Abstract
The potentiometric assay for determining the antioxidant activity of ascorbic acid has been developed and validated according to the following parameters: specificity, linearity, accuracy, repeatability, intermediate precision. The linearity was in the concentration range of 0.002 – 0.02 mol L\(^{-1}\) \((r^2 = 0.9993)\). The percentage of recovery was found to be in the range from 95.38 to 105.00 %. The values of %RSD for repeatability and intermediate precision were 1.86 and 1.95 %, respectively. The method is accurate and reliable, with the relative standard deviation of less than 2 %. It has been proven that the method developed is express, rapid, highly sensitive, accurate and sufficiently reliable.

Keywords: antioxidant activity; potentiometric method; validation; ascorbic acid

Metrological Characteristics of the Potentiometric Assay Developed for Determining the Antioxidant Activity of Ascorbic Acid

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Метрологічна характеристика розробленої методики визначення антиоксидантної активності аскорбінової кислоти

Анотація
Потенціометричну методику визначення антиоксидантної активності аскорбінової кислоти було розроблено і валідовано відповідно до таких параметрів: специфічність, лінійність, точність, прецізійність, внутрішня прецізійність. Лінійність зберігалася в діапазоні концентрацій 0,002 – 0,02 моль л\(^{-1}\) \((r^2 = 0,9993)\). Визначено, що відсоток відновлення становить 95,38 – 105,00 %, прецізійність та внутрішня прецізійність – 1,86 % та 1,95 %, відповідно. Методика характеризується як точна і надійна, має відносне стандартне відхилення менше 2 %. Доведено, що розроблена методика експресна, проста, високочутлива, точна і достатньо надійна.

Ключові слова: антиоксидантна активність; потенціометричний метод; валідація; аскорбінова кислота

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Introduction

Reactive oxygen species are generated endogenously in the mitochondrial oxidative phosphorylation, or they may arise from exogenous sources, such as xenobiotic compounds [1, 2]. Oxidative stress results in indirect or direct damage of nucleic acids, proteins, and lipid, and has been implicated in carcinogenesis, neurodegeneration, atherosclerosis, diabetes and aging [3, 4].

Ascorbic acid is a six-carbon lactone obtained from glucose in the liver. Human is not able to synthesize it due to the lack of L-gulonolactone oxidase [5]. Therefore, vitamin C must be obtained from the diet to maintain a normal metabolic functioning of the body. In the body of a human, vitamin C reacts with free radicals as an antioxidant [6]. The ascorbyl radical is formed when ascorbic acid loses one electron, the second form is called dehydroascorbic acid formed with losing an electron by the ascorbyl radical [7, 8].

Most of the physiological functions of vitamin C are related to its reduction properties [9, 10]. Ascorbic acid is involved in the synthesis of collagen, carnitine and neurotransmitters. In addition, ascorbic acid accelerates the healing process, affects the synthesis of a number of hormones, regulates hematopoiesis and normalizes capillary permeability [11–13].

According to Pubmed and ScienceDirect databases, the number of publications dealing with the study of the antioxidant activity of different substances is growing steadily (Figure 1). Known assays for determining the antioxidant activity are based on the oxidation of the test sample with oxidizing agents of various nature. Oxidizing agents can be inorganic compounds – K₃[Fe(CN)₆], H₂O₂, KMnO₄, and organic compounds – 2,2′-diphenyl-1-picrylhydrazyl (in DPPH assay), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (in ABTS assay), Fe(III)-tripyridyltriazine (in FRAP assay) [14]. The antioxidant activity of ascorbic acid has been determined by different assays, such as DPPH [15], ABTS [16], FRAP [17], chemiluscent and potentiometric method [18].

S. Martinez et al. [19] applied the potentiometric assay in determining the antioxidant activity of wine. The potentiometric assay they use is based on titration of test samples with an electrogenerated chlorine. Chlorine has the ability to enter into various reactions (radical, redox, electrophilic substitution and addition to multiple bonds). Due to this, titration with chlorine allows to cover a wide spectrum of biologically active components possessing antioxidant properties. However, in our opinion, this method has some disadvantages. Firstly, in the assay the pH equals 2. At the same time, for studies of the antioxidant activity related to living organisms, the pH value should be maintained in the range of 7.2–7.4 since it is physiological. Secondly, chlorine is rather toxic compound.

Figure 1. The number of published articles in the period of 2003–2022 years
Currently, ascorbic acid is found in numerous plants, e.g., rose hips, mountain ash, viburnum, currants, raspberries and citrus fruits. In addition, vitamin C is also an ordinary “guest” in caplets, tablets, capsules, drink mixes, multivitamin, antioxidant formulation and dietary supplements. Hence, the development of methods for determining the antioxidant activity of ascorbic acid is a promising task today. Thus, the aim of the present study was to develop and validate the potentiometric assay for determining the antioxidant activity of ascorbic acid.

## Materials and methods

### Reagents

Ascorbic acid ≥ 98.0% (Sigma-Aldrich). Reagents K₃[Fe(CN)₆], K₄[Fe(CN)₆], NaHPO₄, KH₂PO₄ were of analytical grade and purchased from Reakhim (Kharkiv, Ukraine).

### Instruments

A pH meter Hanna 2550 (FRG) with a combined platinum electrode EZDO 50 PO (Taiwan) were used during potentiometric measurements. The digital analytical balance AN100 (AXIS, Ukraine) with d = 0.0001 g was used for weighing.

### Preparation of standard solutions

A stock solution of ascorbic acid (0.05 mol L⁻¹) was prepared by dissolving ascorbic acid in distilled water.

Model solutions of ascorbic acid were prepared by dilution of the stock solution of ascorbic acid (0.002, 0.006, 0.01, 0.014, 0.02 mol L⁻¹).

### Preparation of phosphate buffer solutions

8.00 g of NaCl, 0.20 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄ were dissolved in 800 mL of distilled water, and pH to 7.4 was adjusted with hydrochloric acid or sodium hydroxide, and the solution was diluted with distilled water to 1 L.

### The procedure for the antioxidant activity determination

2 mmol L⁻¹ solution of K₃[Fe(CN)₆] was prepared by weighing 0.8232 g into a 25.0 mL volumetric flask, dissolving the compound in distilled water, and diluted to the volume with the same solvent. 0.02 mmol L⁻¹ of K₄[Fe(CN)₆] was prepared by weighing 0.0921 g into a 250.0 mL volumetric flask, dissolving the compound in distilled water, and diluted to the volume with the same solvent. Then 5.00 mL of the aliquot of both prepared solutions was taken and transferred into a 250.0 mL volumetric flask and diluted to the volume with 0.067 mmol L⁻¹ phosphate buffer solution. 50.00 mL of the prepared intermediate solution was transferred into an electrochemical cell. The initial potential of the intermediate solution was measured after the initial one was determined, 1.00 mL of the aliquot of the solutions obtained was added, and a final potential was measured. The difference (ΔE) between the initial (E₁) and final (E₂) potentials was found [20].

The antioxidant activity was calculated according to the following equation and expressed as mmol L⁻¹:

\[
\text{AOA} = \frac{C_{\text{ox}} - \alpha \cdot C_{\text{red}}}{1 + \alpha} \cdot K_{\text{diff}} \cdot 10^3
\]

where \(\alpha = C_{\text{ox}}/C_{\text{red}} \times 10^{\Delta E/\text{E(ethanol)inF2.3RT}},\) \(C_{\text{ox}}\) is the concentration of \(K_4[\text{Fe(CN)}_6]\), mol L⁻¹; \(C_{\text{red}}\) is the concentration of \(K_3[\text{Fe(CN)}_6]\), mol L⁻¹; \(E_{\text{ethanol}} - 0.0546\times C_{\text{ox}} - 0.0091; C_{\text{ox}}\) is the concentration of ethanol; ΔE is the change of the potential; \(F = 96485.333 \text{ C mol}^{-1}\) is the Faraday constant; \(n = 1\) is the number of electrons in the electrode reaction; \(R = 8.314 \text{ J mol}^{-1} \text{K}^{-1}\) is the universal gas constant; \(T = 298 \text{ K}\); \(K_{\text{diff}}\) is the coefficient of dilution.

### Validation method

Validation of the potentiometric method for determining the antioxidant activity of ascorbic acid was performed according to the International Conference on Harmonization (ICH) [21]. The method proposed was validated by the following parameters: specificity, linearity, accuracy, repeatability and intermediate precision.

The specificity of the method was studied by the potentiometric titration of the solvent.

The linearity of the method was studied at 5 concentration levels (0.002, 0.006, 0.01, 0.014, 0.02 mol L⁻¹). The antioxidant activity was evaluated by the potentiometric method. The linearity was determined by a linear relationship between the logarithm concentrations of the ascorbic acid solutions prepared. The linear regression was calculated by the method of least squares to obtain the regression equation and determine the correlation coefficient \((r^2)\). According to the requirements of ICH, the value of the correlation coefficient when studying the linearity should not exceed 0.999.

The accuracy was evaluated by calculating recovery of ascorbic acid using the standard addition method. Three levels of ascorbic acid concentration corresponding to 50, 100, 200% of the working concentration of ascorbic acid 0.1 mol L⁻¹.
were taken. The acceptance criteria were \( \text{RSD} \) and should not exceed 2%.

The precision of the method was assessed by repeatability and intermediate precision. In the case of repeatability, the working solution of ascorbic acid with the concentration of 0.01 mol L\(^{-1}\) was analyzed six times at the same day. The intermediate precision was evaluated with the same concentration of ascorbic acid at different days. The acceptance criteria were \( \text{RSD} \) and should not exceed 2%.

The statistical processing of the experimental data obtained was performed in accordance with the monograph “Statistical analysis of the results of a chemical experiment” of the State Pharmacopeia of Ukraine.

## Results and discussion

The potentiometric method for determining the antioxidant activity is based on the interaction of a mediator system with an antioxidant. The mediator system consists of an oxidizing agent and a reducing agent. When an antioxidant is added to the mediator system, the ratio of the oxidizing agent and reducing agent changes, which leads to a shift in the potential of the electrochemical cell. After that, the potential difference and the antioxidant activity of the antioxidant studied are calculated.

In order to develop a potentiometric assay for determining the antioxidant activity of ascorbic acid, it was necessary:

1. to choose the optimal redox electrode;
2. to choose the optimal ratio of \( K_{\text{Fe(CN)6}} \) and \( K_{\text{Fe(CN)4}} \);
3. the potential of the platinum electrode should correspond to the Nernst dependence of the potential change.

The main criteria for choosing the redox electrode are the range of measuring the potential of the electrochemical cell and the time of establishing the potential.

The potential of the electrochemical cell is measured by platinum, gold and glass-carbon electrodes. Thus, to choose the optimal electrode we analyzed characteristics of three electrodes mentioned above.

**Table 1. Measuring ranges of potentials of electrodes**

<table>
<thead>
<tr>
<th>No.</th>
<th>Electrode</th>
<th>Measuring ranges, V</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Platinum electrode</td>
<td>from -0.1 to +0.9</td>
</tr>
<tr>
<td>2</td>
<td>Gold electrode</td>
<td>from -0.1 to +0.3</td>
</tr>
<tr>
<td>3</td>
<td>Glass-carbon electrode</td>
<td>from -0.9 to +0.8</td>
</tr>
</tbody>
</table>

**Table 2. The time of the establishing the potential of the electrochemical cell**

<table>
<thead>
<tr>
<th>No.</th>
<th>Electrode</th>
<th>Time, sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Platinum electrode</td>
<td>10±0.3</td>
</tr>
<tr>
<td>2</td>
<td>Gold electrode</td>
<td>1800±36.0</td>
</tr>
<tr>
<td>3</td>
<td>Glass-carbon electrode</td>
<td>300±6.0</td>
</tr>
</tbody>
</table>

**Note:** \( n = 5, p < 0.05 \)

Table 1 demonstrates that the platinum electrode is the fastest (10 sec) in establishing the potential of the electrochemical cell, followed by the glass-carbon electrode (300 sec), and the gold electrode has the longest time (1800 sec).

According to the results obtained, the platinum electrode met all the requirements to the redox electrode and was the most suitable for determining the antioxidant activity of ascorbic acid. Thus, it was used in the study.

The accuracy of determining the redox potential of the system depends on how the Nernst dependence follows the potential change. **Figure 2** shows the dependence of the potential of the platinum electrode EZDO 50 PO in the sodium phosphate buffer with the pH 7.4 and various concentrations of \( K_{\text{Fe(CN)6}}/K_{\text{Fe(CN)4}} \). The pre-logarithmic coefficient in this dependence is 58.5 mV (Figure 2), which is close to the theoretical value of RT/nF = 59.16 mV in the Nernst equation for a one-electron process at 25°C. From mentioned above, it can be concluded that the platinum electrode is completely suitable according to the criteria set.

To select the optimal concentration and ratio of \( K_{\text{Fe(CN)6}} \) and \( K_{\text{Fe(CN)4}} \), the following criteria were used:

1. the minimum change in the system potential must be at least 20 mV;
2. the high speed of equilibration;
3. the potential of the system must be stable over time.

**Table 3** demonstrates the change in the potentials of the system with different ratios of \( K_{\text{Fe(CN)6}} \) and \( K_{\text{Fe(CN)4}} \) concentrations after the introduction of the test samples (1.0–5.0 mol L\(^{-1}\) of ascorbic acid). It is easy to see that in the...
system 1 and 2 the minimum potential change is greater than 20 mV. However, the redox potential of system 2 is not stable for 30 minutes, this may be due to the fact that the concentration of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ is low enough for the potential of the system to be stable over time. System 1 turned out to be the most stable over time, and its potential did not change over the course of an hour. Therefore, this mediator system was chosen to determine the antioxidant activity of the samples under study.

The linearity was proven in the concentration range from 0.002 to 0.02 mol L$^{-1}$. The regression equation of the curve had the following form: $y = 2.5896x + 7.4011$. The value of the correlation coefficient ($r^2$) was equal to 0.9993 (Figure 3).

The accuracy of the method was assessed by the percentage of recovery. The percentage of recovery was found to be in the range from 95.38 to 105.00% (Table 4).

The precision of the method was confirmed by repeatability and intermediate precision. The values

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**Table 3.** The change in the potential after the introduction of ascorbic acid (1.0 and 5.0 mol L$^{-1}$) into the mediator system of various compositions

<table>
<thead>
<tr>
<th>Mediator system</th>
<th>$K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$, mol L$^{-1}$</th>
<th>$\Delta E$ for concentration of 1.0 mol L$^{-1}$, mV</th>
<th>$\Delta E$ for concentration of 5.0 mol L$^{-1}$, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>System 1</td>
<td>0.002/0.00002</td>
<td>25.0±0.3</td>
<td>40.0±0.3</td>
</tr>
<tr>
<td>System 2</td>
<td>0.001/0.00001</td>
<td>40.0±0.3</td>
<td>100.0±0.3</td>
</tr>
<tr>
<td>System 3</td>
<td>0.1/0.001</td>
<td>2.0±0.3</td>
<td>12.0±0.3</td>
</tr>
<tr>
<td>System 4</td>
<td>0.05/0.001</td>
<td>10.0±0.3</td>
<td>25.0±0.3</td>
</tr>
</tbody>
</table>

**Note:** $n = 5$, $p < 0.05$

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**Table 4.** Recovery studies using the standard method of addition

<table>
<thead>
<tr>
<th>AO</th>
<th>AOA$^*$, mmol-eq. L$^{-1}$</th>
<th>Amount added of AO, mmol-eq.L$^{-1}$</th>
<th>Amount taken of AO ($C_1$), mmol-eq.L$^{-1}$</th>
<th>Amount recovered ($C_2$), mmol-eq.L$^{-1}$</th>
<th>%, Recovery $R = \frac{C_2}{C_1} \cdot 100$ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>1.95</td>
<td>0.98</td>
<td>2.93</td>
<td>3.00</td>
<td>102.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.02</td>
<td>103.07</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.80</td>
<td>95.56</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.00</td>
<td>102.39</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>4.11</td>
<td>105.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.72</td>
<td>95.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.00</td>
<td>102.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.14</td>
<td>105.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.80</td>
<td>98.36</td>
</tr>
</tbody>
</table>

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$y = 58.5x + 215$

$R^2 = 0.9977$

**Figure 2.** The dependence of the potential of the platinum electrode EZDO 50 PO in the sodium phosphate buffer with the pH 7.4 and various concentrations of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. 

**Table 4.** Recovery studies using the standard method of addition
of %RSD for repeatability and intermediate precision were 1.95 and 1.86%, respectively. The %RSD values were less than 2%. It proves that the method is precise (Tables 5 and 6).

**Conclusions**

The potentiometric assay for determining the antioxidant activity of ascorbic acid has been developed and validated according to the following parameters: specificity, linearity, accuracy, repeatability, intermediate precision. The method proposed can be used for routine analysis to determine the antioxidant activity of different objects under research and quality control purposes since the method developed is express, rapid, highly sensitive, accurate and sufficiently reliable.

**References**


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