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ARTICLES

Voltammetric Evaluation of Polyphenol–Protein Interactions and Their Influence on the Antioxidant Capacity of Tea

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Abstract—A voltammetric method for evaluating polyphenol–protein interactions was developed based on the oxidation of polyphenols at an electrode modified with multi-walled carbon nanotubes and electropolymerized quercetin. Tea polyphenols (catechin and epigallocatechin gallate) and milk proteins (casein, bovine serum albumin, and β-lactoglobulin) were considered as model systems. It was found that proteins bind the test polyphenols (from 10 to 60%) with increasing protein content (from 1 : 0.25 to 1 : 1.5, by volume) in the mixture by decreasing the fraction of free polyphenol. The developed approach was used to assess the effect of milk on the antioxidant properties of tea (green, white, oolong, and black teas), which were expressed through the antioxidant capacity (AOC) parameter. A statistically significant decrease in the AOC of tea by a factor of 1.8–23 in the presence of 20 vol % milk was demonstrated.

Keywords: voltammetry, chemically modified electrodes, electropolymerization, polyphenols, antioxidant capacity, food analysis

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Food products containing a wide range of biologically active substances of various natures, which have mutual effects, are the main source of polyphenols in the human diet; these mutual effects can change the properties of individual compounds, including their bioavailability and bioaccessibility. Thus, the direct interaction of polyphenols with lipids, polysaccharides, and matrix proteins is possible, the latter being the most significant because it noticeably affects the antioxidant properties of phenolic compounds [1, 2]. Obviously, the development of methods for the assessment of polyphenol–protein interactions is of practical interest. Among proteins, milk proteins should be distinguished because the most common sources of polyphenols are tea and coffee, to which milk is often added when consumed.

Spectroscopic and colorimetric methods, mass spectrometry, and microscopy are used to study polyphenol–protein interactions [3]. In recent years, increasing attention has been paid to in-silico methods, which make it possible to identify active centers and binding sites of molecules using bioinformatics and also to predict their biological activity [3, 4].

Polyphenols easily enter into electron-transfer reactions, and this fact provides an opportunity to use electroanalytical methods for solving the problem. Thus, methods for the coulometric evaluation of polyphenol–protein interactions by the reactions of polyphenols with electrogenerated hexacyanoferrate(III) ions [5] and bromine [6] have been developed. In this case, the hexacyanoferrate(III) ion as a soft single-electron titrant–oxidizing agent is preferable because it is selective to phenolic compounds. Electrogenerated bromine interacts with a wide range of antioxidants and milk proteins and requires a preliminary assessment of their contribution to the experimental results.

Voltammetry has not found wide application to the assessment of polyphenol–protein interactions. Milk proteins do not exhibit electrochemical activity in the range of potentials characteristic of the electrooxidation of polyphenols; therefore, the oxidation currents of the test polyphenols could be used as an analytical signal. However, only one example of the use of cyclic voltammetry with a glassy carbon electrode (GCE), which made it possible to establish the effect of milk on tea and coffee polyphenols, was described by Kilmartin and Hsu [7]. With consideration of the shape of voltammograms, the charge passed through solution on potential scanning from –0.1 to 0.4 V was used for the calculations.

For the voltammetric determination of natural polyphenols (gallic acid, catechin, and epigallocatechin gallate (EGCG)) and the evaluation of the AOC of tea, Ziyatdinova et al. [8] proposed a GCE modified with multi-walled carbon nanotubes (MWCNTs) and electropolymerized quercetin (polyquercetin/

MWCNT/GCE) to increase the sensitivity to analyte responses.

In this work, we demonstrated the applicability of the polyquercetin/MWCNT/GCE to the assessment of polyphenol–protein interactions and their effect on the AOC of tea.

EXPERIMENTAL

Reagents and solutions. We used 98% quercetin dihydrate, 98% catechin hydrate, and 80% EGCG (Sigma, Germany) and prepared their standard solutions (10.0 mM quercetin and 3.00 mg/mL of catechin or EGCG) by dissolving their accurately weighed portions in 5.0 mL of rectified ethanol. More dilute solutions were prepared immediately before measurements. The standard solutions of β-lactoglobulin (Sigma, Germany), β-casein from cow's milk, and bovine serum albumin (BSA) (Fluka, Germany) with concentrations of 3.0, 3.0, and 0.5 mg/mL, respectively, were prepared by dissolving accurately weighed portions in distilled water. The mixtures of polyphenols with milk proteins were prepared based on volume ratios. The other reagents were of chemically pure grade.

Electrode modification. The working surface of a GCE (surface area, 3.14 mm^2) was renewed mechanically by polishing with alumina with a particle size of 0.05 μm. Then, the electrode was rinsed with acetone and distilled water. To modify the electrode surface, we used MWCNTs with an external diameter of 40–60 nm, an internal diameter of 5–10 nm, and a length of 0.5–500 μm (Aldrich, Germany). Their homogeneous suspension (0.5 mg/mL) in a 1% solution of sodium dodecyl sulfate was obtained by ultrasonic dispersion for 30 min. The working electrode was modified by the drop evaporation of 2 μL of the suspension of MWCNTs.

The polyquercetin layer was obtained from a 1.0 mM solution of quercetin under conditions of potentiodynamic electrolysis from –0.1 to 1.0 V at a potential scan rate of 100 mV/s in a 0.1 M solution of NaOH [8].

Voltammetric measurements. The voltammetric measurements were performed on an Autolab PGSTAT 12 potentiostat/galvanostat with the GPES 4.9.005 software (Eco Chemie BV, the Netherlands) in a three-electrode cell containing a polyquercetin/MWCNT/GCE working electrode, a saturated silver–silver chloride reference electrode, and an auxiliary platinum electrode. For calculations in voltammetry, baseline correction was applied using the GPES 4.9.005 software. A supporting electrolyte (a phosphate buffer solution with pH 7.0) and the aliquot portions of the standard solutions of polyphenols (10–750 μL) were introduced into the electrochemical cell, and differential pulse voltammograms were recorded in a range from –0.2 to 0.8 V at a pulse amplitude of 50 mV, a pulse time of 50 ms, and a potential scan rate of 10 mV/s. To assess the effect of proteins, the concentration of polyphenols in the cell was 30 μg/mL, and the fraction of proteins was varied.

The AOC of tea was estimated as described previously [8]: a 250-μL sample was introduced into the electrochemical cell, and differential pulse voltammograms were recorded in a range from –0.2 to 0.8 V at a pulse amplitude of 50 mV, a pulse time of 50 ms, and a potential scan rate of 10 mV/s. The AOC of tea was expressed in EGCG equivalents in 100 mL of the beverage.

An Ekspert-001 pH meter (OOO Ekoniks-Ekspert, Russia) was used to determine the pH of the supporting electrolyte.

Sample preparation. Tea was prepared according to a standard procedure: a weighed portion of tea (2.0000 ± 0.0001 g) was filled with 200 mL of boiled water and infused for 5 min; then, it was filtered, and the solution was used for further studies. Pasteurized cow's milk of 3.2% fat content was used without preliminary sample preparation.

Statistical processing. The statistical processing of the results was carried out for five measurements at a confidence level of 0.95. The results were presented as $X \pm \Delta X$, where X is the average value and ΔX is the confidence interval. Random determination error was estimated based on the relative standard deviation (RSD). Regression analysis was performed using the OriginPro 8.0 software (OriginLab, the United States).

RESULTS AND DISCUSSION

The main polyphenols of tea are catechins; therefore, catechin and EGCG were chosen as model compounds. It was shown that catechin and EGCG are oxidized on polyquercetin/MWNT/GCE at 0.18 and 0.540 V (Fig. 1a) and at 0.084, 0.180, and 0.590 V (Fig. 1b), respectively. The oxidation of catechin and EGCG to the corresponding *o*-quinones in ring B proceeds with the participation of two electrons and two protons [9–11]. The difference in the oxidation potentials at the first step for catechin and EGCG is due to the presence of a gallate fragment in the B ring of the EGCG molecule, which is more easily oxidized than the catechol fragment in the structure of catechin [7].

Both of the analytes exhibited the linear concentration dependence of oxidation peak areas. With consideration for the shape of the voltammograms of EGCG, we used the total oxidation peak areas at 0.084 and 0.180 V for quantification. The analytical ranges were 0.0290–2.90 and 2.90–72.6 μg/mL for catechin (Eqs. (1) and (2)) and 0.0458–4.58 and 4.58–45.8 μg/mL for EGCG (Eqs. (3) and (4)), and the detection limits were 0.0070 and 0.0064 μg/mL, respectively.

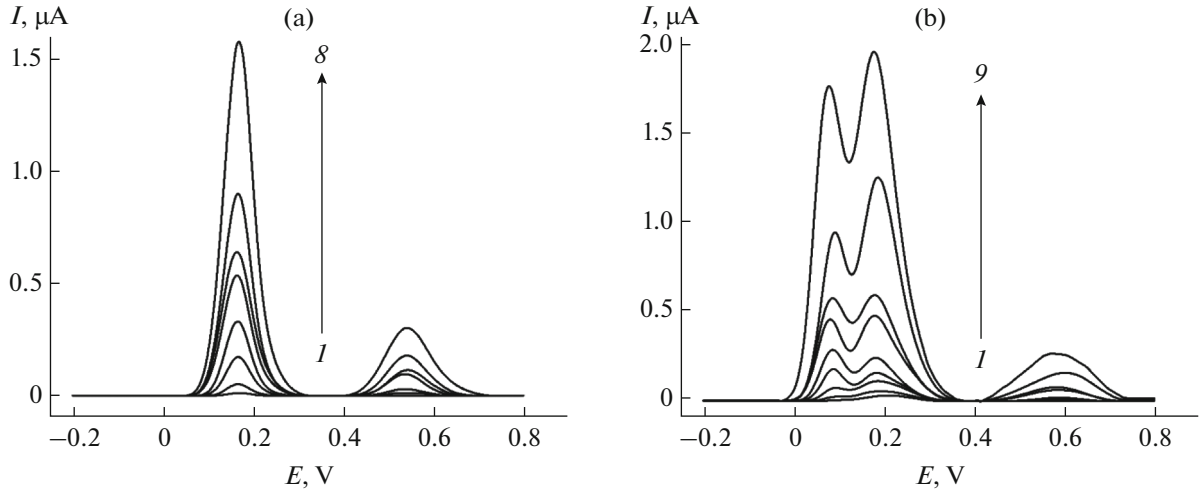

Fig. 1. Differential pulse voltammograms with baseline correction for (a) (*1*) 0.0290, (*2*) 0.290, (*3*) 1.45, (*4*) 2.90, (*5*) 7.26, (*6*) 14.5, (*7*) 21.8, and (*8*) 29.0 μg/mL of catechin and (b) (*1*) 0.0458, (*2*) 0.229, (*3*) 1.15, (*4*) 2.29, (*5*) 4.58, (*6*) 11.5, (*7*) 22.9, (*8*) 34.4, and (*9*) 45.8 μg/mL of EGCG on polyquercetin/MWCNT/GCE in a phosphate buffer solution with pH 7.0. Pulse amplitude, 50 mV; pulse time, 50 ms; and potential scan rate, 10 mV/s.

$$S = (7 \pm 2)\times 10^{-10} + (8.4 \pm 0.2)\times 10^{-9} c_{\text{catechin}}\ (\mu\text{g/mL}), \quad (1)$$
$$R^2 = 0.9986,$$

$$S = (1.93 \pm 0.07)\times 10^{-8} + (2.50 \pm 0.02)\times 10^{-9} c_{\text{catechin}}\ (\mu\text{g/mL}), \quad (2)$$
$$R^2 = 0.9996,$$

$$S = (-2 \pm 3)\times 10^{-10} + (1.41 \pm 0.01)\times 10^{-8} c_{\text{EGCG}}\ (\mu\text{g/mL}), \quad (3)$$
$$R^2 = 0.9995,$$

$$S = (2.8 \pm 0.6)\times 10^{-8} + (6.5 \pm 0.2)\times 10^{-9} c_{\text{EGCG}}\ (\mu\text{g/mL}), \quad (4)$$
$$R^2 = 0.9964.$$

To assess the effect of proteins on polyphenols, we examined model systems consisting of the test catechins and milk proteins (β-casein, BSA, and β-lactoglobulin). The fractions of proteins in the mixtures were varied from 0.25 to 1.5. In this case, the voltammograms of the mixtures exhibited a marked decrease in oxidation peaks with the retention of the shapes of curves (Fig. 2) for all of the test proteins. It was found that 1 min was sufficient for the binding of polyphenols by proteins; therefore, the measurements were carried out 1 min after the addition of proteins. The fraction of free polyphenol in a mixture is a quantitative characteristic of polyphenol–protein interactions. It was established that the concentration of accessible polyphenol decreased as the fraction of protein in the mixture was increased (Fig. 3). The degree of binding of catechin and EGCG to milk proteins decreased in the order β-casein > BSA > β-lactoglobulin, and EGCG was bound to a greater extent than catechin due to the presence of gallate fragments in its structure. The experimental results are consistent with the previously described data for quercetin and rutin [5]. It is well known [2] that polyphenols can interact with proteins due to covalent or intermolecular bonds. Covalent interaction is characteristic of tannins [2]. Noncovalent binding occurs due to the formation of hydrogen bonds (between the carbonyl group of a peptide bond and the hydroxyl groups of polyphenols) and hydrophobic interactions due to the aromatic rings of polyphenol molecules and the aliphatic and aromatic fragments of proteins [12, 13]. The main role in hydrophobic interactions is assigned to the amino acid residues of proline, histidine, arginine, phenylalanine, tryptophan, lysine, cysteine, and methionine in the protein structure. The mechanism of binding depends on the protein structure, and the size and stoichiometry of the resulting aggregates depend on the concentrations of polyphenols and proteins and their ratio [14].

The study of the mixtures of EGCG and catechin with milk showed that the fractions of free catechin and EGCG sharply decreased to 21 ± 1 and 30 ± 1%, respectively, even at a polyphenol to milk ratio of 1 : 0.2. It should be noted that milk is electrochemically inactive under the conditions of a voltammetric experiment.

The developed approach was applied to assess the effect of milk proteins and milk on the antioxidant properties of tea, which is a source of natural polyphenols in the human diet. AOC was used as a parameter

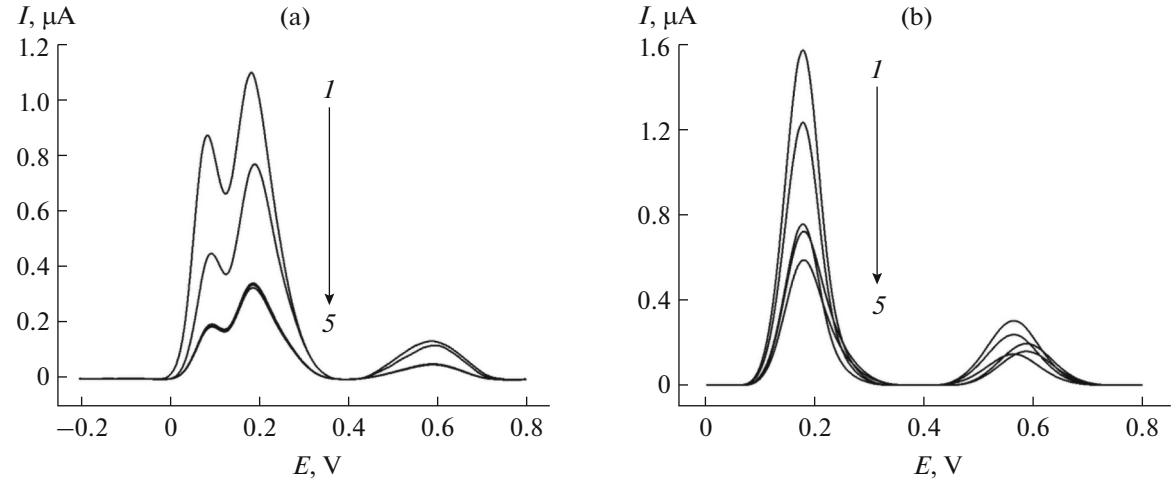

Fig. 2. Typical differential pulse voltammograms with baseline correction for (a) epigallocatechin gallate and (b) catechin in the presence of milk proteins (on example of β-casein) on polyquercetin/MWCNT/GCE in a phosphate buffer solution with pH 7.0. Protein fractions in the mixtures: (*1*) 0, (*2*) 0.25, (*3*) 0.5, (*4*) 1.0, and (*5*) 1.5. Pulse amplitude, 50 mV; pulse time, 50 ms; and potential scan rate, 10 mV/s.

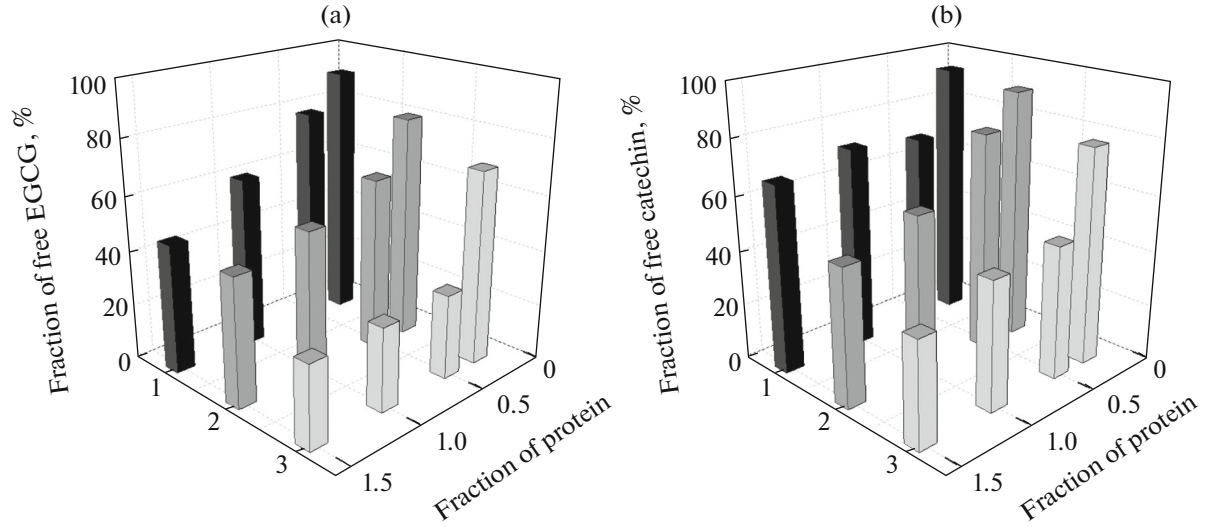

Fig. 3. Fractions of (a) free epigallocatechin gallate and (b) catechin in the presence of (*1*) β-lactoglobulin, (*2*) bovine serum albumin, and (*3*) β-casein depending on the protein fraction in the mixture.

reflecting the bioaccessibility of polyphenols in the presence of milk proteins and milk.

With the use of green and black teas as an example, we found that milk proteins bind their polyphenols to cause a statistically significant decrease in their AOC for all of the test proteins (Table 1). β-Casein and BSA exhibited the highest binding capacity for black and green teas, respectively. It is likely that this was due to the oxidation of black tea catechins by polyphenol oxidase at the stage of fermentation with the formation of theaflavins and thearubigins [15], which are more hydrophobic and bind to proteins mainly due to hydrophobic interactions [2]. Green tea catechins interact with proteins to a greater extent due to hydrogen bonds [16, 17]. In general, the AOC of tea is statistically significantly decreased in the presence of proteins (by factors of 1.6–2.3 and 1.1–1.3 for black and green teas, respectively, even at a 5% protein content of the mixture).

The voltammograms of tea with milk exhibited a gradual decrease in the oxidation currents of tea and the splitting of an oxidation peak at 0.18–0.20 V with the appearance of a shoulder at 0.23–0.25 V as the fraction of milk in the mixture was increased (Fig. 4).

Table 1. Antioxidant capacity of tea in the presence of milk proteins according to voltammetry on polyquercetin/MWCNT/GCE in a phosphate buffer solution with pH 7.0 ($n = 5$, $P = 0.95$)

Protein fraction in the mixture	AOC of tea in the presence of proteins, (mg EGCG)/100 mL					
	β-casein	RSD, %	BSA	RSD, %	β-lactoglobulin	RSD, %
			Green tea			
0	58 ± 1	2	58 ± 1	5	58 ± 1	2
0.05	48 ± 1	2	45.8 ± 0.7	1	53.2 ± 0.8	1
0.1	42.6 ± 0.4	0.7	46.4 ± 0.7	1	45.3 ± 0.8	1
0.2	24 ± 1	4	42.3 ± 0.7	1	38 ± 1	3
0.3	18.0 ± 0.3	2	37.9 ± 0.9	2	29.0 ± 0.8	2
			Black tea			
0	11.3 ± 0.2	1	11.3 ± 0.2	1	11.3 ± 0.2	1
0.05	6.9 ± 0.2	1	4.9 ± 0.2	4	5.9 ± 0.1	2
0.1	5.1 ± 0.3	4	3.5 ± 0.1	3	4.4 ± 0.3	6
0.2	4.0 ± 0.1	5	3.5 ± 0.1	3	4.0 ± 0.2	4
0.3	3.75 ± 0.09	5	3.2 ± 0.3	5	3.70 ± 0.06	1

It was found that the AOC of tea statistically significantly decreased in the presence of milk regardless of the type of tea (Fig. 5), and the main part of polyphenols was bound at a 5% milk content of the mixture. A further increase in the fraction of milk led to a gradual decrease in the AOC of tea. The difference was statistically insignificant for some samples, probably, due to their characteristics. The experimental results are in good agreement with the data obtained by galvanostatic coulometry [5, 6].

The experimental studies showed that the voltammetric approach developed for assessing polyphenol–protein interactions based on the electrooxidation of tea polyphenols at an electrode modified with polyquercetin is a simple and rapid method, which can be considered as an alternative to currently available methods for the evaluation of polyphenol–protein interactions to be used in food analysis.

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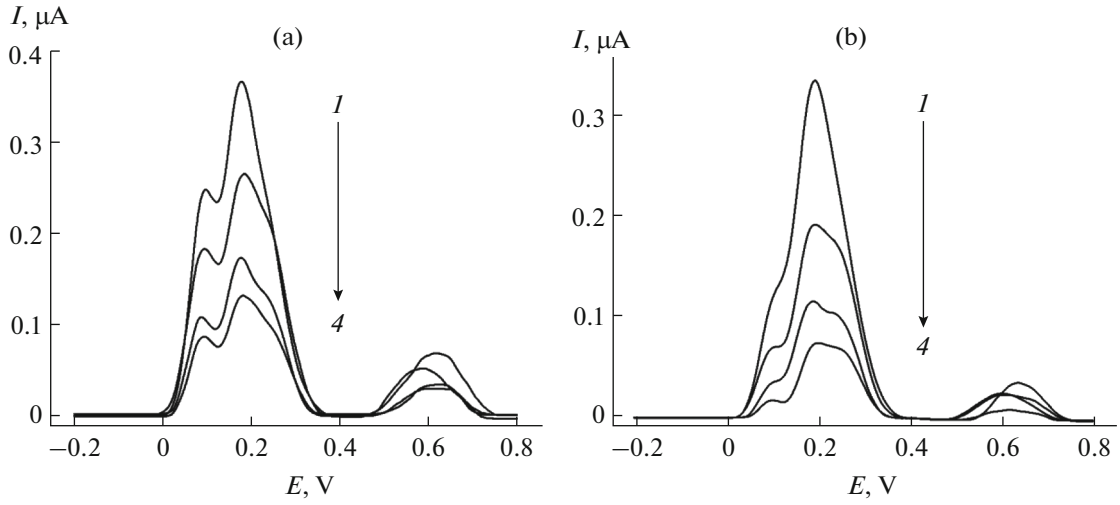

Fig. 4. Typical differential pulse voltammograms with baseline correction for (a) green and (b) black teas in the presence of milk on polyquercetin/MWCNT/GCE in a phosphate buffer solution with pH 7.0. Milk fractions: (*1*) 0, (*2*) 0.05, (*3*) 0.1, and (*4*) 0.2. Pulse amplitude, 50 mV; pulse time, 50 ms; and potential scan rate, 10 mV/s.

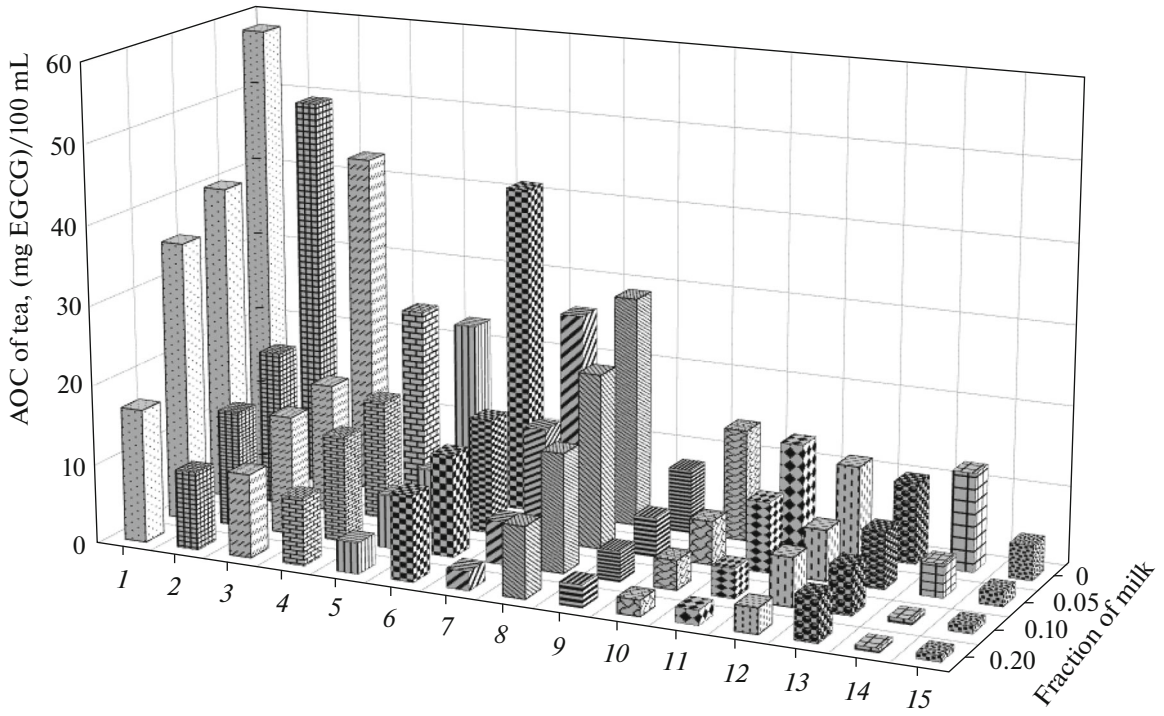

Fig. 5. Effect of milk on the antioxidant capacity of (*1–5*) green, (*6, 7*) white, (*8, 9*) oolong, and (*10–15*) black teas according to voltammetric data on polyquercetin/MWCNT/GCE in a phosphate buffer solution with pH 7.0.

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