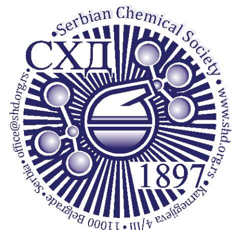

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Electrochemical analysis of antioxidant status of biological media in different sampling and storage conditions

IRINA V. GORONCHAROVSKAYA*, ANATOLY K. EVSEEV, ASLAN K. SHABANOV and SERGEY S. PETRIKOV

N. V. Sklifosovsky Research Institute for Emergency Medicine, B. Sukharevskaya sq., 3, 129090 Moscow, Russia

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Abstract: The use of an electrochemical approach for assessment of the oxidative stress severity is a promising direction for point-of-care testing development, which is especially important for critically ill patients. The aim of this study was to determine the influence of different types of blood collection tubes (with clot activator and separating gel, lithium heparin, sodium citrate and K_2EDTA) and storage conditions (at 25, 4 and –23 °C up to 5 days) on the electrochemical analysis of the antioxidant status of blood plasma, assessed by measuring the open circuit potential of platinum electrode (*OCP*) and antioxidant capacity *via* cyclic voltammetry method (q). It was obtained that blood collection tubes with lithium heparin and clot activator are the most suitable for the electrochemical analysis of antioxidant status of blood plasma, since they do not affect the results of measurements. Furthermore, data obtained during storage blood plasma samples in different temperature conditions indicate that it is preferable to perform electrochemical analysis in fresh samples.

Keywords: potentiometry; voltammetry; antioxidants; sample processing.

INTRODUCTION

Determination of antioxidant status of biological media in patients with various pathologies has an important diagnostic value since it allows to assess the severity of oxidative stress, which in turn affects the disease course and outcome.[1,2] At the present time electrochemical analysis is being actively introduced for the assessment of redox properties of biological media, in particular, methods of potentiometry and cyclic voltammetry (CV), which make it possible to assess both the overall redox balance and the content of low molecular weight antioxidants in analyte.[3–7]

* Corresponding author. E-mail: GoroncharovskayaIV@sklif.mos.ru
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Important preanalytical phase in these studies include the blood collection pro– cedure, sample preparation and storage prior to analysis. In clinical practice, a large number of vacuum blood collection systems are used with various additives for the biological samples stabilization or isolation a certain blood fraction (Table I).

TABLE I. Blood collection tubes types and their uses[8]

Tube cap color	Specimen type	Additive	Analysis
Yellow	Serum	Clot activator (SiO_2) + gel	Biochemistry
Red	Serum	No / Clot activator (SiO_2)	Biochemistry Immunology
Lavender	Whole blood Plasma	K2EDTA K3EDTA	Hematology test Immunology Molecular diagnostics (PCR etc.)
Green	Plasma	Lithium heparin Sodium heparin	Urgent biochemistry Immunology
Light blue	Whole blood Plasma	Sodium citrate 9:1; 0.109 M (3.2 %)	Coagulation tests Hemostasis
Grey	Plasma	Sodium fluoride + Potassium oxalate Sodium fluoride + EDTA	Glucose

However, while maintaining the stability of some substances, these additives may affect the results of analysis of other substances.[9]

There is a lot of data in the literature on the influence of the type of blood collection tubes used on the results of various routine tests,[10,11] however, there are significantly fewer studies on the influence of the type of blood collection tubes on the results of analysis of oxidative stress markers. For example, Bastin *et al.*[12] showed that the analysis of the total antioxidant status of blood serum using the ferric reducing antioxidant power (*FRAP*) assay turned out to be sensitive to the components of the test tube – in plastic tubes with a separating gel, the values were significantly lower than in a glass tubes, but were within acceptable limits from a clinical point of view.

A comparison of the redox potential of blood plasma collected in tubes with lithium heparin and sodium citrate (3.2 %) in study,[5] showed that the values of the redox potential were more negative in heparinized blood plasma.

Studies of total antioxidant capacity of blood plasma containing the anticoagulants EDTA and lithium heparin showed that lithium heparin had no effect on the antioxidant capacity of blood plasma *in vitro*, while EDTA was an interfering agent.[13] However, the direction of the effect (overestimation or underestimation of results), probably depends on the method for determination of total antioxidant capacity.

Similar results were obtained when studying the influence of the type of blood collection tubes on the analysis of the content of individual low-molecular weigh antioxidants in blood plasma such as ascorbic acid[14] and uric acid,[15] which make a significant contribution to the total antioxidant capacity. It was found that blood plasma ascorbic acid is more stable when stored in tubes with lithium heparin compared with tubes with EDTA. The authors suggest that this phenomenon was associated with the fact that EDTA – chelated iron is redox active and can promote the oxidation of ascorbic acid. Reduced levels of uric acid in blood plasma could also be due to the influence of EDTA on the assay reagents.

It is well known that the duration and temperature of storage of samples before analysis affects the laboratory tests results,[10] especially redox properties of biological media,[16,17] since antioxidants are easily oxidized upon contact with atmospheric oxygen. Jansen *et al.* showed that the antioxidant status of blood serum of healthy people, determined by the total antioxidant status (*TAS*) assay, decreased during sample storage longer than 24 h. The samples stored at 20 °C exhibited greater decrease in the parameter compared with samples stored at 4 °C.[16]

Pawlik-Sobecka *et al.*[17] suggest that *FRAP* assay for determination of antioxidant status of blood serum should be carried out no later than 24 h after blood collection. If deviation of 10 % is acceptable, then the analysis can be carried out within 48 h, provided the samples are stored at 20 °C, and within 120 h (5 days), if the samples are stored at 4 °C.

In our previous study of redox properties of blood plasma stored at –40 °C[18] we observed that during first 14 days of storage there were a fluctuations of open circuit potential (*OCP*) of Pt electrode values, measured in blood plasma, indicating that storage of a biological media at very low temperatures does not exclude the occurrence of redox processes.

It is also important to timely centrifuge the tubes after blood collection to separate the plasma or serum from the blood cells to avoid cell metabolism interfering with the test results.[19]

Thus, studies on the influence of the type of blood collection tubes and sample storage conditions on the electrochemical parameters characterizing the antioxidant status of blood plasma or serum are practically not presented in the literature.

The aim of this work is to study the influence of blood collection tubes additives and sample storage conditions on the results of electrochemical assessment of the antioxidant status of blood plasma.

EXPERIMENTAL

Blood plasma and serum sample preparation

In model experiments blood plasma donated by clinically healthy volunteer and collected by automated apheresis was used and was provided by the Department of Transfusion Medi-

cine of the N.V. Sklifosovsky Research Institute for Emergency Medicine in Moscow. Blood plasma was aliquoted into vacuum blood collection tubes with the following additives: clot activator with separating gel (Zhejiang Gongdong Medical Technology Co., China), lithium heparin, sodium citrate (3.2 %, the ratio of citrate to blood is 1:9) and K_2EDTA (Zdravmed-tech, Russia). Tubes without additives were used as a control. Blood plasma samples were stored at 4, 25 and –23 °C. Electrochemical measurements were carried out on the day of blood plasma collection and after 1 and 5 days of storage.

Blood serum samples were obtained from 23 critically ill patients (average age 55±19 y) and examined before and after 24 h of storage at 4 °C. Blood was collected in vacuum tubes with clot activator and separating gel; after at least 30 min tubes were centrifuged at 1500 g (Allegra X-15R, Beckman Coulter, USA) at 25 °C for 10 min.

The study was conducted in compliance with the Declaration of Helsinki, International Conference on Harmonization and Good Clinical Practice Guidelines, and was approved by the Ethical Committee of N.V. Sklifosovsky Research Institute for Emergency Medicine, Moscow, Russian Federation.

Electrochemical measurements

In electrochemical measurements in aqueous media two isotonic to blood plasma solutions were used: saline solution (154 mM NaCl, pH 6.3 (JSC LenReactiv, Russia) and phosphate buffered saline solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7.4 (JSC LenReactiv, Russia). All reagents were of analytical-reagent grade.

Electrochemical analysis of the antioxidant status of biological media consisted of measuring open circuit potential (*OCP*) of platinum electrode and determining its antioxidant capacity by using cyclic voltammetry. In all experiments a saturated silver chloride electrode (sat. Ag/AgCl; all potentials are reffered to Ag/AgCl scale) was used as reference electrode and platinized titanium mesh was used as the counter electrode. The *OCP* result values were recorded 15 min after platinum electrode immersion in the sample. Total antioxidant capacity of biological media was assessed from overall quantity of electricity, q, spent for the oxidation of low molecular weight antioxidants. The q values were determined by integration the area under the polarization curves in a range of potentials from 400 to 1000 mV.[3]

RESULTS AND DISCUSSION

Electrochemical measurements in aqueous solutions

Initially, measurements in saline solution and phosphate-buffered saline aliquoted into the blood collection tubes were carried out (Table II).

TABLE II. *OCP* of Pt electrode and pH values measured in isotonic solutions in the presence of various additives

Blood collection tube	Saline solution		Phosphate buffered saline	
	OCP / mV	pH	*OCP* / mV	pH
No additive	180.5	6.3	149.0	7.43
Clot activator + gel	183.6	6.2	146.9	7.48
Lithium heparin	182.8	6.1	147.2	7.46
Sodium citrate (3.2 %, 9:1)	146.5	8.76	132.4	7.51
K_2EDTA	208.5	4.7	148.4	6.81

Data presented in Table II show that sodium citrate and K_2EDTA additives significantly affect *OCP* of Pt electrode values measured in saline solution, which may be associated with changes in the pH values of solutions. Thus, *OCP* of Pt electrode value decreased by 34 mV in solution with sodium citrate and increased by 28 mV in solution with K_2EDTA compared to solution without additives. In phosphate buffered saline one can see that pH values in all solutions vary slightly and the *OCP* of Pt electrode values are practically the same except for solution with sodium citrate. In this solution measurements showed a decrease by 16.6 mV of *OCP* of Pt electrode value as compared to control.

Cyclic voltammetry measurements show that sodium citrate and K_2EDTA additives also affect the polarization curves in saline solution (Fig. 1).

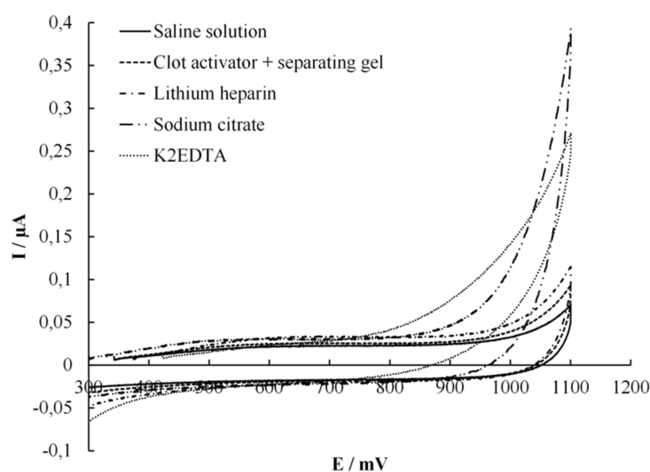

Fig. 1. Cyclic voltammetry in saline solution with various additives.

As can be seen from Fig. 1, the electrochemical oxidation process begins to occur at a potentials more positive than 850 mV in the presence of sodium citrate (dash-double dot line) and more positive than 850 mV in the presence of K_2EDTA (dotted line). According to literature, both sodium citrate[20,21] and EDTA[22,23] are able to adsorb and exhibit electrochemical activity on platinum electrode. Thus, additional electrochemical processes in polarization measurements in biological media will lead to data distortion.

Electrochemical measurements in model blood plasma

After electrochemical measurements in aqueous solutions a model experiments in blood plasma aliquoted into the tubes with various additives and stored at different temperatures were carried out. Fig. 2 shows the results of measurement of *OCP* of Pt electrode and antioxidant capacity of blood plasma *via* CV method during storage at temperature of 25 °C.

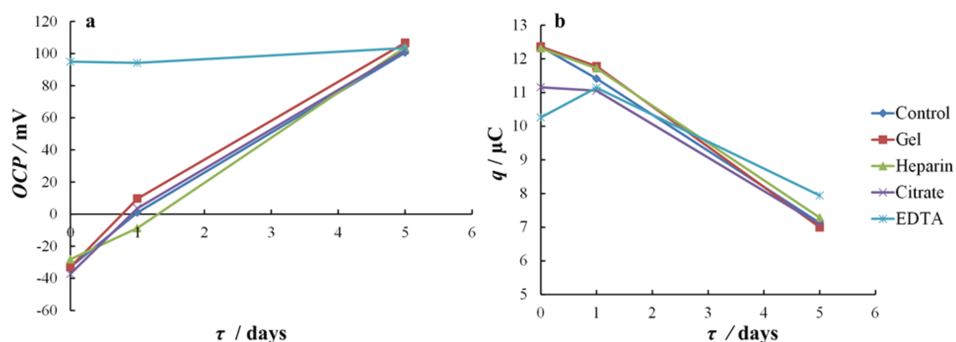

Fig. 2. *OCP* of Pt electrode in blood plasma (a) and antioxidant capacity of blood plasma, measured *via* CV (b) during storage at 25 °C.

From data presented in Fig. 2a it can be seen that the values of *OCP* of Pt electrode measured in blood plasma samples aliquoted into tubes with additives on the day of blood collection practically did not differ from the control sample without additives. On average, the value of *OCP* of Pt electrode was –32.8±4.5 mV. However, this value measured in blood plasma sample with K_2EDTA additive was significantly higher compared with other samples (95 mV, Δ = 128 mV). Throughout the entire storage period, the *OCP* of Pt electrode values measured in all studied blood plasma samples shifted to more positive potentials, which indicates oxidative processes. The shift of *OCP* of Pt electrode value on average was 136.1±3.9 mV, except for the sample with the addition of K_2EDTA, where this value practically did not change during the entire storage period.

As in the case with *OCP* of Pt electrode (Fig. 2b) the presence of such stabilizing additives as lithium heparin and clot activator with separating gel had almost no effect on antioxidant capacity of blood plasma on the day of blood collection. A significant difference in *q* values was observed in blood plasma samples in tubes with K_2EDTA (Δ = 2.1 μC) and sodium citrate (Δ = 1.2 μC).

After 24 h of storage the values of antioxidant capacity in all blood plasma samples varied slightly, and were on average 11.4±0.3 μC. On the 5th day of storage antioxidant capacity significantly decreased, reaching an average value of 7.3±0.4 μC.

Similar electrochemical measurements in samples stored at 4 °C were carried out (Fig. 3).

During storage at a temperature of 4 °C, the values of *OCP* of Pt electrode measured in all blood plasma samples shifted to more positive potentials (Fig. 3a). By the end of the first day of storage, the average value of the *OCP* of the Pt electrode in the blood plasma, measured in the control sample and in samples in tubes with clot activator with separating gel, lithium heparin and sodium citrate, was –7.5±2.5 mV, and in a blood plasma sample in tube with K_2EDTA additive,

the *OCP* of Pt electrode increased to 105.4 mV (*Δ* = 10 mV). The value of the *OCP* of the Pt electrode, measured on the 5th day of storage in the control sample and in blood plasma with additives, shifted to an average of 13±2.2 mV, and in the test tube with K_2EDTA to 106 mV.

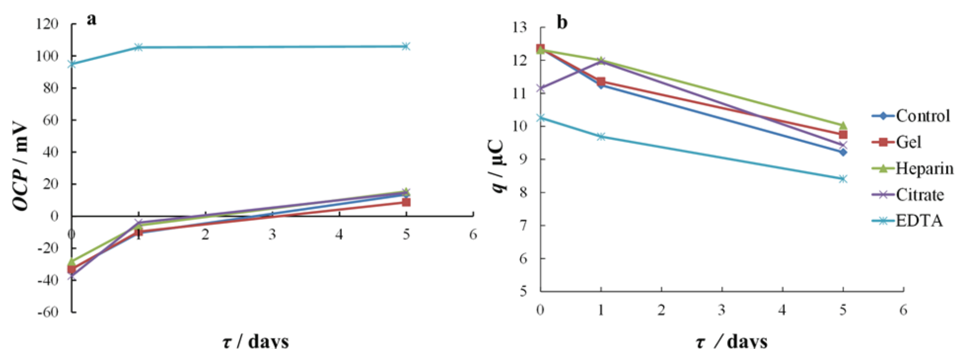

Fig. 3. *OCP* of Pt electrode in blood plasma (a) and antioxidant capacity of blood plasma, measured *via* CV (b) during storage at 4 °C.

The results of measurements of total antioxidant capacity of blood plasma samples demonstrate (Fig. 3b) that the lowest values compared with control were in the samples with K_2EDTA additive, where on the 1st day of monitoring *q* value was 10.3 μC and by the end of the storage period it was 8.4 μC, while in the other samples, the *q* value on the 1st day of storage was on average 11.6±0.3 μC, and on the 5th day – 9.6±0.4 μC.

Next electrochemical measurements were carried out in blood plasma samples stored at –23 °C (Fig. 4).

It can be seen (Fig. 4a) that after 5 days of storage at –23 °C in all studied samples, except the sample with K_2EDTA additive, *OCP* of Pt electrode values shifted towards more positive potentials and reached the values on average –15.0±2.2 mV on the 1st day of storage and 18.1±1.3 mV on the 5th day of storage. The value of *OCP* of Pt electrode measured in blood plasma sample with K_2EDTA additive decreased to a value of 87.6 mV after 24 h of storage at –23 °C, in contrast with the measurements in samples stored at 25 and 4 °C, where this value did not change or increased. After 24 h of storage the values of *OCP* of Pt electrode remained unchanged until the end of storage at all temperatures.

Antioxidant capacity of blood plasma stored at –23 °C (Fig. 4b) decreased to 11.8 μC in control sample and increased to 13.3±0.1 μC in samples with lithium heparin, sodium citrate and clot activator with gel additives after 24 h of storage. In the blood plasma sample with K_2EDTA additive the *q* value increased to 10.8 μC. By the end of storage, the antioxidant capacity of all blood plasma samples decreased, but not as much as when stored in other temperature conditions.

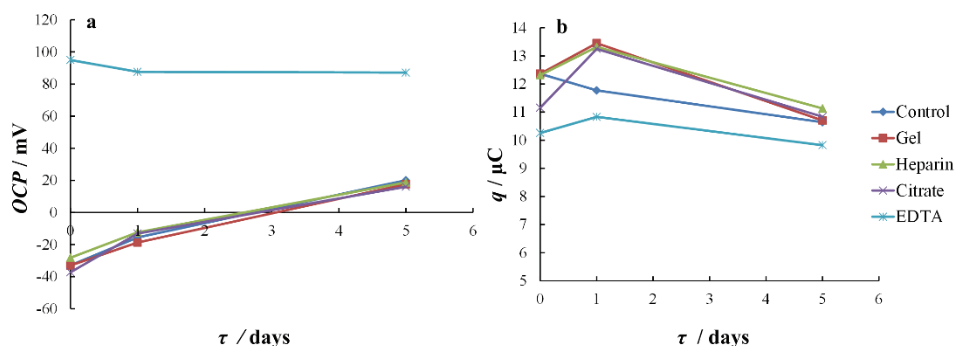

Fig. 4. *OCP* of Pt electrode in blood plasma (a) and antioxidant capacity of blood plasma, measured *via* CV (b) during storage at –23 °C.

Thus we can conclude that parameter of *OCP* of Pt electrode measured in blood plasma turned out to be more sensitive to the storage conditions of biological media. In addition, K_2EDTA and sodium citrate resulted in lower values of total antioxidant capacity of blood plasma measured *via* cyclic voltammetry.

Since both when stored at 4 and –23 °C the *OCP* of Pt electrode values shift towards more positive potentials, and the values of the total antioxidant capacity fluctuate during freezing, it was decided to reduce the measurement time to 24 h and carry out experiments for measuring electrochemical parameters in biological samples from patients under storage temperature of 4 °C in order to avoid additional errors, related to the conditions of the sample thawing.

Electrochemical measurements in critically ill patients' serum

Measurements of *OCP* of Pt electrode and antioxidant capacity in real samples of blood serum collected in tubes with clot activator and separating gel were carried out. In 43 % of cases (n = 10) the *OCP* of Pt electrode values measured in blood serum after 24 h of storage shifted to more negative potentials; in 35 % of cases (n = 8) the *OCP* of Pt electrode values practically did not change; and in 22 % of cases (n = 5) shifted to more positive potentials. Comparison of *OCP* of Pt electrode measurements in model blood plasma of healthy individual and blood serum of several patients in critical condition is presented on Fig. 5.

The data obtained indicate the importance of performing electrochemical measurements in fresh samples, since in real samples the magnitude and direction of the *OCP* of Pt electrode shift during sample storage may be influenced by individual characteristics of the biological media, depending on patients' drug therapy, therapeutic procedures, *etc.*

The value of the antioxidant capacity of blood serum after 24 h of storage at 4 °C significantly (p = 0.00005) decreased from 12.1±4.1 μC to 11.4±4.0 μC. It should be noted that this parameter reflects only the content of low molecular weight antioxidants, while the value of *OCP* of Pt electrode measured in blood

serum is an integral parameter and reflects overall balance between pro- and antioxidants.

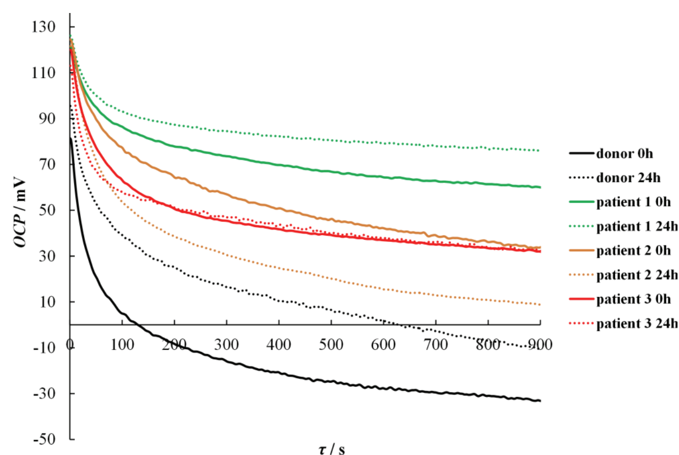

Fig. 5. *OCP* of Pt electrode in blood plasma of healthy individual and blood serum of patients in critical condition before and after 24 h of storage at 4 °C.

CONCLUSION

Electrochemical methods of analysis continue to find their application in laboratory practice for the evaluation of the redox properties of biological media in a group of analyses of oxidative stress markers. The preparation of biological samples is one of the key factors influencing the stability and accuracy of any measurement results, especially the results of assessing redox properties, which is associated with the stability of antioxidants contained in biological media.

In this paper blood plasma and sera samples with various additives stored at three temperatures were studied. It was found that storage conditions have significant effect on the results of electrochemical measurements in blood plasma. This study indicates that blood collection tubes with lithium heparin and clot activator with separating gel are preferable for blood collection and storage due to the least influence on the electrochemical parameters of biological media. Electrochemical analysis in blood plasma should be carried out in fresh samples avoiding storage.

ИЗВОД

ЕЛЕКТРОХЕМИЈСКА АНАЛИЗА АНТИОКСИДАТИВНОГ СТАТУСА БИОЛОШКОГ МЕДИЈУМА У РАЗЛИЧИТИМ УСЛОВИМА УЗОРКОВАЊА И СКЛАДИШТЕЊА

IRINA V. GORONCHAROVSKAYA, ANATOLY K. EVSEEV, ASLAN K. SHABANOV и SERGEY S. PETRIKOV

N. V. Sklifosovsky Research Institute for Emergency Medicine, B. Sukharevskaya sq., 3, 129090 Moscow, Russia

Примена електрохемијског приступа за процену јачине оксидативног стреса је обећавајући правац за развој тестирања на лицу места, што је посебно важно за критично болесне пацијенте. Циљ овог истраживања био је да се утврди утицај различитих типова епрувета за сакупљање крви (са активатором згрушавања и гелом за сепарацију, литијум-хепарином, натријум-цитратом и K_2EDTA) и услова складиштења (на 25, 4 и –23 °C до 5 дана) на електрохемијску анализу антиоксидативног статуса крвне плазме, процењеног мерењем потенцијала отвореног кола платинске електроде (*OCP*) и антиоксидативног капацитета методом цикличне волтаметрије (q). Утврђено је да су епрувете за прикупљање крви са литијум-хепарином и активатором угрушака најпогодније за електрохемијску анализу антиоксидативног статуса крвне плазме, јер не утичу на резултате мерења. Штавише, подаци добијени током складиштења узорака крвне плазме у различитим температурним условима указују на то да је пожељно извршити електрохемијску анализу у свежим узорцима.

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