as -50 mV vs. Ag/AgCl in order to minimize eventual interfering species. Other values investigated were 0, 50 or 100 mV vs. Ag/AgCl. The value of -100 mV vs. Ag/AgCl was also applied but it was found that the low-interferent-to-analyte ratio and the presence of Nafion polymer layer restricted the number of potential interferences and it was decided to fix the value in -50 mV vs. Ag/AgCl. We did not find any influence from interfering species on the ascorbic acid signal with solutions of sucrose or glucose up to 1000 mg.L⁻¹. Samples with high amounts of sugars (not specified by manufacturers, although we found them to be up to 5% w/v sucrose content) resulted in a pronounced drift in the baseline. This effect, which is due to a matrix effect, disappeared after suitable dilution with solution S₃. The noise present during all analyses (Fig. 5) originated from the peristaltic pump which was not suitable for electrochemical experiments. We confirmed this fact using a second, different pump.

Table 2. Optimization of variables (types I, II and III).

Type	Parameter	Investigated range	Selected value
I	Flow rate, mL min ⁻¹	0.4 to 1.6	1.0
	Inj. Volume, μ L	10 to 50	20
	Transmission line, cm	10 to 50	25
II	K ₂ HPO ₄ /KH ₂ PO ₄ , M	0.5 to 5.0	1.0
III	Potential, mV	-100 to 100	-50

I, II and III, hydrodynamic, chemical and electrochemical parameters.

Figure 5. Recorder output of a routine run. From left to right, measurements in triplicate for an ascorbic acid standard solution (50 mg.L⁻¹) followed by duplicate measurements for five ascorbic acid standard solutions (50, 100, 150, 200, 250 and 300 mg.L⁻¹), six medicament samples and two standard solutions. Insert: linear calibration curve ($R^2 = 0.9975$).

In this system, the peak height is the basis of the measurements and is related to the content of ascorbic acid. The flow-injection analysis was designed to provide low dispersion. The carrier stream flow rate was fixed at 1.0 mL.min⁻¹ as a compromise between the sampling rate and the mean available time for the electrocatalytic oxidation by the AgHCF film. Higher flow rates improved the instability of the generated analytical signals and lower flow rates resulted in unacceptable low analytical frequency. The sample aspiration rate was chosen as 1.0 mL.min⁻¹ to fill the sampling loop under good conditions. The sampling loop was generally 5-6 cm long (*ca.* 20 μ L) because lower (10 μ L) or higher values (50 μ L) would deteriorate the precision or linearity respectively.

The system shown in Fig. 1C was used for flow amperometric determination of ascorbic acid with carrier stream defined as 0.25 M of KH₂PO₄ plus 0.13 M K₂HPO₄ (pH 5.5). Ascorbic acid standard (50.0 to 300.0 mg.L⁻¹) solutions were used before the analysis to evaluate the flow system and create the analytical curve. Precision was evaluated by calculating the relative standard deviation of the results of eleven successive measurements of a typical juice and/or medicament sample with an ascorbic acid content of 994.8 mg.L⁻¹. The results obtained from the high performance chromatographic method (ten samples) were compared with those obtained by the proposed approach demonstrating the performance of the amperometric sensor (Tab. 3).

Table 3. Concentration of ascorbic acid determined in real samples by the proposed and reference methods³⁶.

Sample	Ascorbic acid content, mg.L ⁻¹		
	Reference	SPE	SPE _{reused}
Juice 1	146.2	145.9	145.9
Juice 2	182.1	181.1	180.9
Juice 3	19.3	19.1	19.3
Juice 4	182.4	182.2	182.0
Drug 1	496.8	497.0	496.2
Drug 2	53.4	53.2	53.6
Drug 3	40.6	40.4	39.9
Drug 4	1772.2	1774.2	1778.2
Drug 5	942.8	943.0	941.3
Drug 6	439.8	440.8	438.8

For all, mean of two determination ± 0.1

Interference studies

In the studied samples, the amount of ascorbic acid in orange juice was between 150 and 450 mg.L⁻¹ and the sugar between 15.00 and 20.0 g. So, the relation between them was around 50-100. Concerning the drugs this relation was lower. The selectivity of the Nafion/AgHCF/GSPE was studied by detecting the response of the modified electrode for 1 mM AA in presence of

50-100 mM sucrose or 50-100 mM fructose or 50-100 mM glucose. The results showed that the mentioned species did not cause observable interference. It was observed interference (>10%) for concentration higher than 250 times. The samples used in the present propose was diluted between 10 or 250 times and the low-interferent-to-analyte ratio found and the polymer coated onto modified electrode restricted the number of potential interferents.

Applications

After being dimensioned, the flow system was applied to the analysis of orange juice and drugs. The long term stability of the system was evaluated by injecting different samples during 6 to 8-h working periods. To analyze orange juices and drugs, the system was adjusted and the calibration curve was obtained in the concentration range from 50.0 to 300.0 mg.L⁻¹ of AA. For n=6, the typical regression coefficient was 0.99752. Using the proposed system, about 65-70 samples can be run per hour with a detection limit $\approx$ 2.5 mg.L⁻¹ (1.42×10^{-5} M).

The sampling rate can be improved by reducing the mean available time for ascorbic acid oxidation. This aspect is particularly important when sensitivity is not critical. For a typical sample with ascorbic acid content *c.a.* 994.8 mg.L⁻¹, the relative standard deviation of eleven experiments was estimated less than 2-3%. The accuracy was assessed by running ten already analyzed samples by HPLC method as presented by table 3. The linear correlation between both methods was 0.95232.

CONCLUSION

A thin film of silver hexacyanoferrate shows operational stability and potentiality for ascorbic acid determination and opens a new possibility for future determinations of catecholamine, L-cysteine, cytochrome C, dopamine, hydrazine, NADH etc. The electroactivity and potentiality of this sensor can be incremented by forming a composite with a conducting polymer as we demonstrated before or by using nanoparticles. Experiments along this line are in progress. The present approach is an attractive method for the routine measurements of ascorbic acid in instant juices and drugs, and can be well-adjusted for other kinds of samples. The use of screen-printed electrodes and their re-utilization improve potentiality, minimize costs and can be used for large scale analysis and/or for quality control of other beverages and medicaments.

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