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PhD Thesis

UNCONVENTIONAL PROCEDURES OF ELECTROCHEMICAL DETECTION OF CHEMICAL SPECIES OF INTEREST IN BIOTECHNOLOGY AND ENVIRONMENT PROTECTION

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2011

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Thanks

I should first thank God, who, as a wise and skilled chess player, arranged the chess play of my life so that I could enjoy all the opportunities that were given to me and those through whom they came. I therefore thank:

Prof. Cătălin Popescu, who accepted to be my scientific advisor, who actively involved in every step of my PhD thesis preparation, was extremely goodwilling and patient, and showed an extraordinary unique combination of communication styles, high standards and accessibility

Lect. Sorin Dorneanu, who, with patience and friendship, supported me in a large part of my PhD study, by offering me the opportunity to use his experimental device, designed and made by him, by revising many of my articles and reports, but also by helping me understand electrochemistry

The electrochemistry group, formed by professors, associate professors, lecturers, assistants and PhD students, who turned my life into a way sprinkled with challenges and dreams, but also advised me and helped me grow, as a scientific person, but not only

My parents and brother, my relatives and friends who supported me, and, most important, who believed I could finish this challenge that seemed, at the beginning, something too difficult for me to accomplish

List of used abbreviations and symbols

a_A	**activity of analyte A**	**PB**	**Prussian Blue**
AA	**ascorbic acid**	**PBS**	**phosphate buffer**
AOA	**antioxidant activity**	**PCL**	**photochemi-luminescence**
AO	**antioxidant**	**PEGDGE**	**poly(ethyleneglycol) diglicidyl ether**
DCPIP	**2,6-dichlorophenol-indophenol sodium salt**	**pIon**	**activity decade**
DL	**linear domain**	**PVC**	**polyvinyl chloride**
DS	**standard deviation**	**R**	**correlation coefficient**
DSR	**relative standard deviation**	**ROS**	**reactive oxygen species**
E	**equilibrium potential, measured between the work electrode and the reference one**	**RP**	**redox polymer**
ISE-PVC-I	**ion-selective electrode based on PVC and I**	**S**	**sensitivity**
G/PB	**PB-based sensor**	**SOD**	**superoxide dismutase**
G/RP/HRP	**RP and HRP-based biosensor**	**$t_{1/2}$**	**half-life**
G/RP/HRP//XOD	**RP, HRP and XOD-based biosensor**	**t_{95}**	**response time**
HRP	**horseradish peroxidase**	**THF**	**tetrahydrofurane**
I	**ionophore**	**TRAP**	**total radical-trapping antioxidant parameter**
$K_{pot}^{A,B}$	**selectivity coefficient for analyte A as primary ion and B as interfering ion**	**XA**	**xanthine**
KTkClPB	**potassium tetrakis(4-chlorophenil)borate**	**XOD**	**xanthine oxidase**
LD	**detection limit**	**z_A**	**charge of analyte A**
N	**number of registered data**	**ΔI_{AO}**	**reduction current variation observed after AO addition**
o-NPOE	**2-nitropheniloctyleter**	**Φ**	**ISE membrane diameter**
ORAC	**oxygen radical antioxidant capacity**		

Keywords: ion-selective electrode, ionic activity, ionophore, antioxidant, antioxidant activity, amperometric sensor / biosensor

I. Introduction

The selective and accurate assessment of a number of chemical species is the main interest of various laboratories types: clinical analyses, environment protection, quality control, and so on.

The PVC-ionophore-type electrodes (ISE-PVC-I) meet these requirements plainly. In addition, they have the advantages of rapid measurements, simple experimental procedures, possibility of miniaturization, and, often, low cost of the necessary materials and devices. For these reasons, ISE-PVC-I are often preferred to more performant, but very costly devices, such as the inducely coupled plasma mass spectrometer.

Of special interest in nutrition is antioxidant (AO) monitorization; these are substances playing a crucial role in the prevention of various diseases, such as cancer and cardiovascular diseases, and in lowering some destroying processing such as growing old. There is actually a high number of methods used for evaluating the antioxidant activity (AOA). However, due to the high complexity of the AO action mechanism there is no standard method for AOA evaluation yet.

In this context, this work aimed at (i) the characterization of 10 new macrocyclic compounds – a [4.4.4.4]cyclophane and 9 calixarene derivatives – as ionophores for ISE-PVC-I (including the comparative evaluation of their electroanalytical parameters in: Li^+, Na^+, K^+, Cs^+, NH_4^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} and (ii) a comparative study of some amperometric sensors for H_2O_2 (this study aimed at the preparation, optimization, characterization, and utilization of these sensors in synthetic and real samples (wines and fruit juices).

II. Bibliographic study

1. ISE-PVC-I

1.1. Functioning principle of ISE-PVC-I

Starting from 1964, when it was discovered that some antibiotics have the ability to transport alkaline ions in the mitocondria, the interest for the design, development and utilization of ISE-PVC-I has continuously been rising.

The sensitive element of ISE-PVC-I is the ionophore (I), which generally has a macrocyclic structure, in the cavity of which can enter various cations or anions which preferentially bind I as a function of their dimensions and charge[1]. ISE-PVC-I membranes contain, besides I, a plastifier, which plays an essential role in the stability and elasticity of the membrane, a polymer matrix, which assures the mechanic properties of the membrane, and, often, an ionic additive, with a supplementary role in the membrane selectivity. The mass ratio of the membrane components is, generally: I, 0,5-1%; plastifier, 66%; polymer matrix, 33%; ionic additive, max. 0,5%[1].

The ionic activity of the analyte A, havind the ionic charge z_A, is evaluated by measuring the equilibrium potential E between ISE-PVC-I and an external reference electrode. E depends on the activity of A inside the membrane (organic phase) and that in the test solution (aqueous phase), according to the Nernst equation:

$$E = E_A^0 + \frac{RT}{z_A F} \ln \frac{a_{A(aq)}}{a_{A(org)}} \quad (1)$$

where E_A^0 is the standard potential of analyte A and directly depends on the free transfer energy of A, being expressed as a function of the standard chemical potentials in the two phases (scheme 2). If the membrane is sufficiently lipophilic, $a_{A(org)}$ is constant enough and can be included in the expression of the slope in scheme 1. The response of ISE-PVC-I is said to be nernstian if its sensitivity is $59/z_A$ mV / concentration decade (pIon). However, during the generation of E there are various phenomena taking place and causing the deviation of E from the ideal value.

$$E_A^0 = \frac{\mu_{A(aq)}^0 - \mu_{A(org)}^0}{z_A F} \quad (2)$$

If there are more than one ion in the test solution, A being considered the primary one, and B, the interfering one, then the dependence of E on the activities of A and B is given by the Nernst-Nikolskii-Eisenman equation, which is a generalization of equation 1:

$$E = E_A^0 + \frac{RT}{z_A F} \ln(a_A + \sum K_{A,B}^{pot} a_B^{z_A / z_B}) \quad (3)$$

1.2. Utilisations of ISE-PVC-I

1.2.1. ISE-PVC-I for ions of biological interests

Various ionophores of biological interests, such as: Ca^{2+}, K^+ and Na^+, cations for transitional metals, with applications in environment monitorization, such as: Cu^{2+}, Pb^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, but also for molecular ions, such as anionic surfactants: ClO_4^- and NO_3^-, are presented in the following section.

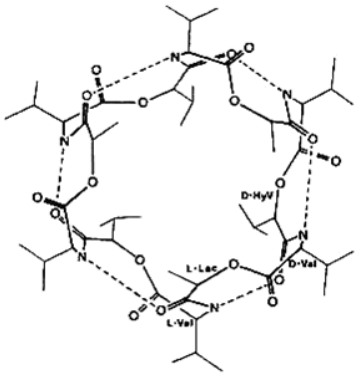


Figure 1. Structure of valinomycin

The clinical monitorization of K^+ is of great importance, especially for ill persons suffering of high and rapid variations of the concentration of K^+, for instance, in the cases of surgery and burns. Several classes of ionophores for K^+ mentioned in the literature are (bis-)crown esters and hemispherand ionophores[2]. The most well-known ionophore for K^+ is valinomycin (Fig. 1), an antibiotic produced from *Streptomyces fulvissimu* cultures. Valinomycin shows excellent

properties concerning selectivity, sensitivity and response time. Its main disadvantage is the low lipophylicity. To solve this problem, several derivatives of valinomycin were immobilized through covalent bonding on a polymer matrix and new valinomycin derivatives are being designed.

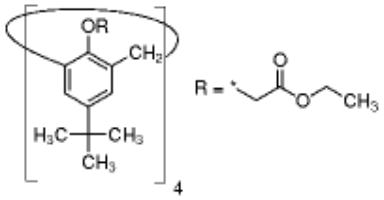


Figure 2. Structure of the tetraethyl tetraacetic 4-tert-butylcalix[4]arenic acid, used as Na^+ ionophore

Na^+ ion plays a fundamental role in the human organism, as many health diseases are caused by the inversion of the normal ration of extracellular Na^+ and intracellular K^+ concentration[3]. The best Na^+ ionophores are calix[4]arene derivatives, such as the so-called „X ionophore" (Fig. 2).

Ca^{2+} assessment is highly important in organ transplant and blood transfusion, phenomena which may cause rapid Ca^{2+} concentrations. The remarkable properties of the ionophore „ETH 1001" (Fig. 3) (especially selectivity) is well known ($\log K^{pot}_{Ca,J}$=-3,4; -4,4, where J means Na^+ and Mg^{2+}, respectively). Compounds with similar properties are the calcium didecylphophate and the calcium bis-di-(1,1,3,3-tetramethylbutyl)-phenyl-phosphate[4].

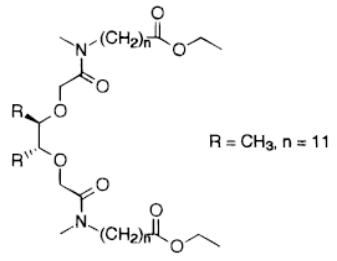


Figure 3. Structure of ETH 1001, used as Ca^{2+} ionophore

1.2.2. ISE-PVC-I for ions of interest in environment protection

The development of agriculture, nutrition, construction, cosmetics, drugs, energy, transport industries, and so on, has created a large number of chemical pollutants of water, air and ground. Among these, many are assessed by ISE-PVC-I: compounds with hard metals, anionic and cationic surfactants, perchlorates, and so on.

Copper is a metal with a large number of industrial applications. Some of the classes of compounds with ionophore properties for the ionic activity of Cu^{2+} are: small crown thiaethers, acyclic ionophores containing dithiocarbamate and N, calix[4]arenes and Schiff bases[5].

Among lead utilisations, we may cite the obtention of alloys, munition, mineral oil, paints and pipes. A large number of calixarenes functionalized with groups containing S, Se, N and P, such as: thioethers, thiaamyloxi-, arylthiaalkloxi-, piridyl, benzothiazoyl, Schiff bases are mentioned in the literature as ionophores for Pb^{2+} [6, 7].

Zinc and its compounds are used for many industrial applications: batteries, alloys, catalysers, explozives, nutrition supplements[8]. The assessment of Zn^{2+} is carried out using ionophores from different classes: disulfide derivatives, calixarene thiamides, and benzopolyethers[9].

Nickel is used to produce steel, magnets, rechargeable batteries, special alloys. Some ionophores for Ni^{2+} assessment are several Schiff bases, 4 N atoms-based amino compounds of various dimensions[10], and so on.

Cadmium is most used for covering steel and iron objects. Some ionophores mentioned in the literature as Cd^{2+}-selective are crown ether derivatives[11].

Cobalt, both in its metallic and ionic form, is used for batteries, alloys, highly resistant glass, drying agents for paints, and so on. Several Schiff bases have been mentioned in the literature as Co^{2+} ionophores[12].

An important application of ISE-PVC-I is the evaluation of the activity of anionic surfactants, compounds largely used as washing agents at home and industry. There are several polyazacycloalcanes and tetraazacyclotetradecanes with good properties as dodelylsulphate- selective ionophores[13].

Waters may be rich in ClO_4^- (≥1000 ppm) coming from the medical industry, due to the tyrostatic action of this ion[14], but also from the fuel and explosives industry. Many Ni^{2+} sau Pt^{2+} complexes are used as neutral ionophores for assessment of the ClO_4^- activity[15].

1.3. Conclusions and perspectives

Due to their excellent properties, concerning selectivity, measurement accuracy and rapidity, simplicity of the experimental procedure, possibility of miniaturization, and so on, ISE-PVC-I is an important and actual subject, and the ISE-PVC-I selective for various species (anions or cations) is constantly rising.

2. Methods of assessment of the antioxidant activity (AOA)

2.1. Generalities about antioxidants (AO) and AOA

AO play a great variety of roles and may have various chemical structures. AOA is defined according to many parameters, such as: (i) the scavenged radical or non-radical species; (ii) the working conditions: hydrophilic or lipophilic media, reaction time, etc.

Among scavenged species we may cite: superoxide radicals ($O_2^{-\bullet}$), hydroxy ($HO^\bullet$), peroxy ($ROO^\bullet$) and reactive oxygen species (ROS)[16], which include non-radical species with oxidant properties, such as H_2O_2 and HOCl.

Research in the subject of AO has developped during the last 20 years especially due to the discovery of beneficial effects of AO on human health. In the human body, the excessive amount of ROS, which may be due to the action of pollution, unhealthy nutrition, smocking, may cause cardiovascular diseases, cancer, diabetes, inflammatory symptoms, and so on[3]. There is evidence of the fact that they may be prevented by AO-rich food consumption. As about AO supplements, there

are various oppinions; is is however known that an excessive AO supplements consumption may cause phenomena such as degradation of the immunitary system[17].

2.2.Methods for AOA evaluation

2.2.1. ORAC method

The „oxygen radical absorbance capacity" (ORAC) method has been proposed by Glazer (1990)[18]; it measures the ability of AO to inhibit the oxidation induced by $ROO^{\bullet}$, generated by thermal decomposition of an azo compound. $ROO^{\bullet}$ reacts with a fluorescent substrate (fluorescein, pyrogallol red), and thus produces a nonfluorescent compound. The ORAC method evaluates AOA by using the areas under the curves describing the variation of the fluorescent substrate in presence / absence of AOA[19]. The ORAC values are usually expressed as Trolox equivalents.

2.2.2. TRAP method

The „total radical-trapping antioxidant parameter" (TRAP) monitors the ability of AO to interfere with the reaction of $ROO^{\bullet}$ and a fluorescent substrate, such as linoleic acid or β-ficoeritrin[20]. The basic reactions are similar to those from ORAC. Unlike ORAC, the TRAP method defines AOA as a function of the inhibition time, which represents the time required for the substrate to be re-oxidized in the presence of AO.

2.2.3. PCL method

This method is based on measuring the photochemiluminescence (PCL) produced by the reaction of radical species with excitable photochemical compounds (luminol, lucigenin). The PCL method PCL has been marketed by Analytik Jena AG (Germany), and the PCL evaluation kit is called „PHOTOCHEM"[21]. This system is extremely effective in terms of cost; in addition, it can be used for both hydrophilic and lipophilic AO, for multiple values of pH and temperature.

2.2.4. DPPH method

The $DPPH^{\bullet}$ radical is used as radical source in the AOA evaluation[22]; it is detected spectrophotometrically or electrochemically[23, 24]. In presence of AO, $DPPH^{\bullet}$ (violet) is reduced to a pale yellow compound; the $DPPH^{\bullet}$ absorbance variation is measured at 517 nm. AOA values are expressed as the amount of AO that produces a $DPPH^{\bullet}$ absorbance decrease of 50%. DPPH method is simple, fast, and it is based on a simple principle of detection, but a possible problem is the evaluation of the AOA of AO whose spectrum overlaps with that of $DPPH^{\bullet}$ (eg carotenoids).

2.2.5. Folin-Ciocâlteu method

The Folin-Ciocâlteu method is based on polyphenols oxidation using a molibdowolframate (Na_2WO_4 / Na_2MoO_4). From the reaction result $O_2^{-\bullet}$, which react with molybdate to form the Mo^{4+} ion (blue), whose absorbance is followed spectrophotometrically between 745 and 750 nm. The reaction takes place in strongly alkaline medium. As AO reference, galic acid is used[25]. FC method is simple and rapid. However, it is necessary that the working pH is not acidic, which would cause slow and nonspecific reactions. One problem is the lack of specificity of the method to polyphenols; monophenols and other compounds that can be reduced arer also detected. There is a good correlation in the literature between the results obtained by the Folin-Ciocâlteu method and those obtained by other methods (eg DPPH), which assess a total AOA[26].

2.3. Conclusions and perspectives

There are a variety of ways to determine AOA. However, this is a relative term, taking into account the reaction conditions: the type of neutralized species, the mechanism of interaction between AO and neutralized species (electron transfer or H atom), the type of AOA monitored (global or specific polyphenols), detection method (spectrophotometric, electrochemical, etc.), the reaction type (hydrophilic or lipophilic), reaction time, the interpretation of results (parameters taken into account). This variety of methods to assess the AOA has, besides the disadvantage of making it difficult to establish standardized methods, the advantage of taking different approaches, i.e., complementary aspects, accessible only to certain methods.

III. Original experimental results

1. Electrochemical behavior of some ISE-PVC-I

1.1. Goal of the study

The first part of this study consisted of the electroanalytical characterization of 10 new macrocyclic compounds - a [4.4.4.4] cyclophane[27] synthesized by the research team of Prof. Ion Grosu (Department of Organic Chemistry, Faculty of Chemistry and Chemical Engineering, Cluj-Napoca) and 9 calixarenic derivatives synthesized by the research team led by Dr. Elisabeth-Jeanne Popovici (Research Institute "Raluca Ripan")[28] (Fig. 3, Table 1) - as ionophores for the ISE-PVC-I. For this purpose, ISE-PVC-I on the 10 compounds were prepared and used for a series of cations: Li^+, Na^+, K^+, Cs^+, NH_4^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}.

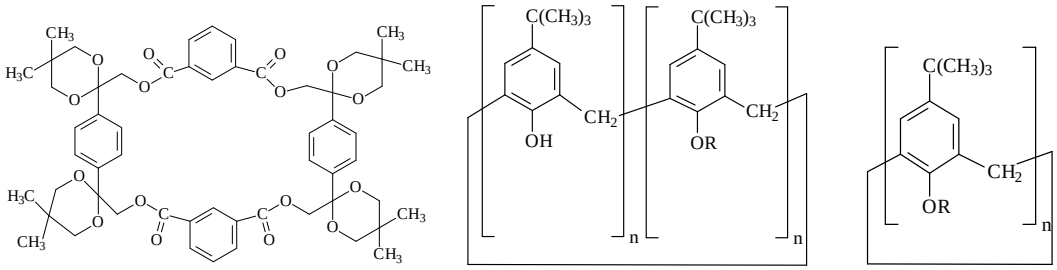
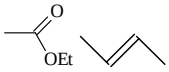
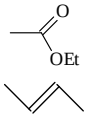
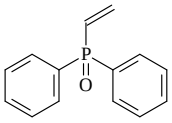
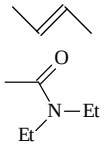
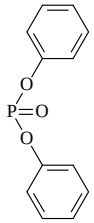
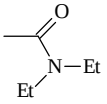


Figure 3. Chemical structures of the compounds used as ionophores: left: cyclophane M7F2, middle, right: calixarene derivatives

Table 1. Chemical structures R and their number (n) present in the tested calixarenic compounds

Name of the calixarenic compound	R	Name of R	n	Name of the calixarenic compound	R	Name of R	n
C4Es2Cr2		ester, crotyl	2	**C6Es3Cr3**		ester, crotyl	3
C4P4		diphenyl-phosphate	4	**C6Cr3Am3**		crotyl, amide	3
C4PO4		diphenyl-ester-phosphate	4	**C6Am3**		amide	3
C6Es6		ester	6	**C8Es8**		ester	8
C6Es3			3				

1.2.Evaluated electroanalitical parameters

Sensitivity

Sensitivity (S) is the parameter expressing the change in electrode potential according to the logarithm of the analyte ion activity and is expressed in mV / pIon. The formula of S is given below:

$$S = \frac{E_1 - E_2}{\log(a_1 / a_2)} \tag{9}$$

where E1 and E2 are the values of the potential between which the evaluation is made, both taken in the field of linearity (see below), and a_1 and a_2 are the ionic potential activities corresponding to E1 and E2, respectively.

S values, ideally 59/zA mV/pIon[34] were evaluated from linear fitting of calibration curves representing the dependence of E on the logarithm of the ionic activity of the species of interest or, if

there were not sufficient values of E, the difference between the ionic activities was taken into account.

Detection limit

The detection limit (LD) is the ionic activity found from intersection of the extrapolations of the linear portions of the calibration curve[35, 36].

Linearity domain

The linearity domain (DL) is the range of analyte activities between which the electrode potential depends linearly on the logarithm of the analyte. DL was calculated as the interval between the LD and the upper limit of the range of activities, otherwise, the upper activity which would meet this criteria was taken into account, for which the square of the correlation coefficient linear range was at least 0.99.

Selectivity coefficient

The selectivity coefficient ($K_{A,B}^{pot}$) is a parameter expressing the ability of the electrode to respond preferentially to the primary ion A, compared to the interfering ion B[37]. According to Nernst-Nikolskii-Eisenmann equation, if the term has a value less than unity, the electrode is selective for A; if it is higher than one, the electrode is selective for B[38]:

$$E = E_A^0 + \frac{RT}{z_A F}\ln(a_A + \sum K_{A,B}^{pot} a_B^{z_A / z_B}) \quad (10)$$

Evaluation of ISE-PVC-I selectivity was achieved in mixed solutions containing both the primary and interfering ion; one concentration varied and the other one was modified. The calculation formula is the following:

$$K_{A,B}^{pot} = \frac{a_A}{a_B^{z_A / z_B}} \quad (11)$$

where a_A and a_B is the ionic activity of the primary and interfering ions, respectively, in the moment of interference detection

1.3.Experimental conditions

Experimental device

Potentiometric measurements were made using a computer-controlled measuring device for electrochemical measurements (Fig. 4)[39] that allowed 17 automatic additions of standard concentrate

dsolution concentrated into a diluted one. Volumes are preset so as to allow uniform distribution of the experimental points as a function of the logarithm of ionic activity.

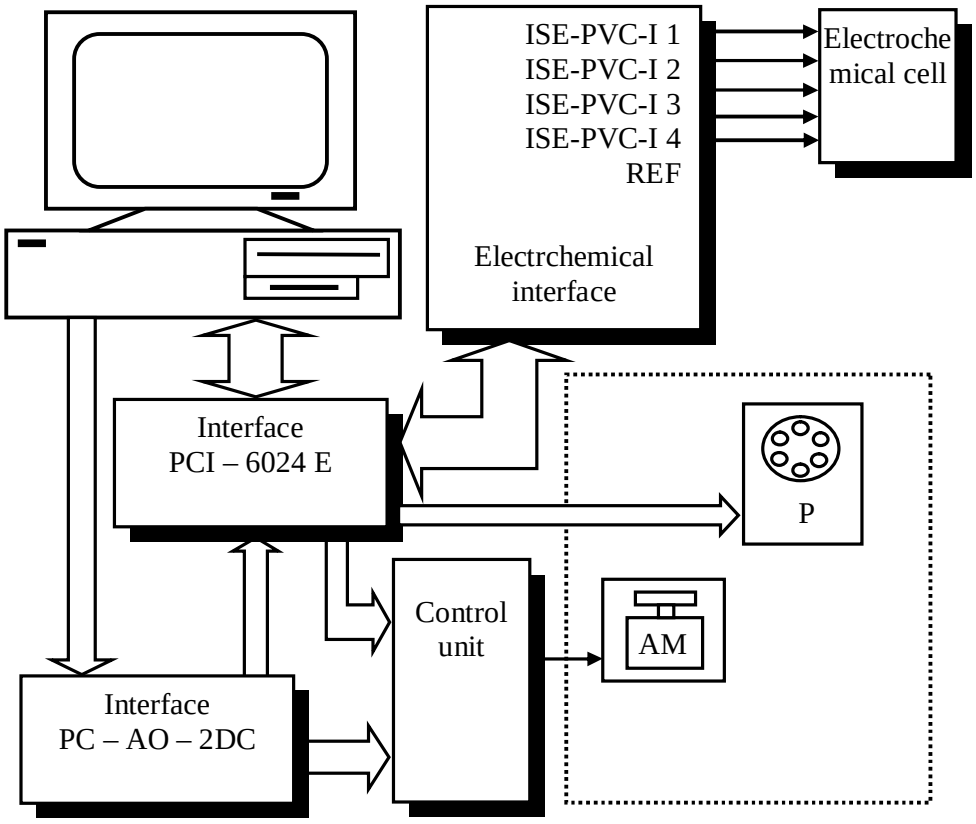


Figure 4. Computer-controlled experimental device used for the potentiometric meaurements

ISE-PVC-I were connected to the data acquisition system via an interface with a very high impedance input (> 1012 ohm). Electrochemical interface and the peristaltic pump (P) used for performing standard solution additions were connected to the computer via a PCI-6024 E (National Instruments, USA). Homogenization of the solution after each addition was made with a magnetic stirrer (MA), whose operation was controlled by a control unit connected to the computer via PC-AO-2DC interface (National Instruments, USA)

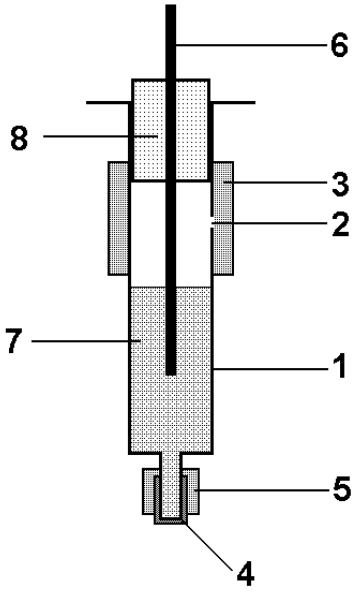


Figure 5. Components of ISE-PVC-I: 1-ISE-PVC-I body, 2- filling hole, 3- rubber ring, 4-ion-selective membrane, 5- rubber ring, 6- internal electrode reference Ag/AgCl, 7- internal reference solution, KCl, 8- screw for fixing the reference electrode

The system control and the data acquisition were done using LabView 5.1 applications (National Instruments, USA), and the experimental data obtained were processed using the Origin 6.0 program.

Electrochemical cell contained four ISE-PVC-I and an external saturated calomel double junction reference electrode (Radelkis, Budapest) with, as the internal junction, a saturated KCl solution and as the external junction solution CH_3COOLi 0.1 M. ISE-PVC-I (Fig. 5) were composed of a syringe body (1 ml volume), and were filled with internal reference solution (cation chloride ion of the investigated solutions, ~ 5 mM). At the bottom of the syringe were fixed membranes in the form of discs of 8 mm diameter.

Reactants

The following reagents were used for membrane preparation: the studied macrocyclic compounds (ionophores), potassium tetrakis (4-chlorophenyl) borate (KTkClPB), Fluka, code 60591, (ionic additive), 2-nitrophenyl octyl ether (o-NPOE), Fluka, code 73732 (plasticizer), polyvinyl chloride (PVC), Fluka, code 81392 (matrix polymer), tetrahydrofurane (THF), Fluka, code 87369 (solvent).

Solutions were prepared using the following salts: LiCl, NaCl, KCl, NH4Cl, CsCl, $CaCl_2$, $MgCl_2$, $BaCl_2$, $CoCl_2$, $NiSO_4$, $ZnSO_4$, CH_3COOLi, using PA reagents obtained from Fluka, Merck, Sigma or Chimopar.

ISE-PVC-I membrane preparation

The membrane composition of ISE-PVC-I was as follows: 0.7 or 1% ionophore (1% for all calixarenic compounds and 0.7 or 1% for M7F2) (corresponding electrodes being called M7F2 (a) and M7F2, respectively); 0,3% ionic additive (KTkClPB) if for the M7F2(a) membrane; 33% polymer matrix (PVC) and 66% plasticizer (o-NPOE) (mass percentages). The total mass of a membrane was ~ 1.5 g.

The required weights of ionophore, ionic additive and matrix polymer, weighed with an analytical balance of ± 0.1 mg precision and the amount of plasticizer required (1.0 ml) were successively dissolved in 2 ml of THF. The mixture was subjected to stirring until solid components dissolved, then was poured into a cylindrical glass ring (Φ = 4.8 cm) and allowed to dry slowly for 24

hours under a glass bell, in an atmosphere of saturated THF vapors to prevent formation of bubbles in the membrane mass. After drying, the membrane was "matured" 24 more hours outdoors in the dark until complete evaporation of the solvent. Later, with a perforating device, membrane discs were cut, with a diameter of 8 mm, which were fixed on the electrode bodies, after which they were conditioned in 1 mM of chloride solutions of the investigated cation.

Working procedure

Evaluation of sensitivity, linearity domain and detection limit was achieved using separate cationic solutions. Measurements consisted of recording of electrode potential versus time and the corresponding calibration curves were drawn as the measured potential versus the corresponding ionic activity. Potential measurement was performed using either the so-called automatic standard addition method (as described in paragraph 1.3.1 of the technical procedure), or the manual standard addition, which requires a simplified experimental procedure, consisting of performing manual measurements of two successive standard additions in a diluted solution ionic. Using this method, the concentrations 10^{-4}, 10^{-3} and 10^{-2} M were considered, and the slope was read between 10^{-3} and 10^{-2} M. For all measurements, two or four ISE-PVC-I with similar composition were parallelly used. Each measurement was repeated at least twice successively under the same experimental conditions, the results being given as an average and standard deviation (SD).

1.4.Results and discussion

Depending on the number and type of groups that make up the tested macrocyclic compounds used as ionophores, their cavities get different dimensions, which determine specific affinities for different cations. Based on these correlations, the studied macrocyclic compounds were used in the preparation of ISE-PVC-I membranes for different classes of mono-and divalent cations belonging to the category of alkali, alkaline earth and transition metals, plus the ammonium ion, which has special interest in biological and ecological applications. A synthetic representation of the performed measurements is shown in Table 3, where "P" indicates that the ion has been used as primary ion for the corresponding macrocyclic compound, "am" and "aa" indicate the used method - manual addition [ex. Fig. 7 (A)] or automatic one [eg. Fig. 7 (B)], "I_{Me}" indicates that ionic interference was studied, Me is the primary ion, "-" indicates that this type of experiment was not conducted. Tables 4-6 present the evaluated electroanalytical pararmeters: sensitivity, detection limit, linearity domain and selectivity coefficient.

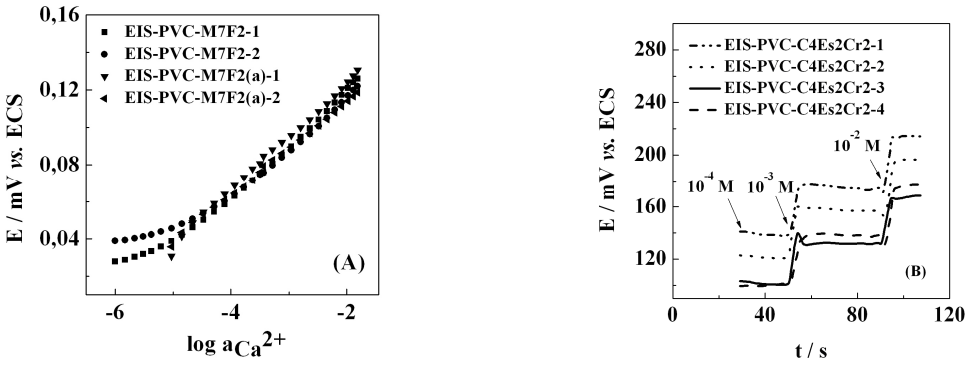


Figure 6. (A) Dependence of the electrode potential on the logaritm of the ionic activity measured with ISE-PVC-M7F2 and ISE-PVC-M7F2(a); (B) Variation with time of the electrode potential measured with ISE-PVC-C4Es2Cr2 in Na^+ 10^{-4} M solutions to which standard Na^+ 1 M additions were made manually

Table 3. Summary of potentiometric measurements using ISE-PVC-I based on the studied macrocyclic compounds

Cation	M7F2, M7F2(a)	C4Es2Cr2	C4P4	C4PO4	C6Es6	C6Es3	C6Es3Cr3	C6Cr3Am3	C6Am3	C8Es8
Li^+	-	-	-	-	P (aa)	-	-	-	-	-
Na^+	P (aa), I_{Ca}	P (am)	P (am), I_{Ca}	P (am)	P (aa), I_K	P (aa)	P (aa)	P (am)	P (am)	P (am)
K^+	P (aa), I_{Ca}	P (am)	P (am), I_{Ca}	P (am)	P (aa)	P (am), I_{Na}	P (am), I_{Na}	P (am)	P (am)	P (am)
Cs^+	-	-	-	-	P (aa), I_K	-	-	-	-	-
NH_4^+	-	-	-	-	P (aa), I_K	-	-	-	-	-
Mg^{2+}	P (aa), I_{Ca}	P (am)	P (am), I_{Ca}	P (am)	P (aa)	P (am), I_{Na}	P (am), I_{Na}	P (am)	P (am)	P (am)
Ca^{2+}	P (aa)	P (am)	P (am)	P (am)	P (aa)	P (am), I_{Na}	P (am), I_{Na}	P (am)	P (am)	P (am)
Ba^{2+}	-	P (am)	P (am), I_{Ca}	P (am)	-	-	-	P (am)	P (am)	P (am)
Co^{2+}	-	-	-	-	-	P (aa)	P (aa)	-	P (aa)	-
Ni^{2+}	-	-	-	-	-	P (aa)	P (aa)	-	P (aa)	-
Zn^{2+}	-	-	-	-	-	P (aa)	P (aa)	-	P (aa)	-

Table 4. S values (mV/pIon) evaluated for ISE-PVC-I based on the studied macrocyclic compounds

Cation	M7F2, M7F2(a)	C4Es2Cr2	C4P4	C4PO4	C6Es6	C6Es3	C6Es3Cr3	C6Cr3Am3	C6Am3	C8Es8
Li^+	-	-	-	-	25,8 ± 3,6	-	-	-	-	-
Na^+	57,7 ± 7,0 39,8 ± 5,4	52,0 ± 0,5	20,8 ± 1,2	33,5 ± 0,5	55,8 ± 3,4	66,2 ± 4,1	57,8 ± 3,2	44,0 ± 2,0	42,5 ± 4,0	40,2 ± 0,2
K^+	37,0 ± 5,1 39,3 ± 4,9	46,5 ± 2,0	25,0 ± 0,5	6,0 ± 2,0	51,9 ± 2,5	47,8 ± 0,7	44,5 ± 1,5	53,8 ± 1,2	46,0 ± 7,0	51,8 ± 0,8
Cs^+	-	-	-	-	29,2 ± 2,2	-	-	-	-	-
NH_4^+	-	-	-	-	52,9 ± 3,0	-	-	-	-	-
Mg^{2+}	5,2 ± 0,4 32,6 ± 5,2	11,8 ± 0,2	18,0 ± 2,5	5,0 ± 2,0	12,2 ± 1,0	7,3 ± 1,9	8,8 ± 1,3	7,5 ± 0,5	4,5 ± 2,0	8,5 ± 0,5
Ca^{2+}	29,0 ± 2,0 26,8 ± 1,1	21,0 ± 0,0	22,5 ± 1,0	4,5 ± 2,0	16,3 ± 2,7	11,4 ± 2,5	11,9 ± 3,6	8,0 ± 0,0	24,2 ± 0,2	11,5 ± 3,5
Ba^{2+}	-	23,0 ± 0,0	14,8 ± 0,8	6,8 ± 0,2	-	-	-	11,5 ± 1,0	25,8 ± 0,8	18,2 ± 0,2
Co^{2+}	-	-	-	-	-	28,2 ± 9,5	21,1 ± 6,5	-	23,2 ± 1,2	-
Ni^{2+}	-	-	-	-	-	48,5 ± 0,7	40,0 ± 2,8	-	43,5 ± 0,7	-
Zn^{2+}	-	-	-	-	-	38,5 ± 1,7	23,3 ± 0,6	-	29,0 ± 1,2	-

Table 5. Linear domains (pIon) and detection limits (mM) evaluated for ISE-PVC-I based on the studied macrocyclic compounds

Para-meter	DL (pIon)					LD (mM)				
Cation	**M7F2 M7F2(a)**	**C6Es6**	**C6Es3**	**C6Es3Cr3**	**C6Am3**	**M7F2 M7F2(a)**	**C6Es6**	**C6Es3**	**C6Es3Cr3**	**C6Am3**
Li^+	-	3,55	-	-	-	-	$9,33*10^{-2}$	-	-	-
Na^+	3,04 1,58	2,45	2,68	2,66	-	$3,02*10^{-1}$ 8,62	1,17	$6,88*10^{-1}$	$7,24*10^{-1}$	-
K^+	1,92 3,43	3,37	-	-	-	4,00 $1,23*10^{-1}$	$1,41*10^{-1}$	-	-	-
Cs^+	-	4,17	-	-	-	-	$2,23*10^{-2}$	-	-	-
NH_4^+	-	3,59	-	-	-	-	$8,60*10^{-2}$	-	-	-
Mg^{2+}	- 1,78	2,79	-	-	-	- 5,42	$5,41*10^{-1}$	-	-	-
Ca^{2+}	4,17 4,23	2,17	-	-	-	$2,22*10^{-2}$ $1,92*10^{-2}$	2,24	-	-	-
Co^{2+}	-	-	1,93	2,04	1,64	-	-	3,91	3,02	7,52
Ni^{2+}	-	-	2,33	2,20	1,91	-	-	1,56	2,07	4,05
Zn^{2+}	-	-	2,11	2,45	1,98	-	-	2,54	1,18	3,49

Table 6. Logaritms of selectivity coefficients evaluated for ISE-PVC-I based on the studied macrocyclic compounds

Cation interferent	M7F2 M7F2(a)	C4P4	C6Es6	C6Es3	C6Es3Cr3
Na^+	$\log K^{pot}_{Ca,Na} = 1,4$ $\log K^{pot}_{Ca,Na} = 2,2$ ($[Ca^{2+}] = 5*10^{-3}$ M)	$\log K^{pot}_{Ca,Na} = -0,6$ ($[Na^+] = 10^{-1}$ M)	$\log K^{pot}_{K,Na} = -1,2$ ($[K^+] = 10^{-2}$ M)	-	-
K^+	$\log K^{pot}_{Ca,K} = 1,3$ $\log K^{pot}_{Ca,K} = 4,6$ ($[Ca^{2+}] = 5*10^{-3}$ M)	$\log K^{pot}_{Ca,K} = 1,8$ ($[K^+] = 10^{-2}$ M)	-	$\log K^{pot}_{Na,K} = 1,4$ ($[K^+] = 10^{-4}$ M) $\log K^{pot}_{Na,K} = 0,4$ ($[K^+] = 10^{-2}$ M)	$\log K^{pot}_{Na,K} = 1,5$ ($[K^+] = 10^{-4}$ M) $\log K^{pot}_{Na,K} = 0,1$ ($[K^+] = 10^{-2}$ M)
Cs^+	-	-	$\log K^{pot}_{K,Cs} = -0,5$ ($[K^+] = 10^{-2}$ M)	-	-
NH_4^+	-	-	$\log K^{pot}_{K,NH_4} = -0,8$ ($[K^+] = 10^{-2}$ M)	-	-
Mg^{2+}	$\log K^{pot}_{Ca,Mg} = -0,3$ $\log K^{pot}_{Ca,Mg} = -0,4$ ($[Ca^{2+}] = 5*10^{-3}$ M)	$\log K^{pot}_{Ca,Mg} = -0,6$ ($[Mg^{2+}] = 10^{-3}$ M)	-	$\log K^{pot}_{Na,Mg} = -3,6$ ($[Mg^{2+}] = 1$ M)	$\log K^{pot}_{Na,Mg} = -3,2$ ($[Mg^{2+}] = 1$ M)
Ca^{2+}	-	-	-	$\log K^{pot}_{Na,Ca} = -3,1$ ($[Ca^{2+}] = 1$ M)	$\log K^{pot}_{Na,Ca} = -2,5$ ($[Ca^{2+}] = 1$ M)
Ba^{2+}	-	$\log K^{pot}_{Ca,Ba} = -2,1$ ($[Ba^{2+}] = 10^{-1}$ M)	-	-	-

1.5. Conclusions

From the study of the electroanalytical parameters of the 10 new studied macrocyclic compounds, namely one [4.4.4.4] ciclophane synthesized by the research team of prof. Ion Grosu (Department of Organic Chemistry, Faculty of Chemistry and Chemical Engineering, Cluj-Napoca) and 9 calixarenic derivatives prepared in the Research Institute "Raluca Ripan" (Cluj-Napoca), it was found that the vast majority of them present ion-selective properties, as follows:

- M7F2, both in the form with and without additive, shows selectivity for Ca^{2+} against Mg^{2+} and it could be exploited as ionophore for the evaluation of Ca^{2+} in solutions with low Na^{+} and K^{+} contents
- C4Es4Cr2 can be used in the production of ISE-PVC-I for determination of Na^{+} activity, requiring optimization of membrane composition and experimental conditions
- C4P4 is a compound used in the construction of ISE-PVC-I for determining Ca^{2+} and K^{+} activities, but only if the two cations are not present simultaneously in the environment investigated
- C4PO4 has weak electroanalytical properties, as all recorded responses net were under-nernstian
- C6Es6 showed a quasi-nernstian response for K^{+}, NH_4^{+}, Na^{+} and K^{+}, so could be used in the development of ISE-PVC-I for the determination of these ions
- C6Es3 and C6Es3Cr3 present a significant selectivity for alkali ions in relation to the alkali earth ones, suggesting that ISE-PVC-I developed based on these compounds could be used to determine Na^{+} in samples with low K^{+} contents. In addition, there was a quasi-nernstian response of ISE-PVC-I based on C6Es3 for Co^{2+} and ISE-PVC based on C6Am3 for Zn^{2+}, thus anticipating the possible applications in monitoring of waste water from metallurgic industry
- ISE-PVC-I based on C8Es8 and C6Cr3Am3 presents a quasi-nernstian response for K^{+}, thus, can be used for the determination of the activity of this ion, but only after optimizing the membrane composition and experimental conditions

2. Evaluation of AOA using amperometric sensors for H_2O_2 detection

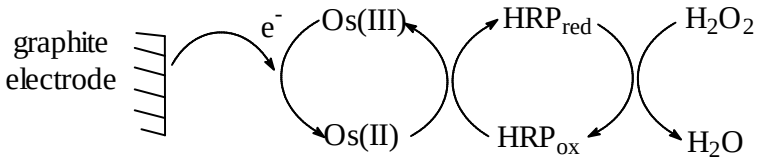
2.1. Functioning principle

2.1.1. HRP-based biosensor for H_2O_2

An amperometric biosensor named G/RP/HRP was prepared and used for detection of H_2O_2. It consisted of a pyrolytic graphite rod modified with horseradish peroxidase (HRP) and a redox

polymer, poly (1-vinilimidazol) complexed with [Os (4-4'-dimethylbipyridine)2Cl)$^{II/III}$ "(redox polymer, RP), used as electric mediator. These components were adsorbed on graphite using the crosslinker poly (ethylene glycol) ether diglicidil (PEGDGE).

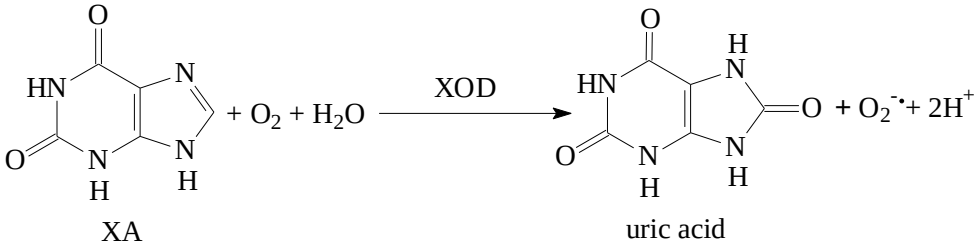
Through the electrocatalytic cycles described by scheme 2, H_2O_2 from the volume phase is reduced. A reduction potential was applied between the modified electrode and the reference one; the resulting current is proportional to the concentration of H_2O_2 reduced on the surface of G/RP/HRP. An important role in producing the current signal is played by the enzyme loading (the amount of HRP deposited on graphite) and mediator efficiency (rate of electron transfer).



(16)

2.1.2. HRP and XOD-based biosensor for XA

The working principle of G/RP/HRP//XOD[41] is similar to that of G/RP/HRP, except the fact that the source of H_2O_2 is $O_2^{-\bullet}$, produced by the reaction of xanthine (XA) with O_2, which is catalized by xantin-oxidase (XOD) (scheme 17). Further on, $O_2^{-\bullet}$ dismutation takes place, either spontaneously, or by superoxide dismutase (SOD) catalization, resulting H_2O_2:

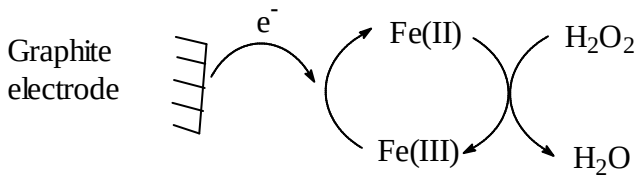


(17)

2.1.3. Prussian Blue-based sensor for H_2O_2

Prussian Blue (PB) is a hexacyanoferate obtained from an equimolar mixture of $K_3Fe(CN)_6$ and $FeCl_3$. It is successfully used as a redox mediator, especially for H_2O_2 reduction[42, 43]. For this reason it is used for many sensors, for the detection of glucose, lactate, persulphate, etc[44]. PB may be adsorbed on graphite, platinum, glassy carbon, carbon paste, „carbon ink", etc., alone or in combination with other materials. Among the advantages of PB, we may cite the low necessary working potential and the low cost[42].

The presented PB-based sensor consisted of a PB layer deposited on pyrolytic graphite (G/PB). PB is used as a redox mediator for H_2O_2 electroreduction through the redox couple $Fe^{II/III}$ (scheme 5)[45].



(18)

Among the three above-presented sensors, G/RP/HRP//XOD has the advantage of reflecting the most well-known AOA definition, i.e., that of ability to scavenge free radicals.

2.2.AOA defitinion

AOA is generally defined as the ability of AO to scavenge various radicals, such as: $O_2^{-\bullet}$, $HO^{\bullet}$ and $ROO^{\bullet}$ (see II.2.1). Some researchers define AOA as ability of AO to oxidize in the presence of H_2O_2[16]. In this study, both approaches were used. Thus, G/RP/HRP//XOD reflects the ability of AO to scavenge $O_2^{-\bullet}$, and the other two sensors reflect the oxidant activity against H_2O_2. The results of these three methods were compared to those obtained by three reference methods: (i) the Folin-Ciocâlteu method for the evaluation of polyphenols; (ii) the method based on the electrochemical detection of $DPPH^{\bullet}$; (iii) the titrimetric metod for ascorbic acid (AA) content evaluation by using the 2,6-dichlorophenolindophenol sodium salt (DCPIP).

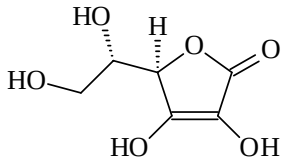


Figure 9. Chemical structure of AA

AA was chosen as a reference AO (Fig. 9) for the following reasons: (i) is is highly water soluble; (ii) its ability to react with H_2O_2 is well known[46]; (iii) it is one of the most common AO in nature, in both fruits and vegetables; (iv) it is used as a food quality index, due to its high sensitivity to food degradation through processing and storage; (v) it plays an extremely important role in human body, as it is an essential vitamin.

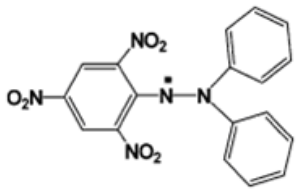


Figure 4. Chemical structure of $DPPH^{\bullet}$

For the three amperometric sensors, G/RP/HRP, G/RP/HRP//XOD and G/PB, AOA evaluation of synthetic and real samples was performed using a calibration curve for AA, consisting of the registration of the reduction current variation as a function of AA concentration in presence of a constant H_2O_2 concentration. Separately, amperometric measurements were performed, consisting of AO additions in presence of a constant H_2O_2 concentration. AOA evaluation formula uses the

sensitivity of the calibration curve (S_{AA}) and the current variation observed as a consequence of AO addition, ΔI_{AO} (scheme 15). AOA results were expressed as equivalent AA concentrations – absolute and relative values (divided to the maximum value).

$$AAO = \Delta I_{AO} * \frac{1}{S_{AA}} \quad (15)$$

For the total polyphenol content evaluation through the Folin-Ciocâlteu method, the UV spectra of each sample in mixture with the Folin-Ciocâlteu was measured, and AOA were expressed as absorbances corresponding to the maximum point. Also, AOA results were expressed as relative values, obtained by dividing each value to the maximum AOA value of all.

The method based on the electrochemical detection of $DPPH^{\bullet}$ (Fig. 7) was based on the same working principle as above; AO additions were performed in presence of a constant $DPPH^{\bullet}$ concentration. The reference AO was the (±)-6-hydroxi-2,5,7,8-tetramethyl cromane carboxilic acid (Trolox)[23].

Following the titrimetric method, the AA conctent of the samples is given by the DCPIP volume necessary to neutralize the samples; this volume is divided by the DCPIP volume which is necessary to neutralize a known AA quantity. Results are expressed as both AA concentrations and as relative values, obtained by dividing each concentration to the highest concentration.

2.3.Experimental conditions

Reactants and apparatus

All reactants were used without prior purifying: horseradish peroxidase 325 U/mg, superoxide dismutase from bovine erithrocites 5030 U/mg, catalase from bovine liver 2950 U/mg solid, xantin oxidase from microbial source 8 U/mg, xanthine sodium salt, L-ascorbic acid – fine cristals (20-200 mesh), Folin-Ciocâlteu reagent 2N, $DPPH^{\bullet}$, citric acid (Sigma - Germany); xantin oxidase from buttermilk 0,5 U/mg protein (Calbiochem – USA); poly(ethylene glycole) diglicidyl ether (Polysciences - USA); poly(1-vinylimidazole) complexed with [osmium (4-4′-dimethylbipyridine)$_2$Cl)]$^{II/III}$ (Sweden – donation of the Analytical Chemistry Department, Lund University); H_2O_2 30%, KH_2PO_4, K_2HPO_4 (Merck - Germany); (±)-6-hydroxy-2,5,7,8-tetramethyl chroman carboxylic acid, $FeCl_3$ (Fluka - Germany); $K_4[Fe(CN)_6]$ (Polskie Odczynniki Chemiczne Gliwice - Poland); H_3PO_4 89% (Loba Chemie - Austria); 2,6-dichlorophenol-indophenol sodium salt, ethanol (Riedel-de Haën - Europe); KCl, Na_2CO_3 (Reactivul - Romania) and HCl 1 N (Microchim - Romania).

For all amperometric and voltammetric measurements the used electrochemical systems consisted of: (i) working electrode - pyrolytic graphite rod (Ringsdorff, Germany) with a diameter of 3 mm, bare (DPPH method) or modified (G/RP/HRP, G/RP/HRP//XOD and G/PB), embedded in a teflon cylindrical body, (ii) reference electrode - Ag wire coated with electrochemically deposited AgCl, immersed in saturated KCl aqueous solution and placed in a Luggin capillary filled with the same solution, (iii) counterelectrode - Pt wire embedded in a plastic body, with Cu contact. Electrodes were immersed in a glass cell (volume 30 ml). For amperometric measurements we used a rotating disk electrode body - Tachyprocesseur (Radiometer Analytical, Germany) or a magnetic stirrer (Heidolph MR 3000, Heidolph Instruments, Germany). The used potentiostats were PARSTAT 2276 (Princeton Applied Research, USA) and BioLogic SP-150 (Science Instruments, France) - suitable for measurement of weak currents. To clean the surface of graphite by ultrasonic bath was used ultrasonic bath Elma S10 (Elmasonic, Germany). For spectrophotometric measurements were used a UV/Vis spectrophotometer Jasco V-530 and standard quartz cuvettes (1.0 * 1.0 * 4.5 cm^3).

Data recording was done with the help of Power Suite 2.56 (for PARSTAT), EC-Lab V9.76 (Biology) and Spectra Manager (for Jasco) programmes, and interpretation of data was done using the program Origin 8.

Sensors preparation

G/RP/HRP and G/RP/HRP//XOD

The graphite bar was cleaned by polishing with emery paper and paper filter, followed by ultrasonicating for 2 minutes. The absence of any redox species on the graphite surface was checked by potential scanning through cyclic voltammetry in the range -0.200 to 0.500 V vs. $Ag/AgCl,KCl_{sat}$ at a speed of 50 mV/s, using phosphate buffer solution (PBS) pH 7.5, prepared from (KH_2PO_4 + K_2HPO_4) 50 mM + 50 mM KCl. If graphite was clean, only the signal corresponding to the reduction of O_2 was observed.

On the graphite bar were deposited: 4.7 U HRP, 7.2 mg and 1.8 mg RP PEGDGE form of a mixture prepared within not more than 15 minutes before use. The thus prepared biosensor was stored at ~5°C, in atmosphere of PBS vapours.

G/PB

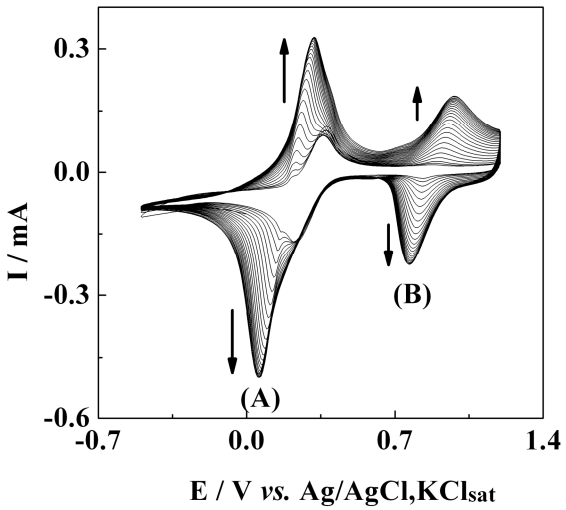


Figure 11. Electrochemical deposition of PB by cyclic scanning of potential in a mixture of $K_3Fe(CN)_6$ 0.1 M and $FeCl_3$ 0.1 M; supporting electrolyte, PBS, pH 3.1; cycles number, 25; v, 25 mV/s

The G/PB sensor was prepared by cyclic scanning of potential. After activation of the graphite surface by application of +1.700 V vs. Ag/AgCl/ KCl_{sat} for 3 minutes in PBS pH 3.1, the potential scanning was performed during 25 successive cycles between -0.500 and +1.200 V vs. Ag/AgCl/KCl_{sat} at 25 mV/s in a mixture of $K_3Fe(CN)_6$ and $FeCl_3$ 0.1 M (prepared in 10 mM HCl) [47] (Fig. 11).

The observed peaks describe the redox processes taking place according to equations 21 and 22, corresponding to the pairs of peaks (A) and (B), respectively.

$$\underset{\text{(soluble PB)}}{KFe^{III}Fe^{II}(CN)_6} + K^+ + e^- \rightleftharpoons \underset{\text{(Prussian White, Everitt salt)}}{K_2Fe^{III}Fe^{II}(CN)_6} \quad (21)$$

$$\underset{\text{(soluble PB)}}{KFe^{III}Fe^{II}(CN)_6} \rightleftharpoons 2/3\ K^+ + 2/3\ e^- + \underset{\text{(Prussian Green)}}{K_{1/3}(Fe^{III}(CN)_6)_{2/3}(Fe^{II}(CN)_6)_{1/3}} \quad (22)$$

In conclusion, it can be said that of the sensors G/RP/HRP, G/RP/HRP//XOD and G/PB, G/PB has the advantage of a the most simple, fast and at low cost preparation.

2.4.Results and discussion

2.4.1. Stability study of G/RP/HRP

Knowing how the electroanalytical performances of the G/RP/HRP biosensor varies with time was considered particularly important. Therefore, we studied its operational stability, for different biosensors with the same composition but different storage temperature and frequency, by performing amperometric measurements in PBS before and after addition of H_2O_2, followed or not by the addition of AA at various intervals of time. Results were expressed as values divided by the current reduction in the maximum current for each biosensor.

There have been two types of measurements: (i) addition of 130 mM H_2O_2 in PBS, (ii) addition of H_2O_2 40 μM in PBS, followed by one addition of AA 330 μM. Working and storage conditions are shown in Table 10. Frequency of measurements ranged from 1 to 7 days for the G/RP/HRP-1-3 identical biosensors, decreasing with increasing duration of use.

Table 10. Conditions of use and storage of G/RP/HRP to study the operational stability

Stability type	Measurement frequency	Storage temperature (°C)	Test solution	Biosensor code
Long term	First and 63-th day	5	H_2O_2 130 μM	G/RP/HRP-0
	1 – 7 days	23		G/RP/HRP-1
		5		G/RP/HRP-2
			H_2O_2 40 μM + AA 330 μM	G/RP/HRP-3
Short term	1 hour	23	H_2O_2 130 μM	G/RP/HRP-1
		5		G/RP/HRP-4
	7 – 10 minutes	23		G/RP/HRP-5

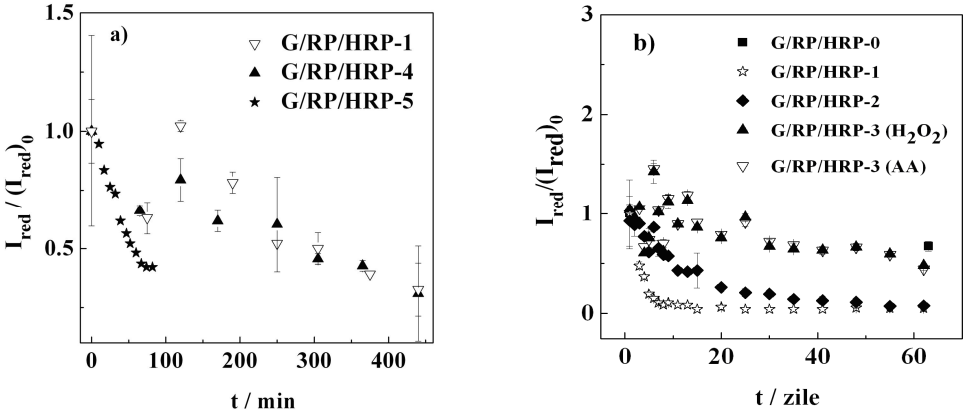


Figure 12. Short-term (7.5 hours) (a) and long-term (63 days) stability (b) for G/RP/HRP-0-3 in presence of H_2O_2 130 μM and for G/RP/HRP-3(AA) in H_2O_2 40 μM + AA 330 μM. Supporting electrolyte, PBS, pH 7.5; E_{apl} = -0.100 V *vs*. Ag/AgCl/KCl_{sat}; ω = 1000 rpm

From the current variations (Fig. 12), the following were observed:

- for 7,5 hours, no significant current variation was observed as a function of storage temperature (23°C for G/RP/HRP-1 and 5°C for G/RP/HRP-4); after 10 days, the current decrease was 52% higher for G/RP/HRP-1 as compared to G/RP/HRP-2
- the performance of the biosensors decreased with increasing the frequency of measurements (G/RP/HRP-0, -2 and -3) and the used H_2O_2 concentration (G/RP/HRP-2 and -3)
- half-lives ($t_{1/2}$) were: 3 days, 11 days, 61 days, 54 minutes and 5.2 hours for the biosensors G/RP/HRP-1 (long-term), -2, -3, -4 and -5 respectively, according to a polynomial interpolation type.

2.4.2. Electroanalytical characterization

The electroanalytical performances of the sensors G/RP/HRP, G/RP/HRP//XOD and G/PB were evaluated for different concentrations of H_2O_2, XA or AA in the presence of H_2O_2 or XA, using their calibration curves: sensitivity (S), linear concentration domain (DL), detection limit (LD), response time (t_{95}) and relative standard deviation (RSD).

A voltammetric study performed on bare graphite in presence of 10 mM AA showed an oxidation process around 0 mV / Ag/AgCl/KCl_{sat}. Therefore, at the potential chosen for the amperometric measurements, -0.100 V *vs*. Ag/AgCl/$KCl_{sat,}$ there is a insignificant level of AA oxidation, and so, this potential is well chosen for amperometric tests in presence of AA.

G/RP/HRP

The electroanalytical characterization of G/RP/HRP was preceded by a study aimed at optimizing the HRP loading and the pH of the PBS used as support electrolyte. The study showed that the performance of the biosensor is optimal for of 4.7 U HRP / electrode and the pH value 7.5.

Amperometric measurements were performed to evaluate electroanalytical parameters of G/RP/HRP at the potential of -0.100 V vs. Ag/AgCl/KCl_{sat} for different H_2O_2 concentrations. The electroanalytical parameters were evaluated for maximum repeatability, i.e., using 2 biosensors with similar composition simultaneously (Table 11). Thus, G/RP/HRP can be used with good results in the H_2O_2 concentration range 28-159 mM.

A set of amperometric measurements using G/RP/HRP was performed in presence of in AA at constant H_2O_2 concentration of 0.3 mM and at -0.100 V vs. Ag/AgCl/KCl_{sat} potential.. From Table 12 it results that G/RP/HRP can be used in good condition in the AA concentration range 93-185 mM.

Table 11. Electroanalytical parameters of biosensor G/RP/HRP evaluated for H_2O_2

Linear fitting			MM fitting				t_{95} (s)	DSR* (%)	LD (µM)
S (mA/M)	$\underline{R^2}$ N	DL (µM)	I_{max} (µA)	K_m^{ap} (µM)	S (mA/M)	$\underline{R^2}$ N			
59.1 ± 1.7	<u>0.9983</u> 7	23.8 – 159.1	37.7 ± 1.1	0.49 ± 0.02	77.3 ± 4.1	<u>0,9992</u> 17	13	2	0,5

*calculated for H_2O_2 300 µM

Table 12. Electroanalytical parameters of G/RP/HRP evaluated for AA in presence of H_2O_2 0.3 mM

Linear fitting			MM fitting				t_{95} (s)	DSR* (%)	LD (µM)
S (mA/M)	$\underline{R^2}$ N	DL (µM)	I_{max} (µA)	K_m^{ap} (µM)	S (mA/M)	$\underline{R^2}$ N			
45,6 ± 3,2	<u>0,9951</u> 3	93 – 185	21,6 ± 1,1	0,3 ± 0,04	74,6 ± 0,2	<u>0,9952</u> 17	4	13,2	5,2

* calculated for AA 94.,0 µM

Amperometric measurements similar to those performed in presence of AA were carried out in the presence of caffeic acid and in Trolox. It should be mentioned that a voltammetric study was previously performed, showing that both caffeic acid and Trolox are in the reduced state at the applied potential -0.100 V vs. $Ag/AgCl/KCl_{sat}$, so they can be used without the risk of interferences in combination with H_2O_2. Electroanalytical parameters of the two AO, presented in Tables 13 and 14, shows that the current dependence of reduction of the concentration of AO is linear throughout the range of concentrations 0,9 – 7,0 mM for Trolox and 0,9 – 6,3 mM for caffeic acid.

Table 13. Electroanalytical parameters of G/RP/HRP, obtained in presence of Trolox and caffeic acid in presence of H_2O_2 0,04 mM

	Linear fitting			t_{95} (s)	DSR (%)	LD (μM)
AO	S (mA/M)	$\frac{R^2}{N}$	DL (mM)			
Trolox	0,7 ± 0,0	$\frac{0,9999}{24}$	0,9 – 7,0	22	0,1*	39,5
Caffeic acid	1,0 ± 0,0	$\frac{0,9991}{17}$	0,9 – 6,3	65	27,9**	40

* calculated for Trolox 6,0 mM
** calculated for caffeic acid 6,0 mM

G/RP/HRP//XOD

Table 14 presents the electroanalytical parameters of the biosensor G/RP/HRP//XOD, evaluated from measurements performed at -0.100 V vs. $Ag/AgCl/KCl_{sat}$ at different XA concentrations, using as support electrolyte PBS containing 0.1 mU XOD/ml. By comparison with the parameters obtained for G/RP/HRP in the presence of H_2O_2, there is some advantage of the previously studied one: (i) the sensitivity obtained from linear fitting is 21% higher; (ii) the linear domain is 70% larger; (iii) the response time is 4 times shorter, (iv) the relative standard deviation for 300 mM analyte is 7 smaller; (v) the detection limit is 3 times lower. These differences are mainly due to higher complexity of the system G/RP/HRP//XOD.

Table 14. Electroanalytical parameters of G/RP/HRP//XOD evaluated for XA

Linear fitting			MM fitting				t_{95} (s)	DSR* (%)	LD (μM)
S (mA/M)	$\frac{R^2}{N}$	DL (μM)	I_{max} (μA)	K_m^{ap} (μM)	S (mA/M)	$\frac{R^2}{N}$			
49,0 ± 2,1	$\frac{0,9924}{5}$	23,8 – 93,0	17,7 ± 0,5	0,24 ± 0,01	76,9 ± 3,8	$\frac{0,9979}{17}$	55	14	1,4

* calculated for 300 μM XA

Table 15 includes the electroanalytical parameters evaluated from the calibration curves in the presence of AA at -0.100 V vs. $Ag/AgCl/KCl_{sat}$ in the presence of XA 0.07 mM having as support electrolyte PBS pH 7.5 containing 0.1 mU XOD/ml. This yields a sensitivity similar to that of G/RP/HRP, which confirms the similarity of processes occurring in the two systems. It may be concluded that AA reacts with H_2O_2 formed by $O_2^{-\bullet}$ dismutation, which is a very rapid process.

Table 15. Electroanalytical parameters of G/RP/HRP//XOD in presence of AA at constant XA concentration of 0,07 mM using PBS pH 7.5 containing XOD 0,1 mU/ml

Linear fitting			MM fitting				t_{95} (s)	DSR* (%)	LD (µM)
S (mA/M)	$\underline{R^2}$ N	DL (µM)	I_{max} (µA)	K_m^{ap} (µM)	S (mA/M)	$\underline{R^2}$ N			
43,6 ± 0,8	<u>0,9983</u> 7	23,8 – 159,1	4,7 ± 0,1	0,1 ± 0,0	58,3 ± 3,3	<u>0,9976</u> 16	90	12,1	0,7

*calculated for AA 95 µM

G/PB

The electroanalytical parameters of the G/PB sensor (Table 16) were estimated using calibration curves performed in the presence of H_2O_2 at constant potential, -0.100 V vs. $Ag/AgCl/KCl_{sat}$ in supporting electrolyte solution PBS, pH 3.1.

Compared with biosensors G/RP/HRP, G/BP has a linear domain about 13 times larger, which is explained by the fact that the active surface at which the H_2O_2 reduction occurs is much higher than of the biosensor. In addition, the sensitivity, response time, standard deviation and detection limit are better than those of G/RP/HRP.

Table 16 presents the electroanalytical parameters evaluated from the calibration curve performed in presence of AA; for this, amperometric measurements were performed for various AA concentrations at constant potential of -0.100 V vs. $Ag/AgCl/KCl_{sat}$, at constant H_2O_2 concentration of 0.8 mM. There is a relatively low efficiency for the detection of AA in the presence of H_2O_2, given the low sensitivity relative to that obtained with biosensors G/RP/HRP and G/RP/HRP//XOD.

Table 16. Electroanalytical parameters of G/PB evaluated for H_2O_2

Linear fitting			MM fitting				t_{95} (s)	DSR* (%)	LD (µM)
S (mA/M)	$\underline{R^2}$ N	DL (mM)	I_{max} (mA)	K_m^{ap} (mM)	S (mA/M)	$\underline{R^2}$ N			
89 ± 2,2	<u>0,9917</u> 15	0,03 – 17,3	4,4 ± 0,01	35 ± 4,5	126 ± 20,1	<u>0,9978</u> 16	7	4	1,2

*calculated for H_2O_2 300 µM

The data presented in this chapter showed that among the investigated sensors G/PB is optimal in terms of the electroanalytical parameters.

Table 17. Electroanalytical parameters of the G/RP/HRP//XOD biosensor evaluated for AA in presence of XA 0.8 mM

Linear fitting			MM fitting				t_{95} (s)	DSR* (%)	LD (µM)
S (mA/M)	$\frac{R^2}{N}$	DL (mM)	I_{max} (mA)	K_m^{ap} (mM)	S (mA/M)	$\frac{R^2}{N}$			
0,9 ± 0,0	$\frac{0,9916}{11}$	0,5 – 44,9	0,1 ± 0,0	72,3 ± 20,3	1,4 ± 0,4	$\frac{0,9892}{12}$	10	4,6	0,5

*calculated for AA 8,5 mM

2.4.2.1. Evaluation of the AOA of some real samples

Amperometric methods

The used real sample were: the red wine "Cabernet" (Recaş, harvest date: 06.05.2009), white wine "Feteasca Regală" (Jidvei, harvest date: 06.05.2009), concentrated apple juice "Pektirom "(Dej, harvest date: 04.05.2009) (provided by the Center for Applied Plant Biotechnology "Proplanta", Cluj-Napoca) and fresh juice, obtained directly from fruits, without any further processing and used immediately after obtention: lemon, grapefruit, red orange, 'Seckel' pear, nectarine, kiwi, orange, "Red Delicious" apple, mandarin, white grapes and black grapes.

The assessment of the above-listed AOA samples was performed using amperometric measurementst carried out in PBS solutions containing H_2O_2 (0.1 mM for G/RP/HRP and 0.8 mM G/PB) or 0.05 mM + XA XOD 0.1 mU/ml (for G/RP/HRP//XOD), to which the sample was added. Interpretation of results was done by comparing the current variations for the samples obtained after addition of H_2O_2 or XA solution to the calibration of AA (as described in Section 2.1). The results of AOA evaluation using the amperometric sensors: G/RP/HRP, G/RP/HRP//XOD and G/PB and the three reference methods are listed in Table 19, and the correlations among results using these methods are presented in Table 20.

Reference methods

For the method based on the reduction of $DPPH^{\bullet}$, real samples additions were carried out in a solution of $DPPH^{\bullet}$ 50 mM and the applied potential was -0.100 mV / Ag/AgCl/Ag/AgCl/KCl_{sat}. To verify that the current variations are exclusively derived from the reaction between the sample and $DPPH^{\bullet}$, amperograms were carried out, similar to those described above, but in the absence of $DPPH^{\bullet}$.

For none of the samples were there any current variations, which confirmed that the change in current reduction of $DPPH^{\bullet}$ was exclusively given by the interaction between AO and $DPPH^{\bullet}$.

For AOA assessment using the Folin-Ciocâlteu method, the absorbance of samples (diluted so that absorbance to be between 0 and 1) containing 0.026 N Folin-Ciocâlteu reagent and Na_2CO_3 13.2 g/l was carried out after incubation at dark and room temperature for 1.5 hours. Absorbances were measured at their peak value, ie, 668 nm to 662 nm for orange and grapefruit, respectively. The blank, against which sample measurements were performed was the Folin-Ciocâlteu reagent solution containing Na_2CO_3 in concentrations equal to those of the samples, and the juice was replaced with distilled water.

Assessment of concentrations of AA in real samples was tested by using the DCPIP method, in the presence of AA is reduced to compound DCPIPH DCPIP (Fig. 10), the mixture is colorless in acid. The total consumption of AA in the system corresponds to turn color from colorless to pink. The results were evaluated in 1 mM DCPIP volume necessary to neutralize a sample volume of 1 ml, compared to that required to neutralize a 1 mM AA ml (0.4 ml)

Table 19. AOA of the real sample evaluated using G/PB, G/RP/HRP and G/RP/HRP//XOD, in comparison with those obtained with the 3 reference methods

Sample / Method	G/PB (mM AA, %)	G/RP/HRP (mM AA, %)	G/RP/HRP//XOD (mM AA, %)	Folin-Ciocâlteu (absorbance, %)	DPPH (mM Trolox, %)	DCPIP (mM, %)
Lemon	43 ± 12 30 ± 9	0,7 ± 0,3 31,8 ± 13,6	-	-	1,8 ± 0,0 8,8 ± 0,0	9,3 ± 0,0 27,9 ± 0,0
Grapefruit	44 ± 14 31 ± 10	2,2 ± 0,2 100,0 ± 9,1	2,2 ± 0,3 75,9 ± 10,3	0,5 ± 0,1 79,6 ± 0,2	3,3 ± 0,3 16,1 ± 1,3	6,1 ± 0,6 18,3 ± 1,8
Red orange	46 ± 5 33 ± 4	1,3 ± 0,03 59,1 ± 1,4	-	-	3,6 ± 0,2 17,6 ± 1,0	19,0 ± 4,1 57,1 ± 12,3
“Seckel“ pear	51 ± 11 36 ± 8	-	-	-	-	-
Nectarine	57 ± 12 40 ± 9	-	-	-	1,4 ± 0,3 6,8 ± 1,6	0,6 ± 0,0 1,8 ± 0,0
Kiwi	59 ± 7 42 ± 5	1,2 ± 0,3 54,5 ± 13,6	-	-	1,8 ± 0,2 8,8 ± 1,2	4,0 ± 0,3 12,0 ± 0,9
Orange	61 ± 13 43 ± 9	1,2 ± 0,2 54,5 ± 9,1	2,9 ± 0,4 100,0 ± 13,8	0,7 ± 0,0 100,0 ± 0,3	3,7 ± 0,2 18,0 ± 0,8	8,3 ± 0,5 24,9 ± 1,5
“Feteasc㠔 Regal㠓 white wine	61 ± 8 43 ± 6	0,1 ± 0,04 4,5 ± 1,8	-	-	1,2 ± 0,2 5,9 ± 0,8	1,1 ± 0,3 3,3 ± 0,9
“Red Delicious“ apple	63 ± 5 45 ± 4	-	-	-	1,4 ± 0,2 6,8 ± 1,0	1,9 ± 0,0 5,7 ± 0,0
Black grapes	72 ± 1 51 ± 1	0,04 ± 0,00 1,8 ± 0,0	-	-	0,8 ± 0,05 3,9 ± 0,3	2,0 ± 0,7 6,0 ± 2,1
White grapes	73 ± 9 52 ± 6	0,08 ± 0,03 3,6 ± 1,4	-	-	0,9 ± 0,04 4,4 ± 0,2	0,4 ± 0,0 1,2 ± 0,0
Mandarine	78 ± 8 55 ± 6	0,3 ± 0,07 13,6 ± 3,2	-	-	2,0 ± 0,4 9,8 ± 2,0	5,1 ± 0,6 15,3 ± 1,8
“Cabernet“ red wine	87 ± 29 62 ± 21	0,3 ± 0,2 13,6 ± 9,1	-	-	20,5 ± 4,9 100,0 ± 24,0	33,3 ± 8,2 100,0 ± 24,6
“Pektirom“ apple juice concentrate	141 ± 6 100 ± 4	0,2 ± 0,08 9,1 ± 3,6	-	-	17,3 ± 2,1 84,4 ± 10,2	9,6 ± 0,0 28,8 ± 0,0

Table 20. AOA correlation between values of samples of wine and fruit juices observed between the various methods used

Method	**G/PB**	**G/RP/HRP**	**G/RP/HRP//XOD**
G/RP/HRP	lemon < kiwi ≈ orange (R^2 = 0,9890) black grapes < white grapes < mandarine (R^2 = 0,9984) lemon < grapefruit lemon < red orange „Feteasca Regală" white wine < „Cabernet" red wine „Feteasca Regală" white wine < „Pektirom" apple juice concentrate	-	-
G/RP/HRP//XOD	grapefruit < orange	-	-
Folin-Ciocâlteu	grapefruit < orange	-	grapefruit < orange
DPPH	black grapes < white grapes < mandarine (R^2 = 0,9934) lemon < grapefruit < red orange < orange < (R^2 = 0,9788) „Feteasca Regală" white wine < vin roşu „Cabernet" „Feteasca Regală" white wine < „Red Delicious" apple nectarine < kiwi orange < „Pektirom" apple juice concentrate	black grapes < white grapes < white wine „Feteasca Regală" < mandarine (R^2 = 0,9844) kiwi < red orange „Feteasca Regală" white wine < kiwi „Feteasca Regală" white wine < orange „Pektirom" apple juice concentrate < „Cabernet" red wine	-
DCPIP	nectarine < white wine „Feteasca Regală" < „Red Delicious" apple (R^2 = 0,8995) kiwi < madarine < „Pektirom" apple juice concentrate (R^2 = 0,9987) grapefruit < orange măr „Red Delicious" < black grapes măr „Red Delicious" < mandarine red orange < vin roşu „Cabernet" white grapes < suc conc. de mere „Pektirom"	„Feteasca Regală" white wine < „Pektirom" apple juice concentrate < „Cabernet" red wine (R^2 = 0,9274) black grapes < orange kiwi < grapefruit white grapes < mandarine lemon < red orange	grapefruit < orange

According to the Table 20, many correlations were established between the results using G/PB and G/RP/HRP, DPPH and the DCPIP-based titrimetric AA content measurement. There are, therefore, similarities in the functioning principles of G/PB and G/RP/HRP. The numerous correlations established with the DCPIP-based method indicates that the main AO detected is AA.

There are numerous results well correlated for G/RP/HRP and DCPIP. However, all AOA estimated using G/RP/HRP are 3 to 50 times smaller than those obtained by DCPIP. This can be explained by the existence of secondary reactions involving AA present in the system, thus decreasing AA concentrations.

For the samples tested with G/RP/HRP//XOD (orange, grapefruit), there was a good correlation with all three reference methods. DPPH method highlights the reduction capacity of the tested AO. Low values obtained compared with those for AA determination method with DCPIP could be explained, as in biosensor G/RP/HRP, by the existence of secondary reactions involving AA reactions, and not involving the consumption of H_2O_2

2.5.Conclusions

This study includes a description of the principle of comparative operating performance, optimization, characterization and utilization for the evaluation of AO of synthetic and real samples using two biosensors (G/RP/HRP and G/RP/HRP//XOD) and an amperometric sensor (G/PB).

In principle, G/RP/HRP, G/PB and G/RP/HRP//XOD are H_2O_2 sensors. AO react with H_2O_2, giving rise to a variation of the current reduction. This variation is compared to the same variation observed in presence of AA – for this, the sensitivity of the calibration curves for AA is used.

G/PB has the advantage of simplicity, rapidity and low cost of preparation. G/RP/HRP//XOD preparation requires is the most laborious among amperometric (bio)sensors, but it has the advantage of more accurately reflecting the concept of "antioxidant capacity" as defined by most of the researchers, namely, that the antiradical activity.

The optimization studies revealed the following conclusions: (i) the maximum sensitivity is obtained for G/RP/HRP in presence of H_2O_2 at neutral pH (7.5) and average enzyme loading, 4.7 U HRP / electrode, (ii) the optimal electroanalytical parameters in presence of XA are obtained for biosensor G/RP/HRP//XOD when XOD was dissolved in the XA solution, and in absence of SOD.

Characterization study included the following: lifetime (G/RP/HRP), electrocatalytic effect for H_2O_2 (G/RP/HRP and G/PB) and XA (G/RP/HRP//XOD) and electroanalytical parameters.

The operational stability, evaluated from the halflife, measured as the time required to decrease the sensitivity of the biosensor G/RP/HRP to half of the initial sensitivity, revealed that the

halflife decreases with measurement frequency, increase of the storage temperature and of the H_2O_2 concentration.

The only electrocatalytic effect for H_2O_2 was highlighted in the case of G/PB. For the two biosensors, no major differences were observed between the peaks recorded in absence / presence of H_2O_2.

In terms of electroanalytical parameters: sensitivity, linearity domain, detection limit, response time and standard deviation, the best performance was obtained for G/PB.

The amperometric sensors were used for AA, Trolox and caffeic acid. For AA, the largest working range was obtained for G/RP/HRP but showed a maximum sensitivity in the case of G/PB. Applications in real samples consisted of AOA evaluation of wines and fruit juices, using AA as reference AO. The results were compared with those obtained by three reference methods: Folin-Ciocâlteu, $DPPH^{\bullet}$ and the titrimetric determination of AA using DCPIP. Most correlations were obtained among G/PB, G/RP/HRP, $DPPH^{\bullet}$ and the titrimetric determination of AA, indicating that there are similarities in the mechanism of the interactions between AO and the neutralized species.

Data concerning the electroanalytical characterization and AOA evaluation shows that G/RP/HRP, G/RP/HRP//XOD and G/PB might be used for evaluating AOA of wine and fruit juices.

IV. General conclusions

This work aimed at the characterization of 10 new macrocyclic compounds (one [4.4.4.4]-cyclophane and 9 calixarenic derivatives) as ionophores for ISE-PVC-I; for this purpose, the electrochemical parameters were evaluated: sensitivity, detection limit and linearity domain - using separate ionic solutions, and selectivity coefficients - evaluated using solutions containing two ions simultaneously. In the case of M7F2, for which membranes were prepared both with and without ionic additive (KTkClPB), there were no significant differences between the parameters of the two types of electroanalytical membranes. M7F2 and C4P4 compounds showed selectivity for Ca^{2+} ion; C4Es2Cr2, C6Es3Cr3 and C6Es3 showe selectivity for Na^{+}; C4P4, C6Es6, C8Es8 C6Cr3Am3 and responded preferentially to K^{+}.

The second goal of this thesis was in preparation, optimization, characterization and utilization of synthetic (AA, Trolox and caffeic acid) and real samples (wines and fruit juices) using amperometric sensors for the evaluation of three AOA (G/RP/HRP, G/RP/HRP//XOD and G/PB). Considering the simplicity of preparation procedure, the costs and the electroanalytical properties, the sensor G/PB is optimal. Taking into account the relevance of the evaluated AOA, the G/RP/HRP-XOD biosensor is preferable because it detects an antiradical capacity. The operational stability study conducted for the biosensor G/RP/HRP showed that its lifetime decreases with increasing storage

temperature, H_2O_2 concentration used and frequency of measurements. Using these sensors and three reference methods (DPPH, Folin-Ciocâlteu and DCPIP), the AOA of 2 wine samples (a white wine, " Feteasca Regală" and a red one, "Cabernet") and fruit juices (lemon, grapefruit, blood orange, pear 'Seckel', nectarines, kiwi, orange, apple "Red Delicious", black grapes, white grape, tangerine, apple juice concentrate "Pektirom") was evaluated. Multiple correlations were obtained between the methods used, which shows that in some cases, the proposed methods can be used successfully in assessing AOA.

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VI. List of results

List of participations at national and international scientific events

1. Lidia Varvari, Liana Mureşan, Ionel Cătălin Popescu, Antioxidant capacity determination in wines and fruit juices using an amperometric biosensor, The 59th Meeting of the International Society of Electrochemistry, Sevilla, Spain, 07-12 September **2008**, poster
2. Lidia Varvari, Liana M. Mureşan, Ionel Cătălin Popescu, New amperometric biosensor for determination of the total antioxidant capacity, International Conference on Metrology of

Environmental, Food and Nutritional Measurements, Budapest, Hungary, 09-12 September **2008**, oral presentation

3. Lidia Varvari, Sorin Aurel Dorneanu, Ionel Cătălin Popescu, Calix[6]arene esters as ionophores for ion-selective electrodes, 2nd International Conference on Advanced Materials and Systems, Bucharest, Romania, 23-24 October **2008**, oral presentation
4. Lidia Varvari, Sorin Aurel Dorneanu, Ionel Cătălin Popescu, Cation-selective electrode based on a calix[6]arenic compound, 8th International Symposium on Metal Elements in Environment, Medicine and Biology, Timişoara, 05-06 December **2008**, oral presentation
5. Vasilica Lateş, Lidia Varvari, Ionel Cătălin Popescu, Biocapteur ampérométrique pour la détermination de la capacité antioxydante, Journées d'Electrochimie, Sinaia, România, 06-10 July **2009**, oral presentation
6. Lidia Varvari, Gabriella Szabo, Ionel Cătălin Popescu, Comparative study on the antioxidant activity evaluation of fruit juices and wines by using amperometric and spectrophotometric methods, Molecular Modeling in Chemistry and Biochemistry, Cluj-Napoca, România, 28 May **2010**, oral presentation
7. Lidia Varvari, Ionel Cătălin Popescu, Comparative study on the antioxidant evaluation of fruits by using amperometric and bioamperometric methods, 3rd EuCheMS Chemistry Congress, Nürnberg, Germania, 29 August-02 September **2010**, poster

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