ARTICLES

Amperometric Determination of Ascorbic Acid in Real Samples Using a Disposable Screen-Printed Electrode Modified with Electrografted *o*-Aminophenol Film

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Ascorbic acid (AA) is an antioxidant considered to play a crucial role in human health. Therefore, diverse methods for the determination of AA in foods have been developed, most of them time-consuming and requiring costly instrumentation. A simple and sensitive method for the quantification of AA in fresh fruits and vegetables and commercial juices using an amperometric sensor is presented on the basis of disposable screen-printed carbon electrodes (SPEs) modified with an *o*-aminophenol (*o*-AP) film selective for the detection of AA. The sensor exhibited a linear response for AA from 2−20 μM, with a correlation coefficient $r^2 = 0.998$ and a limit of detection of 0.86 μM. Common possible interferents of the sample matrices were tested, and results showed high selectivity of the *o*-AP SPEs toward AA. The sensor exhibited an excellent reproducibility (RSD% = 1.98, $n = 8$) and surface stability. The method was validated by a comparison to a reference method, and excellent correlation is obtained.

KEYWORDS: Amperometric sensor; ascorbic acid; electrocatalytic oxidation; diazonium salt; modified electrode; screen-printed electrodes; fruits; vegetables; commercial juices

INTRODUCTION

Ascorbic acid (AA, vitamin C) is a water-soluble antioxidant essential for life with widely recognized benefits (*1*). It is present in relatively high amounts in fresh fruit and vegetables and it is also added to pharmaceutical products and foodstuffs as an antioxidant and a stabilizer of aroma and color. Additionally, AA is used to cure scurvy (*2*). The content of AA in biological fluids is used to assess the amount of oxidative stress in human metabolism, a process that has been linked to cancer, diabetes mellitus, and several liver diseases (*3*). Epidemiological studies have shown that diets high in fruits and vegetables are associated with lower risk of cardiovascular failure, stroke, and cancer and with increased longevity, supporting the hypothesis that anti-oxidant activity may help to decrease the risk of such diseases (*4*). Thus, the development of inexpensive and easy methods

for the determination of AA is particularly important in the pharmaceutical and food industry.

There are several reported methods for the detection of AA in foodstuffs, such as chromatography (*5*), spectrophotometry (*6*), capillary electrophoresis (*7*), and most recently, electro-chemical methods (*8, 9*). Electrochemical methods have been performed using different electrode materials. The amperometric determination of AA is based on its electrochemical oxidation, which occurs at high potentials at carbon or metal electrodes, and fouling by oxidation products leads to poor reproducibility (*10*). Numerous attempts to decrease the high working potentials and improve reproducibility have been made by modifying the electrode surface with various active mediators for the electro-chemical oxidation of AA (*10, 11*). Different approaches have been recently used for electrode modification and applied to the detection of AA, such as glassy carbon electrodes (GCEs) modified with a cellulose acetate film bearing 2,6-dichlorophe-nolindophenol (*12*) or by electropolymerization of *N,N*′-dim-ethylaniline (*13*), as well as carbon paste electrodes modified with calixarenes (*14*) and congo red dye absorbed on a silica/aniline xerogel (*15*). However, electrochemical AA determina-

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Amperometric Determination of Ascorbic Acid

J. Agric. Food Chem., Vol. 56, No. 22, 2008 **10453**

Table 1. Weight and Volumes Extracted from Fresh Fruits and Vegetables for the Preparation of the Samples

sample	weight (g)	volume extracted (mL)
Fruits		
apple	310.1	120
kiwi	143.7	74
lemon	130.3	90
orange	146.4	45
pineapple	387.6	224
strawberry	83.3	50
Vegetables		
green pepper	302	197
tomato	266.2	224

Figure 1. Amperometric response of *o*-AP-modified electrodes to 15 μM AA (1) followed by the addition of 0.15 mM citric acid (2). The arrows indicate the moment of the additions.

tion in foodstuffs has been very limited (*16*−*18*). In particular, to the best of our knowledge, there is only one report on the application of the screen-printing technique for AA determination in fruits, despite the several advantages that they offer in terms of large-scale fabrication of disposable sensors, low cost, versatility, and miniaturization (*19*).

The modification of conductive surfaces through the grafting of organic groups (*20*) via the electrochemical reduction of aryl diazonium salts was first reported by Pinson in 1992 (*21*). We have recently use this strategy to electrograft *o*-aminophenol (*o*-AP) films on GCEs (*22*) and studied the electrocatalytic oxidation of AA in the presence of uric acid (*11*). Here, we report the applicability of modified *o*-AP screen-printed carbon electrodes (SPEs) for the detection of AA in a wide variety of fresh fruits and vegetables and commercial juices. Sensor selectivity, reproducibility, and stability have also been tested.

MATERIALS AND METHODS

Reagents. All chemicals were of analytical grade and used as received without any further purification. Solutions were prepared with MilliQ water (Millipore, Inc.; $\Omega = 18$ MΩ cm^{-1}). L-Ascorbic acid (99%) was purchased from Sigma Aldrich (Spain); 2-amino-4-nitrophenol was purchased from Acros (Spain); and disodium oxalate was purchased from Probus (Spain). Stock solutions of AA (1 mM) were prepared daily in degassed 0.1 M phosphate buffer at pH 7.2. For the spectrophotometric measurements, 2,6-dichlorophenolindophenol disodium salt (DCIP) was purchased from Fluka (Spain).

Instrumentation. Electrochemical experiments were carried out using an Autolab model PGSTAT 12 potentiostat/galvanostat controlled with the general purpose electrochemical system (GPES) software (Eco Chemie B.V., The Netherlands). The commercial screen-printed electrodes used (DropSens, Oviedo, Spain, ref 110) consisted of a carbon working electrode (4.0 mm in diameter), a Ag pseudo-reference electrode, and a carbon counter electrode.

Spectrophotometric measurements were performed with a Cary 100 Bio (Varian), UV−vis spectrophotometer at 520 nm.

Sample Preparation. Fresh fruits (apple, kiwi, lemon, orange, pineapple, and strawberry) and vegetables (green pepper and tomato) samples were obtained from a local supermarket (Mercadona). The commercial juices analyzed were lemon juice (Lemonada, Minute Maid), tomato juice (Hacendado), and refrigerated orange juice (Hacendado).

Fruit juices were first mixed with an extraction solution [1% (w/v) disodium oxalate] and centrifuged prior to dilution with the working buffer solution (0.1 M phosphate buffer at pH 7.2). For fruit and vegetable samples, a specific amount (**Table 1**) was weighed and mixed with 25 mL of 1% (w/v) disodium oxalate. The mixture was homogenized for 5 min with a Braun homogenizer. The extract was centrifuged at 3000 rpm for 15 min, and the supernatant was filtered through a 0.22 μm nylon filter. An aliquot of the recovered filtrate volume was diluted according to the AA content of the sample in 10 mL of 0.1 M phosphate buffer at pH 7.2 and applied to the electrochemical cell.
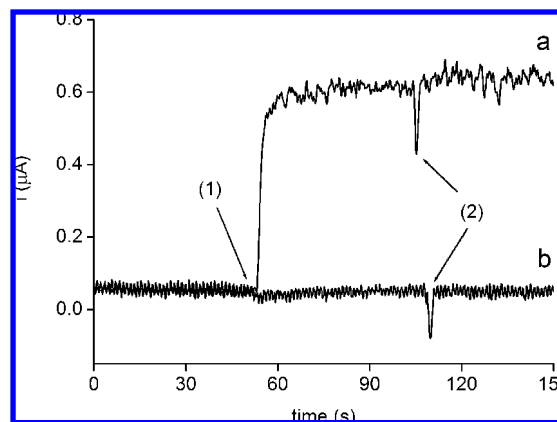
Electrode Modification. Briefly, surface-activated Dropsens SPEs of known area were immersed in 5 mL of a 6 mM 2-amino-4-nitrophenol diazonium salt solution, and 60 potential cycles were carried out between 0.6 and −0.2 V at 50 mV s^{-1} to electrochemically reduce the in situ generated *o*-nitrophenol diazonium salt. After modification, electrodes were washed with Milli-Q water, transferred to 1 M H$_2$SO$_4$, and subjected to five potential scans between −0.1 and −0.85 V at 100 mV s^{-1}, for complete reduction of the nitro group, providing an electrografted film of *o*-AP on the carbon SPE surface (*11*). The modified electrodes were subsequently subjected to a potential scan between −0.1 and 0.6 V for 10 cycles at 100 mV s^{-1} versus Ag in phosphate buffer (0.1 M, pH 7.2) to remove any physically adsorbed compounds and used.

Electrochemical Measurements. Hydrodynamic amperometric measurements were performed at a fixed potential of +200 mV (versus Ag) in a magnetically stirred solution at pH 7.2. After baseline stabilization, an aliquot of the AA containing solution was added to the stirred buffer solution, and the current response was measured after achieving steady state (typically 1−2 s).

Spectrophotometric Measurements (*6*). The instrument was adjusted to zero using a mixture of disodium oxalate 0.6% (w/v) (125 μL) and acetate buffer (300 g of anhydrous sodium acetate plus 700 mL of miliQ water plus 1000 mL of glacial acetic acid) (125 μL). The absorbance (A_1) of a mixture of disodium oxalate (50 μL) plus acetate buffer (50 μL) plus 12 mM DCIP (400 μL) was recorded at 15 s. The absorbance of a 10 ppm standard AA solution (50 μL) plus acetate buffer (50 μL) plus 12 mM DCIP (400 μL) was recorded as A_2. Values for A_2 were recorded for standard solutions (20, 30, 40, and 50 ppm). $A_1 - A_2$ values (the absorbance for each working standard) were calculated, and a calibration graph was constructed plotting $A_1 - A_2$ versus the AA concentration (data not shown). For the absorbance measurements of the samples, A_2 was the absorbance of sample solution (50 μL) plus acetate buffer (50 μL) plus 12 mM DCIP (400 μL). All of the measurements were recorded at 520 nm.

RESULTS AND DISCUSSION

Effect of Interference on the Determination of AA. To evaluate the selectivity of the *o*-AP SPE sensor, the effect of common potentially interfering compounds present in vegetable, fruit, and juice samples (organic acids, antioxidants, and sugars) was investigated. Concentrations typically higher than those of the natural levels expected in the food samples were tested by measuring the AA content of a 15 μM stock solution in the presence of the potential interferent (**Figure 1**). **Table 2** summarizes the percent of signal recovery of the determination of 15 μM AA in the presence of several organic hydroxyacids and sugars (citric acid, tartaric acid, glutamic acid, and oxalate and glucose, fructose, and sucrose). The average recovery ranged from 97.3 to 103.3%, indicating that the tested species did not
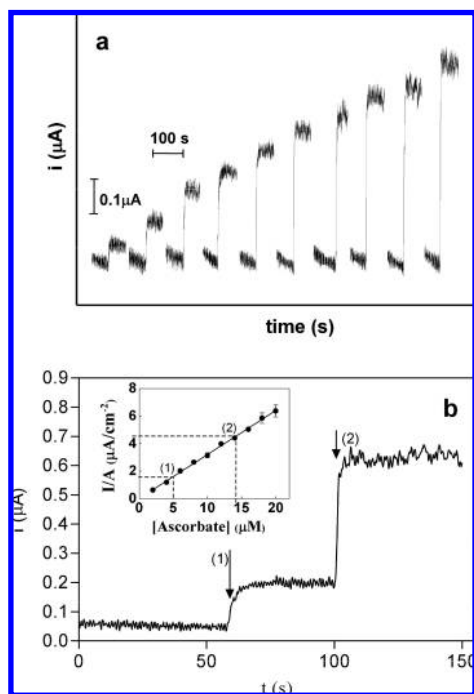
Figure 2. (a) Amperometric responses in 0.1 M phosphate buffer at pH 7.2 to successive injections of AA (2–20 μM) at *o*-AP-modified SPEs. (b) Amperometric response of (1) a diluted sample ($^{1.5}/_{1000}$) of freshly prepared orange juice sample and (2) a recovery test for the addition 15 μM AA. (Inset) Interpolation of the current values obtained in 1 and 2 in the calibration plot.

Table 2. Recovery of AA in the Presence of Some Possible Interferents at *o*-AP-Modified Screen-Printed Electrodes

		AA concentration (μM)		recovery of AA (%)
additive	concentration (mM)	added	found	
glucose	1.5	15	14.9	99.3
fructose	1.5	15	14.8	98.7
sucrose	1.5	15	14.6	97.3
citric acid	0.15	15	15.5	103.3
tartaric acid	0.15	15	14.8	98.7
glutamic acid	0.15	15	14.9	99.3
disodium oxalate	0.15	15	14.7	98.0

cause any interference and demonstrating the specificity and selectivity of the *o*-AP SPCE sensors to AA over a wide number of interfering compounds.

Sensor Calibration. Plots of the current response versus the concentration of AA over a dynamic range of 2–20 μM were obtained in triplicate (**Figure 2a**). They showed a linear behavior in the studied range with a sensitivity of 0.32 μA μM^{-1} ($r^2 = 0.998$). The precision of the method was excellent, with RSD of 1.98% ($n = 8$). The limit of detection (LOD) was 0.86 μM, which is lower than other reported methods using poly(*N*-methylaniline)-modified Pt (*23*) and copper hexacyanoferrate deposited on carbon (*24*) (LOD = 5 and 2.1 μM, respectively). Although there are also methods describing lower LOD values than that obtained in this work [0.015 (*16*), 0.2 (*25*), and 0.3 μM (*11*)], it allows for detection of AA at levels to $^1/_{10}$ those deemed permissible by regulatory agencies. In addition, our aim has been to optimize the method based on accuracy and precision rather that seeking very low detection limits, which would require higher dilution factors, thus introducing potential errors and possibly increasing the time required for the determination.

Table 3. Determination of the Content of AA in Fresh Fruit and Vegetables and Commercial Juices ($n = 3$) Using *o*-AP-Modified Sensor and Recovery Test Results

	amount of AA					
sample	found (μM)	added (μM)	total (μM)	recovery (%)	total amount (mg/kg)	using reference method (mg/kg)
Fruits						
lemon	4.3 ± 0.1	15	19.2	99.5	351 ± 4	343 ± 14
orange	5.0 ± 0.1	15	20.3	101.5	182 ± 4	174 ± 2
pineapple	15 ± 2	15	29.7	99.0	371 ± 8	363 ± 12
strawberry	4.7 ± 0.2	15	19.7	100.0	547 ± 6	565 ± 7
apple	3.4 ± 0.1	15	18.2	98.9	116 ± 1	118 ± 20
kiwi	3.4 ± 0.1	15	18.3	99.5	205 ± 3	ND[a]
Vegetables						
tomato	15 ± 2	15	29.8	99.3	148 ± 13	115 ± 8
green pepper	1.7 ± 0.1	15	15.5	92.8	13.0 ± 0.4	13.8 ± 0.3
Commercial Juices						
lemon	4.3 ± 0.1	15	19.3	100.0	15.1 ± 0.6[b]	12.2 ± 0.8[b]
orange[c]	3.9 ± 0.6	15	18.4	97.4	28 ± 1[b]	26 ± 1[b]
tomato	2.8 ± 0.2	15	17.9	100.6	13.2 ± 0.8[b]	14.02 8 ± 0.5[b]

[a] Not determined (see the text for details). [b] In units of mg/100 mL. [c] Refrigerated sample.

Analysis of Fruit Juices and Fresh Fruits and Vegetables. All amperometric measurements exhibited a stable and extremely rapid response (1–2 s) for AA oxidation at the selected potential. **Figure 2b** shows a typical amperometric response obtained for the sequential addition of orange juice and an AA stock solution and the interpolation of the response in the calibration plot. Verification of the method was carried out by measuring the AA content of the samples using a spectrophotometric method as indicated in ref *6*.

The AA content of the samples analyzed is detailed in **Table 3**. The method was applied to fruits and vegetables containing between 10 and 300 mg of AA per 100 g of the fresh sample. The comparison of the results obtained by the proposed method to those of the reference method demonstrated an excellent correlation. In the case of kiwi, the spectrophotometric measurement of AA contents was not possible because of a persistent turbidity on the extracted (homogenized) samples, which could not be possible to remove by either centrifugation (at 16 000 rpm for 30 min) or filtration through 0.2 μm nylon filters. This resulted in extremely high absorbance values for the blank solution (>2.5), making the measurement impossible to carry out. However, the obtained value for kiwi (205 mg/kg) matches well with the reported content of AA (40–260 mg/kg) in different species of this fruit (*26*).

In addition, different types of commercial juices were tested to determine the potential interfering effect of preservatives contained in the fruit juices. The results (**Table 3**) show that the detected amount of AA with the *o*-AP SPE of the refrigerated orange juice sample, which is the most natural juice of the tested ones, is in good agreement with the expected values. For lemon and tomato juice samples, which are more treated to ensure a longer shelf life of the products, the amounts of AA found were in good correlation with those given by the reference method.

To validate the sensor performance, recovery studies were carried out by adding standard solutions of AA to the sample and measuring the response of the sum of both the sample and standard concentrations. In all cases, recoveries of 92.8–101.5% were obtained, as shown in **Table 3**.

Stability of the *o*-AP SPE. To test the stability of the sensors, *o*-AP-modified electrodes were prepared and stored under vacuum at room temperature. Amperometric measurements were carried out weekly over a 5 months period, and a loss of 16%

Amperometric Determination of Ascorbic Acid

J. Agric. Food Chem., Vol. 56, No. 22, 2008 **10455**

of the initial activity was observed at the end of the fifth month. This characteristic indicates a high degree of robustness of the *o*-AP SPE, making them an attractive tool for the analytical determination of AA.

In conclusion, the applicability of the newly developed amperometric *o*-AP SPE sensor for the detection of AA in real samples has been demonstrated. The sensor exhibited high sensitivity and selectivity toward AA with excellent storage and operational stability, as well as a quantitatively reproducible analytical performance. It could be used for the facile, selective, rapid, and precise determination of AA in a large number of fruits and vegetables containing any amount of AA.

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