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Biosensors Applications on Assessment of Reactive Oxygen Species and Antioxidants

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1. Introduction

The importance of the subject dealing with oxidative stress and antioxidants protection against oxidative stress was increasing in the last three decades, a proof of this assertion being the huge number of publications appeared since 2000 (meaning 150582 publications) devoted, for example, to antioxidant.

Addressing such a topic like Reactive Oxygen Species (ROS) and/or antioxidants (Aox) assessment it has to be clearly described the mean of both these terms and, concomitantly, the close inter-dependence between their actions and, respectively, effects. To support the assertion we would like to mention the fact that two of the four recommended steps in protocol for antioxidant assessment (Becker et al., 2004) are that regarding the evaluation of compound activity as inhibitor of lipid peroxidation in biological model systems and the study of the compound efficiency against relevant oxidative markers, these providing the evidence of strict correlation between ROS toxicity and antioxidant efficacy.

As consequence, in this point it could be found the main reason in the attempt to develop flexible (versatile) bio-analytical tools applicable both in ROS toxicity assessment and antioxidant analysis, such are the sensors/biosensors.

Reactive oxygen species (ROS) toxicity assessment was a subject of highly interest in all types of publications about the oxidative stress because in the last decades was proven an increasing occurrence of pathologies associated to ROS presence (Dalle-Donne et al. 2006, Butterfield et al. 2001). The ROS "attack" arises on specific receptors from a cellular component which is the oxidizable substrate, producing as result an oxidized molecular product. Generally, this is the key-event in several diseases evolution like Alzheimer's (Jaeger et al, 2008) cardiovascular diseases (Knopp et al. 2008) and others age-related diseases (Wang et al., 2008).

In order to status a common basis of the used terms the beginning of this chapter is devoted to a short description of each of them, with examples, further followed by the biosensors development and application on assessment of ROS, respectively antioxidants.

Up to date all reported biosensors employed various approaches, from direct analysis of compounds with characteristics antioxidants, to measuring the antioxidant enzymes activity and detection of free radicals. Most reported biosensors use immobilized enzymes in

combination with electrochemical transducers, mainly amperometric devices (Mello & Kubota, 2007).

We are presenting in this chapter the use of biosensor in such determinations underlining with several experimental critical points that have to be tackled when a biosensor is developed and are implemented in ROS and antioxidants assessments. All examples that we are giving in this chapter are performed using some case study, significant for each category, namely for ROS, respectively antioxidants. It has to be highlighted from the very beginning that all examples are fully applicable only *in vitro* and useful as screening tools for providing information eventually helpful *in vivo* , but the *in vivo* determinations request other deep investigations strictly corroborated to metabolic pathways and pathological substantiation.

In the above mentioned context the versatility of sensors and biosensors application in both the analysis of antioxidants and evaluation of reactive oxygen species is defined by their extended use as analytical tool either in quantification of phytochemical compounds acting as antioxidants (especially phenolics and phenolic derivatives) and their known applications in the evaluation of antioxidant properties with respect to relevant oxidative markers. Moreover, several studies reported the electrochemical sensors application when it has to be evaluated the inhibition/ending of lipid oxidation.

2. Basics on reactive oxygen species and antioxidants

Reactive oxygen species (ROS) describe very reactive molecules containing oxygen, their high reactivity being given by the presence of unpaired electrons in the valence shell. As underlined by Halliwell (Halliwell, 2007 Halliwell Barry, Gutteridge J.M.C, Free Radicals in Biology and Medicine, Fourth edition, Oxford University Press, 2007), ROS is a general term that includes both oxygen radicals and several non-radical derivatives of oxygen, being overall acceptable that all oxygen radicals are ROS but not all ROS are oxygen radicals. A brief review of the most important ROS, mainly those with proved *in vivo* action, is given below, either radicals or non-radicals ROS.

The first produced free radical in the aerobic organisms is the superoxide radical $O_2^{\bullet -}$, a very reactive radical that afterwards generates hydrogen peroxide and can lead to lipid peroxidation, DNA and RNA damage, etc.

Another radical ROS is hydroperoxyl, $HO_2^{\bullet}$ which generally exists in traces in equilibrium with $O_2^{\bullet -}$ at physiological pH, its reactivity being mainly related to its higher capability to membrane cross than superoxide anion radical.

Hydroxyl radical OH• is the most reactive among ROS and can be formed by interaction of superoxide anion O_2•- and hydrogen peroxide H_2O_2 with cellular compounds through reactions like Fenton or Haber–Weiss.

Peroxyl radicals $RO_2^{\bullet}$ and alkoxyl radicals, $RO^{\bullet}$ are oxygen centered radicals formed by various routes, like reaction of carbon centered radicals with oxygen, or by decomposition of organic peroxides, being extremely important in lipid peroxidation reactions, especially as reaction product.

Hydrogen peroxide is a non-radical ROS, widespread *in vivo*, generated as product in various enzymatic reactions (that involving xanthine oxidase, superoxid dismutase, D-aminoacid oxidases etc.), mitochondria being one of the main sources for hydrogen peroxide on cellular level.

Singlet oxygen, existing normally in two states, is produced via photosensitization reactions or by decomposing peroxyl radicals, both reactions being very important *in vivo.*
Anyway, usually ROS are produced in metabolic reactions all the time, their level being maintained at certain level limits by the same metabolic reaction and by the action of so-called antioxidants defence. When an imbalance between ROS and antioxidants occurs, it results in the generation of oxidative stress, defined by Sies as: a disturbance in the pro-oxidant – antioxidant balance in favour of the pro-oxidants, leading to potential damages. Oxidative stress plays an important role in the pathogenesis of many diseases such as atherosclerosis, diabetes, hypertension, cancer and in the ageing process.
Halliwell gave the general accepted definition of the antioxidant as any substance that, when present in low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substance (Halliwell 1990).
The antioxidants classifications support various points of view, depending on the antioxidant source, antioxidant action (mechanism) during radical chain reactions, or depending on antioxidant mechanisms as hydrogen or electron transfer reactions. As sources antioxidants can be divided into endogenous (internally synthesized, enzymatic ones -superoxide dismutase, catalase, glutathione peroxidase- or non-enzymatic - uric acid, bilirubine, albumine, glutathione etc.) and exogenous (diet-derived, like polyphenols, anthocyans, vitamin E, carotens, ascorbic acid etc.), while depending on their mechanism of action they can be divided into chain breaking antioxidants and preventive antioxidants (Somogyi, et al. 2007). Obviously, a classification criterion does not exclude another. Therefore, it has to be stressed that preventive antioxidants include enzymes such as superoxide dismutase that scavenge, for example, superoxide radical, blocking the initiation of chain reactions, while chain breaking antioxidants destroy free radicals after that they were formed, thus inhibiting the propagation of chain reactions, such as tocopherol (vitamin E) action against peroxyl radicals, during lipo-peroxidation.
The assessment of free radicals (FR) toxicity became very important due to the increasing degree and sources of pollution as long as the increasing occurrence of pathologies associated to the presence of free radicals in living organisms (Dalle-Donne et al. 2006, Butterfield et al. 2001).

3. How to design electrochemical sensors for ROS assessement. Case study HO•, superoxide and lipoperoxides radicals' assessment

When developing a protocol to assess free radicals toxicity it has to be taken into account that the FR "attack" takes place against a substrate producing an oxidized molecular product, this being generally the key-event in several diseases evolution. Usually, the amount of oxidized product is proportional to FR concentration and strictly related to the intensity of the damaging effect.
All existing methods able to evaluate effects of the ROS against cellular membrane components bear the drawback of the ROS short life-time, making difficult any attempt of direct assay; therefore it is usually preferred to monitor the molecular product of the oxidative stress reaction (Dalle-Donne et al. 2006, Butterfield et al. 2001). This monitoring approach is possible due to the fact that the degree of substrate oxidative damaging correlates strictly to the ROS concentration.
Generally highly sensitive procedures employed in such type of measurements are based on hyphenated techniques like high-performance chromatography (HPLC) with mass

spectrometry (MS) detection, either coupled mass spectrometry detection (HPLC-MS/MS), or with mass – spectrometry detection with resolved time of flight signal (MS-ToF); the most complex such technique is that based on Matrix Assisted Lased Desorption-Ionization-Time of Flight detection (MALDI ToF) (Dalle-Donne et al. 2006). Another measuring procedure reported for the molecular product determination of the oxidative reaction is based on enzyme immunoassay (EIA) detection as reported, for example, by Kohno (Kohno et al., 2000) which realized a sandwich EIA suitable for the measurement of human oxidized LDL (Ox-LDL) in blood, using mouse monoclonal antibody specific for oxidized phosphatidylcholine as the capture antibody, and a horseradish peroxidase (HRP)-labeled goat anti-human apolipoprotein-B (Apo-B) IgG for detection. The use of such kind of detection devices is costly in order to achieve reasonable analytical information.
Therefore, the logical outcome is that, by using an electrochemical device as screening tool, it is possible to appear an alternative to the mentioned expensive analysis for determination of certain free radicals toxicity.
This approach is more interesting when is undoubtedly necessary to obtain biological significant information about free radicals toxicity or about antioxidant efficacy, especially considering the Halliwell criteria about how to assess an antioxidant (Halliwell, 2006).
Direct determination of ROS extent in biological systems requires highly sensitive methods, at least on nanomolar level since, for example, superoxide anion physiological level is about 10^{-10} molL^{-1} and it can be performed by different methods as fluorescence assays (Benov et al., 1998), electron spin resonance (ESR) (Roubaud et al., 1997) or chemiluminescence (Yao et al., 2004). In the same time, the exact amount of reactive oxygen from complex samples using various types of sensors and biosensors was reported, starting with fluorimetric sensors (Pastor et al., 2004) and ending with electrochemical ones, either amperometric – with mediated or direct electron transfer (Tian et al., 2002, 2005; Ohsaka et al., 1995, 2001, 2002; Campanella et al., 2000; Dharmapandian et al, 2010) or voltammetric (Fan et al., 2004; Cortina-Puig et al., 2009). From all these analytical tools the highly sensitive ones should be mentioned, able to perform the determination of nitric oxide with a detection limit of 20 pmolL^{-1} (Fan et al., 2004) while the fluorimetric biosensor for superoxide anion exhibited a quantification limit of 20 nmolL^{-1}(Pastor et al., 2004). There are data reporting the simultaneous detection of reactive species of oxygen and nitrogen in macrophage cells using multi-step amperometric method (Amatore et al., 2008, 2010).
In this work we are presenting new electrochemical devices -electrochemical sensors- as alternative for ROS toxicity screening analysis, based on the use of bio-mimetic systems as oxidizable substrates.
It has to be emphasized once again that the biomimetic systems were developed for applications addressing information on ROS toxicity for ROS concentration levels with physiological signification, but without a sensitivity competing with *in vivo* measurements, since hyphenated techniques are used for this purpose.
The biomimetic systems use two substrates highly susceptible to lipoperoxidation, namely human low-density lipoprotein (LDL) and phosphatidylcholine (PC).

3.1 Why lipoprotein and phospholipids as biomimetic systems?

Lipoproteins in general and especially low-density lipoproteins (LDL), are the main target of free radicals "attack" on cellular level because lipoperoxidation is considered to be responsible of main damage of both proteins and lipids from cellular membranes (Halliwell & Gutteridge, 2007). LDL are considered to have an important role in biological process that

initiates and accelerates the development of cardiovascular diseases (Knopp et al. 2008) and influence Alzheimer's (Jaeger & Pietrzik 2008) and others diseases (Irshad, 2004; Staples et al. 2008; Parhami, 2003).

This is the main point from which we started the development of a new bio-mimetic model, thought to be able to help in understanding the structural and conformational modifications occurring on LDL subsequent to oxidative damage induction without using *in vivo* assessments. Other data supporting the use of LDL as bio-mimetic model are those related to the evidence that a key event in development of the various pathologies is the LDL oxidation step. The LDL oxidative modification has a unique pathway with respect to other lipoproteins (Parthasarathy et al., 2008), LDL being more sensitive to oxidation (Lam et al., 2004). LDL oxidation is dependent on the antioxidants within the cellular medium (Fierth et al., 2008).

In the same time, another peroxidation process, membrane phospholipid peroxidation, was incriminated in relation to oxidative damage occurring during pathological changes such as immuno-functional modulations, atherogeneses, and aging (Nagakawa et al., 1996) Phospholipid hydro-peroxide-the main product of membrane phospholipid peroxidation, may accumulate when the oxidative damage takes place in cellular membrane. In the same time, it was found that considerably elevated levels of phospholipid hydroperoxides occurred in blood cells of patients with Alzheimer's disease as compared to healthy volunteers. As a result, it seems very important to find out the level of ROS inducing such oxidative damage, and consequently, which are the effective antioxidants that can inhibit the formation of phospholipid hydroperoxides to prevent the disease. All data supporting phopholipid implication in key-events of important degenerative diseases made us to attempt to built up a second bio-mimetic system, based on another substrate highly susceptible to lipoperoxidation, namely phosphatidylcholine (PC).

Phosphatidylcholine, despite of a simple structure, is following in main steps the same oxidation pathway as LDL, generating lipoperoxides in presence of peroxyl radicals (see even figure 1).

3.2 Development of biomimetic systems to be used in sensors/biosensors construction

When dealing with development of biomimetic systems used in construction of sensors for ROS assessment, we decided to exemplify by the mean of the easiest methods available for oxidative substrates immobilization.

Therefore, **when oxidative substrates containing –SH groups** are used (as example LDL or DNA) two simple immobilization methods are available:

a. Direct deposition on solid support (Au sheet) by solution casting from oxidative substrate suspensions using suspensions containing a known mass of lipoprotein. Our protocol, already reported (Litescu et al., 2002) supposes the use of 60 ppm LDL suspension in KCl, 12 hours immobilization by solution casting on solid support, then further washed, dried and stored under vacuum, at 4°C.
b. Co-immobilization of substrate from suspensions on gold nanoparticles (25 mg mL^{-1}) and further attachment on conductive solid support. According to our experience, for LDL the optimal procedure consists in using of 1000 μg mL^{-1} LDL in 0.1 mol L^{-1} KCl allowed to immobilize 12 hours on AuNP then washed, dried under vacuum and stored under vacuum at 4°C.

When phospholipids are the oxidation substrate (example phosphatidylcholine, PC) the immobilization procedure consisted in suspending an exact amount of phospholipid in a solvent containing the best supporting electrolyte for electrochemical sensors, followed by chemisorption in controlled conditions on conductive support: inert atmosphere, optimum deposition time (able to ensure the appropriate thickness for the substrate layer and sensor operational stability), appropriate temperature. For example, our protocol used to build up a PC-based biomimetic system consists in the following: suspending of 72 mg of PC in 5 mL KCl 0.1 mol L^{-1}, suspending 24 mg of magnetic nanobeads (Fe_3O_4) in the previous solution, vortexed for 24 hours. After that PC-modified nanobeads (PC-Fe_3O_4) were separated, washed repeatedly, dried at 60°C for 30 minutes and stored at 4°C. The voltammetry experiments were performed applying a magnetic field in order to focus the same population of PC-Fe_3O_4 on the surface of working electrode.

It should be emphasized that the **main critical point when the biomimetic systems are used in the ROS toxicity evaluation** (and, consequently in assessment of the structural damaging induced by lipoperoxidation) consists in compulsory preservation of main functional groups availability toward ROS "attack". As consequence, checking the proper immobilization by surface analysis techniques (like Fourier Transformed Infrared Spectroscopy, XPS etc.) is essential at least in the first steps of elaboration of the protocols for sensors construction.

In the same time, it has to be mentioned a *sine qua non* condition that is mandatory when electrochemical sensors for ROS assessments are developed: it is necessary that ROS attack generate a significant change in electroactive properties of the used substrate. This means that the structural changes of the substrate have either gave birth to electroactive oxidation products or modify in a measurable way the substrate genuine electroactivity. Of course the amount of oxidized product (the amount of modified charge) is proportional to FR concentration and strictly related to the intensity of the damaging effect, raising an electrochemically measurable signal (amperometric, conductometric or voltametric).

3.2 Substrates oxidation

There are three simple possibilities to induce a fast and controlled lipo-peroxidation: heating, making the substrate (lipoprotein, phospholipids) to react with peroxyl radicals produced by azo-initiators or generating $HO^{\cdot}$ radicals, or using the classical Fenton reaction.

In our protocol , thermally generated peroxyl radicals ($ROO^{\cdot}$) by the decomposition of AAPH reacted with the substrates (LDL, respectively PC) as shown in figure 1 (where LH is the unoxidized lipoprotein, while PC is the unoxidized phosphatidylcholine); subsequently are generated the lipoperoxides ($LOO^{\cdot}$, respectively $PCOO^{\cdot}$) electrochemically active (see also figure 2). The lipoperoxides reduction electrode process is further monitored electrochemically because the LDL, respectively PC, structural modifications induced by oxidation gave rise to a measurable signal.

In our experiments lipoperoxidation of the two substrates (LDL and PC) was initiated using peroxyl radicals obtained at a controlled rate by a known procedure: an aqueous solution of free radical azo-initiator, 2,2'-azobis (2methylpropionamidine) dihydrochloride, AAPH (83,8 mg to 10 mL of KCl 0.1 mol L^{-1}) was left at 37°C for 10 minutes, these conditions inducing the generation of ROS according to reactions given in figure below. It was demonstrated (Nikki, 1990) that aqueous solution of AAPH, 10 mmol L^{-1}, constantly generates, at 37°C, $1.36x10^{-6}$ mol L^{-1} sec^{-1} of free radical.

The generated ROS, ROO·, is further capable to induce lipids peroxidation.

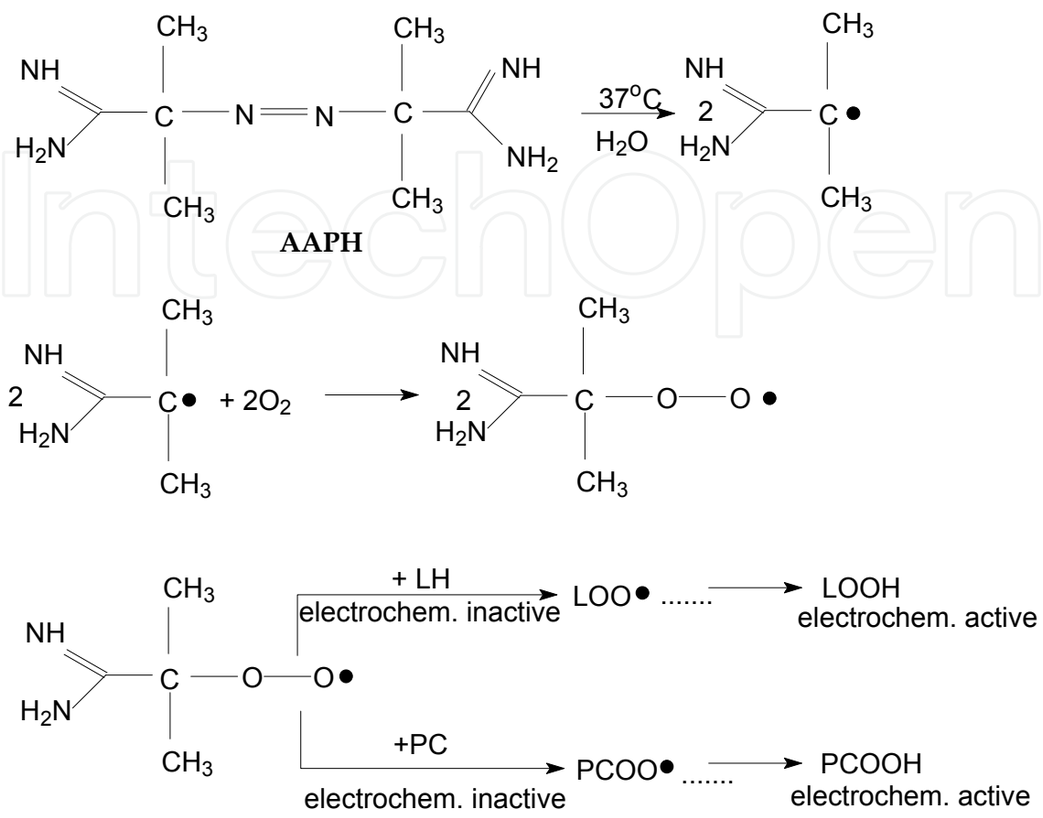

Fig. 1. Pathway of peroxidation induced by thermal decomposition of azo-initiator AAPH.

As noticed from figure 2, both phosphatidylcholine and LDL exhibit a similar behavior, proving no electroactive characteristics in solution or deposed, while an anodic reduction peak around + 0.395 ± 0.020 V raises in the presence of AAPH generated peroxidation.

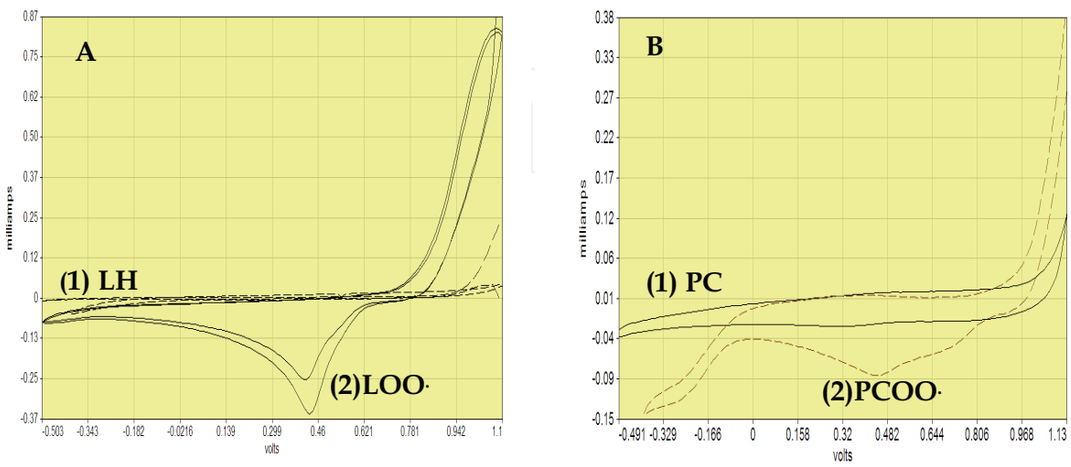

Fig. 2. Cyclic voltammograms of LDL (A) and PC (B) un-oxidized (1) oxidized (2);
v = 100mVs^{-1} (WE=Au) KCl 0.1 molL^{-1}.

3.3 Applications of sensors based on biomimetic systems

3.3.1 Application of sensors based on biomimetic systems to ROS assessment

The variation of the current intensity of the lipoperoxides peak with the ROS concentration was proved to be linear for both substrates on a concentration range of physiological significance 10^{-7} - $1.6x10^{-6}$ molL^{-1}; the equation of the linear domain was I(μA) = 160,14xC(μ molL^{-1})+ 49.72 (R^2 = 0,9958) for LDL and, respectively, I(μA) = 211.15xC (μ molL^{-1})+ 85.38 (R^2 = 0,9636) in the case of PC use, the obtained detection limit for both models calculated as 3 x S/N being $4x10^{-7}$ mol L^{-1}.

In order to accomplish the goal of providing physiological significant data, the electrochemical device response has to be sensitive both to ROS concentration and to oxidizable substrate concentration. As consequence, chronoamperometric determinations using reduction potential of lipoperoxide were performed for various LDL concentrations and the same AAPH (in fact ROO·) concentration.

It was noticed that the lipoperoxides formation increased with the increasing of the substrate concentration in the range 200 to 500 μg mL^{-1}, concentrations higher than 500 μg mL^{-1} causing the electrode passivation (see table 1).

Substrate concentration (μg mL^{-1})	I peak ± SD nA, (E=0.395V±0.020)	
	WE= LDL/Au	WE = PC/Au
200	108 (± 26)	324 (± 32)
420	250 (± 18)	708 (± 29)
1000	302 (± 12)	600 (± 44)

Table 1. Dependence of LOO· signal on oxidizable substrate concentration (oxidation initiated by AAPH $10x10^{-3}$molL^{-1}; results are the mean of 5 measurements)

These responses proved that the designed system is equally sensitive to substrate amount.

The obtained electrochemical data demonstrating the suitability of the built model in the study of lipoprotein ROS oxidation and assessment of the degree of ROS damage against lipoproteins biomimetic system were confirmed by FTIR and MALDI-ToF analysis. Subsequent to AAPH attack FTIR spectra performed on the LDL-Au surface confirmed the lipoperoxides formation: band corresponding to ester groups from lipid residues at 1740 cm^{-1} changed, and new HO absorption bands at 3600-3700 cm^{-1} and 917 cm^{-1} appear, these proving the lipoperoxides formation (FTIR data not shown) and a structural modification of the LDL molecule with respect to amide II absorption band that is modified. The MALDI-ToF analysis was also performed in order to obtain another confirmation of the modifications observed in the LDL structure, as result of oxidation by ROS attack. As could be observed from figure 3, the significant mass region for LDL is ranging between 1 and 10000 Da, because the signals in the mass region 10000 – 25000 were very weak and wide, while from 25000 to 80000 no signal was observed. The characteristic mass fragments for LDL itself are 1509 Da, 2569 Da, 4394 Da, 6420 Da, 6637 Da, 7647.5 Da and the 9432 Da. When the oxidation using AAPH initiator was performed it was noticed a 60% decrease in the intensity of the molecular fragments from 4394 Da, and for 1509 Da, while signals from 7647.5 Da and 9432 Da not only decreased significantly, but even are significantly shifted toward 7600 Da and, respectively 9492 Da, corresponding to mass modification multiples of peroxyl mass (Tache et al., in press).

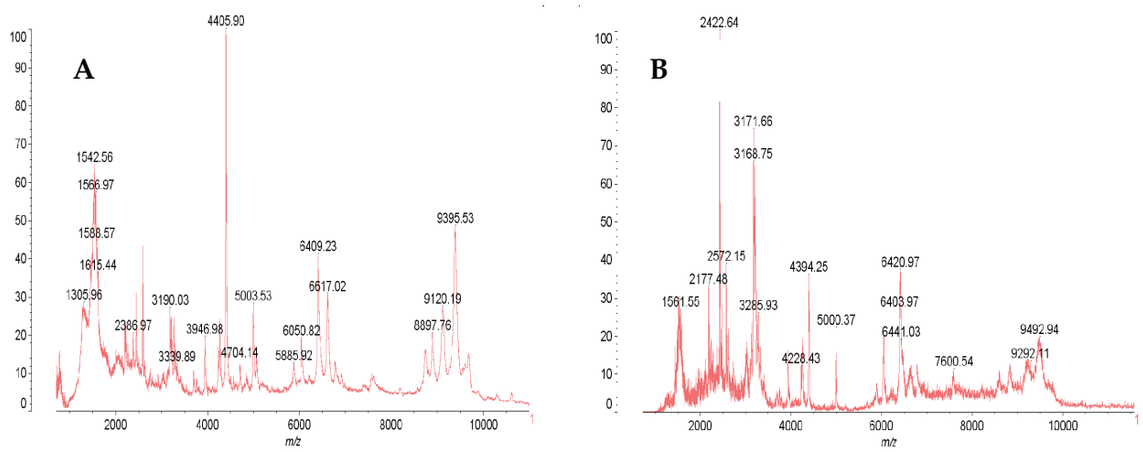

Fig. 3. MALDI-ToF spectra of un-oxidized LDL (A) and oxidized LDL (B), oxidation initiated by AAPH mmolL^{-1}

3.3.2 Application of sensors based on biomimetic systems to antioxidant efficacy assessments

As mentioned earlier a bio-analytical tool devoted to assessment of ROS has to be applicable both in ROS toxicity assessment and antioxidant analysis. As consequence, further was studied the potential application of realized sensors to assess the efficacy of few antioxidants against lipoperoxidation.

Taking into account the fact that an antioxidant is specific for a certain type of free radical that induces the oxidative stress (or, eventually to a narrow class of radicals) several antioxidants were tested against lipoperoxidation of LDL, using the LDL/Au sensor. Determinations were performed using an antioxidant concentration of 10^{-6} mol L^{-1}, the AAPH concentration being $10x10^{-3}$mol L^{-1} (respectively ROO$\cdot$ concentration $1.63x10^{-3}$mol L^{-1}), and the modified electrode being incubated in the antioxidant solution for 20 minutes prior oxidation. The efficacy of antioxidants preservation was monitored in time, and the relative percent of lipoperoxide formation was calculated according to the formula (1), where %LOO is the percent of formed lipoperoxides, $i_{FR}^{LOO\bullet}$ is the current intensity of the peak corresponding to lipoperoxides formation after ROS attack, and $i_{ROS+Aox}^{LOO\bullet}$ is the current intensity of the same peak, when both ROS and antioxidant are in the measuring system (Litescu et al., 2002).

$$\%LOO\bullet = 100 - \left(\frac{i_{ROS}^{LOO\bullet}}{i_{ROS+Aox}^{LOO\bullet}}\right) \quad (1)$$

Essential oils from *Salvia species* (*Salvia*-EO) and astaxanthine (from *Haematoccocus pluvialis*) were used as lipo-soluble antioxidants and their efficacy against lipoperoxides formation was compared with that of two recognized lipophilic antioxidants, coenzyme Q10 (CoQ10) and vitamin E. An efficacy index against lipoperoxidation was established: astaxanthine > CoQ10 ≅ *Salvia*-EO > vitamin E.

Hydro-alcoholic extracts of the same *Salvia* species (Salvia extract) were used as antioxidants, and their efficacy was compared with that of known polyphenolic antioxidants: rosmarinic acid, caffeic acid and gallic acid, an index of efficacy being drawn: Caffeic acid > Rosmarinic acid > Salvia extract > Gallic acid.

It has to be stressed that when the substrate deposition is performed by solution casting, in optimal conditions, from suspensions containing a known mass of lipoprotein but without a controlled reproducibility of the deposition because of the unknown reproducibility of the deposition process, consequently affecting the data reproducibility.

Considering all obtained results it was proven that these new types of electrochemical sensors are applicable both to ROS assessment of and to assessment of antioxidant efficacy against lipoperoxidation. In the same time, the antioxidant efficacy depends strictly even on the nature of the antioxidant, the amount of antioxidant and on eventually occurring synergetic or antagonic effects exerted by a mixture of antioxidants. This is the reason of presenting further the development of biosensors devoted to a certain antioxidant class identification and quantification.

4. How to design biosensors for antioxidants quantitative determination. A case study- polyphenols antioxidants analysis

As mentioned in the introductory part, the majority of data published on antioxidant determination is based on biosensors that use immobilized enzymes (Mello & Kubota, 2007). In an overall acceptation of terms, biosensors are a sub-group of chemical sensors which could be defined as self-contained devices able to supply specific information. The provided analytical information is either quantitative or semi quantitative and is based on the use of a biological recognition element, which is in direct and spatial contact with a transduction element.

Several critical points have to be tackled when biosensors have to be developed:

a. the choice of the biological material and the choice of the transducer depend on the sample properties and on the type of physical magnitude to be measured.
b. the type and the nature of the bio-recognition component determine the degree of selectivity or specificity of the biosensor while the transducer correlates with biosensor sensitivity.

A general overview on designing of such type of biosensors involves following steps:

Quantification and identification of the compound of interest; this step concerns providing the appropriate bio-recognition element, able to supply accurate, sensitive, selective and reproducible information concerning samples composition. Appropriate bio-recognition elements are redox enzymes, the main advantage of using redox enzymes in amperometric biosensor construction being the value of the potential applied to monitor reduction or oxidation of the species at the electrode surface. This value generally occurs in -0.2V-0V range and allows reaching a minimum of possible electrochemical interferences (Mello et al., 2003)

Transduction, that supposes to transform the signal provided by the bio-recognition element into a measurable one, that could be current intensity (or charge), specific fluorescence and/or maximum absorbency.

Sensor performances assessments, that involves to provide the associate values of several performance parameters. This means to set up parameters like: the domain of applicability, selectivity/specificity, the linearity range of the sensor response, detection limit (LoD) and

determination/quantitation limit (LoQ), accuracy of the determination, reproducibility, life time, operational stability, storage stability, and validation of the biosensors sensors response.

Validation of the built up biosensors, that means to validate the biosensors response with respect to ″classic″ methods of phytochemicals assessment.

4.1 Quantification and identification step; biological recognition element immobilization

This step is dealing with appropriate choice of bio-recognition element.

Polyphenols are one of the most important classes of antioxidants, naturally antioxidants, commonly occurring in fruits, vegetables and medicinal plants, and have been found to have a protective role against many chronic human diseases associated with oxidative stress. Polyphenols are divided in three large groups: phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), flavonoids (anthocyanidins, flavonols, flavononas, flavonas, isoflavonas and chalconas) and tannins (hydrolyzable tannins and condensed tannins) (Escarpa & Gonzales, 2001). Due to the importance of this class of compounds, many analytical strategies to evaluate the total phenolic content from plant extract and to establish phenolic profile have been reported, biosensors based on enzymes being proposed as an alternative device for total phenolic content (TPC) assessment. It has to be stressed that, in many cases, it is more important to measure the total content of polyphenols compounds than to determine each of them individually. The term 'total phenol' refers to all the phenols that are responsible for the total antioxidant capacity of a specific sample.

Biosensors based on various polyphenol-oxidases immobilisation are versatile devices in TPC assessment, due to class specificity. The main reaction consists in the oxidation of the substrate (phenols; poly-phenols) in the presence of enzymes molecules (phenol oxidases) the oxidation product – a quinone- being later reduced. While the tyrosinase biosensors are restricted to the monitoring of phenolic compounds with at least one free otho-position those based on laccase are applicable to a wider group of polyphenols, including ortho and para substitution or conjugated phenols with other functional groups.

The immobilisation of biological recognition element is considered as one of **the critical steps** that dictate the effectiveness of the enzymatic biosensor, due to the fact that biosensors performances -in terms of quantification and identification- are ensured by the preservation of the specific structure of the bio-component. For redox enzymes it is important that subsequent immobilization the active site remains available and, more, that immobilization did not affect the electron transfer from the enzyme active site and the electrode surface; this electron transfer could be affected by the insulating effect exerted from the protein structure. Data on laccase immobilization on different solid supports were reported. It has to be underlined that the immobilization matrix has to be, if possible, chemically inert with respect to biological element, stabile and with suitable conductive properties (there are cases when the immobilization matrix is, in fact, a conductive polymer).

Following, we exemplify two procedures of laccase immobilization on the surface of screen-printed working electrodes that lead to successful construction of versatile biosensors for polyphenols analysis. The first procedure is based on laccasse embedding in an electrochemically generated chitosan matrix, the second one being based on Laccase entrapment in a Nafion stabilizing membrane:

Laccase immobilisation on solid modified supports via chitosan matrix. It was envisaged the immobilisation of *Laccase* on a stabilizing matrix deposed on the surface of a solid support;

the chosen solid support was gold. Two types of embedding matrix were used: one consisting only in chitosan (Chi), the other one involving the deposition of a composite matrix of Chi and multi-wall carbon-nanotubes (MWCNT). Chi and MWCNT-Chi films were electrodeposited on gold electrode surface using a -1.5 V controlled potential, deposition time was 5 minutes. Optimum conditions for electrodeposition of MWCNT –Chi film on a gold electrode were established taking into consideration the value of the layer capacity. Laccase immobilization was carried out by entrapment into the Chi-MWCNT nanocomposite film from multi-wall carbon nanotubes (MWCNT)-chitosan (Chi) solution containing 25U/mL enzyme during electrodeposition process (Diaconu et al., 2010).
Laccase immobilisation on screen-printed electrodes via Nafion membrane. Biosensors development was performed on the base of DROPSENS screen-printed electrochemical cell. The screen printed (either gold AuSPE or carbon CSPE) working electrodes of a three electrodes were modified by drop-casting from stock solution of 3 to 5μL of Lacccase solutions of exactly known activity, allowed to quickly dry, than followed by the immobilisation in Nafion membrane. It were used stock solutions of different Laccase activities, in order to obtain different set of biosensors that has different specific activities on the electrode surface, the units deposed ranging between 0.1U/electrode to 1 U/electrode. The Nafion membrane was obtained from aqueous/alcoholic solutions, 0.1%, 0.3 % or 0.5% perfluorinated Nafion. The cells were stored between measurements at 4°C (Litescu et al, 2010).
In order to check the efficiency of immobilisation the apparent Michaelis-Menten constant has to be determined and compared with the value of Michaelis-Menten constant for free enzyme; if the magnitude order is the same for both constants, then the immobilized enzyme preserve the affinity toward substrate exhibited by the free enzyme. The corresponding data related to performed immobilizations are presented further, in the section devoted to **biosensor performances assessment.**

4.2 Biosensors performances assessment

The assessment of biosensors performances is related to several specific characteristics, like: the domain of applicability, selectivity, the linearity range of the sensor response, detection limit (LoD) and the quantitation limit (LoQ), accuracy of the determination, reproducibility, life time, operational stability, storage stability (Thevenot et al., 2001).
When amperometric biosensors are developed the device performances evaluation is performed in optimal measuring conditions for the monitored enzymatic reaction. This means that first it is accomplished the electrochemical characterization of the product of the enzymatic reaction (for reaction in solution) in order to ascribe the corresponding oxidation and reduction potential peak values. It should be mentioned that the potential value differs according to the used conductive material. This electrochemical characterization is achieved by cyclic voltammetry (CV) experiments and it is important to be performed even for substrate, because, by this way, it is possible to solve a critical point by ascertain, from the very beginning, if substrate plays as electrochemical interferent in determination.
A suggestive example, related to laccase biosensor construction, in given in figure 4, where are presented the cyclic voltammograms of caffeic acid (a polyphenolic substrate for laccase) and that of the product of the enzymatic reaction.
After CV experiments it could be concluded which are the corresponding oxidation and reduction potentials. In the case of the considered example (figure 4) caffeic acid has an oxidation peak potential around +200 mV and a reduction one around 0 mV, while the

quinone resulted from laccase catalyzed reaction has an oxidation peak potential around +63 mV and the reduction one is around -180 mV. The amount of enzymatic reaction product is direct proportional with the amount of substrate, in our case caffeic acid. As evident from figure one at about -200 mV the whole amount of quinone produced in enzymatic reaction is reduced. At this potential value, if the catalyst, namely the laccasse enzyme, is not in sufficient amount, correctly is not sufficiently active to ensure the complete substrate oxidation, then the substrate, caffeic acid, is able to electrochemically interfere in chronoamperometric determination of polyphenols. As consequence, the used enzyme amount, better activity is another critical point in biosensors development. By performing the same tests for several possible substrates it was established the optimal working potential, and further the performances characteristics for both constructive variants presented above. The investigated substrates were the polyphenolic antioxidants used to evaluate the LDL based sensors applicability to antioxidant efficacy assessment: caffeic acid, rosmarinic acid, gallic acid and, in addition chlorogenic acid.

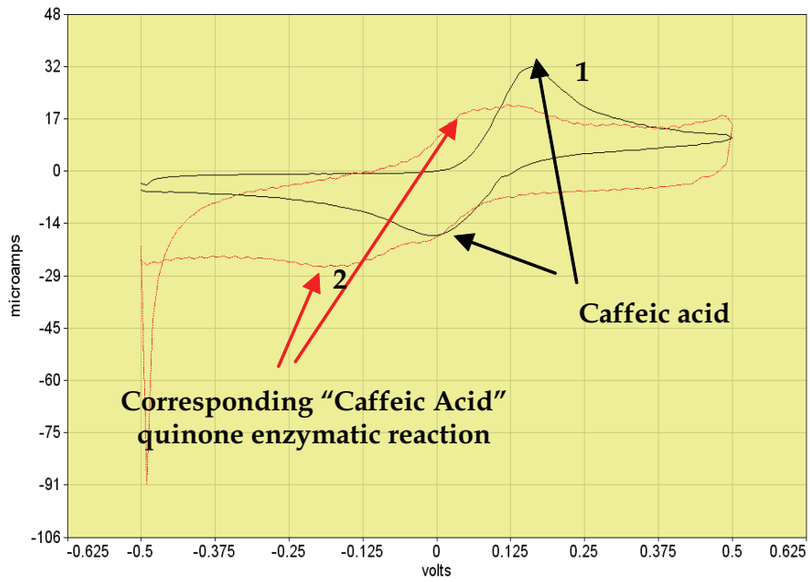

Fig. 4. Overlaid cyclic voltammograms of caffeic acid (1), respectively of caffeic acid in the presence of 1U Laccase (2); experiments performed on Au bare electrode

Obtain performance characteristics are given in table 2 and 3.

Substrate	Sensitivity nA/μmolL^{-1}	Linear range molL^{-1}	LoD molL^{-1}	K_M^{app} molL^{-1}
Caffeic acid	1446	10^{-6} –$1.5x10^{-5}$	$7.7x10^{-7}$	$2.52x10^{-5}$
Chlorogenic acid	1725	$2x10^{-6}$ –$1x10^{-5}$	$1x10^{-6}$	$2.41x10^{-5}$
Gallic acid	72.3	$2x10^{-6}$ –$1x10^{-5}$	$1.5x10^{-6}$	$1.2x10^{-5}$
Rosmarinic acid	788.6	10^{-6} –$1.5x10^{-5}$	$4x10^{-7}$	$1.1x10^{-5}$

Table 2. Performance assessment of Laccase-CHIT-MWCNT biosensor, buffer citrate-acetate pH=4.50, applied potential –0.200 V vs Ag/AgCl

Substrate	Sensitivity nA/μmolL^{-1}	Linear range molL^{-1}	LoD molL^{-1}	K_M^{app} molL^{-1}
Caffeic acid	245.3	3x 10^{-6} –1.5x10^{-5}	2.5x10^{-6}	6.6x10^{-6}
Chlorogenic acid	255.0	2x10^{-6} –7x10^{-6}	2.8x10^{-6}	4.3x10^{-6}
Gallic acid	72.3	3x 10^{-6} –1.5x10^{-5}	1.55x10^{-6}	4.12x10^{-5}
Rosmarinic acid	173.6	3x 10^{-6} –1.5x10^{-5}	2.4x10^{-6}	4.3x10^{-6}

Table 3. Performance characteristics of laccase-Nafion based biosensor 1U/Au-SPE, pHĽ4.50, applied potential _0.200V vs. Ag=AgCl

The apparent Michaelis constant was determined using Lineweaver-Burk method, the obtained values proving that both devices could be applied to determination of the polyphenolic secondary metabolites (as shown in tables 2 and 3).

After these assays, the next to be established is biosensors stability. The operational stability of a biosensor response may vary depending on the sensor geometry, method of preparation, the used receptor and transducer. For operational stability determination, it has to be used a suitable analyte concentration (within dynamic range), the same type of biosensor contact (continuous or sequential) with the analyte solution, the same measuring parameters as temperature, pH, buffer composition, presence of organic solvents, and sample matrix composition. Even if some biosensors have been reported usable in laboratory conditions for more than one year, their practical lifetime is either unknown or limited to days or weeks when they are incorporated into industrial processes.

For storage stability assessment, significant parameters are the state of storage (dry or wet), the atmosphere composition, pH value, buffer composition, temperature and presence of additives. Whilst it is easy to determine the laboratory worktable stability of biosensors -either during storage and operational in the presence of analyte- the procedures for assessing their behavior during several days when biosensors were introduced in industrial reactors is very complex and difficult to handle. In both cases –lab or industrial set-ups-, it should be specified if lifetime is a storage (shelf) or operational (use) lifetime, the storage, respectively working conditions and specific substrate(s) concentration(s), as compared to the apparent Michaelis-Menten constant.

In general, after a raw assessment of the performances characteristics for biosensors, as in the above described protocols, several steps of biosensor optimization have to be performed, in terms of:

1. the amount (the activity) of the immobilized bio-recognition element
2. biosensor operational and storage stability

These two approaches drive out, in fact, due to necessity of reaching a compromise between: the amount of the immobilized units, the associated noise (because it is known that usually, with protein amount increasing the noise increases too, due to partially insulation owed to the protein itself) and the envisaged stability of the built up biosensors, both storage stability and operational one (because, in certain limits, the increase of the active units of enzyme on the working electrode generates a better stability).

1. re-evaluation of interferences, chemical and electrochemical interferences.
2. working electrode material, due to two main issues: the goal of diminishing of noise and interferences, and last but not least the costs of the built up biosensors

Continuing with the examples of laccase immobilization, different amounts of laccase were deposed on the working electrode surface and immobilized using Nafion membrane, the

best responses, no matter the working electrode material, are obtained for an immobilized Laccase activity of 300 mU (Litescu et al., 2010).

In a simplistic way, it could be said that the biosensors operational stability is evaluated by repeated measurements performed using solutions of known concentration (within dynamic response range) of a certain standard substrate, while storage stability is evaluated by repeated measurements in time, at very well-established moments, in the same measuring conditions as optimum defined, using solutions of known concentration of a certain standard substrate.

The storage lifetime is defined as time necessary to decrease the biosensor sensitivity by a factor of 10 % or 50 %. The operational stability could be assessed as reproducibility or accuracy, and expressed as standard deviation of the measured signal for a certain number of determinations.

Laccase-Nafion biosensor operational and storage stability were checked in the following working conditions: MCIlvaine buffer pH = 4.50, 0.3 U Lacc/electrode (+0.1% Nafion), rosmarinic acid concentration level 5 $\mu molL^{-1}$. The operational stability is fair up to 10 measurements, being very good for the first 7 measurements, with a RSD up to 4.00%, after that, for the next 3 determinations the stability decreased.

Operational stability of the Lacc/Chi-CNT electrode was checked for a rosmarinic acid concentration level of 5 $\mu molL^{-1}$, in MCIlvaine buffer, pH = 4.50. Ten consecutive determinations gave a mean current of 890 nA with a relative standard deviation of 5.62 %. After 15 measurements a 10% decrease of the registered current was observed.

Another parameter to be defined is the **biosensor response selectivity**, two methods being generally used to determine biosensor selectivity. One method suppose the drawing of calibration curves for each of the possible interfering substances from sample matrix using the same measuring conditions as for the analyte determination and after that comparing the slopes of the curves with the slope of the analyte calibration curve, the selectivity being related to the ratio between slopes. Yet, for polyphenols analysis, considering the high-significance of the concentration range for phytochemicals, another method is the most important and has to be applied. This method consists in adding of the interfering substance at the expected concentration in the measuring cell which already contains a usual concentration of the analyte, the selectivity being expressed as the percentage of variation of the biosensor response.

The reliability of biosensors for given samples depends both on their selectivity and their reproducibility and it has to be determined under real operating conditions. This means that in the presence of possible interfering substance, the biosensor response should be directly related to the analyte concentration and should not vary with fluctuations of interfering substances concentrations.

For the same example of Laccase-Nafion biosensor, since the applied potential, -0.030V allows the avoidance of electrochemical interferences (is commonly accepted that the window of potential free of electrochemical interferences is ranging between -100 mV and +100 mV), the main problem that rest to be solved is that of chemical interferences. Supposing that the biosensor has to be applied for determination of polyphenols from *in vitro* cultivated plants, the main occurring interferences are those from growth media. Using the second method of selectivity determination and taking into account that an inhibition of 9-10 % of the enzyme activity itself was noticed when the Laccase activity was checked spectrophotometrically (against ABTS as unspecific substrate), it was concluded that no interference is taking place despite of the lowering of biosensors response with 10%.

4.3 Validation of the built up biosensors

Generally, when a new analytical method has to be validated, several principles should be satisfied and characteristic values of a number of parameters should be provided; we are mentioning as most important the following: method suitability to the analysis purpose, method specificity, precision and accuracy, repeatability, linear domain of response, limit of detection, limit of quantification, method traceability etc. Taking into account the peculiarity of applying analytical methods based on biosensor measurements, when discussing of validation for biosensor analysis the validation parameters addresses more the biosensor response than the overall method.

Using the same example of the built up laccase biosensors, the validation of the biosensors for phytochemical antioxidants analysis was supported by comparing the biosensor response with the high performance liquid chromatography-diode array-mass spectrometry (HPLC-DAD-MS) response obtained for the same samples, using the same measuring procedures. An in-house obtained example of feasibility of polyphenol-oxidases based biosensor application in polyphenols analysis is given in the table below and refers to two types of *Salvia* callus, *Salvia Maxima* and *Salvia verde*. The results are expressed as total rosmarinic acid equivalent, the main issue in validation step being precisely this one, namely to clearly define which is the main component of the sample in order to report data as main component equivalent.

Method used	Limit of Detection (mol L^{-1})	Amount of polyphenolic content, RAEC (μg/ g fresh material)	
		S maxima	*S verde*
HPLC-DAD-MS	3.36×10^{-7}	103 μg/g	174 μg/g
Laccase – Nafion Biosensor	4.2×10^{-7}	97.8 μg/g	162.2 μg/g

Table 4. Determination of polyphenolic secondary metabolites as „total rosmarinic acid equivalent" in two callus samples

When the biosensors responses, expressed again in equivalent of rosmarinic acid, were compared with the response obtained by HPLC-DAD--MS for real samples of extracts of *Salvia officinalis* and *Mentha Piperita* good results were obtained, the biosensor response is about 94-95% from HPLC response (Litescu, 2010; Diaconu, 2011).

5. Conclusions

Application of sensors using lipoproteins in ROS determination is a feasible approach for lipoperoxides and peroxyl radicals, ensuring a fair measure sensitivity and specificity, but being strongly affected by the matrix complexity. In the same time such sensors are useful bio-analytical tools in *in vitro* assessing of the antioxidants efficacy against lipoperoxidation.

Based on reported data, comparing the results accuracy between biosensors based determinations and LC–DAD–MS determinations it could be concluded that the versatility of biosensors application in determination of phytochemical antioxidants content was

proven by numerous publications, the critical point in ensuring a reliable result being the choice of the most suitable biological recognition element and of a transduction mode able to support the necessary measure sensitivity. Moreover, if biosensors designed for superoxide anion radical or hydrogen peroxide determinations are introduced in measuring solutions containing plant extracts, the antioxidants effects of phytochemicals could be assessed by the mean of radical scavenging monitoring.

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Environmental Biosensors

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This book is a collection of contributions from leading specialists on the topic of biosensors for health, environment and biosecurity. It is divided into three sections with headings of current trends and developments; materials design and developments; and detection and monitoring. In the section on current trends and developments, topics such as biosensor applications for environmental and water monitoring, agro-industry applications, and trends in the detection of nerve agents and pesticides are discussed. The section on materials design and developments deals with topics on new materials for biosensor construction, polymer-based microsystems, silicon and silicon-related surfaces for biosensor applications, including hybrid film biosensor systems. Finally, in the detection and monitoring section, the specific topics covered deal with enzyme-based biosensors for phenol detection, ultra-sensitive fluorescence sensors, the determination of biochemical oxygen demand, and sensors for pharmaceutical and environmental analysis.

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