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Development and Characterization of an Ascorbate Oxidase-based Sensor–Biosensor System for Telemetric Detection of AA and Antioxidant Capacity in Fresh Orange Juice

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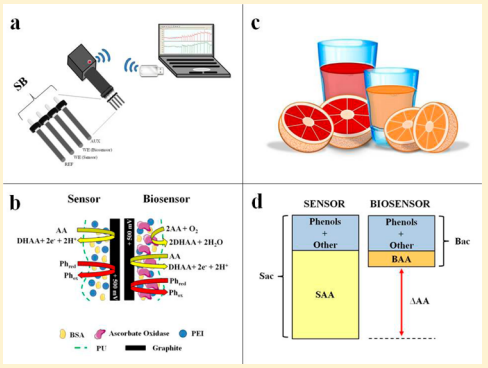
Supporting Information

ABSTRACT: A new carbon ascorbate oxidase-based sensor–biosensor system (SB) was coupled to a dual-channel telemetric device for online simultaneous electrochemical detection of ascorbic acid (AA) and antioxidant capacity in Hamlin, Sanguinello, and Moro orange varieties. The electrocatalytic performances of the SB were investigated by cyclic voltammetry and amperometric techniques. The phenol composition of orange juice of each variety, and the cyclic voltammetries of the most represented phenols, were provided. The *in vitro* calibrations were performed in PBS (pH 5.6), applying a constant potential of +500 mV. A standard mixture of phenols, based on orange juice composition, was used as reference material for studying SB behavior. SB works at an applied potential of +500 mV, in a concentration range comprised between the LOD 0.26 μM and 20 μM. In this concentration range, limiting the data acquisition time to 2 min, the problems of electrode passivation due to phenols polymerization were overcome. AA calibration showed that the biosensor registered statistically lower currents than the sensor since the enzyme oxidized AA before it reached the electrode surface. Standard mixture calibration showed that currents registered by sensor and biosensor did not statistically differ. The difference between sensor and biosensor AA registered currents was used to calculate an *AA selectivity index* and, consequently, to determine the AA content and the antioxidant capacity in the juices. The novelty of the SB is its ability to distinguish between AA and phenols contribution to antioxidant capacity. The obtained results were in accordance with reference methods.



Antioxidants are able to protect, directly or indirectly, a biological target from oxidation acting against the adverse effects of reactive oxygen and nitrogen species on physiological functions.[1−3] Many complex disease such as cardiovascular diseases, inflammatory disorders, cancer, Parkinson's disease, diabetes mellitus, and stroke are linked to an imbalance between generation of oxidants and the antioxidant system.[4] Oranges, like most fruit and vegetables, are an excellent source of antioxidants, being rich in ascorbic acid and phenols.[5,6] Thousands of publications reported studies on antioxidant capacity determined with different methods based on spectrophotometric, chromatographic, fluorescence, and electrochemical techniques.[7−9]

The direct electrochemical determination of antioxidants in food has been largely used since those molecules are easily oxidized at bare electrodes, such as glassy carbon,[10] graphite,[11] or platinum.[12] Cyclic voltammetry (CV) has probably been the most used electrochemical method for antioxidant capacity evaluation.[13] The potential at which the oxidation starts enables the identification of the type of antioxidant involved, whereas the peak potential is an indicator of the antioxidant capacity. A low oxidation potential indicates a high reducing power[8] since the ionization potential is the main factor that determines the efficiency of antioxidants.[1] Pilijac-Žegarac et al.[14] estimated the antioxidant capacity in tea infusions by CV integrating the area under the peak up to 0.6 V. The CV method was also applied to study the profile of flavonoids in onions.[15] The total phenol content and the flavonol level in red and white wines were similarly estimated.[16] Unfortunately, CV provide information about total antioxidant capacity, without the specific determination of the contribution of each individual component.[17]

With a different approach, working in constant amperometric mode, the reducing power of tea infusions was assessed and the total phenol content was quantified by measuring the oxidation currents at 0.5 and 0.8 V, respectively.[11] In the same direction, biosensors are promising tools for the assessment of antioxidant

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capacity. Enzyme-based biosensors show high selectivity for bioactive compounds and can directly detect antioxidant substances in biological samples without requiring a prior separation step.[18] Several enzymes were coupled in various ways with different transducers. A lot of examples were reported where laccase, tyrosinase, and many other enzymes were entrapped, cross-linked, covalently immobilized, incorporated, adsorbed, or coated on gold, platinum, and carbon working electrodes.[8,19,20]

Ascorbate oxidase-based biosensors were also largely used for ascorbic acid (AA) detection in different matrices. The enzyme was immobilized on a Pt electrode in a gel-like kappa-carrageenan membrane, to determine the AA content in red and white wines.[21] An egg shell membrane bounding *Lagenaria siceraria* fruit ascorbate oxidase was mounted over a gold working electrode for determination of AA in serum, fruit juices, and vitamin C tablets.[22] Wang et al.[23] coated an oxygen permeable hydrophobic ascorbate oxidase micelle membrane on glassy-carbon and gold electrodes in order to evaluate the AA content of some commercial fruit juices. The enzyme was also immobilized on a nylon membrane by glutaraldehyde linking and attached to the polyethylene membrane of a Clark oxygen electrode for AA chronoamperometric detection in natural fruit juices and soft drinks.[24] A flow injection system, based on the capacity of ascorbate oxidase to oxidize AA before it reached a glassy carbon detector, was set up for a selective assay of AA in multivitamin tablets and white wines.[25]

The idea of this study grew out when a rapid and inexpensive detection of AA in orange juice and in fresh-cut melon, kiwi, and pineapple fruits was performed with a simple graphite working electrode at an applied potential of +120 mV. That system was highly specific for AA due to the low applied potential.[26,27] The results of Buratti et al.[11] suggested that applying a working potential up to +500 mV or more would make it possible to determine the antioxidant capacity of samples containing different classes of compounds (phenols, sugars, organic acids, and, in general, every antioxidant molecule). Our research aims to demonstrate that, with a new ascorbate oxidase-based sensor−biosensor system (SB) at an applied potential of +500 mV, it is possible to detect the AA content and the antioxidant capacity of fresh orange juice and simultaneously distinguish between the AA and the phenols contribution. The SB system was coupled with a dual channel telemetric device for on line measurement of AA and antioxidant activity. A previous design of a one-channel telemetric device has been successfully used for online detection of AA in orange juice[26] and in the juice of fresh cut fruits during storage.[27]

EXPERIMENTAL SECTION

Reagents and Solutions. All chemicals were of analytical grade and used as received without any further purification.

Ascorbic acid (99%) was purchased from Merck (Germany); stock solutions of AA were prepared daily in phosphate buffer (PBS) at pH 5.6. The phosphate buffer saline solution was made using Milli-Q water (Millipore, Inc.; 18 MΩ /cm), NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (8.1 mM), and KH_2PO_4 (1.47 mM) from Sigma-Aldrich and then adjusted to pH 5.6. For titrimetric measurements, oxalic acid dihydrate and 2,6-dichlorophenol-indophenol sodium salt dihydrate, and sodium hydrogen carbonate ($NaHCO_3$) were purchased from Merck (Germany).

Gallic acid, caffeic acid, chlorogenic acid, p-coumaric acid, ferulic acid, sinapic acid, naringin, and hesperidin were purchased from Sigma-Aldrich. Kuromanin chloride (cyanidin 3-glucoside), naringin, and narirutin were purchased from Extrasynthese. Ethanol, methanol, dimethylformamide, polyethylenimine (PEI), bovine serum albumin (BSA), polyurethane (PU), and tetrahydrofuran were purchased from Sigma-Aldrich. PEI solutions were prepared by dissolving the enzyme stabilizer in Milli-Q water. BSA powder was dissolved in PBS in order to obtain 10% solutions. PU solutions were prepared by dissolving the polymer in tetrahydrofuran.

Ascorbate oxidase (AOx) from *Cucurbita* sp. (EC 1.10.3.3) was purchased from Sigma-Aldrich. The enzyme was dissolved in the BSA solution (one unit of AOx will oxidize 1.0 μmol of AA to dehydroascorbate per min at pH 5.6 at 25 °C).

Fruit Samples and Juice Preparation. Oranges (*Citrus sinensis* (L.) Osbek) of cultivars Hamlin, Sanguinello, and Moro were hand-harvested, when commercially mature, in the experimental orchard of the Institute of Sciences of Food Production, in central-western Sardinia, Italy. Fruits were delivered to the laboratories immediately after harvest. There, oranges free from rind defects, were randomly selected, placed in plastic trays, and left overnight at 17 °C. The juice, prepared by squeezing fresh oranges, was filtered with a kitchen strainer, immediately stored in ultrafreezer at −80 °C and then lyophilized. Freeze-dried samples were fully rehydrated before chemical composition analysis (AA and phenols determination), in vitro calibrations, and antioxidant activity determination.

Sensor−Biosensor System Description and Preparation. The SB assembly was composed of four rod carbon (length = 30 mm; Ø = 300 μm; 2H Staedler graphite pencil leads) electrodes: two working electrodes (WEs), a pseudoreference electrode and an auxiliary electrode (Figure 1a).

The two WEs, the sensor and the biosensor, were previously coated with an insulating thin layer of epoxy resin, and their active surface (0.071 mm^2) was disc-shaped using a high speed drill equipped with an aluminum oxide grinding wheel as described in Barberis et al.[26] The pseudoreference electrode and the auxiliary electrode were not insulated.

The biosensor, schematically represented as C/(PEI+BSA-AOx)$_{10}$/PU (Figure 1b), was obtained as follows:

Step 1. A layer of PEI, an enzyme stabilizer,[28] was deposited on the sensor surface by dip-evaporation from a 1% PEI aqueous solution.

Step 2. BSA and AOx were loaded on the sensor surface, immediately after step 1, by dip-evaporation from a 25 μL of 10% BSA solution containing 25 U of enzyme. The sequence step 1−step 2 was repeated 10 times, with a time interval of 5 min for water evaporation after step 2.

Step 3. The biosensor was quickly dipped in a 0.2% PU solution in order to immobilize the enzyme. After 30 min for tetrahydrofuran evaporation, the biosensor was stored at 4 °C until employment.

The sensor, schematically represented as C/(PEI+BSA)$_{10}$/PU (Figure 1b), differed from the biosensor only for the absence of AOx.

The image of the biosensor surface was obtained using an ESEM Zeiss EVO LS10 in variable pressure (VP) modality at a pressure between 50 and 80 Pa (Figure 1c).

Determination of Phenol Composition of Orange Samples. Phenol composition was determined by LCMS

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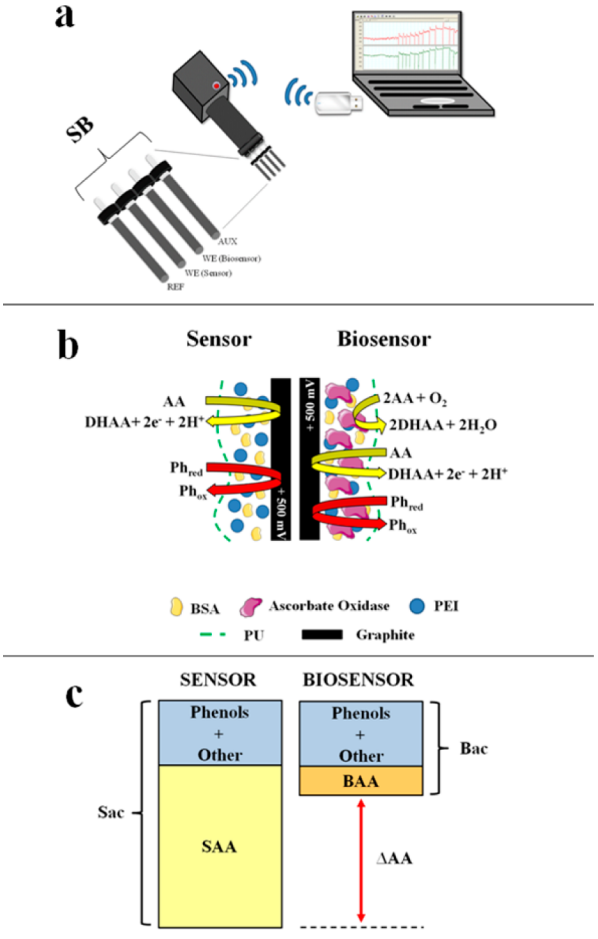

Figure 1. (a) Schematic drawing of the telemetric device interconnected with a laptop, used in this study. (b) Schematic drawing of the sensor and the biosensor surface: AA and phenols are oxidized on the carbon electrode surface of the sensor, while a quota of AA is oxidized by ascorbate oxidase before it reaches the transductor surface of the biosensor. (c) Scheme of the working principle of SB. Abbreviations: SB = sensor–biosensor system; WE = working electrode; REF = reference electrode; AUX = auxiliary electrode; Ph_{ox} = oxidized form of phenols; Ph_{red} = reduced form of phenols; AA = ascorbic acid; DHAA = dehydroascorbic acid; AOx = ascorbate oxidase; BSA = bovine serum albumin; PEI = polyethylenimine; PU = polyurethane; Sac = sensor antioxidant current (is the total current registered by the sensor at the applied potential of +500 mV); Bac = biosensor antioxidant current (is the total current registered by the biosensor at the applied potential of +500 mV); SAA = the AA current registered by the sensor; BAA = the AA current registered by the biosensor; ΔAA = the quota of AA oxidized by AOx before it reaches the transductor surface of the biosensor.

analysis. The detailed methodology and relative results were reported in the Supporting Information (S-1.1)

Electrochemical Behavior Investigation of AA and Phenols Identified in the Orange Juice. All electrochemical characterizations and calibrations were performed using a four channel system (eDAQ Quadstat, e-Corder 410 and Echem software, eDAQ Europe, Poland) placing the SB in a glass beaker containing 20 mL of air-bubbled PBS at pH 5.6. AA calibrations were made the day after the sensor preparation, applying a positive potential of +500 mV (vs carbon pseudoreference electrode) after a stable baseline was achieved. A 10 point calibration was performed by adding known amounts of AA in order to have concentrations comprised between 0 and 100 μM in the cell.

CVs of AA and of the most represented phenols identified in the juice of the three orange varieties were carried out in order to investigate their electrochemical behavior at the sensor surface vs the carbon pseudoreference electrode. The suitability of carbon as a pseudoreference electrode has already been demonstrated by our previous study.[26] An additional CV of AA was performed to investigate its electrochemical behavior at the biosensor electrode surface. AA CVs were executed starting from −200 mV to +600 mV, while phenols CVs went from −200 mV to +1000 mV, at a scan rate of 100 mV/s.

The effect of the phenols and of orange juice on AA currents was investigated by spiking a phenols standard mixture (see next paragraph) and the orange juice with known amounts of AA (standard addition method). Five 20 μL aliquots, containing a 10 μM standard mixture, were spiked with increasing quantities of AA, in order to have AA concentrations comprised between 0 and 10 μM in the 20 mL PBS cell. Similarly, five aliquots of Hamlin orange juice (60 μL) were spiked with increasing quantities of AA and then added to 20 mL of PBS (in order to have AA concentrations comprised between 0 and 10 μM in the cell). Both the experiments were repeated 8 times, and linear regression analyses were performed on baseline subtracted data.

Preparation of the Standard Mixture. An AA free standard mixture containing multiple phenol phytochemicals was prepared according to Luthria and Vinyard.[29] to evaluate if and how phenols affect the SB behavior (for more details, see Supporting Information S-1.2)

Quantification of AA and Antioxidant Capacity in the Orange Samples with the SB. The quantification of AA and antioxidant capacity was attained by coupling the SB to the dual channel telemetric device. A total of 60 μL of orange juice followed by 10 μL of 10 mM AA were injected into a 20 mL PBS solution. The resulting currents were used to calculate the values of AA content and of antioxidant capacity of fresh orange juices, distinguishing between the AA and the phenols contribution to antioxidant capacity. The experiment was repeated eight times for each variety.

In order to have a further assessment of phenols' contribution to antioxidant capacity, AA was completely eliminated from juice by adding 50 U of AOx to 60 μL of orange juice of each variety. The solutions were stored in the dark, at room temperature for 2 h, then injected into 20 mL of PBS, and the resulting currents were registered.

Dual Channel Telemetric Device. The telemetry system used in this study (Figure 1a) was based on previous designs.[26,28] SB data were acquired second-by-second, averaged, and transmitted to the notebook every 15 s. A detailed description of the telemetric device is provided in the Supporting Information (S-3).

Evaluation of AA and Antioxidant Capacity with Reference Methods. Methodologies are in the Supporting Information (S-1.3)

Statistical Analysis. AA and phenol phytochemical currents were expressed in nanoamperes and given as mean ± standard error of the mean (SEM) of absolute oxidation currents (nA) or baseline-subtracted currents (ΔnA). AA and phenol juice content were expressed as mg/100 mL to be comparable with reference methods. Antioxidant activity was expressed as ascorbic acids equivalents in accordance with Kim et al.[30]

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Statistical analysis was performed with GraphPad Prism 5 for Windows software (GraphPad Software, Inc., La Jolla, CA 92037, USA). The Student's t test was performed to compare means from data obtained with the sensor and the biosensor. Analysis of variance (one-way ANOVA) was performed to compare results obtained with different analytical methods, using a unifactorial complete randomized block design. Mean comparisons were calculated by Fisher's least significant difference test at $P \leq 0.05$.

RESULTS AND DISCUSSION

Preliminary Characterization of Sensor and Biosensor. As previously described, the sensor and biosensor of SB differ only for the presence or the absence of the enzyme. The influence of PEI, BSA, and PU on the electrochemical behavior of both sensor and biosensor was studied. The results of Rocchitta et al.[28] indicated that an increase in the number of 1% PEI dips on a Pt electrode resulted in an increase of AA oxidation, and that the use of 1% PU, as an enzyme immobilizer, conferred high AA shielding properties to the biosensor. But, whereas the authors considered AA as an interference to shield, in our case AA is the target. Our purpose was to obtain good enzyme stabilizing and immobilizing effects and, at the same time, to use PEI and PU concentrations able to confer, to the sensor, performances as similar as possible to a bare electrode. With this aim, several AA calibrations, in an AA range comprised between 0 and 100 μM, in the absence and in the presence of PEI (5 dips of 1% PEI and 10 dips 1% PEI) and PU at different concentrations (1%, 0.5% and 0.2%), were performed. Results indicated that, using 1% PEI and 0.2% PU solution, the performances of the C/PEI$_{10}$/BSA/PU electrode were not statistically different from a bare electrode (r^2 = 0.999 and slope 0.656 ± 0.021 vs r^2 = 0.993 and slope = 0.665 ± 0.02 respectively for bare electrode and C/PEI$_{10}$/BSA/PU; n = 4). The BSA was reported to enhance the sensitivity toward ethanol when used in combination with glutaraldehyde;[28] in our work BSA was added to have a proteic element on the sensor too, to be as similar as possible to the biosensor.

Operating Principle of the SB. The SB works in a concentration range comprised between the LOD and 20 μM, at Eapp = +500 mV vs carbon pseudoreference. Its working principle, schematized in Figure 1b and c, is based on two assumptions: (A) The sensor and biosensor record different currents when exposed to the same quantity of AA since AOx oxidizes a quota of AA before it reaches the biosensor transductor surface. *Sac* (sensor antioxidant current) and *Bac* (biosensor antioxidant current) are the total current registered by the sensor and the biosensor, respectively, at the applied potential of +500 mV (Figure 1d). (B) The sensor and biosensor record the same current values in an AA free sample.

Assumption A was demonstrated by results showed in Figure 2 where SAA is the AA current registered by the sensor, BAA is the AA current registered by the biosensor, and ΔAA = SAA − BAA is the quota of AA oxidized by AOx before it reaches the transductor surface of the biosensor.

AA oxidation occurred at the sensor surface at about +50 mV, and at about +300 mV at the biosensor surface, vs carbon pseudoreference electrode. At +500 mV, the sensor (dotted red line) and the biosensor (blue dotted line) registered AA currents of 0.91 μA and 0.41 μA, respectively. These values, baseline subtracted (the current value of the baseline at +500 mV is 0.20 μA), represent SAA (0.91 − 0.20 = 0.71 μA) and BAA (0.41 − 0.20 = 0.21 μA) respectively, and their difference

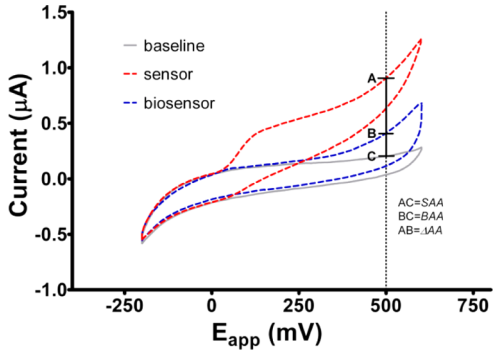

Figure 2. AA cyclic voltammetries, with a scanned potential range (E_{app}) comprised between −200 and +600 mV vs carbon pseudoreference, in the absence (gray line) and in the presence of 1 mM AA, performed by the sensor (dotted red line) and the biosensor (dotted blue line). The AC segment represents the difference between the current values registered, at +500 mV, by the sensor and the baseline: this value corresponds to SAA (the AA current registered by the sensor). The BC segment represents the difference between the current values registered, at +500 mV, by the biosensor and the baseline: this value corresponds to BAA (the AA current registered by the biosensor). The AB segment represents the difference between the current values registered by the sensor and the biosensor: this value corresponds to ΔAA, the quota of AA oxidized by AOx before it reaches the transductor surface of the biosensor.

is ΔAA (SAA − BAA = 0.71−0.21 = 0.50 μA). The ratio between BAA and SAA values is 0.30, thus indicating that, at +500 mV, the biosensor registered about 30% of the current registered by the sensor.

Similarly, the AA calibration in the inset of Figure 3a (r^2 = 0.996 with a slope = 0.668 ± 0.002 nA/μM and r^2 = 0.998 with a slope = 0.196 ± 0.004 nA/μM for sensor and biosensor, respectively) evidenced that the ratio between the biosensor slope (B_{slope}) and the sensor slope (S_{slope}) was 0.29, confirming what was previously demonstrated in Figure 2.

If AOx was completely inefficient, the same quantity of AA would be oxidized on the sensor and the biosensor transductor surface, and the ratio B_{slope}/S_{slope} would be 1. On the contrary, when the AOx efficiency is high, the ratio B_{slope}/S_{slope} will tend to zero. We called "AA selectivity index" the ratio B_{slope}/S_{slope}, which represents the index of specificity toward AA of our SB system. An AA selectivity index = 1 indicates that the SB is able to determine the antioxidant capacity, but it is unable to distinguish between AA and other compounds in the sample; an AA selectivity index = 0 indicates the highest selecting capacity toward AA of SB, which is able to discriminate between AA and other molecule contribution to antioxidant capacity.

Assumption B was demonstrated in Figure 3b where the standard mixture calibration revealed that there were not any significant statistical differences between sensor and biosensor current values, both with an excellent linearity between 0 and 20 μM (r^2 = 0.997 with a slope = 0.589 ± 0.002 nA/μM and r^2 = 0.996 with a slope = 0.565 ± 0.002 nA/μM respectively; n = 6).

According to these results, we can calculate SAA and BAA as they were the unknown variables of the following "two equation system":

$$\text{SAA} - \text{BAA} = \Delta\text{AA} \quad (1)$$

$$\text{SAA} = \Delta\text{AA}/(1 - \text{AA selectivity index}) \quad (2)$$

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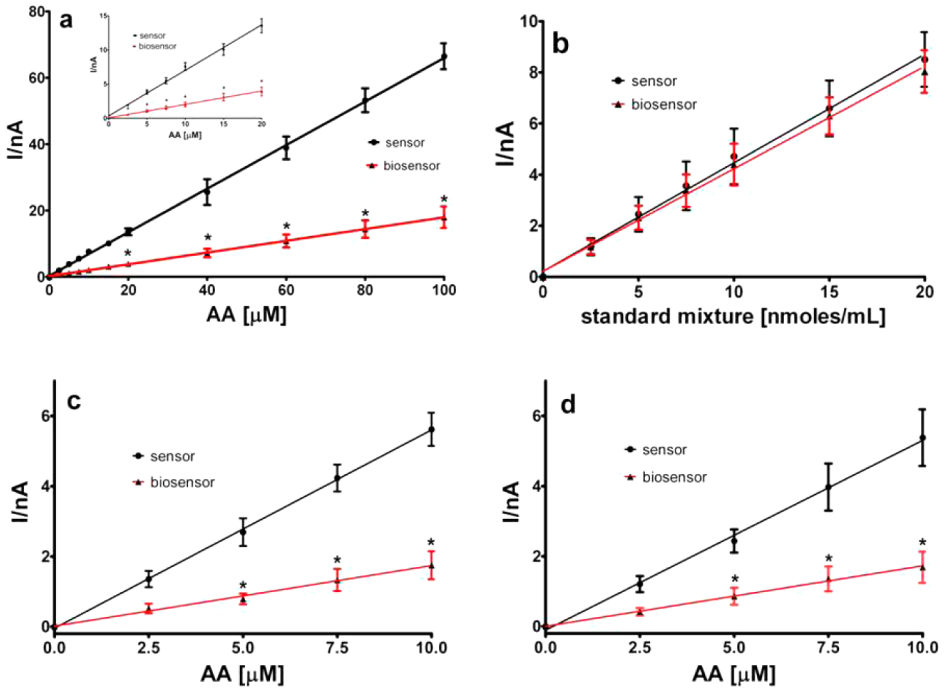

Figure 3. (a) Ten point calibration in PBS and linear regression of AA starting from 0 up to 100 μM performed with the SB. (Inset of a) Six point AA calibration in the 0−20 μM concentration range. (b) Six point calibration in PBS and linear regression of phenols' standard mixture, starting from 0 up to 20 μM, performed with SB. (c,d) AA calibration performed with the standard addition method: (c) The addition of a standard mixture spiked with increasing quantities of AA to the PBS determined a linear increase in the AA registered currents, both of sensor and biosensor. (d) The addition of juice spiked with increasing quantities of AA, to the PBS (d) determined a linear increase in the AA registered currents, both of sensor and biosensor. An asterisk (*) indicates statistical significant difference between the currents obtained with the sensor and those obtained with the biosensor for the same AA concentration, according to Student's *t*-test ($P < 0.05$).

In eq 1, the value of ΔAA is given by ΔAA = Sac − Bac. In eq 2 SAA represents 100% of the AA current that the system is able to detect, and ΔAA is proportional to 1 − AA selectivity index.

The current value SAA, converted as mg/100 mL, measures all AA content in the samples. The current values Sac, SAA and Sac − SAA, converted as μmol of AA equivalents/mL of juice, represent the antioxidant capacity of the juice, the AA contribution to antioxidant capacity, and the phenols contribution to antioxidant capacity, respectively. The use of an ascorbate oxidase-based biosensor systems is not new,[22,24,25] but all of the cited systems were set up for a selective assay of AA or to shield a biosensor from AA interference.[28] The use of this enzyme to discriminate between the contribution of AA and phenols to antioxidant capacity represents a new approach to monitoring AA in foods.

Phenols Influence on SB Behavior. The capacity of SB to record AA currents is influenced by phenols and/or other compounds of orange juice. The calibrations of AA spiked in the standard mixture (r^2 = 0.999, slope =0.565 ± 0.008 nA/μM; r^2 = 0.993, slope = 0.173 ± 0.007 nA/μM, respectively, for sensor and biosensor; Figure 3c), and in the orange juice (r^2 = 0.997, slope = 0.541 ± 0.016 nA/μM and r^2 = 0.997 slope =0.172 ± 0.005 nA/μM respectively for sensor and biosensor) (Figure 3d), compared with that carried out in PBS, revealed that the exposure to the standard mixture and to juice negatively conditioned the sensor and the biosensor performances but did not affect either the linear response or the AA selectivity index (0.31 and 0.32 respectively vs 0.29 obtained in PBS). The results also indicated that this performance decreasing depends on the polymerization of phenols (and not of other substances) on the electrode surface, since the performances of SB exposed to standard mixture and to orange juice are quite similar.

Phenol Composition of Orange Varieties. The phenol composition of the juice of the three varieties is reported in the Supporting Information (Table S-1). The results are in accordance with previous studies on blonde and blood orange varieties.[6,31]

Voltammetric Behavior of Phenols at Carbon Electrode. CVs, the potential at which the oxidation starts, and the peak potential value of phenols characterizing the juice of the three orange varieties are reported in the Supporting Information (Figure S-2 and Table S-2).

LOD, LOQ, and Aging of the SB. The limit of detection (LOD) and of quantification (LOQ) of the SB were calculated as follows: LOD = 3.3 σ/S where σ is the standard deviation of background noise of the sensor (or biosensor) and **S** is the slope of the linear region of the calibration curve: LOQ = 3*LOD. The LOD and the LOQ of the sensor were 0.26 ± 0.12 μM and 0.77 ± 0.37 μM, respectively.

The reproducibility of responses of the SB was tested by 10 subsequent measurements of a standard mixture (5 μM) alternate to AA (5 μM). Standard mixture currents of 3.20 ± 0.60 nA and 3.08 ± 0.52 nA for the sensor and biosensor, respectively, and AA currents of 2.04 ± 0.52 nA and 0.93 ± 0.32 nA for sensor and biosensor respectively, were registered.

The operational stability over time of sensor and biosensor was tested by subsequent current measurements relative to 40 injections of 60 μL of Hamlin juice (the SB was rinsed after every measurement). Results indicated a good operational stability until the 30th injection, 6.23 ± 0.89 nA and 2.09 ± 0.36 nA for sensor and biosensor, respectively; then the

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currents dramatically decreased to values 70% lower than the initial ones.

Choice of the Working Potential Concentration Range and Time for Data Acquisition. As previously said, the SB works at E_{app} = +500 mV, in a concentration range comprised between the LOD and 20 μM. Two minutes is the time needed to acquire a measure, thus overcoming passivation effects of the electrodes due to phenol polymerization. These working parameters were considered the most appropriate since they satisfied the following conditions:

- E_{app} = +500 mV ensured selectivity toward those compounds effectively able to protect biological targets from oxidation. The antioxidant efficiency is dependent on the ability of a free radical scavenger (FRS) to formally donate a hydrogen atom or an electron to a free radical, and the reduction of a FRS should be lower than +600 mV.[1] +500 mV was the potential applied to detect antioxidant power in several types of tea infusions, and this value was demonstrated to be able to discriminate the compounds with effective antioxidant power.[11]
- The linear responses of sensors and biosensors were not affected by phenol polymerization, despite a slight decreasing in the registered currents being observed during the electro-oxidation of phenol compounds. The formation of a polymeric film on the electrode surface of Au, Pt, and carbon electrodes was demonstrate to promote electrode passivation. High potentials and long oxidation time determined more significant passivating effects, especially in matrices with acidic pH.[32,33]

Performance of the Dual Channel Telemetric Device. The use of the dual-channel telemetric device as a portable workstation allowed the detection of AA content and antioxidant capacity without moving out orange samples from storage rooms. According to previous results,[27] the electronic calibration was made with a linear distance between the transmitter and the receiver unit of about 3 m. Before the measurements with the SB, the analog electronics of the telemetric device had been studied by connecting a dual-channel (25 MΩ) dummy cell (used as Thevenin current source)[34] and generating a stable current of 20 nA per channel (VApp =0.5 V). The signal was recorded for 6 h and showed a stable response over time and a noise around 25 pA. In a successive series of tests, a 10-point linear calibration was performed in a range of currents comprised between 0 and 40 nA (by increasing VApp from 0 to 1 V). The recorded currents were plotted versus the expected current values generating a linear graph with a slope of 0.9927 ± 0.006 nA nA^{-1} (r^2 = 0.9998). These data confirm the experimental results obtained in previous studies.[26,28,34,35]

AA Content and Antioxidant Capacity Determination. Tables 1 and 2 show the results obtained with the SB for each orange variety. The highest AA concentration was found in Hamlin juice, while Moro contained a sensibly lower quantity of AA than the other two varieties (Table 1). These values were in accordance with those achieved with titrimetric and HPLC methods.

Hamlin, the blond variety, showed also the highest antioxidant capacity, statistically higher than Sanguinello and Moro (Table 2). Kelebek et al.[31] found a higher antioxidant activity in Moro than in Sanguinello juice, but in that case the AA content of Moro and Sanguinello juice was quite similar while the phenol content was really different. In our work, Moro and Sanguinello juices have similar phenol content, while Sanguinello has a higher AA content than Moro, thus resulting in a higher antioxidant capacity.

Table 1. Ascorbic Acid Content (mg/100 mL) As Determined by SB at +500 mV, Titrimetric, and HPLC Methods in the Juice of Hamlin, Sanguinello, and Moro Oranges[a]

AA juice content (mg/100 mL)			
	method of detection		
orange varieties	SB	titrimetric	HPLC
Hamlin	79.10 a (a)	74.90 a (a)	76. 32 a (a)
Sanguinello	70.37 b (a)	69.50 b (a)	68.12 b (a)
Moro	45.28 c (a)	n.d.	47.12 c (a)

[a]Means in columns followed by unlike letters differ significantly by Fisher's least significant difference (LSD) procedure, $P \leq 0.05$. Means in rows followed by (unlike letters) differ significantly by Fisher's least significant difference (LSD) procedure, $P \leq 0.05$.

Table 2. Antioxidant Capacity (Ascorbic Acid Equivalents) As Determined by SB (electrochemical) at +500 mV, DPPH (EPR), and ABTS (Spectrophotometric) Methods, in the Juice of Hamlin, Sanguinello and Moro Oranges[a]

antioxidant capacity (μmol equivalents of AA/mL of juice)				
		orange varieties		
method of detection		Hamlin	Sanguinello	Moro
SB	total	4.64 a(a)	4.23 b(b)	3.46 c (b)
	$AA_{contribution}$	4.49 a	4.00 a	2.57 b
	$Ph_{contribution}$	0.15 c	0.23 b	0.89 a
DPPH	total	4.99 a (a)	4.72 a (a)	3.07 a (b)
ABTS	total	5.07 a (a)	4.76 a (a)	3.95 b (a)

[a]AA and phenol contributions to antioxidant capacity were also provided. Means in columns followed by unlike letters differ significantly by Fisher's least significant difference (LSD) procedure, $P \leq 0.05$. Means in rows followed by (unlike letters) differ significantly by Fisher's least significant difference (LSD) procedure, $P \leq 0.05$. n.d. = not detectable.

The results were confirmed by reference methods since measurements achieved with DPPH and ABTS did not statistically differ from those obtained with SB.

AA and phenol contribution to antioxidant capacity of the oranges was very different and flesh color dependent (Table 2). The SB attributed 26% of antioxidant capacity of Moro to phenols: this contribution is due to a high content of anthocyanins and hesperidin (see Tables S-1 and S-2 in Supporting Information). On the other hand, only 3.2% of antioxidant capacity of Hamlin can be ascribed to phenols, in particular to hesperidin. The hesperidin and a little content of anthocyanins seem to be responsible for 5.4% of antioxidant capacity of Sanguinello. Narirutin, which is highly represented in Sanguinello and Hamlin, is oxidized starting from +600 mV, and for this reason, its contribution to antioxidant capacity cannot be detected by SB. Previous studies on the relative contributions of AA and phenols to the antioxidant capacity of orange juices led to opposite conclusions: Gardner et al.[5] indicated that AA accounted for 65−100% of the total antioxidant activity, thus supporting our observation that AA is the major antioxidant in orange juice; on the contrary, the studies of Rapisarda et al.[36] on Moro, Sanguinello, and other blonde orange varieties, affirm that AA plays a minor role and that the antioxidant efficiency should be attributed to phenol content.

A further confirmation of the effectiveness of the proposed system comes from the phenol content evaluation, assessed by

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eliminating all AA from the samples using AOx. The recorded currents in treated samples, transformed in micromole equivalents of AA/mL of juice, were compared to the results in Table 2 relative to phenol contribution to antioxidant capacity: 0.40 ± 0.24 vs 0.44 ± 0.33 for Hamlin, 0.68 ± 0.21 vs 0.70 ± 0.17 for Sanguinello, and 2.73 ± 0.56 vs 2.66 ± 0.80 for Moro; the results did not statistically differ.

CONCLUSION

In this paper, we showed the ability of SB to assess, with a single analysis, AA content and antioxidant capacity, and to distinguish between the AA and phenols contribution to the antioxidant capacity. This is a novelty compared to the most accredited evaluation methods.

We demonstrated that is possible to have highly reproducible analytical performances without expensive instrumentation. The SB is low cost in comparison with traditional methods considering that, with four graphite rods, it can supply a correct measurements of AA content and antioxidant capacity in the juice of different orange varieties.

The system also indicated that the orange varieties with the highest concentration of AA have the highest antioxidant capacity. Considering that there is not a complete agreement, in the literature, about the relative contribution of AA and phenols to the antioxidant capacity of orange juice, our SB seems particularly suitable for this kind of analysis. Furthermore, the SB device is particularly advantageous for colored samples. The presence of pigments, as in blood orange juice, could negatively influence the results of analyses based on color change of redox indicators or cromogen radicals. The SB aims to be a simple and low-cost diagnostic valid alternative for AA and antioxidant capacity detection, indifferently for blonde and blood orange varieties, and for those juices of species containing red pigments.

The use of the telemetry in the postharvest sector of the food industry is still new. The possibility of coupling the SB with the telemetric device makes possible an effective rapid screening of AA and antioxidant capacity at any moment of the productive chain of orange juice, at harvest in the field, later in storage, and refrigerating rooms and during the processing for commercialization.

ASSOCIATED CONTENT

Supporting Information

Additional information as indicated in the text. This material is available free of charge via the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interest.

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