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DETERMINATION OF ANTIOXIDANT CAPACITY USING XANTHINE-XANTHINE OXIDASE SYSTEM COUPLED WITH H_2O_2 AMPEROMETRIC BIOSENSOR

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A new approach for antioxidant capacity (AOC) determination was proposed. In order to reduce the risk of electrochemical interferences, the new method takes advantage of using a H_2O_2 amperometric biosensor (based on horseradish peroxidase wired by Os-redox polymer), operated at low applied potential (-0.100 V *vs.* Ag/AgCl,KCl_{sat}). This biosensor was coupled with the xanthine-xanthine oxidase system which, as a result of the spontaneous dismutation of superoxide radicals ($O_2^{-\bullet}$), generates H_2O_2. The addition of an antioxidant (AOX), by its scavenging ability for $O_2^{-\bullet}$ radicals as well as by consumption of H_2O_2, will decrease the H_2O_2 concentration. This effect was exploited for AOC determination of the investigated AOX. The whole bioanalytical system was optimized regarding to its working principle. The electroanalytical parameters for H_2O_2 and ascorbic acid (AA; selected as reference AOX) detection, were estimated. Finally, the AOC for real samples of citrus juices were assessed by using the new approach, and were found in good correlation with those provided by the method based on electrochemical detection of $DPPH^{\bullet}$, as well as with their polyphenolics content, estimated using Folin-Ciocalteu method, and their AA content, assessed using a titrimetric method.

INTRODUCTION

There is increasing evidence of the fact that antioxidants play a crucial role in the human health, because of their ability to scavenge free radicals.[1,2] Therefore, consumption of fresh fruit and vegetables, which are commonly known to contain a high amount of antioxidants, is highly recommended.

There are various terms used to express the efficiency of an antioxidant (AOX), but one of the most widely used is the antioxidant capacity (AOC). However, it is noteworthy to observe that, despite the importance assigned to this subject, no unique definition was stated for AOC. Mainly, this is due to the complex nature of AOX which depending on the reaction media may play various roles, and, consequently, they cannot be defined by a single type of action. However, two broad meanings for AOC definition are generally accepted: (i) the AOX ability to scavenge free radicals ($HO^{\bullet}$, $O_2^{-\bullet}$, $ROO^{\bullet}$, $DPPH^{\bullet}$, $ABTS^{+\bullet}$, etc.) and/or (ii) the AOX ability to remove nonradical reactive oxygen species (such as H_2O_2, NO_3^-, HOCl, etc).[3-6]

Among the various methods known for AOC evaluation, based on spectrophotometric, colorimetric, fluorimetric, electrochemical, and chemiluminescence measurements,[7-9] the amperometric biosensors, exploiting various enzymes, offer certain advantages due to their high selectivity, good sensitivity, fast response and simple operation mode.

Several amperometric biosensors have already been proposed for AOC evaluation.[6,10-20] Most of them are based on the amperometric detection of H_2O_2, resulting from the catalyzed dismutation of superoxide radicals ($O_2^{-\bullet}$) in presence of superoxide dismutase (SOD), at an applied potential around +0.650 V,[10-13,16] or at +0.300 V *vs.* Ag/AgCl,KCl_{sat} as is the case of SOD - based

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biosensors detecting directly the $O_2^{-\bullet}$ radicals.[15] Another approach consisted in using cytochrome c (Cyt c) for $O_2^{-\bullet}$ detection, requiring a lower applied potential (+0.15 V *vs.* Ag/AgCl,KCl_{sat}).[18-20] However, Cyt c is not specific for $O_2^{-\bullet}$, and ascorbic acid (AA) or glutathione may be oxidized too. Also, Cyt c is less active than SOD, displaying a reaction rate by several orders of magnitude lower.[16] The main drawback of these strategies is due to the high applied potential, which considerably increases the risk of electrochemical interferences. Moreover, all above-presented methods assess the AOC of an AOX mainly as its scavenging activity for $O_2^{-\bullet}$ radicals, neglecting the inherent interactions between the AOX and other reactive oxygen species such as H_2O_2, despite that a good correlation was reported between the AOX ability to consume H_2O_2 and their activity as radicals scavengers. [21-26]

In this context, a new bioelectroanalytical system for AOC evaluation was proposed, by coupling an amperometric biosensor for H_2O_2 detection, obtained by modification of a graphite electrode (G) with Os-polymer wired horseradish peroxidase (HRP), with xanthine oxidase (XOD) – xanthine (XA) enzymatic system, as generator of $O_2^{-\bullet}$ radicals. The new strategy has two important advantages: (i) it works at low applied potential (-0.100 V *vs.* Ag/AgCl,KCl_{sat}), allowing a significant decrease of the risk of electrochemical interferences; (ii) the AOC determination, requiring the monitoring of H_2O_2 concentration in presence of AOX as well as in its absence, will be a global estimation of the free radicals ($O_2^{-\bullet}$) and nonradical reactive species (H_2O_2) interactions with the investigated AOX. The paper presents the preparation, optimization and characterization of the H_2O_2 amperometric biosensor and its utilization for AOC evaluation of some real samples (fresh juice of oranges or grapefruits), using AA as a reference antioxidant. The obtained results were found in good correlation with those provided by three reference methods: the Folin-Ciocalteu method for phenolics content determination, the method based on electrochemical detection of $DPPH^{\bullet}$ and the titrimetric method based on 2,6-dichlorophenolindophenol sodium salt (DCPIP) for AA content estimation.

RESULTS AND DISCUSSION

1. Optimization of the bioanalytical system

1.1. Effect of SOD incorporation

The SOD incorporation in the biosensor construction is expected to enhance the biosensor sensitivity, due to an increase of the detected H_2O_2 concentration resulting from the catalytic activity of SOD on the $O_2^{-\bullet}$ radicals dismutation.[10-13] In order to quantify this effect in the case of our bioanalytical system, two biosensors, G/Os-HRP-SOD and G/Os-HRP, were similarly prepared, excepting the presence/absence of the immobilized SOD in the enzymatic matrix (as described in the Experimental). Both biosensors were calibrated against XA (Fig. 1), and their sensitivities were estimated as the ratio between the kinetic parameters I_{max} and K_M, obtained from the Michaelis - Menten fit. Surprisingly, as can also be observed from Fig. 1, the G/Os-HRP biosensor was slightly more sensitive (-66.2 ± 2.5 μA/mM) than G/Os-HRP-SOD (-58.7 ± 2.3 μA/mM). This unexpected behavior could be due to the fact that the beneficial effect of SOD presence in the sensing matrix is surpassed by its hindering effect on the H_2O_2 transport from the adjacent buffer solution to the electrode surface.

In order to confirm this unexpected effect of the SOD presence in the enzymatic matrix, amperometric measurements were carried out with G/Os-HRP and G/Os-HRP-SOD biosensors, when CAT, XOD and XA were successively introduced into the biosensor adjacent solution (Fig. 2). In the bulk of solution, the H_2O_2 concentration, generated by the XA-XOD system, will be kept low due to the CAT addition. However, in the immediate proximity of the electrode surface, the immobilized SOD will produce a supplementary increase of H_2O_2 concentration due to the catalytic dismutation of $O_2^{-\bullet}$ radicals. Consequently, it can be stated that when XA additions are performed, as a final result of the two contrary effects mentioned above, a higher reduction current should be expected in the case of G/Os-HRP-SOD bioelectrode than in the case of G/Os-HRP one. However, within the experimental errors, the reduction currents observed after the first addition of 23 μM XA were almost identical: 0.07 μA for G/Os-HRP-SOD and 0.08 μA for G/Os-HRP. At the same time, both currents were ~20 times smaller than that recorded in absence of CAT. It was concluded that: (i) the introduction of immobilized SOD in the construction of H_2O_2 biosensor has no beneficial effect on its sensitivity; (ii) despite the successive additions of XA, the presence of dissolved CAT avoids the increase of H_2O_2 concentration in solution; (iii) all experiments should be performed further only with the G/Os-HRP biosensor.

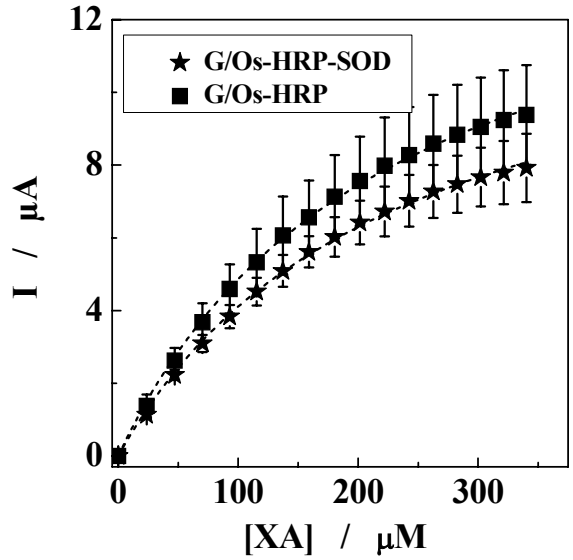

Fig. 1 – Calibration curves for G/Os-HRP and G/Os-HRP-SOD biosensors. Experimental conditions: applied potential, -0.100 V *vs.* Ag/AgCl,KCl_{sat}; supporting electrolyte, 0.1 M PB containing 0.1 XOD U/mL (pH 7.5); stirring rate, 1500 rpm.

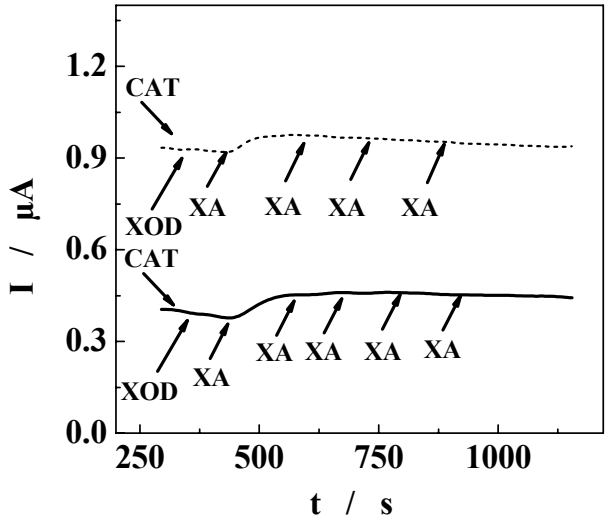

Fig. 2 – Amperometric responses recorded for G/Os-HRP-SOD (-----) and G/Os-HRP (——) biosensors for successive additions of 23 μM XA in PB solution (pH 7.5) containing CAT (295 U/mL) and XOD (0.1 U/mL). Experimental conditions: same as in Fig. 1.

1.2. Effect of XOD immobilization

Further investigations were done in order to establish the best modality to incorporate XOD in the biosensor construction: dissolved or immobilized. Obviously, the most inexpensive variant is that based on immobilized XOD, but this could not be, at the same time, the most efficient one. For this reason, three similar biosensors were constructed: two having different loading of immobilized XOD and one using dissolved XOD in the adjacent buffer solution (as described in the Experimental). The results of the amperometric measurements carried out, in presence of 23 μM XA, with G/Os-HRP-XOD bioelectrodes showed much lower reduction currents [(103 ± 1) μA for G/Os-HRP-XOD(1) and (146 ± 8) μA for G/Os-HRP-XOD(2)] than that recorded for G/Os-HRP bioelectrode (1050 μA) coupled with dissolved XOD. It is worth to mention that: (i) even if the total amount of XOD (17.5 mU) used for immobilization on G/Os-HRP-XOD(2) was close

to that dissolved in the buffer solution (21 mU), in the case of free XOD the biosensor signal was more than 10 times higher; (ii) the lower permeability of the enzymatic matrix, probably due to a higher XOD loading, could explain the significantly smaller reduction current observed in the case of biosensor with higher XOD loading. Taking into account all these observations, the G/Os-HRP biosensor coupled with dissolved XOD (0.1 U XOD/mL of PB) was chosen for all subsequent experiments.

2. Characterization of the bioanalytical system

In order to check the proper functioning of the G/Os-HRP biosensor for H_2O_2 detection, cyclic voltammetry measurements were carried out in PB solution (pH 7.5) without and with dissolved XA-XOD system (Fig. 3). As expected, due to H_2O_2 production by the XA-XOD enzymatic system, the peak current corresponding to the reduction of Os(III) to Os(II) increased significantly (~28%): from 3.2 μA, observed in the absence of XA-XOD, to 4.1 μA recorded in its presence. At the same time, this peak intensity increases with XA concentration increase (data not shown). Additionally, a decrease of the oxidation peak current was observed in presence of the XA-XOD system: from 4.1 μA, observed in absence of XA-XOD system, to 3.7 μA, estimated in its presence. Thus, it can be concluded that a clear electrocatalytic effect for H_2O_2 reduction at G/Os-HRP bioelectrode was evidenced.

The supplementary oxidation peak observed around +0.3 V *vs.* Ag/AgCl,KCl_{sat} was attributed to the uric acid, resulting from the enzymatic reaction. Obviously, this peak does not hinder in any way the H_2O_2 amperometric detection, because it is carried out in the domain of reduction potentials.

Amperometric measurements, carried out with the G/Os-HRP biosensor in presence of the XA-XOD system, allowed the estimation of the characteristic parameters for H_2O_2 detection. Thus, the calibration curve, obtained by using 0.1 XOD U/mL of PB solution and XA of increasing concentrations (from 23 to 340 μM), was fitted to Michaelis-Menten equation and gave the bioelectroanalytical parameters: I_{max}, the maximum current; K_M^{app}, the Michaelis-Menten apparent constant; S, the biosensor sensitivity; DL, the detection limit (estimated for the signal to noise ratio equal to 3); t_{95}, the response time (Table 1).

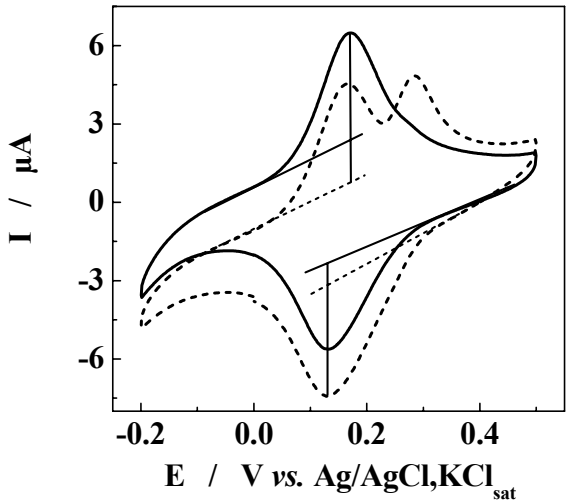

Fig. 3 – Cyclic voltammograms recorded at G/Os-HRP bioelectrode in PB solution (——) and in PB solution containing 0.1 XOD U/mL and 0.41 mM XA (-----). Experimental conditions: potential scan rate, 0.005 V/s; starting potential, -0.2 V *vs.* Ag/AgCl/KCl_{sat}.

Table 1

The bioelectroanalytical parameters of G/Os-HRP biosensor for H_2O_2 detection

I_{max} (μA)	K_m^{app} (μM)	S (mA/M)	R^2/N	DL (μM)	t_{95} (s)	RSD* (%)
17.7 ± 0.5	0.24 ± 0.01	73.7 ± 3.6	0.9978 / 17	1.4	55	8.7

* the relative standard deviation was estimated for 7 mM XA

3. AOC determination

3.1. Evaluation of AA as reference AOX

AA was chosen as reference antioxidant and, consequently, its voltammetric as well as its amperometric behavior were investigated using the G/Os-HRP biosensor. The main goal of these experiments was to establish the optimal experimental conditions for using AA as reference antioxidant, and to characterize the system for its detection.

Cyclic voltammetry measurements performed at G/Os-HRP bioelectrode, in presence of AA, showed that AA oxidation is catalyzed by the Os(II)/Os(III) redox system (Fig. 4). However, taking into account that in the domain of reduction potentials no electrochemical activity was observed, the potential, -0.100 V *vs.* Ag/AgCl,KCl_{sat} was considered suitable as applied potential to carry out amperometric measurements in presence of AA.

The bioelectroanalytical characterization of G/Os-HRP biosensor for AA detection in presence of XA-XOD system was performed by using amperometric measurements. Previously, the absence of amperometric response of the bioanalytical system to AA was confirmed by repetitive additions of AA in PB solution, containing dissolved XOD (data not shown). Successive additions of AA in PB solution containing the XA-XOD system allowed obtaining the calibration curve to AA. Due to the $O_2^{-\bullet}$ consumption by AA, the reduction current decreased continuously. By using Michaelis-Menten and linear fits, the obtained data were processed in order to estimate the bioelectroanalytical parameters for AA detection (Table 2).

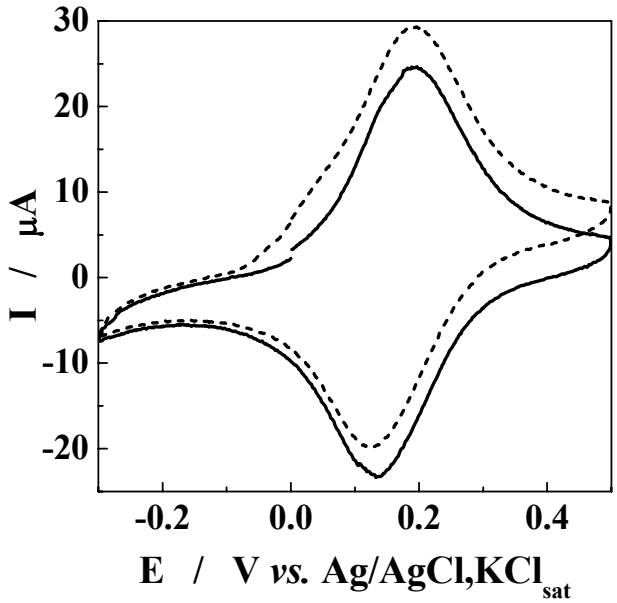

Fig. 4 – Cyclic voltammograms recorded at G/Os-HRP bioelectrode in PB solution (——) and in PB solution containing 1 mM AA (----). Experimental conditions: potential scan rate, 5 mV/s; starting potential, -0.300 V *vs.* Ag/AgCl/KCl_{sat}.

Table 2

The bioelectroanalytical parameters of G/Os-HRP biosensor for AA determination

Michaelis-Menten fit		**Linear fit**	
I_{max} (µA)	4.7 ± 0.1	**S (µA/mM)**	43.6 ± 0.8
K_m^{app} (µM)	80 ± 4	**R^2/N**	0.9983 / 7
S (µA/mM)	58.8 ± 3.2	**LD* (µM)**	7 – 47
R^2/N	0.9976 / 16	**DL (µM)**	1.2
	t_{95} (s)	90	

* linear domain

3.2. AOC evaluation of citrus samples

Finally, the AOC of two real samples (grapefruit juice and orange juice) was evaluated by using the new bioanalytical system, the Folin-Ciocalteu method, the method based on electrochemical detection of DPPH$^{\bullet}$, and the DCPIP-based titrimetric method for assessment of AA content (Table 3). AOC values were expressed as relative values, calculated as percents from the highest measured AOC value. For the method using the G/Os-HRP biosensor and the titrimetric method for AA evaluation, absolute AOC values were also reported as equivalent concentrations of AA. It is noteworthy to mention that all four methods were found in a good correlation.

Table 3

AOC values evaluated using the G/Os-HRP biosensor and three reference methods

Sample / Method	G/Os-HRP	Folin-Ciocâlteu method (%)	Electrochemical detection of DPPH$^{\bullet}$ (%)	Titrimetric determination of AA
Orange juice	(100.0 ± 13.8) % (2.9 ± 0.4) mM AA	100.0 ± 0.1	100.0 ± 4.4	(100.0 ± 7.2) % (8.3 ± 0.6) mM AA
Grapefruit juice	(75.9 ± 10.3) % (2.2 ± 0.3) mM AA	79.6 ± 0.01	89.4 ± 7.2	(73.5 ± 7.2) % (6.1 ± 0.6) mM AA

EXPERIMENTAL

1. Reagents and materials

Horseradish peroxidase (HRP; EC 1.11.1.7, 330 U/mg solid), xanthine sodium salt (XA), catalase from bovine liver (CAT; EC 232-577-1, 2950 U/mg solid), superoxide dismutase from bovine erythrocytes (SOD, EC 232-940-0, 5030 U/mg), L-ascorbic acid fine crystals (20-200 mesh), Folin-Ciocalteu reagent 2N, and 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) were purchased from Sigma (St. Louis, MO, USA). Poly(ethylene glycol) diglicidyl ether (PEGDGE) was obtained from Polysciences (Warrington, PA, USA). Xanthine oxidase from buttermilk (XOD; EC 232-657-6, 8 U/mg solid), potassium dihydrogen phosphate and di-potassium hydrogen phosphate trihydrate were provided by Merck (Darmstadt, Germany). Ethanol and 2,6-dichlorophenolindophenol sodium salt (DCPIP) were obtained from Riedel-de Haën (Hanover, Germany). Potassium chloride and sodium carbonate were purchased from Reactivul (Bucureşti, Roumania). The poly(1-vinylimidazole) complexed with (4-4'-dimethylbipyridine)$_2$ClOs(II/III) was a kind gift from dr. Elisabeth Csöregi (Department of Analytical Chemistry, Lund University, Sweden). All reagents were of analytical grade and were used as received.

2. Biosensors preparation

Biosensors were prepared by using working electrodes made of pyrolytic graphite rods (3 mm diameter), incorporated in Teflon® caps. The graphite rod was thoroughly polished using successively emery papers of 320 and 1200 grit; then the electrode was sonicated (Elma S-10 ultrasonic bath; Elmasonic, Singen, Germany) in distilled water for 2 minutes. The absence of any redox species on the graphite surfaces was checked by cyclic voltammetry performed in the supporting electrolyte (0.1 M phosphate buffer (PB), pH 7.5). The electrode surface was considered clean when two consecutive voltammetric cycles, recorded between -0.300 and +0.500 V *vs.* Ag/AgCl,KCl$_{sat}$ (20 mV/s scan rate), showed no significant redox activity.

Biosensors of different compositions (Table 4) were prepared by spreading 5 µL of the corresponding mixture on the graphite electrode surface. The modified electrodes were allowed to dry at room temperature for 2.0 hours, in a beaker covered with filter paper. Until use the biosensors were kept at ~5 °C in an atmosphere saturated with PB vapors.

3. Methods used for AOC determination

3.1. Folin-Ciocalteu spectrophotometric method

The Folin-Ciocalteu spectrophotometric procedure, used for phenolics content evaluation, is described elsewhere.[27] Briefly, it uses a mixture of phosphotungstic and phosphomolybdic acids (FC reagent), which turns blue when oxidized by polyphenols. The sample AOC is measured as the final absorbance of the sample- FC reagent mixture.

A mixture of 132 µL of 2N FC reagent, 330 µL of 200 g/L Na_2CO_3 and 165 µL of juice sample (1:3 diluted with water) was brought to a total volume of 5 mL using distilled water. The mixture was incubated for 1.5 hours at room temperature; then, the absorbance was measured at 662 and 668 nm for grapefruit and orange juice, respectively. Stable absorbance values were obtained within 5 minutes.

3.2. Method based on electrochemical detection of DPPH$^{\bullet}$

The method is based on the amperometric monitoring of DPPH$^{\bullet}$ concentration. The presence of an AOX will induce a current decrease, allowing quantifying its AOC. The procedure is described elsewhere[28] and it was adapted as follows: in a 5 µL of citrus juice were added in 5 mL of 49.5 µM DPPH$^{\bullet}$ solution (40:60 mixture of ethanol and 0.05 M PB), under continuous stirring. AOC value is given as the value of the current variation recorded after the sample addition.

3.3. Titrimetric evaluation of AA content

The AA content of the citrus juice samples was evaluated by the titrimetric method based on using DCPIP. When DCPIP is reduced by the AA its color turns from dark blue (or pink, in acidic media) to colorless. The AOC value of a sample of AOX is expressed by its AA equivalent concentration.

3.4. Method based on G/Os-HRP biosensor and XOD-XA system

The new bioelectroanalytical system proposed for AOC determination exploits the XA-XOD enzymatic system, for $O_2^{-\bullet}$ generation,[29] coupled with the amperometric detection of H_2O_2 (Fig. 5). It is worth to mention that the detected H_2O_2 is produced either by the spontaneous dismutation of $O_2^{-\bullet}$ radicals or by their catalyzed dismutation, if SOD is introduced in the system.

The working procedure consisted in adding XA and AOX to a volume of 3 mL PB (pH 7.5) containing a specified concentration of dissolved XOD. Before each measurement, air was bubbled for at least 10 minutes in order to ensure oxygen saturation.

Table 4

Composition of the enzymatic matrix for the prepared biosensors

Biosensor type	HRP (U/cm^2)	PVI-Os ($\mu g/cm^2$)	PEGDGE ($\mu g/cm^2$)	XOD (mU/cm^2)	SOD (U/cm^2)
G/Os-HRP	0.7	1.0	0.2	-	-
G/Os-HRP-SOD				-	5.1
G/Os-HRP-XOD(1)				2.5	-
G/Os-HRP-XOD(2)				0.7	-

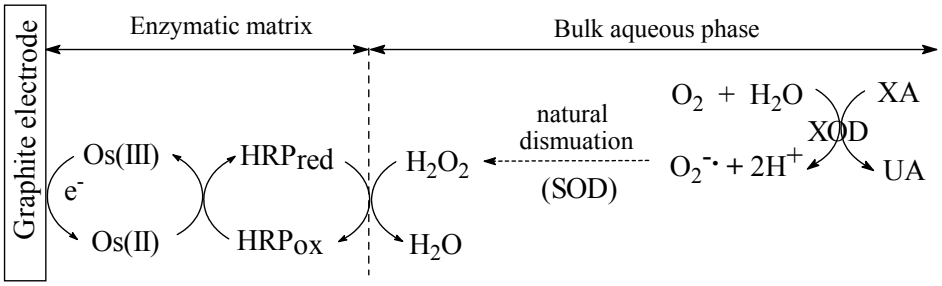

Fig. 5 – Detection scheme of H_2O_2, generated by the XA-XOD enzymatic system.

The presence of an AOX will induce a decrease of both $O_2^{-\bullet}$ and H_2O_2 concentration, resulting in a decrease of the H_2O_2 biosensor signal (current intensity). The corresponding AOC value, expressed as an equivalent concentration of reference antioxidant (RAOX), was calculated using the relation

$$AOC = \Delta I_{AOX} * \frac{1}{S_{RAOX}} * \frac{(I_{XA})_{RAOX}}{(I_{XA})_{AOX}},$$

where: ΔIA_{AOX}, is the current decrease due to the AOX addition in a PB solution containing the XOD - XA enzymatic system; S_{RAOX}, stands for the system sensitivity estimated for RAOX, as the slope of the linear range of the calibration curve; $(IXA)_{RAOX}$ and $(IXA)_{AOX}$ represent the currents due to XA addition, recorded prior to RAOX and AOX addition, respectively. The $(IXA)_{RAOX}$ / $(IXA)_{AOX}$ ratio, represents a correction factor introduced in order to take into consideration the inherent variation of the biosensor response.

4. Equipments and software

Electrochemical measurements were performed using a conventional 3-electrode system: a biosensor or graphite electrode as working electrode, a Ag/AgCl, KCl_{sat} as reference electrode, and a Pt wire electrode as counter electrode.

A computer-controlled potentiostat (PARSTAT 2276 Advanced electrochemical system; Princeton Applied Research, Oak Ridge, TN, USA) was used for cyclic voltammetry and amperometric measurements. In order to assure the solution homogeneity during the amperometric measurements, the working electrode was attached to a rotating disk electrode device (EDI10000; Radiometer Analytical, Copenhagen, Denmark). All electrochemical data were recorded using the Power Suite software (Princeton Applied Research) and were further processed by using OriginPro 7.5.

For spectrophotometric measurements a Jasco V-530 UV-Vis spectrophotometer and standard quartz cuvettes (1x1x4.5 cm^3) were used. The Spectra Manager software was used for collecting spectrophotometric data.

Unless otherwise stated, all results were given as the mean value of two replicate measurements.

CONCLUSIONS

A new bioanalytical system was designed, optimized, characterized and applied for AOC determination. The functioning principle of the new approach is based on coupling the production of $O_2^{-\bullet}$ radicals, generated by the XA-XOD enzymatic system, with the amperometric detection of H_2O_2, by using a low applied potential biosensor involving Os-wired HRP.

The whole bioanalytical system was optimized regarding: (i) the use of a biocatalyst (SOD) for O_2^{-}• dismutation to H_2O_2; (ii) the XOD immobilization. It was found that the simplest and, at the same time, the most efficient design was based on G/Os-HRP biosensor working in tandem with XA-XOD enzymatic system, dissolved in the biosensor adjacent solution. The new bioanalytical system was characterized by cyclic voltammetry, in order to put in evidence the possible interferences for H_2O_2 detection (as for example uric acid and ascorbic acid), and by amperometry, aiming to evaluate the bioelectroanalytical parameters for AA detection as a reference AOX (sensitivity, linear domain, detection limit and response time).

Taking into account the good correlation observed between the AOC values for two real samples of citrus juices obtained by using the new method and provided by Folin-Ciocalteu method, the method based on electrochemical detection of DPPH•, as well as the DCPIP-based titrimetric method, it can be concluded that the new approach represents a simple, fast and sensitive approach for AOC determination.

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