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Measurement of the Antioxidant Capacity of Forage Using the Amount of Superoxide Radical in Ruminal Fluid

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ABSTRACT

The present work shows that the concentration of the superoxide radical is a useful indicator to know the behavior as antioxidant of forages in ruminal fluid. This study consisted of adding increasing concentrations of reductant compound to aerated dimethylsulfoxide solution supplemented with rumen fluid (1%, v/v). The cyclic voltammograms of these solutions show a diminished anodic peak corresponding to the oxidation of superoxide radical (O2-). Through this decrease, it is possible to classify the forage materials according to their potentiality as antioxidants.

Keywords: Forages, Superoxide radical, Rumen fluid, Antioxidants, Phenolics compounds, Oxidation/reduction, Electrochemistry

INTRODUCTION

The basic studies in biology sciences on the topics of antioxidants have increased significantly in recent times [1,2]. Among the causes of this fact we can mention the important role that these compounds have in maintaining the conditions that define the so-called antioxidant status or reducing status in the different environments of cell and tissues [3-6].

The work described here is focused on the study of the antioxidant capacity of a natural system (ruminal fluid solution) when increasing concentration of phenolic redox molecules were incorporated into it. Phenolics (hydroxy cinnamic and p-hydroxy benzoic acid derivatives) and quinone compounds are ubiquitous compounds in plants, and they have an important redox behavior in different natural ecosystems [7-11]. They were described as antioxidant molecules that may reduce several important metabolic compounds and affect the redox status of the microbial ecosystem, and by this path can be changed many biochemical processes, either assimilative processes (biomass production) or dissimilative processes (e.g. denitrification, volatile fatty acids or gas production). In this study, as model for phenolic compounds, caffeic acid (CA) (derived from cinnamic hydroxy acid) and syringic acid (SYR) (derived from hydroxy benzoic acid) and as an example of quinone molecule, 9, 10-anthraquinone-2,6 disulfonate (AQDS) were used (Figure 1).

In this study, a voltammetric technique (Current/Voltage curves) was used. In voltammetric techniques, the high of current peak can be used to determine the concentration of the reactant in the bulk solution [12-15]. Within the voltammetric techniques, cyclic voltammetry [12-15], which can provide valuable information on redox pairs (study of reaction mechanisms, identification of intermediate compounds), appears especially hierarchized in the recent literature [16,17]. In addition, the development of low-cost equipment allows these studies to be performed by laboratories with reduced budgets [18].

The cyclic voltammograms of an aerated aprotic medium clearly show the electrochemical reduction of oxygen to superoxide radical ($O_2 + e^- \rightarrow O_2^-$) (cathodic wave) and reverse wave re-oxidation of the latter compound (anodic wave) [19,20]. If an antioxidant compound is added to this solution, a decrease in the anodic peak height corresponding to the decrease in the amount of superoxide radical present is observed. This fact allows to define the impact of an antioxidant compound when introduced into the solution and makes it possible to perform comparative studies in terms of redox capacity factor or redox status of the system (intensity and capacity redox factors) [4]. In this regard,

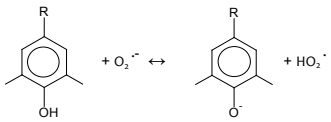
the antioxidant concentration necessary to reduce the anode signal to a certain percentage of control signal (without the addition of antioxidant) provides a useful tool to estimate and compare the oxidative capacity of the potential antioxidant compounds.

REDUCTION OF THE SUPEROXIDE RADICAL

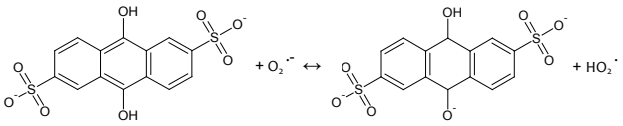
In an aprotic solvent such as dimethyl sulfoxide (DMSO) and at low electrochemical potentials, oxygen is reduced to stable superoxide radical by a reversible process involving an electron [21-23]. The reaction is:

$$O_2 + e^- \leftrightarrow O_2^{\cdot -} \qquad (E^{\circ\prime} = -0.75 \text{ V vs. SCE})\ ^{14a}$$

From a thermodynamic standpoint, the radical $O_2^{\cdot -}$ is a mild reducing agent [21-23]. Moreover, phenolic acids used in this work, have relatively high reduction potentials ($E^{0'}_{CA}$= 0.174 V [24,25]; $E^{0'}_{SYR}$=0.480 V [26] and $E^{0'}_{AQDS}$=0.561 V [27]). In systems with low electrochemical potentials (as the ruminal environment, for exemple) (E´<-0.4 V vs. SCE) the considered compounds are present in reduced form. This situation is derived from the Nernst equation [28] (see Supporting Information). In the conformed system, it is thermodynamically unfavorable for the radical $O_2^{\cdot -}$ to oxidize these phenolics by which the direct electron transfers. However, as showed by Sawyer et al. [21] in pioneering studies, phenolic acids can be oxidized by $O_2^{\cdot -}$ radical. These studies showed that phenolic acids transferred at an early stage a proton to $O_2^{\cdot -}$ radical, which radical peroxide and the corresponding phenolate anion are produced [19,20]. The general mechanism can be summarized as follows:



The same reasoning can be applied to the protonated form of 9,10-anthraquinone-2,6 disulfonate (AH_2DS^{2-}) [27] and this step can be written as follows:



In view of the above, in the presence of these phenolic compounds, the amount of $O_2^{\cdot -}$ in the system may be significantly decreased.

EXPERIMENTAL OVERVIEW

The study described here aims to determine, by cyclic voltammetry, the effect of an increasing of concentration of phenolic redox molecules models incorporated on DMSO-ruminal fluid solution (1%; v/v) on the decrease of the anodic signal corresponding to $O_2^{\cdot -}$ oxidation compared with control (without phenolics). Then, by this way the antioxidant capacity of these compounds were estimated.

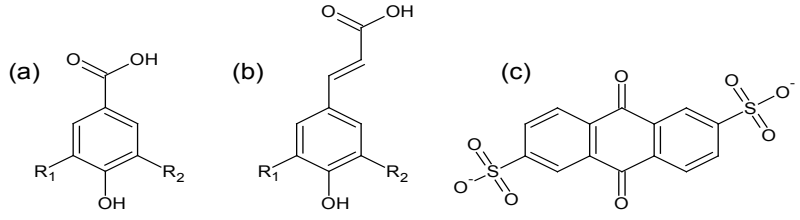


Figure 1: Structure of the tested phenolic compounds. (a) Syringic acid (SYR) (p-hydroxibenzoic derivative; R1 and R2: OCH3); (b) Caffeic acid (CA) (hydroxycinnamic acid derivative; R1: H and R2: OH); (c) 9,10-anthraquinone-2,6-disulphonate (AQDS)

MATERIAL AND METHODS

Chemicals and solutions

Caffeic acid (CA), syringic acid (SYR) 9, 10-anthraquinone-2,6 disulfonate (AQDS), hydroquinone and KCl were pro-analysis grade from Aldrich. These chemicals were used without further purification. The stock solutions of phenolic compounds were prepared daily (0.1%, w/v). These solutions were used as a starting point to calculate the volume needed to reach a final concentration in the voltammetric cell (5100 µl) equal to 0.05, 0.1 and 0.15 mM for each compound. Five replicates were made for each studied phenolic compound solutions. KCl solution 2 N to be used as supporting electrolyte in the voltammetric scans is also prepared daily. The organic solvent dimethyl sulfoxide (DMSO) was purchased from Sigma (ACS Reagent grade, <0.1% H_2O). The aliquots of rumen fluid employed in this study were obtained from the same rumen fluid sample in order to diminish the normal composition heterogeneity between rumen fluid samples. This sample was obtained from sheep with rumen fistula in Rumen Laboratory, Faculty of Veterinary Sciences, National University of Rosario (Programa SecyT-UNR-Res CS Nº 054/2013; CONEAU, Res.1104/15) and was strained directly with muslin [29]. The sheep were given a diet of hay ad libitum.

Voltammetry

Cyclic voltammograms (CVs) in DMSO containing the supporting electrolyte (KCl 0.02 mM) were obtained with a voltammetric equipment fitted with a microcell (5-10 ml capacity), a glassy carbon working electrode, a Pt electrode and a calomel electrode (SCE) with a liquid junction protection tube (Radiometer Analytical). Between scans, the working electrode and the Pt electrode were polished and washed with distilled water and dried. All potentials were measured against the SCE at 20°C ± 0.1°C. In all cases the CVs were recorded at a scan rate of 0.1 $V.s^{-1}$ and t=20 ± 1°C. Currents are reported in accordance with IUPAC convention (anodic current is positive and cathodic current is negative). DMSO containing the supporting electrolyte was saturated by dry air during 5 min. before each scan (the solubility of oxygen was estimated 1.10^{-3} $mol.l^{-1}$). CVs were initiated by cathodical scanning starting at +0.50 V and the switching potential ($E_{reverse}$) was -1.77 V [24]. Each voltammetric scanning was repeated five times on the same sample until reproducible cycle was achieved. The current signals corresponding to each reduction potential value were considered equal if they had differences smaller than 40 nA. Voltammetric studies with each solution of the phenolic compounds studied were performed in triplicate. Proper performance of the overall system was periodically verified by reproducing CVs for 4mM Hydroquinone in water buffered solution at pH 7.0.

Comparison of antioxidants capacity by the $C_{60\%}$ values

The measure of C60% values [19,20] which is the compound concentration needed to decrease the anodic peak current ($I_{a.p}$) to 60% of its initial value in control solution (solution without antioxidant), provides a useful tool to estimating and comparing the antioxidant capacity either of the pure forage or mixtures (e.g. extract of plants) or in animal fluids (e.g. oral, vaginal or rumen fluid). When the ratio $I_{a.p}/I_{control\ a.p}$ is plotted against concentration, the antioxidant potential of various compounds can be compared using the C60% parameter.

Hazards

Caffeic and syringic acid produce skin irritation and severe eye irritation. They can cause respiratory irritation. Avoid breathing dust. Anthraquinone-2,6 disulfonate (AQDS) may be harmful if inhaled or absorbed and may cause eye irritation. Use personal protective equipment. Avoid release to the environment. Dimethyl sulfoxide (DMSO) by itself has low toxicity.

RESULTS AND DISCUSSION

The superoxide ion has a great significance among the radical species associated with the functioning of numerous chemical and biological systems. Numerous studies have been carried out in recent years on this subject, favored by the significant advances in technology and instrumentation.

Antioxidants take advantage of the presence of free radicals, including $O_2^{\cdot-}$. The results presented in this paper allow us to propose a useful procedure in understudied systems such as ruminal liquid, to determine the presence of antioxidants from the reduction of current signal which indicates the presence of $O_2^{\cdot-}$. The use of an aprotic medium such as DMSO avoids the disproportionalization of the electrogenerated anion $O_2^{\cdot-}$. This allows this radical to remain stable even in voltammetric sweeps at low scanning rate [22,23]. In our case, the stability of $O_2^{\cdot-}$ in DMSO is tested by the anodic oxidation current observed during the reverse scan performed (-1.7 to +0.5 V) (Figure 2).

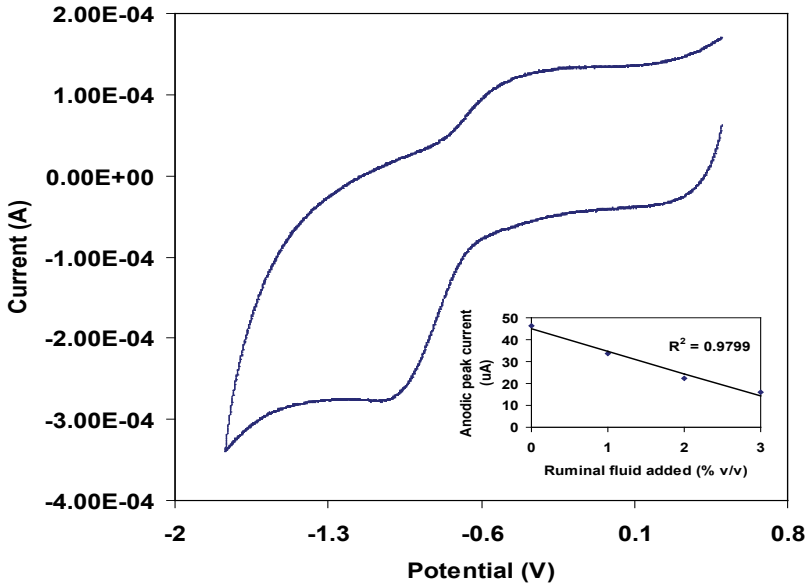


Figure 2: Cyclic voltammogram of O_2/O_2^- system at the glassy-carbon electrode (2-mm diameter) in presence of ruminal fluid (1% v/v) in DMSO+KCl 0.02 mM solution. Previous to ruminal fluid addition, this solution was saturated by dry air during 5 min. within voltammetric cell (in our experimental conditions the solubility of oxygen can be estimated at around 2.0 10^{-3} mol.l^{-1}). Scan rate=0.1 V.s^{-1}. The figure insert shows the anodic peak intensity values of superoxide ($I_{a.p}$; µA) when increase the amount of ruminal fluid in the analyte. For other explanations, see Section Experimental Overview and Material and Methods

The results obtained can be applied to other ubiquitous molecules in fodder compounds such as soluble lipid vitamins and compounds such as α-tocopherol and retinol containing OH groups acting as proton donors [30].

Electrochemical behavior of the radical O_2^- in the presence of ruminal fluid

The first cyclic voltanogramms were recorded to characterize the electrochemical behavior of ruminal fluid. To accomplish this, a solution of DMSO containing supporting electrolyte (5000 µl; 0.02 mM KCl) was saturated with dry air for 5' and then supplemented with ruminal fluid (0, 1, 2 and 3%, v/v). Five replicates were performed for each concentration. Figure 2 shows a reversible reduction wave with a peak at -1.1 V (cathodic peak potential; $E_{c.p}$), which corresponds to the reduction of oxygen to superoxide radical O_2^- [19,20,30]. The potential of the corresponding peak in the anodic wave ($E_{a.p}$) shows a large difference from the $E_{c.p}$ indicating a slow kinetics of the heterogeneous electron transfer [19,20]. Moreover, the anodic peak current values (Ia.p) according to supplementations with ruminal fluid (regression curve insert in Figure 2) clearly show the negative relationship between ruminal fluid supplementation and O_2^- concentration ($r=-0.99$; $p<0.01$) (Supporting Information; Table SI-1). This behavior is expected since the ruminal fluid per se is a source of reducing power, so it acts quickly decreasing the amount of oxygen that can exist in the system and this is clearly seen in the decrease of the current peak corresponding to the cathodic wave ($E_{c.p}$) when the concentration of ruminal fluid is increased in aerated DMSO+KCl solution 0.02 mM (Supporting Information; Figure SI-2).

Effect of reduced compound on electrochemical behavior of the radical O_2^- in the system ruminal fluid-DMSO

The results presented allow to define a system mainly composed of an aprotic solvent (DMSO) (which favors stability of O_2^-) [21-23] and a small amount of aqueous solution supplemental to ruminal fluid (1%; v/v). In this system, the electrochemical signal indicating the amount of superoxide in the system appears well defined. This system is useful to study the impact and comparatively dimensioning the antioxidant capacity of incorporated reducing compounds.

In this study, the system ruminal fluid-DMSO was modified by the presence of increasing concentrations (0, 0.05, 0.10 and 0.15 mmol. l^{-1}) of the selected phenolic compounds (Figure 3 and Supporting Information, Table SI-2) Results show that the peak corresponding to superoxide decreases with the addition of phenols in the following order: SYR; CA>AQDS. While the phenolic compounds used are oxidizable in quite high potential (see Introduction) therefore its electrochemical signal must not interfere with this study. In the case of CA and SYR the appearance of a new anodic peak is observed in the range -0.1 to 0.0 V. This behavior was observed by various authors and it awarded to the oxidation reaction of ion phenolate and its electrochemical characteristics would depend upon the nature of the phenolic compound [19,20].

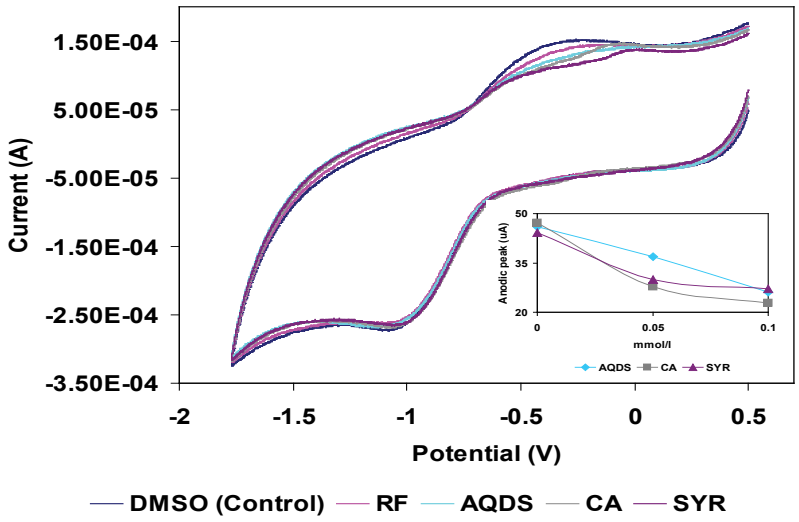


Figure 3: Cyclic voltammograms of the system O_2/O_2^- in DMSO (+0.02 mmol.l^{-1} KCl) in the presence of rumen fluid alone (RF) and supplemented with 0.05 mmol.l^{-1} of selected phenolic compounds (9,10-anthraquinone-2,6-disulfonate (AQDS); caffeic acid (CA) and syringic acid (SYR)). Scan rate=0.1 V.s^{-1}. The figure insert shows the anodic peak intensity values of superoxide (Ia.p; µA) when increasing the amount of phenolics in the analyte. For other explanations, see Section Experimental Overview

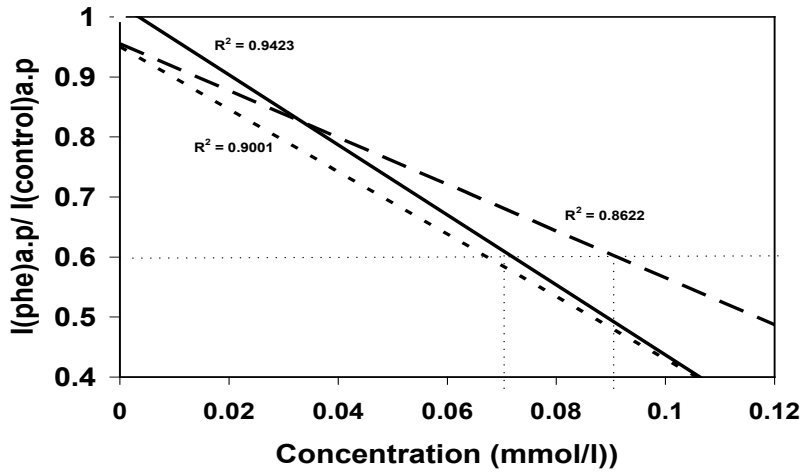


Figure 4: Determination of $C_{60\%}$ values[13]. Current dimensional parameters associated with the decrease in the anodic current corresponding of O_2^- concentration versus phenolic substrates: AQDS (—), SYR (– –) and CA (- - -). I(phe)a.p: anodic peak current with phenolic supplementation; I(control) a.p: anodic peak current in absence of phenolic supplementation. For other explanations, see Section Material and Methods

$C_{60\%}$ value as an indicator to compare the antioxidant capacity of phenolic compounds in the rumen fluid

The value $C_{60\%}$ was proposed by René et al. [19,20] as an interesting indicator that allows the comparative classification of reactivity of antioxidant compounds against superoxide. It was defined by these authors as the concentration of an antioxidant compound required to decrease the value of the anodic peak current ($I_{a.p}$) to 60% of the value observed in the system when the compound is absent ($I_{control\ a.p}$) [31]. Lowest $C_{60\%}$ values correspond to compounds with greater reactivity towards superoxide radical [24]. Figure 4 shows that within the compounds studied, the syringic acid showed the lower reactivity towards O_2^- (0.9 mmol.l^{-1}) and although some differences are observed at low concentrations, caffeic acid and AQDS had similar values for $C_{60\%}$ (0.7 mmol.l^{-1}).

This paper intends to show the usefulness of the study of the radical O_2^- in areas where its use is scarce as is the case of physiology, pharmacology and animal production. On the other hand the conceptual analysis presented in this work (See Supporting Information) can be used to stimulate the causal and non-descriptive treatment of the subject of the oxido-reduction in the teaching of the veterinary and zootechnical science. In these disciplines the treatment of this subject can present difficulties due to a theoretical base bounded by the students.

The result obtained in this study provides the platform for interesting discussions regarding the potential impact of modifying the oxygen content in the rumen fluid by the presence of phenolic compounds. While considering the

rumen environment as a strict anaerobic system in the rumen gases is a small proportion of oxygen (0.5-1.0%). It was suggested that this amount of oxygen enters the rumen with food, saliva, or by diffusion from the blood [32]. Different authors showed that oxygen can be utilized by rumen microorganisms. For instance, Czerkawski and Breckenridge [33] showed that when a small amount of oxygen was added to the ruminal system on the one hand, there was net oxygen consumption by the microorganisms. On the other hand, when the oxygen supplementation was reduced to half, the production of methane increased about 50%. About these results, it is important to remember that the production of an undesirable gas for animal production as methane (means a considerable loss of energy and contributes to the greenhouse effect) appear inversely related to the low concentration of oxygen that may exist in the ruminal fluid. According the results achieved in this study, the same decreasing effect on the oxygen concentration which produces syringic acid would occur with 20% less, both caffeic acid or AQDS. That is, at equal molar concentration by dry weight, forage that contains caffeic acid would reduce ruminal methane production more efficiently than other that contains syringic acid.

CONCLUSION

The study shows the usefulness of using superoxide radical oxidation in an aerated aprotic medium, to classify the potential as antioxidants of the phenolic compounds in ruminal fluid. In this study, the decrease in superoxide electrochemical signal is clearly seen when it was incorporated caffeic acid, syringic acid and 9, 10-anthraquinone 2,6 disulfonate. This study may adapt to other biological or environmental aqueous solutions.

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SUPPORTING INFORMATION

Information on basic concepts in connection with the study is included. This information serves as framework proposals, for the development of discussions related with redox behavior in biological solutions. In addition, tables with analytical data corresponding to Figures 2 and 3 are included.

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