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Short communication

Exploration of the global antioxidant capacity of the stratum corneum by cyclic voltammetry

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Abstract

Cyclic voltammetry is proposed as a new method for evaluating the antioxidant capacity of skin based on the reducing properties of low molecular weight antioxidants (LMWA). Experiments were performed simply by recording the anodic current at 0.9 V/SCE of a platinum microelectrode placed directly on the epidermis surface without any gel or water. This method ensured a direct, rapid (less than 1 min), reliable (accuracy 12%) and non-invasive measurement of the global antioxidant capacity of the stratum corneum with a high spatiotemporal resolution. At the same time, the pH of the skin surface was determined by recording the cathodic current at 0 V/SCE. Based on an exploratory study involving nine volunteer subjects, the evolution of the amperometric response of the microelectrode with time revealed a periodic modification of the redox properties.

Keywords: Skin; Antioxidants; Microelectrodes; Cyclic voltammetry; Oxidative stress

1. Introduction

Oxidative stress has been one of the most widely studied biochemical processes for several years as it has been considered as an entity similar to the inflammation syndrome. It is involved in the aging process and is also the cause of cataracts, cancers, cardiovascular or degenerative diseases (Parkinson's, Alzheimer's) [1,2]. Oxidative stress is the consequence of an imbalance between pro- and antioxidant species. The former essentially concerns highly reactive oxygen or nitrogen species (ROS or RNS), e.g., superoxide anion $O_2^{\bullet -}$, hydrogen peroxide H_2O_2, hydroxyl radical $OH^{\bullet}$, nitric oxide NO or peroxynitrite $ONOO^-$ [3]. Naturally produced by the organism, these metabolites play a role in many physiological processes: cell respiration, neurotransmission, blood vessel dilatation, cytochrome P450-mediated or immune reactions [4–6]. Nevertheless, excessive production leads to the oxidation of lipids, proteins and nucleic acids, and contributes to the cellular dysfunction and death [7]. To avoid oxidative stress, cells are equipped with two major antioxidant defense systems. The first concerns enzymes which catalyze ROS or RNS degradation, such as superoxide dismutase, catalase or glutathione reductase. The second involves low molecular weight antioxidants (LMWA) like Vitamins A, C, E and glutathione, which reduce ROS and RNS by oxido-reduction

Abbreviations: ABTS, 2,2′-azino-(3-ethyl-benzothialozine-6-sulfonic acid); *C*, concentration of electroactive species; *D*, diffusion coefficient; *F*, Faraday's constant; FRAP, ferric reducing ability of plasma; H_2O_2, hydrogen peroxide; I_{lim}, limiting current; KCl, potassium chloride; KH_2PO_4, potassium dihydrogen phosphate; K_2HPO_4, dipotassium hydrogen phosphate; $K_3[Fe(CN)_6]$, potassium ferricyanide; LMWA, low molecular weight antioxidant; *n*, electron number; NADPH, nicotinamide adenine dinucleotide phophate; NO, nitric oxide; $OH^{\bullet}$, hydroxyl radical; $ONOO^-$, peroxynitrite; $O_2^{\bullet -}$, superoxide anion; Pt, platinum; PtO, platinum oxide; RNS, reactive nitrogen species; ROS, reactive oxygen species; SCE, saturated calomel electrode; TEAC, trolox equivalent antioxidant capacity; UV, ultraviolet

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reactions [8,9]. Skin, which constitutes the interface between the human body and the environment, is the largest organ and its outermost barrier. It represents a major target of oxidative stress since it is continuously exposed to external oxidant aggressions, such as ultraviolet (UV) radiation, ozone or chemicals. Repeated exposure to such aggressions is responsible for skin damage and contributes to the development of cutaneous diseases like psoriasis and cancers [10]. The epidermis provides the first line of defense against exogeneous ROS by means of many antioxidant systems [11].

Numerous analytical techniques are available to evaluate oxidative stress namely, electron spin resonance, chromatography, spectroscopy or mass spectrometry [2,12–15]. All these techniques need expensive materials, involve complex protocols, often need tissue biopsy and provide delayed results. Electrochemistry has been considered for a few years as a promising alternative approach. Electrochemistry deals with electron transfer phenomena between an electrode and oxidized or reduced molecules. It therefore represents a suitable methodology to study the redox properties of solid, liquid or gaseous media and to detect oxidant and antioxidant species. Cyclic voltammetry was for example, tested to evaluate the global antioxidant capacity of real samples like wine, biological fluids or tissues [16–18]. The anodic part of the voltammogram provided information concerning the ability of LMWA to transfer electrons to an anode, and thus to act as reducing agents. Kohen recently applied this technique successfully to skin analysis [19,20]; nevertheless, the method used suffered from two major drawbacks. Firstly, the analysis was either invasive because it involved skin homogenates, or indirect as it was performed in an electrolytic solution in contact with the skin surface. Secondly, the measurements used macroelectrodes with surface areas in the range of a square centimeter; they presented low sensitivity and did not allow localized measurements. The present paper shows preliminary works allowing the direct evaluation of the global antioxidant capacity of the stratum corneum. Cyclic voltammograms were performed directly on the skin surface without adding water or gel. Platinum disk microelectrodes with surface areas lower than 2×10^{-3} mm^2 were used in order to perform rapid, sensitive, non-invasive and localized measurements on the skin surface. The accuracy of the amperometric signal was studied, as well as the evolution of the electrochemical response in time.

2. Material and methods

2.1. Chemicals

All chemicals were reagent grade and used as-received. Potassium ferricyanide $K_3[Fe(CN)_6]$ and potassium chloride KCl were purchased from Acros Organics (Noisy Le Grand, France). Potassium dihydrogen phosphate KH_2PO_4 and dipotassium hydrogen phosphate K_2HPO_4 were purchased from Sigma–Aldrich (Lyon, France). Unless otherwise indicated, all solutions were prepared in phosphate buffer (KH_2PO_4/K_2HPO_4 0.1 mol L^{-1}, pH 7.0).

2.2. Apparatus and material

The electrochemical experiments were carried out with a PAR model 283 potentiostat (Princeton Applied Research) interfaced to a microcomputer and controlled by the CorrWare software (Ametek, Trappes, France), or with an Autolab Metrohm potentiostat controlled by the General Purpose Electrochemical System software (Metrohm, Courtaboeuf, France). The accuracy of the amperometric response was evaluated by using a VMP2/Z multipotentiostat controlled by the ECLab software (Ametek, Trappes, France). A 50 μm diameter or a 1 mm diameter platinum wire (ref. PT025110 and LS263293, respectively) was used as working electrode (purchased from Goodfellow, Lille, France). They were inserted in a glass capillary (GC-120F-10) from Clark Electromedical Instruments (Phymep, Paris, France). A microelectrode puller (Model PC10) and a microgrinder (Model EG44) were also used (purchased from Narishige, London, UK). A large surface area platinum disk was used as counter-electrode. All potentials were measured and expressed versus a saturated calomel reference electrode (SCE). A glass membrane electrode purchased from Hanna Instruments (VWR, Fontenay-sous-Bois, France) was used to measure the skin pH.

2.3. Microelectrode fabrication and electrochemical characterization

Glass-encased platinum microelectrodes were used [21]. Briefly, the 50 μm diameter platinum wire was inserted in a glass capillary. The capillary was pulled into two microelectrodes with the microelectrode puller. A tapered end was produced and warmed up again to consolidate the cohesion between glass and platinum. Then the tip was polished on the diamond particle whetstone microgrinder at an angle of 90° for several minutes. The resulting microelectrodes presented a disk geometry with a total radius of less than 120 μm. The exact platinum electrode radius was determined by cyclic voltammetry performed at 50 mV s^{-1} between −0.5 V and 0.4 V/SCE in a 5 mmol L^{-1} deaerated ferricyanide solution (see below).

2.4. Cyclic voltammetry on skin

All measurements were made on the inside of the forearm of the volunteers. The reference electrode, counter-electrode and one or several working microelectrodes were placed directly on the skin surface, without adding water or gel. The saturated calomel reference electrode was connected to the skin by a felt-tip pen used as a Luggin capillary, since it allowed a more pleasant application on skin than a glass cap-

illary. Cyclic voltammograms were plotted between −0.4 V and 1.2 V/SCE, thus avoiding the reduction and oxidation of water, with a potential scan rate varying between 10 and 500 mV s^{-1}.

2.5. pH measurements

pH measurements were made by applying the glass membrane on the inside of the forearm.

3. Results and discussions

3.1. Electrochemical determination of the electrode radius

The radius of the platinum disk microelectrodes was determined by plotting the current-potential curve in a 5 mmol L^{-1} deaerated ferricyanide solution. Fig. 1 shows the steady-state voltammogram corresponding to the electrochemical reduction of ferricyanide into ferrocyanide: $Fe(CN)_6^{3-} + e \rightarrow Fe(CN)_6^{4-}$. The current generated at a microelectrode is dependent on its geometry. For a disk microelectrode, the limiting current is directly proportional to the disk radius r (cm) [22] : $I_{lim} = 4nFrDC$, where I_{lim} is the limiting current (Amperes), n the number of electrons exchanged, F the Faraday's constant (96,500 C), D the diffusion coefficient ($cm^2\ s^{-1}$) and C is the concentration of the electroactive species (mol cm^{-3}). The diffusion coefficient of the ferricyanide being estimated at 7×10^{-6} $cm^2\ s^{-1}$ [23,24], the radius of the microelectrodes was deduced from the limiting current recorded at −0.4 V/SCE. More than 300 microelectrodes were produced; the average radius was 30.5 ± 2.9 μm, a value close to the platinum wire radius commercially indicated. As the microelectrodes were handmade, each radius was determined experimentally. In order to compare the results obtained on the skin with the different microelectrodes, all the amperometric responses were referenced to the same surface area of the electrode.

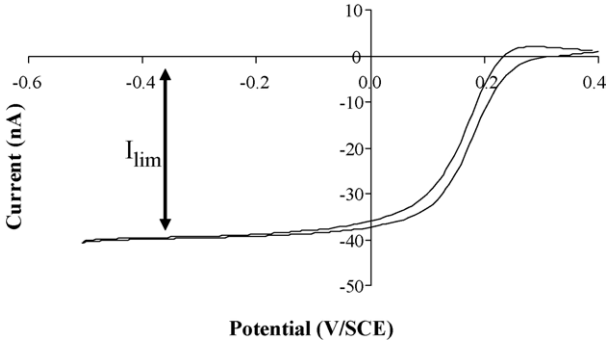

Fig. 1. Cyclic voltammogram performed with a 50 μm diameter platinum microelectrode in a deaerated 5 mmol L^{-1} $Fe(CN_6)^{3-}$ solution (pH 7.0). Potential scan rate: 50 mV s^{-1}.

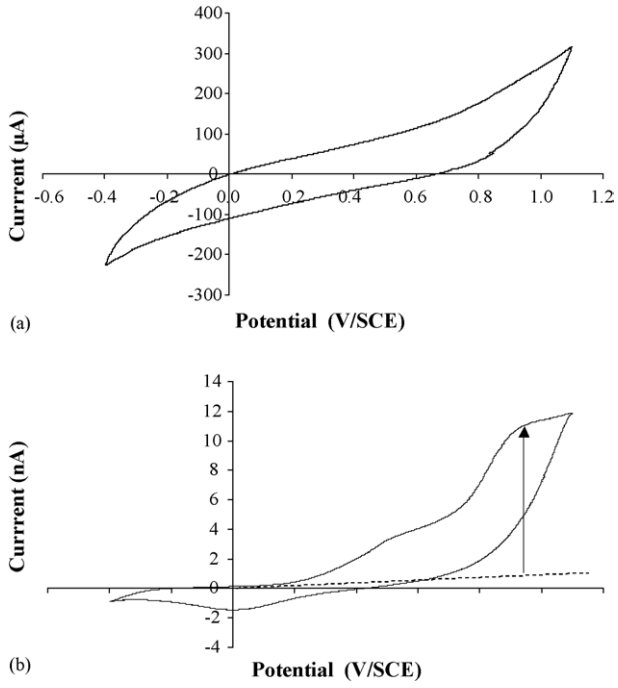

Fig. 2. Cyclic voltammograms obtained directly on the forearm: (a) with a 1 mm diameter platinum electrode and (b) with a 50 μm diameter platinum electrode. Potential scan rate: 50 mV s^{-1}.

3.2. Cyclic voltammogram on skin

3.2.1. Protocol validation

Fig. 2a and b show cyclic voltammograms obtained on the skin with a 1 mm diameter disk platinum electrode and with a 50 μm diameter disk microelectrode, respectively. In the former case, the curve displayed a current with high resistive and capacitive components, leading to unusable data. In comparison, a well defined sigmoid steady-state voltammogram was obtained with the microelectrode showing different faradic current waves. Comparison of the two results clearly demonstrates that microelectrodes offer access to cyclic voltammetry experiments, which are impossible with conventionally-sized macroelectrodes. In addition to analyses in small volumes or at microscopic locations, microelectrodes allow measurements in resistive media and make it possible to perform high scan rate voltammetry [25]. These differences are caused by the modification of mass transport conditions in the vicinity of the electrode surface. With macroelectrodes, the electrode size is greater than that of the diffusion layer in solution; the electrode geometry can therefore be considered flat and the mass transport unidirectional, mostly perpendicular to the surface. In contrast, the dimensions of a microelectrode are of the same order of magnitude as the diffusion layer thickness; mass transport takes on a hemispherical profile, increasing the amount of electroactive species diffusing to the electrode. In the present application, the miniaturized size of the working microelectrode enhanced mass transport and improved the sensitivity of the response [26]. Well defined anodic and cathodic currents were observed even with

electroactive species at very low concentrations. It was not necessary to deposit water or gel on the skin surface. For all the volunteer subjects, the ionic conductivity of the skin was high enough owing to the hydro-cutaneous film present on the surface. The current generally recorded being in the range of a few nano-Amperes, no Ohmic drop between the working and the reference electrodes was generated. It was verified that the current–potential curves were identical whether the experiments were performed with or without water added. It was thus been considered that applying the microelectrode directly on the skin surface would give more reliable results than adding water, which could modify the cutaneous surface.

3.2.2. pH measurement

The voltammogram obtained with the microelectrode (Fig. 2b) presented two major waves. At potentials near 0 V/SCE, a cathodic wave corresponded to the reduction of platinum surface oxides : $PtO + 2H^+ + 2e \rightarrow Pt + H_2O$ [27]. The electrochemical reaction rate being dependent on the concentration of protons, the corresponding amperometric response could be affected by the pH of the skin surface. Fig. 3 shows that a linear correlation was effectively obtained by plotting the cathodic current as a function of the skin pH measured with a glass membrane electrode. The absolute value of the amperometric response increased as the pH increased, i.e. as the concentration of protons decreased; this shows that the platinum oxide reduction is more complex than the global reaction indicates. In each experiment, the lower and upper potentials of the cyclic voltammogram were chosen in order to avoid oxidation and reduction of water, thus excluding the variation of pH induced by the electrochemical reactions. Experiments carried out with different groups of subjects confirmed this correlation. Moreover, previous works done in our laboratory have clearly shown that this cathodic current is correlated with the skin pH [28].

3.2.3. Evaluation of stratum corneum global antioxidant capacity

The anodic part of the voltammogram showed two anodic waves with limiting currents recorded at 0.6 V and 0.9 V/SCE. These anodic waves are related to electrochemical–oxidation reactions which are due to the presence of reduced species at the electrode/skin interface. After deduction of the residual current estimated by the extrapolation of the tangent at 0 V/SCE as shown in Fig. 2b, amperometric signals were evaluated at 0.9 V/SCE. Indeed at this potential, the signal includes the global antioxidant level. Complementary experiments were performed by immersing the microelectrode in 0.1 mol L^{-1} phosphate buffer or 20 mmol L^{-1} potassium chloride solutions. The anodic charge corresponding to the oxidation of the platinum surface into platinum oxide ($Pt + H_2O \rightarrow PtO + 2H^+ + 2e^-$), as well as that corresponding to the oxidation of chloride ions ($2Cl^- \rightarrow Cl_2 + 2e^-$), were recorded at 0.9 V/SCE (data not shown). It was verified that these amounts of charge contributed at most to 30% of the anodic charge recorded when the experiment was performed with the microelectrode applied on the skin surface. Consequently, the anodic currents observed with the skin cannot be principally due to either the oxidation of the electrode material or the chloride ions naturally present in the sweat. One can better deduce that the amperometric response is principally correlated to the reduced species on the cutaneous surface, thus playing a role in its global antioxidant capacity. Kohen et al. previously showed cyclic voltammograms using macroelectrodes introduced in a buffer solution left in contact with the skin [19,20]: anodic waves were observed at potentials (0.6 V and 1.0 V/SCE) close to those indicated here. Furthermore, they showed by complementary analyses by HPLC that these electrochemical responses were correlated to antioxidant species like ascorbic acid and uric acid. Similar experiments were successfully carried out with our microelectrodes (data not shown). All these results demonstrate the potential offered by cyclic voltammetry to reveal the global antioxidant properties of skin using microelectrodes.

In addition, according to Fick's law, the thickness of the diffusion layer is directly proportional to the square root of the diffusion coefficient: $x = (4Dt)^{1/2}$, where x is the thickness (cm), D the diffusion coefficient ($cm^2\ s^{-1}$) and t is the measurement time (s). Knowing that the diffusion coefficients of hydrophilic molecules within the stratum corneum were estimated between 10^{-8} and $10^{-10}\ cm^2\ s^{-1}$ [29–31], direct measurement allows the exploration of the extracellular medium of the stratum corneum on a thickness from 1 to 10 μm.

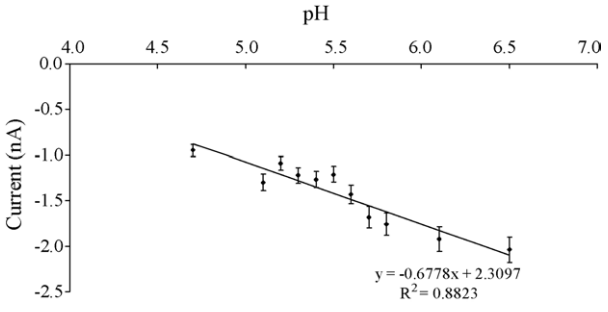

Fig. 3. Correlation between the skin pH and the cathodic current measured at 0 V/SCE. The error bars give the mean standard deviation.

3.2.4. Measurement accuracy

Fig. 4a shows four successive cyclic voltammograms obtained on the skin surface. The microelectrode was applied on the forearm and its location was unchanged during the whole experiment. The anodic current recorded at 0.9 V/SCE decreased as scan number increased. This result cannot be due to an inconstant amount of platinum oxide generated at the electrode surface, since the successive cathodic waves at 0.0 V/SCE corresponding to the reduction of platinum oxides remained essentially unchanged. These variations more probably result from a modification of the electrode surface state

induced by the electrochemical oxidation of the antioxidant species. In order to validate this assumption, the experiment was repeated with different potential scan rates. In each case, a new microelectrode was used and only the first voltammogram was recorded. Fig. 4b shows the amperometric response measured at 0.9 V/SCE as a function of the potential scan rate. The current increased linearly with the scan rate, indicating that the electrochemical reaction was limited by the adsorption of one or several species on the platinum surface [32]. These adsorbed species progressively covered the electrode surface and modified its electrochemical properties.

Obtaining reliable measurements therefore implies using each microelectrode once only. The evaluation of repeatability, by means of the standard deviation of the signal obtained with the same electrode placed on the same skin surface, was therefore impossible. In order to validate the methodology, a multipotentiostat was used to determine the accuracy of the measurement. Three cyclic voltammograms were recorded simultaneously with three different microelectrodes. The anodic current was recorded at 0.9 V/SCE for each electrode. Twenty volunteer subjects were involved. For the first 13 volunteers, the three microelectrodes were placed at 1 cm from each other. For the seven other volunteers, the microelectrodes were placed at 5 cm from each other. A set of three new microelectrodes was used for each subject. The mean standard deviation of the amperometric response was estimated to be 12% for the first group of volunteer subjects and 9% for the second. These results attest to the reliability of the method and also give evidence for the homogeneity of the amperometric response over a large skin surface area (around 11 cm^2).

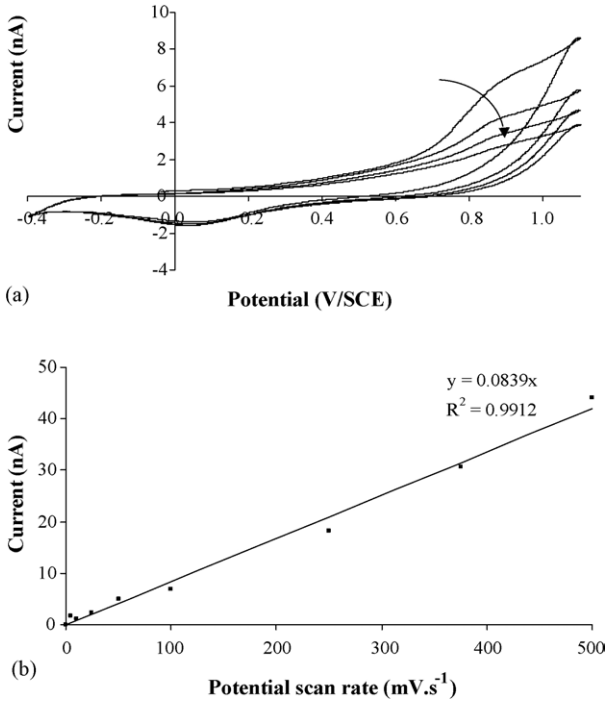

Fig. 4. (a) Successive cyclic voltammograms obtained on the skin surface with a 50 μm diameter platinum microelectrode. Potential scan rate: 50 mV s^{-1}. (b) Influence of the potential scan rate on the current recorded at 0.9 V/SCE.

3.3. Applications: evolution of amperometric signal with time

In perpetual contact with the environment, the skin ensures a protective role and to do so successfully the epidermis surface is in constant renewal [33]. In order to show the evolution of the global antioxidant capacity of the stratum corneum with time, a preclinical study was performed. Nine volunteer subjects were involved, five men and four women between 20 and 25 years old and with a skin phototype II. Successive cyclic voltammograms were performed every 15 min over 7 h. All the experiments started at 9:30 a.m. During the study, the subjects did not wash their arms with toilet soap, remained relaxed in the same room and their diet was checked. Each measurement was realized with a new microelectrode. Fig. 5 shows the typical curve giving the evolution of the anodic current at 0.9 V/SCE as a function of time. Accuracy of the amperometric response is indicated for each measurement. A sinusoidal evolution was observed in all cases. Current values as well as the amplitude and the period of the variations were different for each subject. The amplitude of the values was significantly higher than the accuracy of the amperometric response. Likewise, no variation of the cathodic wave corresponding to the reduction of platinum oxides was observed. Consequently, the variation of the anodic current was actually due to a variation of the redox properties of the skin and was not an artifact of the measurements. The method therefore seems to be suitable to follow the modification of antioxidant levels in real time. This modification could result from enzymatically catalyzed oscillating reactions occurring in the epidermis and involving oxidants and antioxidants. It is thought that the exact period and amplitude of these variations could be determined by continuously recording the amperometric response of the microelectrode and that the mean standard deviation of the amperometric response could be determined by using the multipotentiostat. This work is in progress in our laboratory.

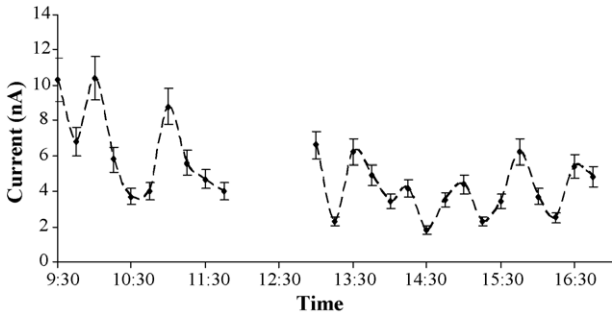

Fig. 5. Evolution of the current at 0.9 V/SCE recorded with a 50 μm diameter platinum microelectrode on forearm skin with time. Error bars correspond to the mean accuracy of the measurement.

4. Conclusion

Cyclic voltammetry allowed direct, rapid, non-invasive, precise and high spatiotemporal resolution measurements to be made on the skin surface by using ultramicroelectrodes. The anodic current recorded at 0.9 V/SCE on platinum was correlated to the global antioxidant capacity of the epidermis surface, whereas pH was determined simultaneously at 0 V/SCE. Involving a multipotentiostat allowed the validation of the measurement and to estimate the mean accuracy at 12%. Evolution of the anodic response with time revealed a periodic modification of the antioxidant properties. This method could be used to evaluate the effect of oxidative stress induced by UV irradiation.

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