Micellar Effects of Cetyl Pyridinium Chloride on Antioxidant Capacity, Voltammetric Response of Serum and Saliva Samples

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Abstract

The production of reactive oxygen species due to increased energy demand during physical exercise can increase the cellular and blood oxidation state. The reducing power of biological samples reflects its antioxidant capacity, largely maintained by Low Molecular Weight Antioxidants (LMWA), which donate electrons to radical species. LMWA include antioxidants such as uric acid, vitamins C and E and lipoic acid, among others. The electroanalytical technique of Differential Pulse Voltammetry (DPV) presents a good methodological alternative to quantify acute and chronic modulations of the antioxidant capacity from biological fluids in response to metabolic adaptations caused by physical exercise. However, when biological samples are analyzed, proteins are an important preanalytical interfering in the technique. The proteins can be adsorbed on the electrode surface during the potential application, resulting in a significant decrease of voltammetric signal. The aim of the present study was to investigate the applicability of cationic surfactant Cetyl Pyridinium Chloride (CPC) as a micellar system for the improvement of DPV technique for serum and saliva analysis. Forty individuals’ samples were analyzed. The obtained data revealed that the use of CPC increased the sensitivity and stability of the voltammetric signal, enabling the application of the method DPV for serum and saliva samples. Our data suggest that the voltammetric signal of samples is influenced mainly by the uric acid concentration.

Keywords: Differential pulse voltammetry; Total antioxidant capacity; Low molecular weight antioxidants; Cetyl pyridinium chloride; Serum; Saliva

Introduction

The quantification of antioxidants has become a topic of increasing interest in interdisciplinary research works involving pathologies and physical exercise [1-5]. Antioxidants can be defined as molecules that can prevent or reduce the extent of oxidative destruction of biomolecules when present in small concentrations compared to those biomolecules [1,6,7].

The antioxidants can be classified in two groups: enzymatic and non-enzymatic or low molecular weight antioxidants (LMWA). The enzymatic antioxidants include a relatively small number of specific enzymes such as superoxide dismutase, catalase and the system of glutathione peroxidase/glutathione reductase. LMWA include many compounds, which can be considered mainly responsible for total antioxidant capacity of tissues and biological fluids [8,9]. LMWA are located within ROS targets and specific sites of ROS generation in the cells. They interact directly with ROS (radical scavengers) and indirectly, chelating metals ions involved in ROS formation. LMWA, synthesized by the cells, can originate from the diet or can be derived from waste products [9,10].

The LMWA that react directly with ROS are reducing agents that donate electrons to radical species. In other words, the antioxidant capacity indicates the reducing power of biological samples. LMWA have electroactivity; therefore, they can be measured by electrochemical methods [1,10].

Differential pulse voltammetry (DPV) and cyclic voltammetry (CV) are widely used methods in analytical chemistry. Both were developed under the same principle, that is, to evaluate the capacity of a sample to donate or to receive electrons from a work electrode that is subjected to a defined potential interval. The voltammetric signal is formed by two parameters: the peak potential, which is determined by the redox potential of the analyzed compounds, and the peak current, which is a direct function of its concentration [11,12].

Validity, sensitivity and reproducibility are three factors that need to be considered to select and to define an analysis method [13]. Validity is the capacity of a measurement tool to reflect what it is designed to measure, in this case, the antioxidant capacity of serum and saliva given by the reducing power of LMWA. The sensitivity is the capacity of the technique to detect small alterations. The reproducibility of an analysis method consists of the absence of systematic and random error in repeated trials [14,15].

Chevion et al. [8] developed a procedure using CV to determine the antioxidant capacity of plasma samples. However, the DPV method has several advantages compared to CV such as good discrimination against background currents, better sensitivity and lower detection limits [12].

The large limitation of the applicability of electroanalytical
methods is the gradual passivation of the working electrode by the species generated in the electrochemical reaction [16]. Bare electrodes often suffer from a fouling effect due to the accumulation of oxidized products on their surfaces. All these result in low selectivity and sensitivity [17]. When we consider biological samples, the proteins are significant interfering agents. They can be adsorbed on the electrode surface during the application of potential and result in a decrease or even a complete loss of the signal during repeated measurements with the same electrode [18]. The passivation can be avoided through the polishing of the working electrode before each analysis [10]. However, our preliminary data showed that the polishing alters the electrode surface, constituting a limitation in the application of the technique of DPV to analyze the reducing power of biological samples.

Several strategies have been developed for overcoming the adsorption interferences, including mineralization of the sample [19], coating of the working electrode with a permselective membrane [20], microwave activation and ultrasonication [21]. These techniques involve different analytical procedures and complex instrumentation [22]. Therefore, it is necessary to develop new techniques that enable the use of biological samples in electrochemical analyses.

To that end, the use of surfactants for the stabilization of voltammetric signal is a simple alternative. The sole required manipulations are the addition of the surfactant and the thermostating of the electrochemical cell. Micellar aggregates assembled from surfactants can be adsorbed on electrodes and may mediate catalytic systems [23,24].

Surfactants have also been used as selective masking agents to improve selectivity and sensitivity of electrochemical analysis [23,25]. Hoyer and Jensen [16] showed that the fall-off of voltammetric serotonin signal could be reduced by surfactant addition in the sample. The stabilization effect of surfactants could be attributed to the competitive adsorption at the electrode surface, which counteracts the accumulation of oxidation products [18].

The behavior of the electrochemistry oxidation of ascorbic acid in aqueous solution with different surfactants has been studied by Szymula and Nazkiewicz-Michalek [26]. It was verified that the cationic surfactant shifts the oxidation peak potential and changes the peak current, mainly due to the surfactant film formed at the electrode/solution interface. The negative charge of ascorbic acid interacts with the positive charge of cationic micelles, which enhances the rate of oxidation and consequently increases the peak current. The use of nonionic and anionic surfactants acted in the opposite way.

Thus, the present work describes a study based on differential pulse voltammetry (DPV) techniques for determination of antioxidant capacity in serum and saliva samples in a cationic micellar system (cetyl pyridinium chloride (CPC)) using a glassy carbon electrode as the working electrode. The other goal was to validate the VPD in a cationic micellar system CPC as a useful method for direct measurement of antioxidant capacity conferred by ABPM serum and saliva.

### Materials and Methods

#### Reagents and solutions

All reagents were of analytical grade and were used as received without further purification. Cetyl pyridinium chloride monohydrate (C_{16}H_{33}NCl•H_{2}O), NaCl, uric acid and ascorbic acid were acquired from Sigma Aldrich. Disodium and monosodium phosphates (Na_{2}HPO_{4} and NaH_{2}PO_{4}) were acquired from Merck (KgaA Germany).

The solutions of cetyl pyridinium chloride monohydrate, uric acid, ascorbic acid and PBS (aqueous phosphate buffer 0.5 molL^{-1}, 0.9% of NaCl, pH=7.4) were prepared using water purified by a millipore milli-Q system (pH>18.2 MΩ).

### Serum samples and saliva

Blood and saliva samples were collected from forty volunteer soccer players (18 ± 1 years old). This study was approved by Committee of Ethics in Human Research (State University of Campinas) (0200.0.146.000-08).

In the morning, 8.0 mL of blood were collected in Vacuette tubes (Greiner Bio-One GmbH, Kremsmuenter, Austria). The volunteers fasted for at least 8 hours. The collected blood was centrifuged at 2000 g for 15 minutes at 4°C. Aliquots of 500 µL of serum were frozen and stored at -80°C until required for assay.

The saliva samples were collected immediately after the blood collection through the Salivates Collection System (SCS) (Greiner Bio-One GmbH, Kremsmuenter, Austria) in agreement with the manufacturer’s instructions. The oral rinsing solution supplied in the SCS kit was used before the saliva collection. Subsequently, the volunteers remained with the saliva extraction solution in their mouth for two minutes. The collection beaker was opened and filled with the solution from the mouth. The saliva extraction solution was transferred into two graduate tubes via vacuum and centrifuged at 2200 g for 15 minutes at 4°C. The graduated tubes measure the exact volume of collected saliva (27). The saliva quantity was determined photometrically from the extracted saliva solution at 450 nm.

The internal standard in the saliva sample was determined by means of saliva calibrators, and the saliva quantity was then recalculated.

### Serum and saliva uric acid concentration

Serum and saliva uric acid concentration was determined by automated equipment (Autolab 800) via an appropriate kit (Uricostat, Wierner lab., Argentina).

### Procedure for voltammetric measurements

The voltammetric measurements were carried out with a PGSTAT-10 potentiostat from Autolab Echo Chemie (Utrecht, Netherlands) interfaced to a PC (software GPES 4.9). An electrochemical cell with three electrodes was used with a saturated calomel electrode as the reference electrode, a platinum wire as the auxiliary electrode and a glassy carbon electrode (3-mm diameter) as the working electrode. The measurements were performed under thermostatic control at 20.0 ± 0.5°C.

For the voltammetric measurements, 120 µL of serum or saliva were used. The samples were diluted in 480 µL of PBS solution (aqueous phosphate buffer 0.5 molL^{-1}, 0.9% of NaCl, pH 7.4). The glassy carbon electrode was polished with 0.3 µm alumina, rinsed thoroughly with deionized water and sonicated immediately before each experimental series in deionized water. The potential for voltammetric experiments was recorded from -300 mV to +1000 mV.

### Statistical analysis

The software Origin 6.0 was used for the statistical analysis of results. The results were expressed as mean ± standard deviation. The Student t-test for paired samples was used to compare the results of CPC addition on voltammetric measurements of serum and saliva samples. Statistical significance was set at p<0.01. The boxplot graphs were used for a descriptive analysis of results. The graphs were made by the Matlab® 7.0 program.
Effects of CPC on biological fluid voltammograms

Figure 1 shows the differential pulse voltammograms obtained for the same serum (A) and saliva (B) samples in the presence and in the absence of CPC in a phosphate buffer solution at pH 7.4.

The presence of 100 mmol L⁻¹ CPC (solid lines) in serum and saliva samples shifted the first peak potential (Ep) toward lower positive values. The value of the second peak potential in the serum remained unaltered. As can be seen, in presence of CPC, two oxidation potentials were observed at 0.272 V and 0.755 V (Figure 1A, solid line). Only one oxidation potential at 0.295 V was observed in the saliva sample voltammogram (Figure 1B, solid line). A significant enhancement of one oxidation potential at 0.295 V was observed in the saliva sample voltammogram (Figure 1B, solid line). The oxidation potentials of saliva sample voltammogram was observed at 0.413 V (Figure 2B, dotted line). A significant enhancement of the first peak potential in the saliva sample voltammogram explains the increased speed of oxidation and the consequent increase in the peak current [31].

Effect of CPC concentrations on biological fluids voltammograms

The influence of CPC concentration on the peak potential (Ep) and peak current (Ip) were investigated in order to define the most effective surfactant concentration to use. Solutions containing different amounts of CPC were prepared and tested for the same serum sample. Twenty subsequent voltammograms were carried out for each CPC concentration. Figure 2 presents the data of the peak potential, Ep (A), and peak current, Ip (B), of a serum sample diluted in PBS with the surfactant concentrations. The values of variation coefficients (%) after 20 measurements for each concentration of CPC are also presented in figure 1 beside the data points, which correspond to average values.

The Ep values decreased while Ip values increased for all CPC concentrations added, when compared with the same sample in absence of CPC. Ep and Ip variation coefficients after 20 scans in the absence of CPC were 6.9% and 29.8%, respectively. The results indicate that the responses with the highest stabilization effect were obtained using 100 mmol L⁻¹ of CPC, which shows variation coefficients of 3.8% for Ep and 9.3% for Ip. This CPC concentration provided effective stability and led to an increase and better definition of peak current. The CPC concentration of 100 mmol L⁻¹ can be applied to saliva samples given that the total protein concentration is low in these samples. The saturated adsorption process of surfactants on solid surfaces generally coincides with critical micellar concentration (CMC) of surfactant [32]. The surfactants form micelles above CMC, and the solute-micelle interaction prevents the adsorption of compounds on the electrode surface. CMCs are difficult to measure with great accuracy; thus, the values found in the literature occasionally present variations [33].

CPC concentrations used in experiments presented in figure 1 were above CMC. CPC concentrations below CMC also were tested. The

![Figure 1: DPV of (A) serum and (B) saliva (diluted in PBS 1:4) in the presence of CPC (100 mmol L⁻¹) (solid line), and absence of CPC (dashed line), PBS+CPC (dotted line).](image-url)

![Figure 2: Variation of the peak potential Ep (A) and peak current Ip (B) in serum (diluted in PBS 1:4) with surfactant concentrations. (pH 7.4).](image-url)
results were not effective, likely due to the low surfactant concentrations, which did not allow for micellar aggregate formation and the necessary solute-micelle interaction (data not shown).

Therefore, the concentration 100 mmol\(\text{L}^{-1}\) was used for all of the results obtained in the presence of CPC.

The repeatability of the results was verified. Seven serum and saliva samples were analyzed in the presence and in the absence of CPC. For each sample, 20 voltammograms were carried out. Figure 3 presents \(E_p\) and \(I_p\) values obtained for each analyzed measure in serum (A-B) and saliva (C-D).

For all serum and saliva analysis voltammograms, the \(E_p\) values shifted toward lower positive potential values in the presence of CPC (\(p<0.01\)), and the results show better repeatability and stability between the first and the twentieth voltammogram. In addition, a significant increase in and better definition of peak current (\(I_p\)) were obtained in the presence of CPC.

Serum and saliva voltammograms analyzed in the absence of CPC presented broader and significantly lower peak currents (\(p<0.01\)), as well as low repeatability. The drop in signal during repeated DPV scans was reduced by CPC addition.

The voltammograms of a specific saliva sample presented significantly lower \(I_p\) values when compared with others saliva samples. Only 10 subsequent voltammograms of this sample were carried out in the absence of CPC due to the low concentration of analyzed compounds and the decrease in voltammetric signal, followed by complete loss of the signal during repeated scans (Figure 4). Twenty subsequent measures of this sample could be accomplished in the presence of CPC due to the increase in the peak current and larger stability of voltammetric signal.

Figure 4 shows the first and twentieth saliva sample voltammograms in the presence and in the absence of CPC.

On saliva voltammograms in the presence of CPC, a small decrease in peak current, \(I_p\), was found after 20 repeated voltammograms without polishing of the electrode (solid line). No differences were seen in peak potential, \(E_p\).

In the absence of surfactant, a decrease in peak current, \(I_p\), and a displacement in peak potential, \(E_p\), toward larger positive values were observed after 20 repeated scans (dashed lines).

The CPC addition produced better repeatability of the measurements. The relative standard deviation for \(I_p\) values obtained after 20 subsequent voltammograms in the presence of CPC was 0.514×10\(^{-7}\) A. \(E_p\) values did not vary. In the absence of surfactant, the standard deviations were 1.172×10\(^{-5}\) A to \(I_p\) and 0.008 V for \(E_p\).

The stability of a thawed sample was verified. Three aliquots of one sample were thawed at the same time. The samples were analyzed three times: immediately (1 min), 40 minutes and 80 minutes after thawing. During that period, the samples remained exposed to light at room temperature (20ºC) in order to evaluate possible oxidation of compounds after thawing.

This same experiment was repeated four times without electrode polishing between the measurements. The objective was to verify the repeatability and to identify possible electrode passivation due to the same successive scans with electrode without polishing. A single polish was performed before Experiment no. 1. The data of \(E_p\), \(I_p\) and area for the four experiments are presented in the figures 5A-5C, respectively.

The independent analysis results of each experiment show that peak potential values, \(E_p\), were not displaced due to thawing time of samples (Figure 5A). However, peak current values, \(I_p\), (Figure 5B) and Area (Figure 5C) can be influenced by the time of thawing, presenting a slight decrease of values because of the oxidation of compounds.

The data presented good repeatability for measurements accomplished immediately after thawing and 40 minutes after thawing. The results indicate that the decrease of voltammetric signal, in this case, can occur mainly due to oxidation of compounds during thawing. The large number of performed measurements did not result in fouling or passivation of electrode surface by adsorption of constituents from the samples.

The higher variability of the voltammogram data from Experiment no. 1 may have occurred due to the initial electrode polishing performed before beginning the experimental series. These data reinforce the interference of electrode polishing on the measurements. To minimize that initial effect, we suggested performing a larger number of scans.
The decrease of peak current observed with ascorbate addition in the presence of CPC (Figure 6A) does not necessarily indicate a low sensitivity of the method; nor is the loss of voltammetric signal associated with fouling on electrode surface or electrode passivation. Vladmirova and Ramenskai [34] showed that CPC interacts with ascorbate and ionic associations of different compositions are formed that can alter the mechanism of ascorbate oxidation. When the ascorbate concentration exceeds the capacity to form ionic associations with CPC, the signal becomes broad, probably due to two mechanisms simultaneously competing for ascorbate oxidation.

**Effects of urate additions in blood serum in the presence of ascorbate**

The urate and ascorbate interaction was investigated through urate additions in serum sample containing ascorbate (100 mmol L\(^{-1}\)) in the presence and in the absence of CPC. The obtained data are presented in figure 7.

In the presence of CPC, the urate additions induced a displacement of Ep to lower positive values (from 0.276 V to 0.150 V) with dose-dependent increases in peak current. Peak current and peak area values increased in response to the antioxidant concentration increase. In the absence of CPC, this effect was not observed. The peak potential showed a slight displacement from 0.40 V to 0.42 V. Tendencies of peak current and peak area to decrease were observed. In the absence of CPC, no sensitivity was observed in response to the increase in urate concentration.

These data reinforce that the first potential peak can be attributed mostly to urate and ascorbate [8]. The DPV method in the presence...
of CPC was sensitive to detect additions of urate and/or ascorbate added in the serum sample, and urate negates the ascorbate effect on voltammograms.

Correlations between DPV and uric acid in serum and saliva samples

Figure 8 presents the correlation data between peak current values (Ip) and uric acid concentration values of 40 serum (A) and saliva (B) samples of young soccer players. The peak current values presented good correlation with uric acid concentration in serum (r=0.6186, p<0.001) and saliva (r=0.803, p<0.0001) (A and B, respectively). The peak current values of serum and saliva did not present a correlation amongst themselves (data not shown). The correlation data between Ip and urate points to urate as being the main determinant of antioxidant capacity, mainly in saliva.

Conclusions

The results presented in this work demonstrate that the DPV method combined with the cationic micellar system of CPC can be considered a direct measure of the antioxidant because the results are based only on the physico-chemical properties of the antioxidant compounds, without the use of reactive species. The urate and the ascorbate are the main compounds of the first peak current.

Due to the complexity of biological samples, the separation of each antioxidant compound and individual study of their actions can be inefficient. The results would not take into account the synergistic interaction between the compounds, which is a very relevant fact when the antioxidant capacity is analyzed in a system. Moreover, the separation processes usually increase the costs.

The surfactant CPC showed highly effective results in terms of stability, sensitivity, selectivity and repeatability of antioxidant detection in serum and saliva. It can substitute for electrode polishing and prevent the electrode passivation and the drop in voltammetric signal. At the evaluated conditions, DPV is a simple method of application. The analysis time is brief, and the cost becomes lower because materials can be reused. The data presented in this study validate DPV as a methodology for serum and saliva redox state evaluation at different physiological or pathological situations.

The determination of reducing potential of biological fluids, such as saliva, can be more reliable because the samples are not influenced by diet or other interferences. The literature does not present studies of antioxidant capacity in saliva using the DPV method. The possibility of determination of antioxidant capacity in saliva is presented as a practical and non-invasive alternative.

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