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Development of a cytochrome *c*-based screen-printed biosensor for the determination of the antioxidant capacity of orange juices

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ABSTRACT

This paper describes the development of an amperometric cytochrome *c* (cyt *c*)-based biosensor and its later application to the quantification of the scavenging capacity of antioxidants. The enzymatic biosensor was constructed by covalently co-immobilizing both cyt *c* and XOD on a mercaptoundecanol/mercaptoundecanoic acid (MU/MUA) mixed self-assembled monolayer (SAM)-modified screen-printed gold electrode. The applicability of this method was shown by analyzing the antioxidant capacity of pure substances, such as ascorbic acid and Trolox, and natural sources of antioxidants, particularly 5 orange juices.

1. Introduction

Free radicals are highly unstable molecules with available electrons that can be generated in vivo during metabolic processes. Reactive oxygen species (ROS) represent the most important class of free radicals generated in living systems. Biologically important ROS include superoxide anion ($O_2^{\bullet -}$), singlet oxygen (O_2^1), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl radical ($OH^{\bullet}$), peroxynitrite ($ONOO^-$), peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals [1]. These ROS can cause damage at various sites in the cell (membranes, cytoplasm and nucleus). In order to avoid damage, the cell contains various enzymes and antioxidants to provide protection in these cell compartments [2]. However, environmental or behavioural stressors, such as pollution, sunlight exposure, cigarette smoking and excessive alcohol consumption, or simply a malfunction of the antioxidant production may lead to a free radical excess, resulting in oxidative stress. It has been established that oxidative stress is involved in the aging process [3] as well as in numerous human diseases, including heart disease [4], cataracts [5], cognitive dysfunction [6] and cancer [7].

Although our body implements different antioxidants, their external supply is essential to countervail the harmful consequences of oxidative stress. Fruits and vegetables have received particular attention because they contain high amounts of known antioxidants, such as vitamins C and E, carotenoids and polyphenols [8]. The consumption of fruits and vegetables has been associated with low incidences and mortality rates of cancer [9] and heart disease [10].

The determination of free radicals and antioxidants has been widely investigated in the food technology field. Traditional techniques, such as spectrophotometry, fluorescence or chromatography [11,12], are being replaced by other innovating technologies. In this direction, electrochemical techniques have opened new possibilities to know the "total polyphenolic" content because of its selectivity [13,14]. In the last years, electrochemical methods have been combined with biosensors. Electrochemical biosensors have become promising tools since they provide the advantage of rather simple equipment and operation protocols [15]. On one hand, several amperometric biosensors for the detection of mono or polyphenols have been developed on the basis of enzymes such as tyrosinase, laccase or peroxidase [16]. On the other hand, biosensors for the assessment of the antioxidant capacity are based on the free radical scavenging ability. There are two main types of electrochemical $O_2^{\bullet -}$ biosensors, based on either superoxide dismutase (SOD) or cytochrome *c* (cyt *c*).

The first group is based on the specific dismutation of $O_2^{\bullet -}$ by SOD to O_2 and H_2O_2. Usually, the electrooxidation of H_2O_2 at the electrode surface generates the detection signal [17,18]. However, this oxidation reaction occurs in a high potential (>0.5 V vs. Ag/AgCl), which results in interference problems, limiting the practical applications of these biosensors.

The second group is based on the reduction of the immobilised cyt *c* by $O_2^{\bullet -}$ radicals followed by the electrooxidation of the redox protein at the gold electrode surface, leading to an oxidation current proportional to the radical concentration [19]. The electron transfer between the electrode and the cyt *c* can be achieved by employing specific

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modifications of the electrode surface, for example with mixed long-chain self-assembled monolayers (SAMs) [20,21]. However, these sensors usually show very small signal responses due to spontaneous dismutation of the $O_2^{\cdot-}$ radical in the way from the bulk where it is generated to the electrode surface where it reacts with the cyt *c*. $O_2^{\cdot-}$ radicals are usually produced in the solution through the enzymatic reaction between the xanthine oxidase (XOD) and the hypoxanthine (HX).

This paper describes the development of a superoxide biosensor based on the co-immobilization of cyt *c* and XOD on a SAM-modified gold electrode and its later application to the determination of the antioxidant capacity of pure substances and several orange juices.

2. Materials and methods

2.1. Reagents

XOD from buttermilk (EC 1.17.3.2), catalase from bovine liver (EC 1.11.1.6), cyt *c* from horse heart and HX, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 11-mercapto-1-undecanol (MU) and 11-mercaptoundecanoic acid (MUA), ascorbic acid, caffeic acid, gallic acid, ferulic acid, curcumin, catechol, quercetin, nitroblue tetrazolium (NBT), potassium hexacyanoferrate (III) and potassium hexacyanoferrate (II) trihydrate were purchased from Sigma (St. Quentin Fallavier, France). N-hydroxysuccinimide (NHS) was provided by Fluka (France) and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) from Aldrich (France). All reagents were of analytical grade and were used without further purification. All solutions were prepared using Milli-Q water.

2.2. Preparation of the antioxidant samples

Pure antioxidant compounds were freshly prepared in 0.1 M potassium phosphate buffer (K-PB) pH 7.0. Four Spanish brand name orange juices and a fresh-pressed one were centrifuged at 10 000 rpm for 5 min at room temperature. The resulting supernatants were used directly as final samples.

2.3. Apparatus

Colorimetric measurements were performed with a Beckman DU520 UV–Vis Spectrophotometer (Beckman Coulter France, S.A., Roissy CDG, France). Cyclic voltammetry (CV) was carried out using an AUTOLAB PGSTAT12 Potentiostat (Metrohm, France). Amperometric measurements for the determination of the scavenging capacity of antioxidants were made using a 641VA potentiostat (Metrohm, France), connected to a BD40 (Kipp & Zonen, The Netherlands) X-t recorder at 150 mV.

2.4. Electrode preparation

Gold screen-printed electrodes (DropSens, Spain) of 12.6 mm^2 were used as working electrodes. A platinum wire and a double-junction Ag/AgCl electrode (Thermo Orion 900200) were used as counter and reference electrodes.

Gold electrodes were electrochemically cleaned by cycling the potential between 0 and +1.4 V at the scan rate of 100 mV s^{-1} in 0.1 M H_2SO_4 until the characteristic cyclic voltammogram for a clean gold electrode was obtained. After this, electrodes were rinsed with water and ethanol and incubated in an ethanolic solution containing 3.75 mM MU and 1.25 mM MUA. The modified gold surface was then rinsed thoroughly with absolute ethanol and with water to remove any unattached thiol molecules. The MU:MUA monolayer was next activated incubating the electrode in an aqueous solution of EDC (200 mM) and NHS (50 mM) for 30 min. The excess of EDC and NHS was eliminated by cleaning with 5 mM K-PB pH 7.0. Cyt *c* and XOD were then immobilized on the modified electrode surface by incubating the electrodes during 30 min in a 50 μM cyt *c* + 50 mU mL^{-1} XOD solution in 5 mM K-PB pH 7.0. Finally, the cyt *c*/XOD-modified electrodes were rinsed again with the same buffer solution to remove non-attached substances.

2.5. Measurement of the superoxide scavenging capacity

2.5.1. Measurement of the superoxide scavenging capacity using the cyt *c*-based biosensor

The detection principle is based on the redox reaction of cyt *c* with $O_2^{\cdot-}$ radicals, which are generated by the HX/XOD system (Fig. 1). The immobilised cyt *c* is reduced by the $O_2^{\cdot-}$ radical and immediately regenerated at the electrode surface polarised at its oxidation potential (150 mV vs. Ag/AgCl). In order to avoid interferences from the H_2O_2, generated as a final product of the enzymatic reaction or by spontaneous dismutation of the $O_2^{\cdot-}$ radical, catalase enzyme is added to the reaction media. The oxidation current might be related to the superoxide concentration, even if some H_2O_2, produced near the vicinity of the modified electrode surface, can still react with the immobilized cyt *c*. The addition of antioxidants reduces the radical concentration and thus the oxidation current, allowing the quantification of their antioxidant capacity.

All amperometric experiments were carried out under constant stirring in 0.1 M sodium phosphate buffer (Na-PB) with 100 μM EDTA pH 7.5. Before measurements, a catalase solution (final concentration in the cell = 10 U mL^{-1}) was added. Once a stable baseline was established, the HX solution was added into the cell (final concentration = 80 μM) and a steady-state superoxide level was

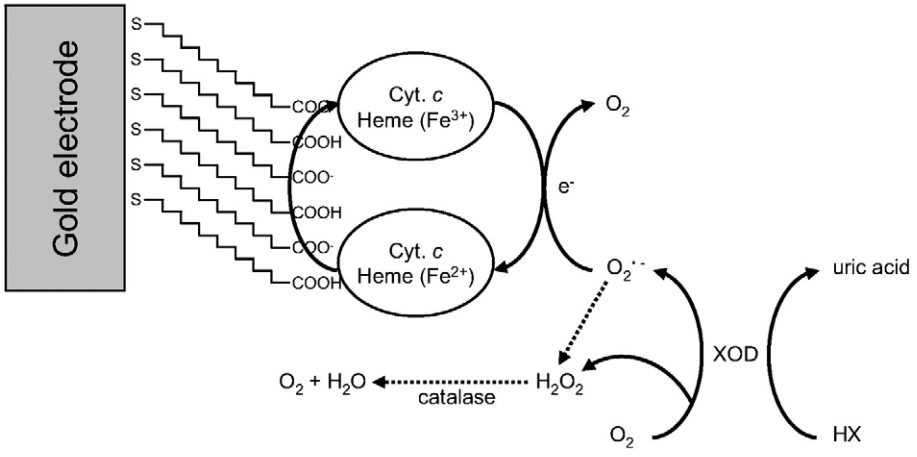

Fig. 1. Scheme of the detection principle of $O_2^{\cdot-}$ produced by the HX/XOD enzyme system, using a cyt *c*-modified electrode.

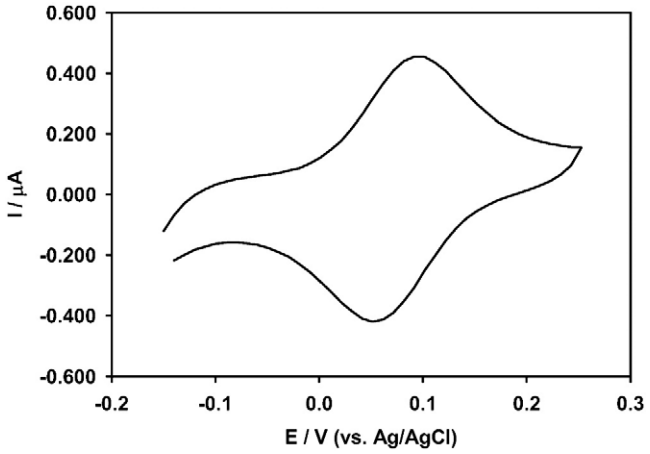

Fig. 2. Cyclic voltammogram of the covalently immobilized cyt *c* on a SAM-modified gold electrode in 5 mM K-PB pH 7.0. Scan rate: 100 mV s^{-1}.

recorded. Stock solutions of HX were prepared in 50 mM K-PB pH 7.5 with 0.01 M KCl and 0.5 mM EDTA.

In the investigation of the role of superoxide scavengers, aliquots of the antioxidant samples were added and the current decrease was recorded after the addition of HX. The decrease of the signal was normalized regarding the initial ROS signal, which minimized the sensitivity variation between sensors. The antioxidant capacity was calculated by considering the sample concentration necessary for 50% signal inhibition (IC_{50}). This value was used for comparison between antioxidants: low IC_{50} corresponded to a high antioxidant capacity.

2.5.2. Measurement of the superoxide scavenging capacity using the NBT method

In this method, the $O_2^{\cdot -}$ radical reduces NBT (yellow) into NBT formazan (purple), which is measured spectrophotometrically at 560 nm. The presence of radical scavengers inhibits the formation of NBT formazan leading to a decrease of its production rate. As before, the $O_2^{\cdot -}$ radicals were generated in vitro by the HX/XOD system.

The experimental procedure was as follows. A reaction mixture was prepared with 50 mM K-PB pH 7.5 containing EDTA (0.1 mM), 25 µM HX, 50 µM NBT, the antioxidant extract (distilled water for the blank) and 0.2 U mL^{-1} XOD, which was added last. The increase in absorbance for 3 min was recorded at 560 nm. Stock solutions of NBT, HX and XOD were prepared in 50 mM K-PB pH 7.5 containing EDTA (0.1 mM).

The antioxidant capacity was also determined by calculating the IC_{50}. This value corresponded to the sample concentration necessary for 50% inhibition of the NBT formazan production rate.

2.6. Colorimetric method for the quantification of ascorbic acid in orange juices

A standard colorimetric method (Roche Diagnostics, Mannheim, Germany) was used for the quantification of ascorbic acid in orange juices. In this case, the ascorbic acid reduces the tetrazolium salt MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] in the presence of the electron carrier PMS (5-methylphenazinium methosulfate) at pH 3.5 to formazan (MTT-formazan), which is determined by means of its light absorbance at 578 nm.

3. Results and discussion

3.1. Electrochemistry of the immobilized cyt c

The electrochemical behaviour of the immobilized cyt *c* was investigated by CV. A characteristic cyclic voltammogram for cyt *c* immobilized on a mixed SAM is shown in Fig. 2. A pair of well-defined redox peaks was observed with a formal potential, $E^{\circ\prime}$, taken as $(E_p^c + E_p^a)/2$, of 62 mV and with a peak-to-peak separation value, ΔE_p, of 41 mV, at a scan rate of 100 mV s^{-1}. The quasi-reversible redox transformation of cyt *c* is the basis of the amperometric superoxide detection.

According to the Laviron method [22], the surface coverage (Γ) and the electron transfer rate constant (k_S) of the immobilized cyt *c* were calculated to be 14.5 pmol cm^{-2} and 42.8 s^{-1}, respectively.

In order to check the blocking characteristics of the prepared SAM, CV was performed in an hexacyanoferrate solution (1 mM $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$ in 0.1 M K-PB pH 7). Fig. 3 illustrates the voltammetric behavior of the bare and the modified electrodes.

As can be seen, the bare gold electrode showed a well defined faradaic response. In contrast, the cyclic voltammogram for the electrode prepared with the mixed SAM showed effective blocking for this redox couple. It was attributed to the repulsing interaction of the negative charge on the SAM and the negatively charged redox couple.

If cyt *c* electrodes were polarised at 150 mV, the amperometric detection of $O_2^{\cdot -}$ radicals was feasible. When the HX solution was added into the cell, containing the modified electrode, an oxidation current of around 20 nA was obtained. This oxidation current corresponded to 1.6 nA mm^{-2}. Thus, the current density of the biosensor based on the co-immobilization of cyt *c* and XOD on a SAM-modified gold electrode was at least 5 times bigger than those reported in the bibliography [20,23,24], where the XOD remained in solution. As cyt *c* and XOD were very close, generated $O_2^{\cdot -}$ radicals could quickly react with cyt *c*, avoiding its spontaneous dismutation. Hence better sensitivities were achieved by using the developed approach.

The need for catalase, when XOD remains in solution, is not clear and some opposite results can be found in the bibliography. On the one hand, Tammeveski et al. [19] stated that no change in the electrode response was seen in the presence of catalase over the range of XOD concentrations used, thus eliminating the possibility of H_2O_2 causing re-oxidation of the reduced cyt *c* and leading to overestimation of the response due to superoxide. On the other hand, Lisdat et al. [23] stated that a stable cyclic voltammogram during the enzymatic radical production by the HX/XOD system was only visible in the presence of catalase. In fact, this enzyme removed the coproduced H_2O_2 which otherwise would interfere with the measurement because of the reaction with the reduced cyt *c*. Therefore, Tammeveski et al. claim that H_2O_2 do not react with cyt *c* while Lisdat et al. claim that both species can react if catalase is not present in the reaction media. In the present work, with both cyt *c* and XOD immobilized on the electrode surface, first experiments were performed

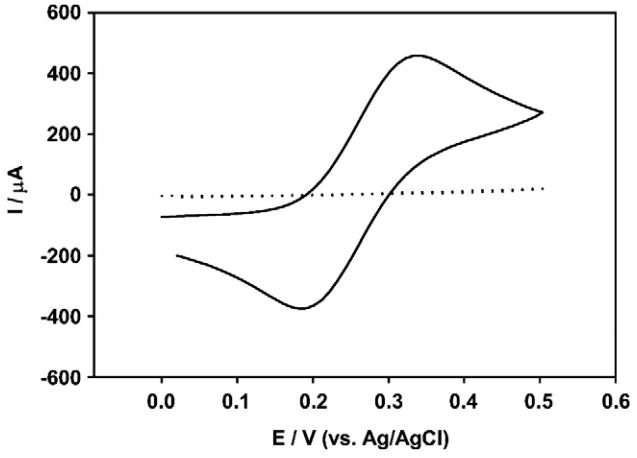

Fig. 3. Cyclic voltammograms of the bare gold electrode (solid line) and the SAM-modified gold electrode (dotted line) in 0.1 M K-PB pH 7.0 containing 1 mM $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$. Scan rate: 100 mV s^{-1}.

without using catalase. In that case, the signal never reached the steady-state. Therefore, new experiments with catalase in the reaction media were carried out, leading to stable and reproducible signal current responses. Hence, all experiments were performed in presence of catalase.

3.2. Determination of the antioxidant capacity of pure substances and natural sources of antioxidants

3.2.1. Antioxidative properties of pure substances

The developed biosensor was first applied to the determination of the antioxidant capacity of pure substances such as ascorbic acid, caffeic acid, gallic acid, ferulic acid, curcumin, catechol, quercetin and Trolox, which is a water soluble derivate of vitamin E.

Amperometric measurements were made by following the protocols detailed in Sections 2.3 and 2.5. In Fig. 4 the current decrease versus acid ascorbic concentration is shown.

The hyperbolic fit allowed the determination of IC_{50}. The data points were fitted according to the Hill equation [25]:

$$\text{Signal inhibition}\,(\%) = \frac{100}{1 + (IC_{50}/[\text{Antioxidant}])^h} \quad (1)$$

where h is the Hill coefficient and IC_{50} is the antioxidant concentration necessary for 50% signal inhibition. These two values were determined for each antioxidant compound using the SigmaPlot 2000 computer software. Thus, the IC_{50} and the Hill coefficient for the ascorbic acid were calculated to be 30.3 ± 0.9 μM and 1.04 ± 0.02, respectively. On the other hand, Trolox was found to be less effective with an IC_{50} value of 162.1 ± 0.7 μM and a Hill coefficient of 1.27 ± 0.05.

These results were compared with those obtained through the spectrophotometric NBT method, described in Section 2.5. For each tested compound, the NBT formazan production rate was plotted versus the antioxidant concentration. The obtained calibration curve for ascorbic acid is shown in Fig. 5. In all cases, IC_{50} values were graphically determined from the plots. Thus, IC_{50} values for ascorbic acid and Trolox were 34.0 ± 0.8 μM and 209.5 ± 0.9 μM, respectively.

No significant results were obtained with the other tested pure compounds by using the developed biosensor. On the one hand, no scavenging capacity against $O_2^{\cdot -}$ radicals was found for both ferulic acid and curcumin. On the other hand, with the other compounds, a steady-state superoxide level was not obtained.

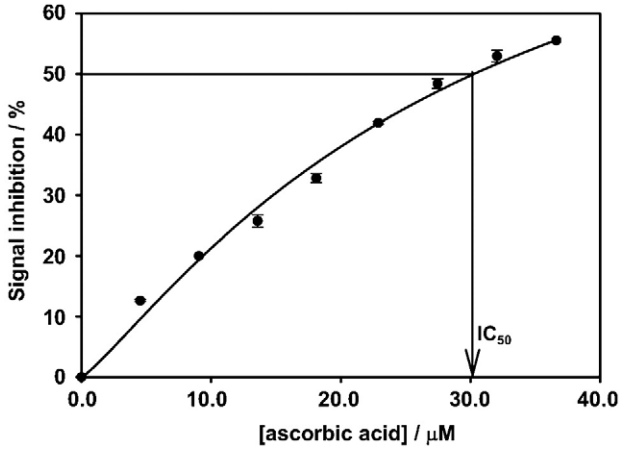

Fig. 4. Representation of the signal inhibition by different concentrations of ascorbic acid. Superoxide generated with 80 μM HX. Error bars are deduced from three repeated measurements.

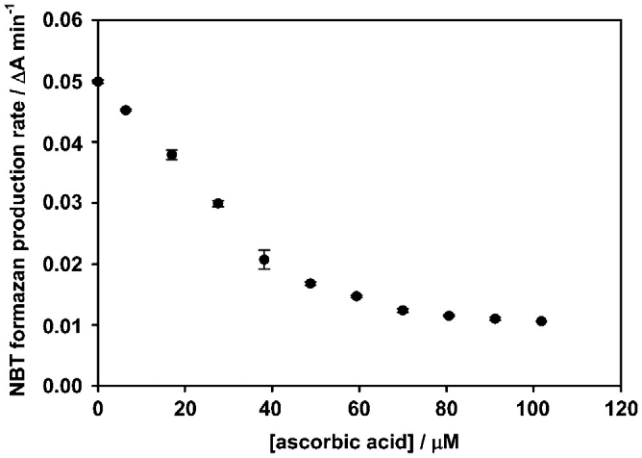

Fig. 5. Representation of the NBT formazan production rate by different concentrations of ascorbic acid. Error bars are deduced from three repeated spectrophotometric measurements.

3.2.2. Antioxidative properties of orange juices

Finally, in order to show the ability of the system to allow quantification of antioxidative properties even in complex media, 5 orange juices were tested by using the developed cyt *c* biosensors as well as the NBT method. Orange juice contains an array of potent antioxidants including flavonoids (hesperetin and naringenin predominantly as glycosides), carotenoids (xanthophylls, cryptoxanthins, and carotenes), and vitamin C in addition to other beneficial phytochemicals, such as folate. Thus, the addition of orange juice samples caused a decrease in current signal, allowing a quantification of their antioxidant capacity.

In order to compare their efficiency against $O_2^{\cdot -}$ radicals, ascorbic acid was used as standard substance. In all cases, the radical scavenging capacity was calculated as IC_{50} and expressed as AEAC (Ascorbic acid Equivalent Antioxidant Capacity):

$$\text{AEAC} = \frac{1/IC_{50}\ \text{sample}}{1/IC_{50}\ \text{ascorbic acid}} = \frac{IC_{50}\ \text{ascorbic acid}}{IC_{50}\ \text{sample}} \quad (2)$$

All resulting values are collected in Table 1. In each case, the ascorbic acid concentration was obtained through the standard colorimetric method described in Section 2.6.

By using the two different methods, almost the same AEAC was obtained for each sample and the following trend could be concluded: Natural juice>>Brand 1>Brand 2>Brand 4>Brand 3.

The agreement between both methods was also evaluated, using the *t*-test, carried out as a bilateral coupled test [26]. The tabulated *t*-value of 2.57 when compared with the calculated one of 2.42 showed the absence of statistical differences for those results obtained by the two different methods at the 95% confidence level.

Table 1
Comparison of the $O_2^{\cdot -}$ scavenging capacity of different orange juices determined by using the developed biosensor and the NBT method.

	Cyt *c*-based biosensor		NBT method	
Sample	IC_{50}/μM	AEAC	IC_{50}/μM	AEAC
Ascorbic acid	30.3 ± 0.9	1.00	34.0 ± 0.9	1.00
Brand 1 (2.27 mM)	17.0 ± 0.8	1.78	16.9 ± 0.8	2.01
Brand 2 (1.56 mM)	17.2 ± 0.7	1.76	17.5 ± 0.8	1.94
Brand 3 (0.91 mM)	26.4 ± 0.8	1.14	29.6 ± 0.9	1.15
Brand 4 (2.22 mM)	18.9 ± 0.9	1.60	20.2 ± 0.8	1.68
Natural orange juice (1.01 mM)	10.6 ± 0.5	2.84	11.5 ± 0.7	2.96

Values for pure ascorbic acid are also included. Ascorbic acid concentrations are given in parentheses. Measurements were performed in triplicate.

The relative antioxidant capacity of orange juices was always bigger than the one obtained with the pure ascorbic acid. This fact may be understood by considering that natural and commercial juices contain other antioxidant substances, although around two thirds of their antioxidant capacity is usually attributed to the vitamin C [27].

Furthermore, the highest antioxidant capacity was obtained with the natural orange juice even if its ascorbic acid concentration was small. In fact, commercial squeezed juices usually contain a higher concentration of vitamin C than domestic squeezed ones, which are richer in soluble phenolic compounds [27]. Therefore, the antioxidant capacity of the natural orange juice could not be mainly attributed to ascorbic acid but to other antioxidant compounds.

4. Conclusions

In this paper, an amperometric biosensor for the quantification of the scavenging capacity of antioxidants has been developed. An MU/MUA-modified gold electrode with immobilized cyt *c* and XOD has been characterized and applied to the antioxidants analysis. The co-immobilization of both species on the electrode surface increased at least 15 times the amperometric current signals in comparison with those where the XOD remained in solution. The concentration which induces a 50% inhibition of the superoxide level has been determined for different substances. The applicability of this method has been shown by analyzing the antioxidant capacity in vitro of ascorbic acid, Trolox and 5 orange juices. The antioxidant capacity of orange juices was always bigger than the one obtained with the pure ascorbic acid. This fact may be understood by considering that natural and commercial juices contain other antioxidant substances.

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