ELECTROCHEMICAL EVALUATION OF POLYPHENOLS PRESERVATIVE EFFECT AGAINST LIPOPEROXIDATION

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Acest articol prezintă un nou instrument electroanalitic – electrod de aur modificat cu lipoproteină umană de densitate mică – capabil să funcționze ca un sistem biomimetic al răspunsului pereților celulari la stresul oxidativ indus prin atacul unor radicali liberi. Sunt prezentate rezultatele obținute la aplicarea acestui instrument în evaluarea eficacității antioxidante a unor polifenoli în prevenirea lipoperoxidării. Acești polifenoliau fost ordonați în funcție de eficiența lor în prevenirea lipoperoxidării: acid cafeic >acid rosmarinic>acid clorogenic.

This paper presents a new developed electro-analytical tool – gold modified electrode with low-density lipoprotein – able to mimic the biological response of cellular wall against the oxidative damage induced by free radicals attack. Results obtained when applying this tool in the assessment of the polyphenols antioxidative efficacy against lipoperoxidation are reported. These polyphenols have been ordered in function of their efficiency in preventing lipoprotein oxidation: Caffeic acid > Rosmarinic acid > Chlorogenic acid.

Keywords: lipoperoxidation, electroanalysis, antioxidants, polyphenols, preservative effect

List of Abbreviations

AAPH	2,2,-Azobis(2-methylpropionamidine) dihydrochloride
DNA	Deoxyribonucleic acid
FR:Aox	Free radical:antioxidant
HPLC	High Performance Liquid Chromatography
LDL	Low-density lipoproteins
MS	Mass spectrometry

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MALDI	Matrix-assisted laser desorption/ionization
ORAC	Oxygen Radical Absorbance Capacity
PDA	Photo Diode Array
ROS	Reactive species of oxygen
RNOS	Related nitrogen oxide species
TEAC	Trolox equivalent antioxidant capacity
ToF	Time of flight

1. Introduction

Free radicals are chemical species with one or more unpaired electrons in the valence shell [1], frequently and ubiquitous associated with the initiation of cellular damaging processes encountered in various pathological states (tumor initiation and growth, degenerative diseases etc). The main defensive system against these oxidative stress related processes is commonly ensured by the antioxidants, either in living organisms or in synthetic polymeric products. At the level of living organisms this defense system has two sources, an endogenous one (body enzymes and plasmatic low weight antioxidants) and an exogenous (diet) source [2].

Usually, the term 'antioxidant' referrers to any compound able to block or delay the reaction of a substrate with molecular oxygen or reactive oxygen species, but the fundamental issue in order to decide if a certain compound could be considered antioxidant is the evaluation of the concentration ratio between the oxidizable substrate and the presumed antioxidant. Emphasizing the definition by Halliwell and Gutteridge [3], an antioxidant is 'any substance that is present in low concentrations compared to those of an oxidizable substrate and significantly delays or prevents oxidation of that substance'.

The assessment of antioxidant efficacy as preservative against oxidative stress represents a challenging task for any chemist, due to several complex parameters that should be taken into account when such an experiment is developed, and due to the fact that the obtained results are calculated using various methods and are expressed via different values of efficacy: Trolox equivalent antioxidant capacity, TEAC (micromollar) [4], IC_{50} [5], peroxidation (lipoperoxidation) induced lag phase etc. Normally to report a compound as an effective antioxidant the concentration ratio free radical: antioxidant (FR:Aox) has to be in the limit of 100:1, molar ratio. In this respect, frequently some 'retarder' compounds – molecules able to diminish the oxidation rate – are often wrongly reported as antioxidants. The confusion is caused by the ability of these compounds to diminish the oxidation rate when it is found in very large amounts.

As a consequence, despite of the important number of methods that have been developed for the evaluation of the molecules antioxidant effect, as many

variables have to be taken into account when measuring the antioxidant characteristics of a compound, the results have to be treated with caution. There is no universal system able to provide information about the 'true' antioxidant power or capacity of a single antioxidant or complex mixture of antioxidants [6, 7], and a comparative evaluation of antioxidant efficacy is difficult to be performed because the activity depends on the substrate, the reaction medium, the oxidation conditions, interfacial phenomena and the antioxidant partitioning properties between phases.

That is why a good radical scavenging activity does not necessarily correlate with a good antioxidant activity, and thus, not all the compounds showing a high radical scavenger effect show good antioxidant properties. In order to ascribe antioxidant properties to a compound, it is also necessary to determine the efficacy in preventing the oxidation of relevant substrates such as lipids, lipoproteins, DNA, etc., against relevant free radical species such as the peroxyl-, superoxide or hydroxyl radical. All these arguments dwell upon methods for the "assessment of antioxidant efficacy using biological significant markers and significant substrates", meaning methods providing information strictly related to the true preservative efficacy of that compound [8-10]. This category of methods involves determining the antioxidant efficacy via the evaluation of the damaging effects on a biological substrate produced by reactive species of oxygen (ROS) or related nitrogen oxide species (RNOS) at the reaction of lipids, lipoproteins, DNA etc. The techniques employed in such type of characterizations are usually based on hyphenated chromatography with multiple detections, like HPLC-MS/MS (or MS-ToF) or HPLC-PDA-MALDI ToF, as all types of detection are based, in fact, on proteomics analysis [11-13].

Using such kind of detection devices involves a huge amount of money spent in order to achieve reasonable analytical information. Therefore, it is a logical outcome to use an electrochemical device as screening tool as an alternative to the expensive analysis, in order to determine the toxicity of certain free radicals. This approach is more interesting when it is undoubtedly necessary to obtain biological significant information about the toxicity of free radicals not at high sensitivities.

In this paper, we are reporting the results obtained in developing a bio-mimetic model that can be used in the assessment of antioxidant efficacy using biological significant markers and substrates, namely low-density lipoproteins.

The reason of LDL use is supported not only from physiological point of view, but even by the fact that there are previous methods to compare the obtained results by electrochemistry, like the spectrochemical measuring of lipoperoxidation, at 234 nm or ORAC assay [14].

2. Materials and methods

The electrochemical experiments were performed using a Princeton Applied Research potentiostat 273A and a three electrodes measuring cell (working electrode: low-density lipoprotein modified gold electrode; counter electrode: platinum wire; reference electrode: Ag/AgCl, KCl 3M); supporting electrolyte KCl 0.1 $molL^{-1}$. The cell volume was 3 up to 15 mL. The used technique was cyclic voltammetry, on a potential range from –0.4 to 1.1 V (*vs.* reference electrode). This technique was preferred despite the lack of sensitivity, in order to avoid the modification of the layer surface charge during electroanalytical procedure.

The modified electrode involves the low-density lipoprotein deposition directly on solid support (gold sheet), accordingly to the optimised reported method [15], from suspensions containing a known mass of lipoprotein.

All the used reagents: potassium chloride, 2,2,-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), rosmarinic acid, caffeic acid, chlorogenic acid were Sigma-Aldrich analytical or HPLC purity grade. Low-density lipoprotein was from human source, lyophilised powder, Sigma provided.

All measurements were performed at 25^0C and the determinations were at least five times replicated.

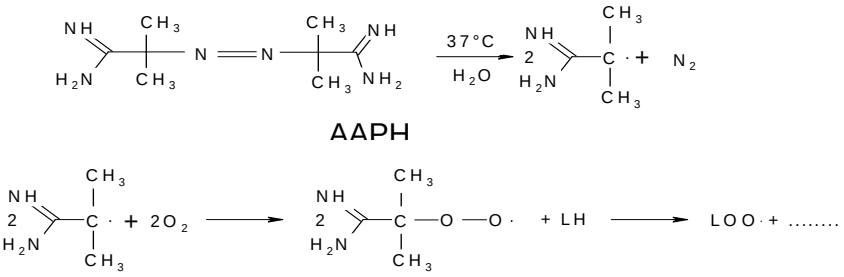
3. Results and discussions

The low-density lipoproteins (LDL) are not electroactive proteins themselves. As previously reported, un-oxidized low-density lipoproteins are not electrochemically active neither in suspension, nor as deposed layer [17, Litescu, 2002]. The free radical attack generates an oxidized product of the substrate that begins to be electrochemically active (LOOH), the extent of damage depending on the free radicals concentration and their toxicity (see scheme 1).

Scheme 1. Monitoring of lipoperoxidation using the electrochemical approach

There are three possibilities to induce simple, fast and controlled lipo-peroxidation: 1. Heating; 2. making LDL to react with peroxyl radicals produced by azo-initiators- an example being peroxyl radicals thermally produced by

AAPH in aqueous media (see scheme 2); or 3. generating $HO^{\cdot}$ radicals, for example using the classical Fenton reaction.



Scheme 2. Lipoperoxide formation using AAPH initiators

In our study, thermally generated peroxyl radicals reacted with lipoprotein and the electrochemically active lipoperoxides are subsequently generated. The lipoperoxide reduction process is monitored on the working electrode by cyclic voltammetry.

The attack of AAPH generated free radicals against the structure of LDL deposed on Au support was studied, taking into account the fact that for a concentration of 10-20 $mmolL^{-1}$ of AAPH, the flux of aqueous radicals calculated on the basis of the known rate of free radical generation from AAPH at 37°C is $[FR] = 1.36x10^{-6}$ [AAPH] mol/liter/s, and the generated FR are physiological-like superoxides radicals as stated by Niki Etsuo [16]. It was noticed that by the increasing of AAPH concentration lipoperoxides were produced in a higher amount, electrochemically quantifiable. It should be emphasized that AAPH itself does not interfere in lipoperoxides reduction voltammogram, in the used conditions of reaction (phosphate buffer, pH=5.50 in potassium chloride 0.1 $molL^{-1}$, 37°C)

When the lipoprotein layer is the subject of free radical attack, a specific lipoperoxide reduction peak appears around +0.385 ±0.015 V (n=7) (see figure 1).

The peak intensity increases with the free radicals amount in the measuring cell.

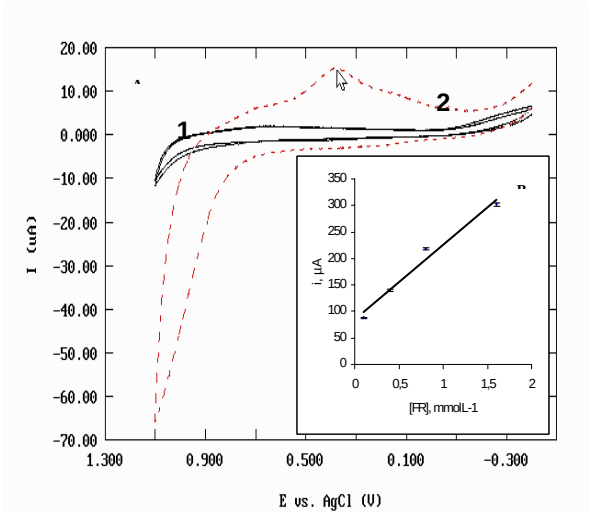


Fig. 1. Cyclic voltammograms of LDL deposed on Au sheet in PBS buffer, pH=5.50 (KCl 0.1 molL^{-1}) before (curve 1) and after free radicals attack (curve 2). (B) Variation of peak current intensity (0.385 ±0.015 V) with FR addition

The tests performed on the free radicals concentration range between 0.1 and 2.5 mmolL^{-1} shown an acceptable linear response for about a decade, namely 0.1 –1.7 mmolL^{-1} (I µA = 142,56xC(mmolL^{-1}) + 84,141, R=0.987, RSD=2.45%, n=5)) (inset figure 1). In the same time, our studies provide evidence that the lipoperoxides formation is related to the concentration of LDL in the used for lipoprotein layer deposition (figure 2).

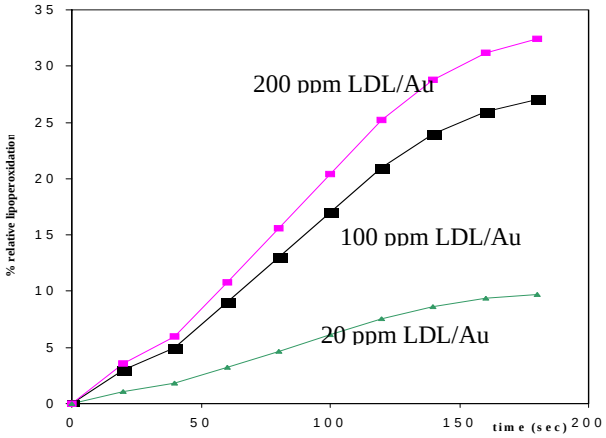


Fig. 2. Variation of lipoperoxide formation with LDL amount

Taking into account the linearity domain of the response of biomimetic model to free radical toxicity, it was concluded that it is appropriate to measure free radical concentration levels of 10^{-3} mmolL^{-1}. This level of concentration is

significant for the envisaged model application – assessment of preservative efficacy of antioxidants against lipoperoxidation – due to the fact that in living organisms the level of formed peroxides ranges around micromolar concentrations.

Since the developed model proved to be sensitive to free radical attack, the LDL structural modification being electrochemically quantifiable, the model was further tested in order to study its applicability as an analytical tool in the assessment of antioxidant efficacy of polyphenolic derivative compounds.

Consequently, another experiment was developed to test the LDL model applicability, using known polyphenolic antioxidants, standard compounds – rosmarinic acid, caffeic acid, gallic acid and chlorogenic acid. A known concentration of polyphenol was added in the system containing the generated peroxyl radicals. The antioxidant addition was performed by respecting the molar ratio free radical: antioxidant of 100:1, and the delay in lipoperoxide formation was measured. Measurements involved the quantification of the intensity current corresponding to the specific peak from +0.385 ± 0.015V. The relative percent of lipoperoxide formation was calculated according to the formula:

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

where %LOO is the percent of formed lipoperoxides, $i_{FR}^{LOO\bullet}$ is the current intensity (in μA) of the peak corresponding to lipoperoxides formation after FR attack, and $i_{FR+Aox}^{LOO\bullet}$ is the current intensity of the same peak, when both FR and antioxidant are in the measuring system. Results are given in table 1.

Table 1

Efficacy of different presumed antioxidants against lipoperoxide formation

Antioxidant	Antioxidant concentration level	AAPH concentration / generated FR concentration	% lipoperoxide formation		
			1 min	5 min	10 min
Caffeic acid	6 x 10-6 $molL^{-1}$	10 $mmolL^{-1}$ AAPH /1.63 $mmolL^{-1}$	0	2± 0.015	15± 0.015
Chlorogenic acid			0	1± 0.03	17± 0.03
Rosmarinic acid			0	4 ± 0.02	15 ± 0.02
No antioxidant	0		30 ± 0.1	50 ± 0.1	60 ± 0.1

It could be noticed that all tested standard compounds proved their capability to preserve the lipoperoxide formation in a certain degree.

Moreover, as it could be observed, an index of efficacy against lipoperoxide formation is thus available for the studied polyphenols: Caffeic acid > Rosmarinic acid > Chlorogenic acid.

Our results obtained using this new developed model, which employs the evaluation of LDL oxidative damage preservation in the presence of polyphenols, are in agreement with data reported by Alonso and co-workers in 2002 [17].

4. Conclusions

A new analytical tool able to provide information on polyphenols preservative efficacy against lipoperoxidation was developed and it proved its applicability in a concentration range significant as physiological level. The use of low-density lipoprotein as biological significant marker in the assessment of antioxidant capacity was proven. Obtained data are in concordance with several other papers reporting polyphenols efficacy as antioxidants. An index of pure polyphenols efficacy against lipoperoxide formation was provided.

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