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DEVELOPMENT AND VALIDATION POTENTIOMETRIC METHOD FOR DETERMINATION OF ANTIOXIDANT ACTIVITY OF EPIGALLOCATECHIN-3-O-GALLATE

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Abstract

There is high interest of scientists in the study of antioxidant activity (AOA) of different objects, which is caused by study influence of free radicals on developing pathological process in human's body. Today, there is no main method that would be used to assess antioxidant activity. In our view, the development and modification of existing methods for determining antioxidant activity is a topical and prospective task of the pharmacy, cosmetic and food industries.

The aim of study was to develop and validate potentiometric method for determination of antioxidant activity of epigallocatechin-3-O-gallate (EGCG).

The object of the study was EGCG. Potentiometric measurements were conducted by pH meter Hanna 2550 (Germany) with a combined platinum electrode EZDO 5010.

For assessing the level of AOA of EGCG was chosen following optimal conditions: $K_3[Fe(CN)_6]$ was used as oxidizer, an electrode was made of platinum material, a 0.067 mol/L phosphate buffer solution with pH 7.2 – 7.4 was chosen as electrolyte, the ratio of $K_3[Fe(CN)_6]$ /$K_4[Fe(CN)_6]$ equaled 0.002/0.00002 mol/L. The linearity was in the concentration range of 0.002 – 0.02 mol/L (r^2 = 0.9992). The percentage of recovery was found to be in the range from 96.01 to 101.61 %. The values of %RSD for repeatability and intermediate precision were 1.86 and 1.34 %.

The potentiometric method for determination of antioxidant activity of EGCG was developed and validated according by the following parameters: linearity, accuracy, repeatability, intermediate precision. It was proved that the developed method is express, rapid, highly sensitive, accurate and sufficiently precise.

Key words: *antioxidant activity, potentiometric method, validation, epigallocatechin-3-O-gallate*

Introduction

Oxidative stress is defined as the imbalance between prooxidants and antioxidants, is characterized by the inability of endogenous antioxidants to counteract the oxidative damage on tissues and organisms owing to overproduction of cellular reactive oxygen species (ROS) that are highly reactive and can cause oxidative modification of biological macromolecules, such as lipid, protein, and DNA, leading to tissue injury, accelerated cellular death, and various diseases such as atherosclerosis, diabetes mellitus, chronic inflammation, neurodegenerative disorders, cardiovascular disease, Alzheimer's disease, mild cognitive impairment, Parkinson's disease and certain types of cancer [1]. That is why, there is a high interest of scientists in studying antioxidant activity of different materials such as plant extracts, tinctures, dietary supplements, various beverages, medicines.

Many AOA methods have been developed over the years, based on different chemical, physicochemical, and biochemical mechanisms. The AOA methods are classified by the type of oxidation source, the compound being oxidized and the way the oxidized compound is measured [2]. According to the way of measuring AOA all methods can be divided into volumetric [3], spectrophotometric [4], chemiluminescent [5], fluorescent [6], electrochemical [7], and a number of more specific ones. Usually, a model reaction proceeding by a radical mechanism (most often - oxidation) of an individual compound is used, according to the influence on the course of which the AOA of either an individual compound or mixture is estimated [8].

Green tea is one of the most widespread beverage over the world, a popularity of tea relates with a high content of polyphenols, which are responsible for AOA [9]. EGCG is a main polyphenolic compound and the most abundant catechins in green tea. In previous studies, EGCG has been investigated quit well as result it has found that it possesses antibacterial, anticancer, antidiabetic, antiangiogenic effects [10].

According to Pubmed and ScienceDirect databases, spectrophotometric methods for determination AOA are widely used ones to evaluate AOA of investigated samples. FRAP [11], DPPH, Folin-Ciocalteu, ABTS [12] assays are the most applied spectrophotometric methods so as to find out antioxidant capacity of investigated objects. These methods are based on reduction of an oxidant, which changes color when reduced. However, the application of spectrophotometric methods is limited in the case if sample is either turbid or initially colored.

Potentiometric method for determining antioxidant activity is underlying on measuring potential shift in the mediator system of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ as a result of its interaction with antioxidants. Unlike spectrophotometric methods this one can be used to study AOA of turbid and initially colored samples. Potentiometric method is quiet express, high sensitive, rapid analysis procedure, not need expensive equipment or reagents [13]. But, the main advantage of described method is no need to use standard for comparison as integrated value of AOA is used in potentiometric method. In addition, according to previous researches this method was successfully used for the analysis of food products, food raw materials and dietary supplements, blood and its fractions [14].

The aim of study was to develop and validate potentiometric method for determination of antioxidant activity of EGCG.

Methods

Potentiometric measurements were conducted by pH meter Hanna 2550 (Germany) with a combined platinum electrode EZDO 5010. Weighing was carried out using digital analytical balance AN100 (AXIS, Ukraine) with d = 0.0001 g. Epigallocatechin-3-O-gallate ≥ 98.0% (Sigma Aldrich), $K_3[Fe(CN)_6]$, $K_4[Fe(CN)_6]$, $NaHPO_4$, KH_2PO_4 were analytical grade and purchased from KharkivReakhim.

For preparation a module solution of EGCG a 1.1450 g of it was accurately weighed and transferred into a 50.00 mL volumetric flask dissolved in 96% ethanol solution and made up to the mark and with the same solvent to give a solution having 0.05 mol/L concentration. A different aliquots of prepared module solution of EGCG were transferred into 50.00 mL volumetric flasks and made up to the mark with distilled water

to give solutions having 0.002, 0.006, 0.01, 0.014, 0.02 mmol/L concentration.

Validation of the potentiometric method for the determination of AOA of EGCG was performed according to the International Conference on Harmonization (ICH) [15]. The proposed method was validated by the following parameters: linearity, accuracy, repeatability, intermediate precision.

In order to develop potentiometric method of analysis of AOA, it was evaluated the effects of the following influencing factors on analysis: a type of electrode, electrolyte, mediator system components concentrations.

Determination AOA of EGCG was made by following assay: a 2 mmol/L solution of $K_3[Fe(CN)_6]$ was prepared by weighing 0.8232 g into a 25.0 mL volumetric flask, dissolving a compound in a distilled water and filling the flask to volume with the same solvent. A 0.02 mmol/L of $K_4[Fe(CN)_6]$ was prepared by weighing 0.0921 g into a 250.0 mL volumetric flask, dissolving a compound in a distilled water and filling the flask to volume with the same solvent. Than a 5.00 mL aliquot of both prepared solutions was taken and transferred into a 250.0 mL volumetric flask and made up to the mark by 0.067 mol/L phosphate buffer solution. A 50.00 mL of prepared mediator solution was transferred in an electrochemical cell. The initial potential of mediator solution was measured after initial one was established, a 1.00 mL of aliquot of the prepared solutions was added and a final potential was measured. The difference (ΔE) between the initial (E_0) and final (E_1) potentials was found. The shift of potential is explained by the change of ratio of oxidized and reduced forms of the mediator system as a result of following chemical reaction:

$$a\cdot[Fe(CN)_6]^{3-} + b\cdot AO = a\cdot[Fe(CN)_6]^{4-} + b\cdot AO_{ox}$$

where AO – antioxidant; AO_{ox} – oxidized form of antioxidant; a, b – stoichiometric coefficients

Antioxidant activity was calculated according to the following equation and expressed as mmol/L [16]:

$$AOA = \frac{C_{ox} - \alpha \cdot C_{red}}{1+\alpha} \cdot K_{dil} \cdot 10^3$$

where, $\alpha = C_{ox}/C_{red} \cdot 10^{(\Delta E - E_{ethanol})nF / 2.3RT}$; C_{ox} – concentration of $K_3[Fe(CN)_6]$, mol/L; C_{red} – concentration of $K_4[Fe(CN)_6]$, mol/L; $E_{ethanol}$ – $0.0546\cdot C_{\%} - 0.0091$; $C_{\%}$ – concentration of ethanol; ΔE – change of potential; F = 96485.333 Cl/mol – Faraday constant; n = 1 – number of electrons in electrode reaction; R = 8.314 J/mol · K – universal gas constant; T – 298 K; K_{dil} – coefficient of dilution.

Statistical analysis was performed in Microsoft Excel 2010 with the accepted significance level α = 0.05.

Results

The oxidized form of iron in the composition of potassium hexacyanoferrate (III) was used as a model for the potentiometric determination of antioxidant activity. The choice of potassium hexacyanoferrate (III) is due to the fact that this compound meets all the requirements for oxidants used to correctly determine the content of antioxidants:

1. $K_3[Fe(CN)_6]$ is an oxidizing agent of medium strength (E_0 = 0.36 V), so there is a possibility of the reaction between oxidizer and antioxidants. By the way, there is no interaction with compounds, which has weak reducing properties;
2. reaction between oxidizer and antioxidants should be provided at at pH ≈ 7, as such pH close to physiological. $K_3[Fe(CN)_6]$ and its reduced form are stable at this medium as the values of the conditional stability constants of significantly exceed 10^8;
3. $K_3[Fe(CN)_6]$ accepts a one electron as result its reaction with antioxidants performs stoichiometrically in accordance with the number of functional groups exhibiting antioxidant properties;
4. the rate of the redox reaction of $K_3[Fe(CN)_6]$ with antioxidants is high enough to get an acceptable analysis duration (3–30 min, depending on the object of research).

The main role in potentiometry is played by the electrode. It should meet the following criteria:

1. the range of the measured potential must be large enough
2. the electrode must provide the Nernst dependence of the change in the potential of the system of concentrations of components
3. rapid equilibration of potential

Currently, in order to measure the redox potential following types electrodes are used

platinum, gold, and glass-carbon. Table 1 shows measuring range of electrodes` potential.

According to this criterion, platinum and glass-carbon electrodes are suitable since their potential measurement ranges are quite wide compared to an electrode made of gold material. Also, an important criterion is the rate of potential establishment, on platinum electrodes the rate of potential establishment is faster (~ 10 sec.) In comparison with glass-carbon electrodes (~ 5 min.). Moreover, the potential on platinum electrodes has higher stability than on glass-carbon electrodes. According to this criterion, a platinum electrode is more suitable.

The accuracy of determining the redox potential of the system depends on the meet of the Nernst dependence of the potential change. Fig. 1. shows the dependence of the potential of the platinum electrode EZDO 5010 in sodium phosphate buffer pH 7.4, containing various concentrations of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. The prelogarithmic coefficient in this dependence is 58.5 mV (fig. 1), which is close to the theoretical value 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.

The electrolyte must provide physiological conditions so that the obtained AOA results can be applied to the human body, therefore the acidity of the medium must be neutral. Extracts, juices can have different pH values. As mentioned above, the potential of the system directly depends on the acidity of the medium. Therefore, for the analysis, it is necessary to use an electrolyte that is able to maintain a certain pH of the medium. In addition, the electrolyte should have a high value of conductivity and constant ionic strength of the solution. Since the test samples contain different amounts of ions. In our view, phosphate buffer pH 7.2-7.4 is an appropriate choice. Fig. 3. shows that the complexes $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ are stable at this pH value. Table 3 demonstrates the buffer capacity and ionic strength of the solution, which corresponds to the total concentration of salts NaH_2PO_4 and K_2HPO_4.

Phosphate buffer with a concentration of 0.067 M provides a high ionic strength of the solution (I = 0.168), and the buffer capacity of this solution (B = 0.031 M) is sufficient to maintain pH when adding samples to the electrolyte solution. A 0.067 M phosphate buffer solution was chosen as the electrolyte for the determination of AOA.

To select the optimal concentration and ratio of $K_3[Fe(CN)_6]$ and $K_3[Fe(CN)_6]$, the following criteria were used:

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

Table 4 demonstrates the change in the potentials of the system with different ratios of the concentrations of $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$, after the introduction of the test samples (1.0 - 5.0 mM of EGCG). It is easy to see that in 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 $K_4[Fe(CN)_6]$ 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 AOA of the samples under study.

Linearity was proved in the concentration range from 0.001 to 0.005 mol/L. The regression equation of the curve has the following form: $y = 9.2299x + 32.828$ The value of the correlation coefficient (r^2) was equal to 0.9992 (fig. 2).

The accuracy of the method was assessed by the percentage of recovery. The percentage of recovery was found to be in the range from 96.01 to 101.61 %. The percentage of recovery of EGCG were not exceeding the range 95 – 105% (Table 5).

The precision of the method was confirmed by repeatability and intermediate precision. The values of %RSD for repeatability and intermediate precision were 1.86 and 1.34 %. The %RSD values were less than 2 %. It proves that the method is precise (Tables 5, 6).

Conclusions

The article presented practical approaches to apply potentiometry in order to evaluate AOA as well as it also explained the choice of the oxidizer, the required conditions for chemical reaction

between antioxidants and mediator system. The potentiometric method for determination of antioxidant activity of EGCG was developed and validated according by the following parameters: linearity, accuracy, repeatability, intermediate precision. The proposed method can be used for routine analysis for determination AOA of different investigated objects and quality control purpose as the developed method is express, rapid, high sensitive, accurate and sufficiently precise.

Acknowledgments

The authors declare that there are no conflicts of interest.

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Table 1. Measuring ranges of potentials of electrodes

Electrode	Measuring ranges, V
Platinum electrode	from -0.1 to +0.9
Gold electrode	from -0.1 до +0.3
Glass-carbon electrode	from -0.9 to +0.8

Table 2. Ionic strength and buffer capacity corresponding to the total concentration of NaH_2PO_4 and K_2HPO_4 salts of phosphate buffer

N	mol/L	I, mol/L	B, mol/L
1	0.010	0.025	0.005
2	0.025	0.063	0.012
3	0.067	0.168	0.033
4	0.134	0.268	0.067

Table 3. Change in potential after the introduction of EGCG (1.0 and 5.0 mM) into the mediator system of various compositions

Mediator system	$K_3[Fe(CN)_6]$ /$K_4[Fe(CN)_6]$, mol/L	ΔE, mV
System 1	0.002/0.00002	30 – 60
System 2	0.001/0.00001	60 – 140
System 3	0.1/0.001	5 – 20
System 4	0.05/0.001	15– 40

Table 4. Recovery studies by standard additions technique

AO	AOA, mmol-eq./L	Amount added of AO, mmol-eq./L	Amount taken of AO (C_1), mmol-eq./L	Amount recovered (C_2), mmol-eq./L	%, Recovery $R = \frac{C_2}{C_1} \cdot 100\%$
EGCG	14.27	7.14	21.41	21.38	99.86
				21.50	101.42
				21.72	101.45
		14.27	28.54	29.00	101.61
				28.30	99.16
				28.00	99.11
		28.54	42.81	43.00	100.45
				42.00	98.11
				41.10	96.01

Table 5. Repeatability results for determining AOA of EGCG

Number of sample	AOA of EGCG, mmol-eq./L
1	14.30
2	14.25
3	14.00
4	14.80
5	14.50
6	14.35
Mean, mmol-eq./L	14.37
SD	0.26771
Confidence interval, (P=95%), mmol-eq./L	0.28

Table 6. Intermediate precision results for determining AOA of EGCG

Number of samples	EGCG	
	The first day	The second day
1	14.35	14.65
2	14.27	14.15
3	14.50	14.17
4	14.20	14.20
5	14.60	14.30
6	14.10	14.45
Mean, mmol-eq./L	14.37	14.32
SD	0.18726	0.19596
Confidence interval, (P=95%), mmol-eq./L	0.20	0.21
RSD, %	1.31	1.37
Mean of RSD, %	1.34	

Figure 1. Dependence of the potential of the platinum electrode EZDO 5010 in sodium phosphate buffer pH 7.4, containing various concentrations $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$

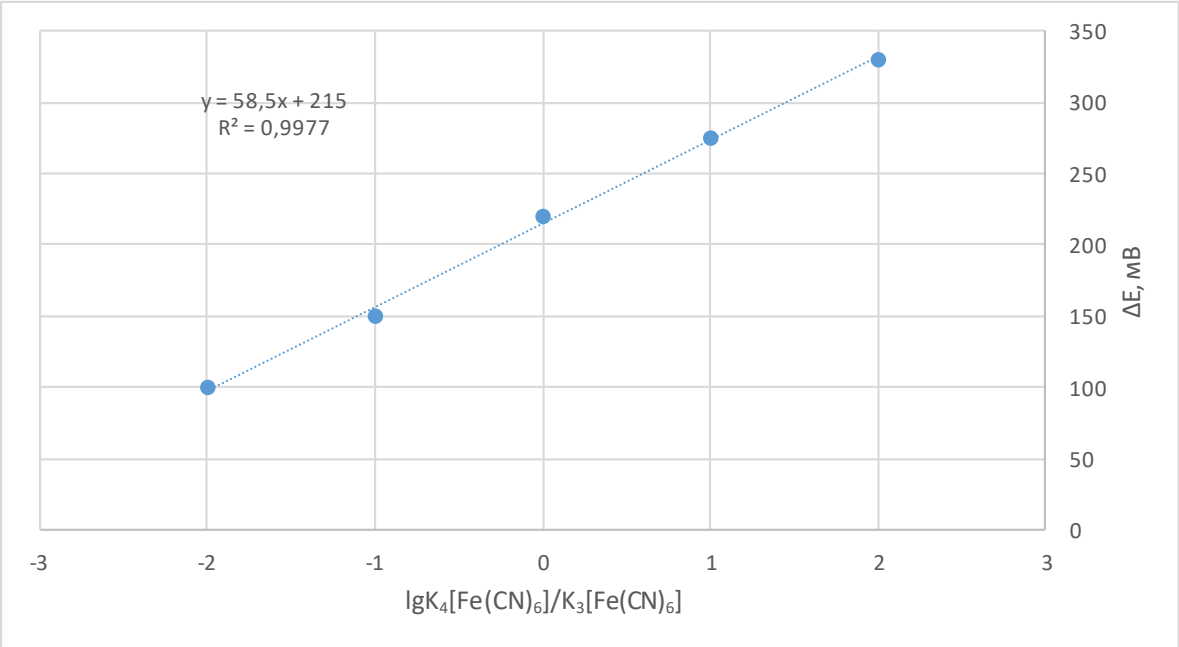

Figure 2. Calibration curve of the concentration logarithm *vs* AOA of EGCG