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Square wave voltammetry based on determination of copper (II) ions by polyluteolin- and polykaempferol-modified electrodes

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ABSTRACT

Applicability of square wave voltammetry for the determination of Cu(II) ions by PolyLut/GC and PolyKae/GC electrodes was evaluated in this study. For this luteolin and kaempferol were electrochemically polymerized on glassy carbon (GC) electrode surface in order to get polyluteolin and polykaempferol-modified glassy carbon electrodes (PolyLut/GC and PolyKae/GC, correspondingly). The formation of polyphenol layer on the GC electrode surface was evidenced by atomic force microscopy. Square wave voltammetry was found to be more sensitive in comparison with differential pulse voltammetry. It was determined that PolyLut/GC and PolyKae/GC electrodes offered great sensitivity towards Cu(II) ions with very low limit of detection, good reproducibility, sufficient stability and excellent selectivity of analytical signal.

1. Introduction

Within the last few years, the number of researches related to modification of the carbon materials has significantly increased [1,2]. Especially high attention has been paid to application of glassy carbon. There are many reasons for such interest since glassy carbon (GC) is inert, has good conductivity and high resistance towards environmental factors and aggressive chemical agents [3]. For this reason differently modified carbon electrodes have been used as a support substrate with a wide potential window suitable for bioanalytical purposes in catalytic biosensors [4–6], immunosensors [7,8], biofuel cells [9,10] and chemical sensors based on complex formation [11–14]. The studies on the modification of carbon electrodes by a wide variety of organic compounds have been reported [11,12,15–17].

The polyphenolic compounds (e.g. flavonoids) have an increasing interest due to their potentially positive curing effect against certain diseases. Some of them are electroactive compounds and are very abundant for human life e.g. luteolin and kaempferol [18,19]. However there are just few scientific reports about the electrochemical investigation of polyphenols especially related to investigation of their oxidative properties on solid electrode surfaces and to their possible analytical applications [20–22]. Differently from other flavonoids more detailed electrochemical investigations of quercetin were performed [23–26].

Recently rapid industrialization and urbanization have led to the contamination of air, soil and water by heavy metal ions and other chemical compounds based on heavy metals. Usually they are toxic and non-degradable [27]. For this reason the evaluation of heavy metal concentration is of special interest and it has attracted considerable attention. Up to now, the electrochemical determination of Cu(II) ions by differently modified electrodes has been realized by some other research groups and the best results have been reported by Freire and Kubota with the limit of detection (LOD) of 1.8×10^{-14} M for Cu(II) detection on gold electrode modified with a self-assembled monolayer of 3-mercaptopropionic acid [28]. In addition to this another sensor with low LOD has been reported by Yang et al. [29]. Reported sensor has been created by covalent attachment of tripeptide Gly-Gly-His to the self-assembled monolayer based on 3-mercaptopropionic acid formed on the gold electrode. This Cu(II) ion sensor has detection limit lower than 0.2×10^{-12} M [30].

Owing the importance of the determination of Cu(II) ions and lack of the studies related to analytical application of electropolymerized luteolin and kaempferol we developed electroanalytical system based on solid surfaces modified by these compounds. Major objectives of this work were: (i) development of GC electrodes electrochemically modified by polyluteolin (PolyLut/GC)

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and polykaempferol (PolyKae/GC), (ii) characterization of PolyLut/GC and PolyKae/GC electrodes (iii) application of PolyLut/GC and PolyKae/GC electrodes coupled to square wave voltammetry (SWV) for the electrochemical determination of Cu(II) ions.

2. Materials and methods

2.1. Chemicals and instruments

Kaempferol (96%) and luteolin (98%) were purchased from Sigma (St. Louis, USA). The other chemicals needed for electrochemical experiments were purchased from Merck, Riedel and Sigma–Aldrich companies and were of reagent grade.

The 1.0 mM solutions of flavonoids were prepared in 100 mM phosphate buffer solution (PBS), pH 7.5. The PBS was prepared by mixing of K_2HPO_4, KH_2PO_4 and KCl followed by adjusting of pH by addition of KOH or H_3PO_4. $CuSO_4$ solutions were prepared at different concentrations (ranging from 1.0×10^{-6} until 1.0×10^{-11} M) in Britton–Robinson (BR) buffer solution, pH 5.0, which was prepared by mixing of H_3BO_3, H_3PO_4, CH_3COOH and KCl and then adjusting of pH by addition of HCl or NaOH. All chemicals for preparation of buffer solutions were of extra pure grade.

A traditional three-electrode cell system was used in all electrochemical experiments. The GC electrode Model MF-2012 obtained from BAS (Kenilworth Warwickshire, United Kingdom) was used as working electrode with a geometric area of 0.071 cm^2. An Ag/AgCl in saturated KCl ($Ag/AgCl/KCl_{sat}$) electrode was used in aqueous media as reference electrode while platinum wire was used as counter electrode. All experiments were carried out inside a Faraday cage at room temperature (25 °C). All solutions used in the voltammetric system were deaerated with argon before electrochemical treatment (99.999%) at least for 5 min. The electrode modification step was performed by cyclic voltammetry (CV) technique applied with PHE 200 software and the application step of the modified electrode surfaces were performed by SWV technique applied with PV 220 software, which is suited for control of Potentiostat/Galvanostat Gamry Reference PCI4/750 from Gamry Instruments (Warminster, USA). The bare and modified GC electrode surfaces were characterized by atomic force microscope (AFM) Multimode II from VEECO Instruments (Santa Barbara, USA) by using tapping mode in air atmosphere with antimony n-doped Si cantilever. The UV–vis spectrophotometer with a double beam was used (UV-1800, Shimadzu, Kyoto, Japan) for spectroscopic detection of the interactions between metal (Cu(II)), polyphenol (luteolin or kaempferol) and chelating agent (ethylenediamine tetraacetic acid).

In order to prove analytical results obtained by here reported electrochemical sensors the Cu(II) ion determination by flame atomic absorption spectrometry (AAS) was chosen as reference method which was performed by AAS (Unicam 929, Cambridge, UK).

2.2. Electrode preparation, modification and characterization procedures

All pretreatment steps mentioned in the published scientific reports were performed before using the GC electrodes in electrochemical experiments in order to avoid contamination by oxidation products and to obtain a clean electrode surface [11–14].

The polished and cleaned GC electrodes were modified by potential cycling in 1.0 mM solutions of flavonoids (luteolin and kaempferol) dissolved in 100 mM PBS, pH 7.5. Electrochemical modification of the GC electrode by flavonoids was performed by 30 potential cycles in the potential range between +100 mV and +900 mV at the scan rate of 100 mV/s. After the modification of the GC electrode, the surfaces of obtained luteolin-modified GC (PolyLut/GC) and kaempferol-modified GC (PolyKae/GC) electrodes were rinsed in water in order to remove all soluble compounds from the electrode surface.

The surface morphology of electrodes was characterized by atomic force microscope (AFM) Multimode II from VEECO Instruments (Santa Barbara, USA) using tapping mode in air atmosphere with antimony n-doped Si cantilever. For all AFM images the used particular cantilevers were 110–140 μm length with a resonance frequency of 230–410 kHz and with spring constant of 20–80 N/m.

2.3. Application of polyphenol-modified GC electrodes

The interaction of Cu(II) ions with PolyLut/GC and PolyKae/GC electrodes was performed in order to approve the formation of polyluteolin and polykaempferol layers on the GC electrode surface. For this aim, the PolyLut/GC and PolyKae/GC electrodes were immersed into solutions with varying concentrations of Cu(II) ions for 1 h at open circuit in order to form Cu/PolyLut/GC and Cu/PolyKae/GC electrodes, respectively. Cu(II) ions on Cu/PolyLut/GC and Cu/PolyKae/GC electrodes were reduced at −900 mV for 1 minute in BR buffer, pH 5.0. The SWV technique was applied for the determination of Cu(II) ions by Cu/PolyLut/GC and Cu/PolyKae/GC electrodes in BR buffer, pH 5.0. A Gamry Reference PCI4/750 potentiostat controlled by PV 220 software was applied for electrochemical measurements. The potential was swept from −400 mV to +200 mV at frequency of 50 Hz, amplitude of 40 mV and a step potential of 5 mV. The equilibration period of 15 s at −400 mV was applied before starting of SWV experiment. Peak potentials and peak currents corresponding to the solutions including various concentrations of Cu(II) ions were calculated by evaluation of square wave voltammograms.

The effect of the interfering ions as Cd(II), Fe(II), Pb(II), Al(III) and Mg(II) on Cu(II) determination was also investigated. For this aim, PolyLut/GC and PolyKae/GC electrodes were treated with 1.0×10^{-6} M interfering ion solution containing 1.0×10^{-8} M of Cu(II) ions for 1 h. The analyte solutions were prepared in BR buffer, pH 5.0.

The polyphenol-modified carbon electrodes were used for the determination of Cu(II) ions in water-based sample. The sample was taken from the tap and tested without any pretreatment to investigate the applicability of PolyLut/GC and PolyKae/GC electrodes in real samples. In order to provide optimum conditions, the pH of the sample was adjusted to pH 5.0 with the addition of diluted acid or base solutions. All procedures for electrochemical determination of Cu(II) ions as it is described above were performed and the results were evaluated. The measurements were repeated for five times in order to calculate statistical values. Concentration of metal ions in the sample was evaluated using calibration curves obtained with known Cu(II) ion concentrations. Flame AAS was applied as reference method.

2.4. Spectrophotometric experiments

The UV–vis spectra were recorded in the range of 220–550 nm with a double beam spectrophotometer model UV-1800, Shimadzu (Kyoto, Japan) using quartz cuvettes (1.0 cm optical path) at room temperature in order to investigate the effect of the addition of ethylenediamine tetraacetic acid (EDTA) to the relationship between polyphenols and Cu(II)ions. The absorbance spectra of 25 μM of luteolin or kaempferol dissolved in PBS, pH 7.5 were registered before any treatment. Then the spectra were recorded after addition of 50 μM of $CuSO_4$ dissolved in BR buffer, pH 5.0. In addition to this, the effect of chelating agent on the interaction between polyphenols and Cu(II) was investigated by the addition of 100 μM of EDTA. Registered spectra were analyzed in order to evaluate the

regeneration of Cu/PolyLut/GC and Cu/PolyKae/GC electrodes and suitability of them for continuous electrochemical measurements.

2.5. The repeatability, reproducibility and stability experiments

In order to check the repeatability of Cu/PolyLut/GC and Cu/PolyKae/GC electrodes, the electrochemical determination of Cu(II) ions was investigated after regeneration of Cu/PolyLut/GC and Cu/PolyKae/GC electrode surfaces with EDTA solution. After each detection/regeneration cycle, electrochemical responses of these electrodes *vs* 1.0×10^{-11} M Cu(II) ions were investigated.

The reproducibility of PolyLut/GC and PolyKae/GC electrodes in electrochemical determination of Cu(II) ions was investigated with 10 different electrodes. Electrochemical responses of each electrode *vs* 1.0×10^{-11} M Cu(II) ions were investigated by SWV as described above. All electrodes were prepared freshly just before their usage for Cu(II) detection.

The stability of PolyLut/GC and PolyKae/GC electrodes were also investigated and for this aim three different control experiments were performed. In the first group of these experiments, the electrochemical responses of PolyLut/GC and PolyKae/GC electrodes were investigated after scanning within "negative" (between 0 and −1.0 V) and "positive" (between 0 and +1.0 V) potential regions by applying of 10 potential sweep cycles in BR buffer, pH 5.0. In the second group, PolyLut/GC and PolyKae/GC electrodes were kept in air, water and BR buffer, pH 5.0, from 1 to 24 h. In the third group, PolyLut/GC and PolyKae/GC electrodes were kept in the room temperature in BR buffer, pH 5.0, for 10, 20, 30 and 40 days. After all these treatments, the ability of the treated modified electrodes in the electrochemical determination of 1.0×10^{-8} M Cu(II) were investigated.

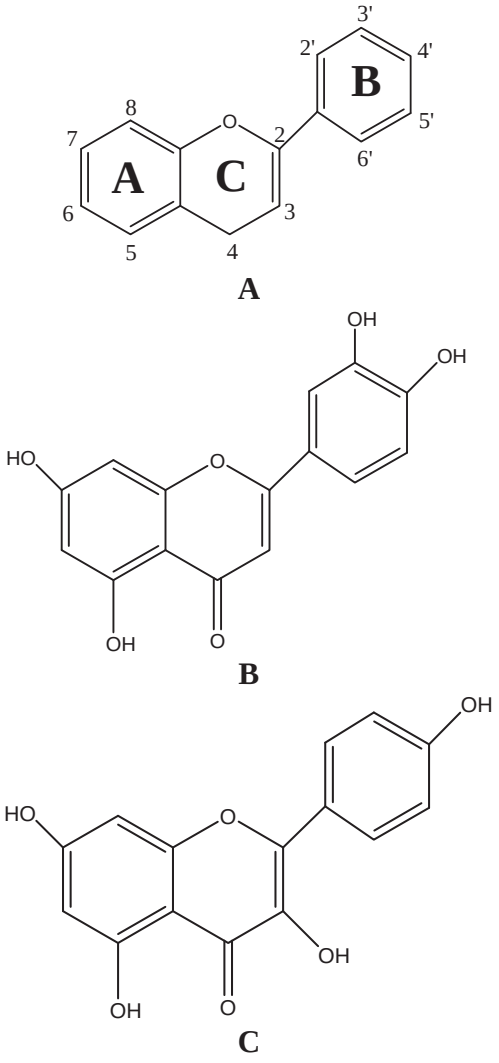
3. Results and discussion

In this study, an application of polyphenol-modified GC electrodes for the detection of Cu(II) ions was investigated. The Cu(II)-ion preconcentration in the form of complexes of Cu(II) with polymerized luteolin and polymerized kaempferol was realized in order to get electrodes suitable for Cu(II) determination. In our previous work we have demonstrated applicability of differential pulse voltammetry for the determination of Cu(II) ions. It has been determined that both polyphenol-modified electrodes showed sensitivity towards Cu(II) with good reproducibility and stability of analytical signal. To improve sensitivity and selectivity in present work square wave voltammetry was applied and it was found more sensitive in comparison with differential pulse voltammetry based detection. Additionally, the evaluation of stability, repeatability, reproducibility, and morphology of modified electrodes was performed.

3.1. GC electrode surface modification

Luteolin and kaempferol selected for this study have the same empirical formula but they have one hydroxyl group at different position as it is presented in Scheme 1.

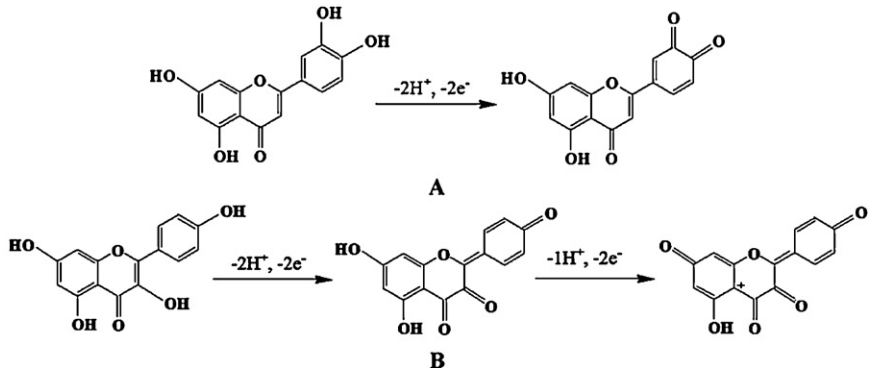
The cyclic voltammograms of luteolin have shown that luteolin on the bare GC electrode exhibited electrochemically irreversible oxidation peak at lower positive potentials (E_{pa} = +475 mV), which is attributed to the oxidation of the 3′-hydroxyl and 4′-hydroxyl groups in the B-ring of luteolin [13]. The formation of quinonic structure by oxidation of luteolin is in agreement with formation of *o*-quinonic structure during oxidation of quercetin as it was reported in some studies based on: experimental and theoretical investigations of quercetin in PBS, pH 4.0 performed by rotating disk electrode [23], electrochemical oxidation of quercetin in hydro-alcoholic solution on the GC electrode [24], formation



Scheme 1. Structures of used flavonoids: (A) general structure; (B) luteolin and (C) kaempferol.

of *o*-quinone during quercetin oxidation on the wax-impregnated graphite electrode [26], electrochemical oxidation of quercetin in the presence of benzenesulfinic acid on the GC electrode [31], investigation of quercetin in the presence of differently charged anionic and/or cationic surfactants [32], investigation of quercetin with organized molecular assemblies of non-ionic surfactant [33] and formation of *o*-quinone during quercetin oxidation on the wax-impregnated graphite electrode [26]. According to our calculations and results presented in references listed above it has been thought that the oxidation of luteolin could be described as two-electron and two-proton electrode reaction (Scheme 2A).

The cyclic voltammograms of kaempferol have shown that kaempferol on the bare GC had two irreversible oxidation peaks (at E_{pa1} = +390 mV, E_{pa2} = +710 mV) [13]. It has been thought that the oxidation peak at +390 mV is attributed to the oxidation of 4′-hydroxyl group in the B-ring and to 3-hydroxyl group in the C-ring, while the second oxidation peak is attributed to oxidation of 7-hydroxyl group in the A-ring. The presence of the second peak in the cyclic voltammograms of kaempferol could be explained by oxidation/reduction ability of 3-hydroxyl group localized in the B-ring and of 7-hydroxyl group in the A-ring of kaempferol. Another reason for the oxidation of 7-hydroxyl group in the A-ring of kaempferol differently from luteolin could be the formation of radicalic species by an electron extraction from A- and C-ring



Scheme 2. Oxidation mechanisms proposed for (A) luteolin and (B) kaempferol.

of kaempferol and a leakage of hydrogen from 7-hydroxyl group present in the A-ring by turning to the oxidized form of 3-hydroxyl group in the C-ring of kaempferol, which then could be easily rearranged into corresponding quinonic form. Here described oxidation process takes place more efficiently for kaempferol in comparison with that for luteolin. According to comments presented in this section and experimental results, the proposed oxidation process for kaempferol is proposed in Scheme 2B.

According to Scheme 2B, the oxidation process of kaempferol includes two major steps: one of them is based on loss of two protons and two electrons; another one is based on loss of one proton and two electrons; while luteolin's irreversible oxidation includes only one step based on loss of two protons and two electrons. The loss of one proton together with two electrons in the second step of kaempferol oxidation caused the formation of cationic structure. This is the main difference in electrochemical oxidation of luteolin and kaempferol. The formation of cationic structure is a result of transfer of one proton together with two electrons, as it was reported by Ghidouche et al. [34] where enzymatic oxidation of some flavonols has been investigated. The authors have showed the enzymatic oxidation of polyphenols to be first promoted by the formation of the radical on the oxygen at C3 position of the C-ring, which was formed by H abstraction. In addition to this the same authors have shown that subsequent reactions with solvent or with another substrate molecule were initiated by a dismutation reaction of two radicals, leading to the formation of a carbocation having positive charge localized at C2 atom of the C-ring. The authors have speculated that formation of carbocation during oxidation of phenolic species is of great importance and it will help to understand better biosynthesis of phenolic oligomers and polymers in plants [34]. On this basis it could be predicted that high polymerization yield, which was achieved in our study, was influenced by the formation of carbocations during the oxidation of kaempferol.

In our previous study related to the formation of the electropolymerized polyluteolin and polykaempferol layers on the GC electrode surface it has been reported that the irreversible peak current decreased with an increasing number of the potential cycles and after some cycles it reached the steady-state conditions [13]. This gradual decrease in the peak current by potential cycling could have been attributed to the formation of the polyluteolin or polykaempferol layer. We thought that the electrochemical oxidation of both polyphenols induced polymer formation on the GC electrode [35–37].

3.2. Characterization of polyphenol-modified GC electrodes

The PolyLut/GC and PolyKae/GC electrodes have been characterized by CV and electrochemical impedance spectroscopy (EIS) technique in the presence of redox probes, ellipsometry and scanning electron microscopy (SEM) [13]. Experimental characterization results by CV technique have indicated that the blockage of oxidation/reduction of redox probe on the PolyLut/GC and PolyKae/GC electrodes was a reliable indication for the modification of GC electrode surface by corresponding polyphenolic species. Ellipsometric measurements have shown that considerable thickness of polymeric layer increased with the concentration of luteolin or kaempferol and it is an important proof for the formation of polyluteolin and polykaempferol layers. The morphology of the surfaces has been investigated by SEM and characteristic features in the images have approved the formation of polyphenol layers on the surface of GC electrode [13].

In addition to these characterization methods, the presence of polymerized layers was assessed by AFM in this study. Fig. 1A and B present three-dimensional images of PolyLut/GC and PolyKae/GC electrode surfaces. The morphology of both polyphenols are different from the bare GC surface and also from each other. The surface of the polykaempferol layer on GC electrode is more uniform and the size of characteristic surface features is significantly lower. These results are in agreement with the ellipsometric measurements and with SEM results presented in our previous study [13].

3.3. Electrochemical characterization of complex formation ability of polyphenol-modified GC electrode:

Electrochemical sensing has been proven as an inexpensive and simple analytical method with remarkable detection, sensitivity, selectivity and reproducibility. Therefore high attention is currently being paid to the development of modified electrodes suitable for determination of trace-elements [1,11–14,38,39]. In order to show analytical applicability of polyphenol–modified GC electrodes and to provide some more evidences on the formation of polyluteolin- and polykaempferol-based layers, the ability of polyluteolin and polykaempferol to form complexes with Cu(II) ions was investigated. For this aim, modified electrodes were prepared as it is described in Section 2.2. It is known that the pH and the concentration of the solution and the incubation period are important parameters in the formation of metal ion based complexes. Therefore the pH of Cu(II) solution and the incubation time of PolyLut/GC and/or PolyKae/GC electrodes were optimized. For this purpose, solutions containing 1.0×10^{-8} M of Cu(II) ions were prepared in BR buffer at different pH values ranging from 3.0 to 9.0 and the modified electrodes were incubated in these Cu(II) solutions. The accumulation of Cu(II) ions on the modified electrodes was performed at open circuit. The pH region over 9.0 was not evaluated since it is known that the pH values, which exceeded pH 11.0, were not suitable for the determination of Cu(II) ions due to the formation of insoluble hydroxide compounds of Cu(II) ions [13]. The BR buffer, pH 5.0, was found as the most suitable solvent for the

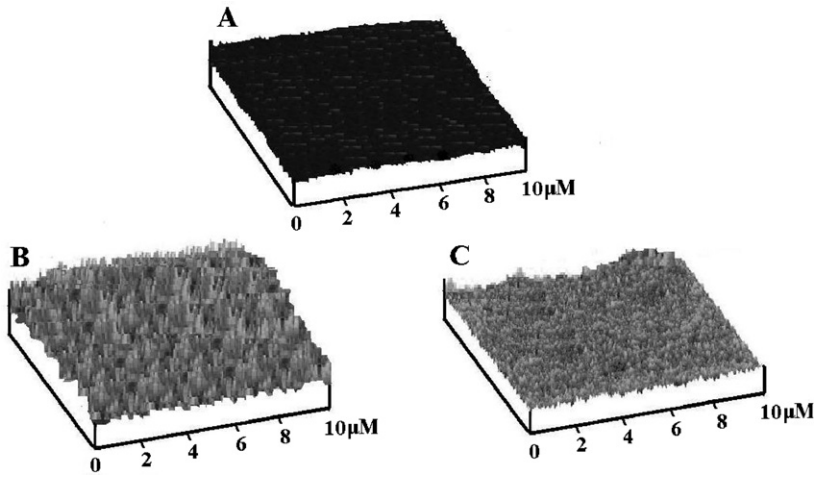


Fig. 1. Three dimensional AFM images (1.0 μm × 1.0 μm) of bare GC (A), PolyLut/GC, (B) and PolyKae/GC (C) electrodes.

determination of Cu(II) ions. To determine the optimal incubation period, the PolyLut/GC or PolyKae/GC electrodes were incubated in BR buffer, pH 5.0, containing Cu(II) ions for different periods: 10; 20; 40; 60; 80 and 100 min. It was observed that SWV signals increased hyperbolically by increase of incubation period but they became steady-state only in the cases if incubation period exceeded 60 min. Moreover even after 20 min of the incubation analytical signals become meaningful even at low Cu(II) ion concentrations. As a result of here presented examinations, the most suitable conditions for the formation of Cu(II) complex on PolyLut/GC or PolyKae/GC electrodes were found in BR buffer, pH 5.0, when PolyLut/GC or PolyKae/GC electrodes were incubated for 60 min. The SWV technique was used in order to investigate Cu(II)-based complex formation. The square wave voltammograms of PolyLut/GC and PolyKae/GC electrodes after incubation in solutions with different Cu(II) ion concentration are presented in Fig. 2.

The SWV investigations were performed and the points of the peak potentials and the current values at these potentials for PolyLut/GC and PolyKae/GC electrodes were evaluated. As it is seen from Fig. 2, the peak-currents obtained by PolyLut/GC and PolyKae/GC electrodes are different from that obtained by unmodified GC electrode. It should be noted that the responses of the bare GC electrode registered by SWV are negligible and they remain at the constant level for any Cu(II) concentration (Fig. 2, curve a). In contrary PolyLut/GC (Fig. 2, curve b) and PolyKae/GC (Fig. 2, curve c) electrodes showed high sensitivity to the Cu(II) ions in broad concentration range and even at very low concentration level (up to 1.0×10^{-11} M). The formation of Cu(II) ion-based complexes was not observed on the bare GC electrode surface, thus it confirms that polyluteolin and/or polykaempferol layer is crucial for the formation of Cu(II)-based complexes.

The sensitivity of PolyLut/GC and PolyKae/GC electrodes for Cu(II) ions was studied at different concentration of $CuSO_4$ ranging from 1.0×10^{-11} to 1.0×10^{-6} M. Square wave voltammograms of PolyLut/GC and PolyKae/GC electrodes for different concentrations of $CuSO_4$ are presented in Fig. 3B and C, respectively. Peak currents registered by SWV measurements increased by increasing of Cu(II)

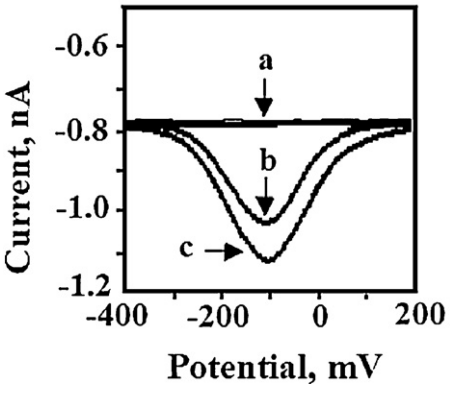


Fig. 2. Square wave voltammograms of: (a) bare GC, (b) PolyLut/GC and (c) PolyKae/GC electrodes, after 60 min incubation in solutions containing 1.0×10^{-11} M M of Cu(II) ions. Measurements were performed in BR buffer, pH 5.0, all potentials are presented *vs* $Ag/AgCl/KCl_{sat}$.

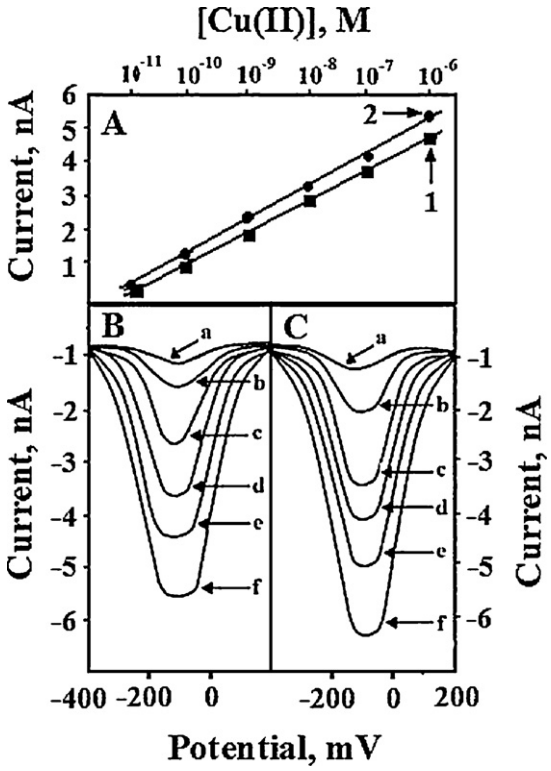


Fig. 3. The linear relationship between the peak current of square wave voltammograms and Cu(II) concentration for (1) PolyLut/GC and (2) PolyKae/GC electrodes (A). Square wave voltammograms of Cu/PolyLut/GC (B) and Cu/PolyKae/GC (C) electrodes at different concentrations of $CuSO_4$: (a) 1.0×10^{-11} M–(f) 1.0×10^{-6} M. Experiments were performed in BR buffer, pH 5.0, potentials are referred *vs* $Ag/AgCl/KCl_{sat}$.

ion concentration (Fig. 3A). The value of the peak current is linearly dependent on the concentration logarithm of Cu(II) ions in the concentration range of 1.0×10^{-11} to 1.0×10^{-6} M. The data were approximated by linear regression and correlation coefficient of SWV current responses for PolyLut/GC and PolyKae/GC electrodes were found to be 0.9927 and 0.9934, respectively.

Limits of detection (LOD) were calculated as low as 0.6×10^{-11} M for the PolyLut/GC and 0.3×10^{-11} M for the PolyKae/GC electrodes and the differences between polyluteolin- and polykaempferol-based electrodes were observed which are also recorded with electrochemical and spectroscopic characterization techniques. The PolyKae/GC electrode showed higher analytical signal in the presence of the same concentration of Cu(II) ions if compared with analytical signals obtained by PolyLut/GC electrode. This can be related to the antioxidant activity of the both polyphenols. The antioxidant activity of kaempferol is higher than that of luteolin [40]. The antioxidant activity of flavonoids is related to their ability to coordinate metal ions, several studies are found in literature regarding the formation of metal complexes of flavonoids, mainly with Cu(II) and Fe(III) ions [30,41]. So, our results on complexation of Cu(II) ions are in well agreement with antioxidant activity of both polyphenols. The interaction of Cu(II) ions with PolyLut/GC and PolyKae/GC electrodes one more time approved the presence of electrochemically formed polyphenolic layers.

Both PolyLut/GC and PolyKae/GC electrodes evaluated in our study showed better sensitivity towards Cu(II) ions than the electrode surfaces reported by some other researchers, e.g.: the LOD of 4.0×10^{-7} M was reported by Niu et al. by gold electrode modified with a self-assembled monolayer of penicillamine using cyclic voltammetry [42]; the LOD of 5.0×10^{-9} M was reported by Betelu et al. by 4-carboxyphenyl-grafted screen-printed electrode using SWV [39]; the LOD of 1.0×10^{-10} M using stripping techniques was reported by Zeng et al. [43] by gold electrode modified with N-acetyl-L-cysteine and the LOD of 1.0×10^{-11} M was reported by Oztekin et al. by polyphenol-modified GC electrode using differential pulse voltammetry technique [13].

Regeneration protocol of PolyLut/GC and PolyKae/GC electrodes suitable for multiple detection of Cu(II) was developed. This protocol was based on the application of EDTA solution during the washing process of Cu/PolyLut/GC and Cu/PolyKae/GC electrodes, since the EDTA is an agent forming complex with Cu(II) ions. In order to study the repeatability of detection/regeneration cycles of Cu/PolyLut/GC and Cu/PolyKae/GC electrodes, the UV–vis spectroscopy technique in the range from 220 nm to 550 nm was applied in the presence of dissolved luteolin and kaempferol. The absorption spectra of both flavonoids have two maximums: one in the UV region (at approximately 270 nm) usually denoted as a band II and the other one in Vis region (between 380 and 420 nm) usually denoted as a band I. Band I and band II are related to optical absorption of B-ring and A-ring, respectively (Fig. 4A and B, curve a). It was observed that after addition of $CuSO_4$ to the cuvette containing luteolin or kaempferol, the absorbance of luteolin and kaempferol shifted from 268 nm and 272 nm to 305 nm and 320 nm, respectively (Fig. 4A and B, curve b). The effect of stepwise increments of $CuSO_4$ concentration on the spectral characteristics of luteolin and kaempferol is described in terms of shifts of absorbance peaks. However, the shifts recorded with the addition of $CuSO_4$ solution missed and the original absorption spectra of luteolin and kaempferol were recovered with the addition of EDTA (Fig. 4A and B, curve c).

Here presented and discussed spectrophotometric investigations confirmed that the Cu/PolyLut/GC and Cu/PolyKae/GC electrodes can be regenerated back to the PolyLut/GC and PolyKae/GC by treatment with EDTA. The repeatability of detection of 1.0×10^{-11} M of Cu(II) was investigated by SWV for five analysis and no significant changes in analytical signal were detected. The variation coefficient for five measurements was 4.4% and 2.7% for luteolin and kaempferol, respectively.

The reproducibility of 1.0×10^{-11} M Cu(II) ion detection was investigated by SWV for 10 measurements and no significant changes in analytical signal were detected. The calculated standard deviation for PolyLut/GC and PolyKae/GC electrodes illustrated good reproducibility of proposed analytical systems. The variation coefficient for 10 measurements was 4.3% and 2.3% for PolyLut/GC and PolyKae/GC electrodes, respectively.

For several flavonoids, e.g. quercetin, taxifolin, catechin and mircetin, possessing a catechol group, which is present at C3′ and C4′ position, has been thought to be suitable for the binding of metal ions. However it is absent in kaempferol molecule. Our previously published electrochemical and spectroscopical investigations have proved that the binding places for the Cu(II) ions are neither 3′-hydroxyl nor 4′-hydroxyl groups [13]. We are predicting that the Cu(II) ions bounded to 5-hydroxyl group located in the A-ring and 4-carbonyl group in the C-ring that are present in both: luteolin and kaempferol. The speculation on the position of interaction places of both polyphenols with Cu(II) ions is in accordance with the results evaluated in some other studies [44–46]. This suggestion is also confirmed by: (i) changes in band II, which is related to the absorption of A-ring and (ii) stable band I, which is related to the absorption spectra of B-ring (Fig. 4A and B) that were observed after incubation in solution containing Cu(II) ions. Here discussed spectrophotometric results were supported by electrochemical investigations, which were presented and evaluated in Section 3.1.

However, the stripping analysis of Cu(II) can be affected by interfering ions among heavy metal ions. In this study, five different metal ions including Cd(II), Fe(II), Pb(II), Al(III) and Mg(II) were chosen as interferences for the investigation of selectivity of PolyLut/GC and PolyKae/GC electrodes. The Cd(II), Fe(II), Pb(II), Al(III) and Mg(II) ions were selected as interfering ions because: (i) cadmium ions can form intermetallic complexes with copper and because of this the Cd(II) has relatively severely interference to Cu(II) stripping analysis; (ii) iron (in its Fe(II) form) is a very strong reducing agent in neutral to basic pH conditions and reduced copper from aqueous media; (iii) lead can occupy coordination sites that are available on the electrode surface and can decrease the peak current induced by Cu(II) ions. Additionally two more widespread metal ions as Al(III) and Mg(II) were selected in order to evaluate analytical applicability of PolyLut/GC and PolyKae/GC electrodes. The square wave voltammograms after incubation in solution of Cu(II) with Cd(II), Fe(II), Pb(II), Al(III) or Mg(II) ions were compared (Figs. 5 and 6). The square wave voltammograms of Cu (II) ions and interfering ions (Cd(II), Fe(II) or Pb(II)) registered with PolyLut/GC (Fig. 5A–C) and PolyKae/GC (Fig. 5D–F) electrodes were evaluated. Both (PolyLut/GC and PolyKae/GC) electrodes showed great selectivity towards Cu(II) ions even in the presence of 100-fold higher concentration of Cd(II) and Pb(II) ions. However in our previous study which was performed with PolyLut/GC and PolyKae/GC electrodes a small peak appears at +90 mV in the presence of 1.0×10^{-6} M of Pb(II) ions [13]. On the other hand, it was observed that analytical signal was affected by interfering effect of Fe(II) ions and the peak current attributed to Cu(II) has decreased. However, the decrease was not found to be very significant, for this reason it is thought that the interfering effect of Fe(II) can be prevented by addition of masking agents. The presence of Al(III) and Mg(II) ions in the sample at relatively low extent negatively effected analytical signals generated by square wave voltammetry for both polyphenol modified electrodes (Fig. 6A–D). The stripping peak current attributed to copper significantly decreased only in the cases if concentrations of Al(III) or Mg(II) exceeded Cu(II) concentration by more than 100-folds. In addition to registered decrease some

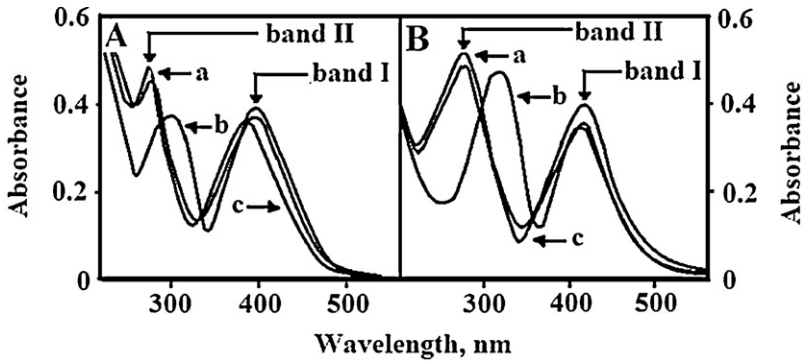


Fig. 4. Absorption spectra of luteolin (A) and kaempferol (B): (a) 25 μM of luteolin or kaempferol, (b) mixture of 25 μM of luteolin or kaempferol and 50 μM of Cu(II), (c) mixture of 25 μM of luteolin or kaempferol, 50 μM of Cu(II) and 100 μM of EDTA.

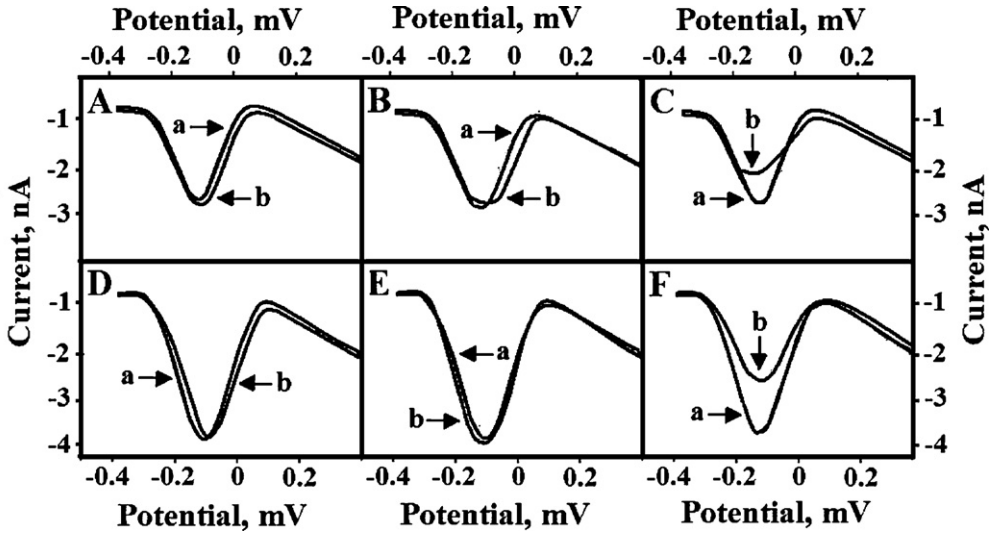


Fig. 5. Evaluation of the influence of Cd(II) (A and D), Pb(II) (B and E) and Fe(II) (C and F) on the SWV signal attributed to Cu(II), which were registered by PolyLut/GC (A–C) and PolyKae/GC (D–F) electrodes. (a) 1.0×10^{-8} M Cu(II), (b) 1.0×10^{-8} M of Cu(II) + 1.0×10^{-6} M of Cd(II), Pb(II) or Fe(II).

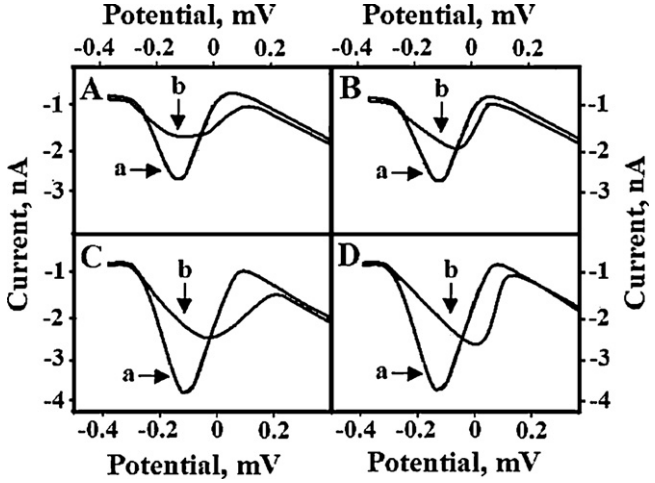


Fig. 6. Evaluation of the influence of Al(III) (A, C) and Mg(II) (B and D) on the SWV signal attributed to Cu(II), which were registered by PolyLut/GC (A and B) and PolyKae/GC (C and D) electrodes. (a) 1.0×10^{-8} M Cu(II), (b) 1.0×10^{-8} M of Cu(II) + 1.0×10^{-6} M of Al(III) or Mg(II).

shifts of stripping peak potentials were observed in the presence of Al(III) and Mg(II) ions. The shifts by 7.3% and 5.7% in the potential scale in the presence of Al(III) and by 10.4% and 9.2% in the presence of Mg(II) were observed for PolyLut/GC and PolyKae/GC electrodes, respectively. The decrease in the peak current was between 30 and 40% for both Al(III) and Mg(II). The reason for observed decrease of peak current could be attributed to the competition for complex-forming sites. As it is known that Mg(II) and Al(III) ions can easily form complexes *via* hydroxyl groups. Due to observed changes in the stripping voltammograms it is thought that after the formation of complexes with Al(III) and Mg(II) ions, some hydroxyl groups (e.g.: 5-hydroxyl group in the A-ring) become blocked. Therefore the sensitivity of the electrodes for Cu(II) would decrease. Moreover, the changes in square wave voltammograms in obtained after incubation of modified electrodes in samples containing Cu(II) and Al(III) were different from that in obtained after incubation in samples containing Cu(II) and Mg(II). Observed differences are related to the lower complexation ability of Mg(II) ions in comparison with Al(III) ions. Clear separation of the peak attributed to Cu(II) in the presence of most common interfering ions as (Cd(II), Fe(II) or Pb(II)) offers the possibility for the sensitive and selective determination of Cu(II) ions. Therefore we could conclude that electrodes modified by polyphenol layer show excellent selectivity towards Cu(II) even in the presence of interfering ions.

Furthermore, the stability of PolyLut/GC and PolyKae/GC electrodes was evaluated. For this aim three different control experiments were performed. In the first group of these experiments, the electrochemical responses of PolyLut/GC and PolyKae/GC electrodes *vs* Cu(II) were recorded after potential sweep in "negative" and "positive" potential regions as it is indicated in Section 2.5.

Any significant differences in square wave voltammograms in the range of −400 to +200 mV (Fig. 2, curves b and c) were observed after Cu(II) ion detection by stripping at "negative" and "positive" potential regions. From these results it can be observed that the modified electrodes showed stability towards treatment by negative and positive potentials. In the second group of experiments, the stability of PolyLut/GC and PolyKae/GC electrodes was evaluated by keeping of modified electrodes in air, distilled water and BR buffer, pH 5.0, each treatment was lasted from 1 to 24 h. After this treatment, electrochemical responses of these electrodes *vs* 1.0×10^{-8} M Cu(II) were investigated by SWV. It was seen that the modified electrodes showed good stability when they were kept in BR buffer, pH 5.0, even for 24 h period, while they did not show the similar or even closer stability if stored in air and distilled water. It was also observed that the PolyLut/GC and PolyKae/GC electrodes lost all their sensitivities towards Cu(II) ions within 16 h after keeping them in air, while they lost whole sensitivity after 24 h if they were kept in distilled water. Since PolyLut/GC and PolyKae/GC electrodes showed good stability in BR buffer, pH 5.0, the long term stability experiments were performed with keeping them at room temperature in BR buffer, pH 5.0, for 10, 20, 30 and 40 days, and their ability for the electrochemical determination of Cu(II) ions was investigated by SWV. It was observed that after 10 days the electrochemical responses of PolyLut/GC and PolyKae/GC electrodes towards Cu(II) ions decreased by 8.2% and 7.1%; after 20 days by 14.1% and 11.6%; after 30 days by 21.8% and 18.7%; after 40 days 30.4% and 25.6%, respectively. These results illustrated that the electrodes prepared by electrochemical polymerization of polyphenols showed sufficient long term stability if stored in BR buffer, pH 5.0.

AAS was selected as reference method to prove results obtained by SWV. The statistical evaluation of electrochemical and spectroscopic determinations showed acceptable precision of both PolyLut/GC and PolyKae/GC electrodes. The determination of Cu(II) ions in tap water, which contains high concentrations of interfering ions, was performed in order to investigate practical applicability of both polyphenol-modified electrodes. The concentration of Cu(II) ions in tap water was calculated to be $3.9 \pm 0.5 \times 10^{-7}$ and $5.4 \pm 0.3 \times 10^{-7}$ M by SWV using PolyLut/GC and PolyKae/GC electrodes, respectively. These results were proved by AAS and detected concentration of Cu(II) was $4.8 \pm 0.2 \times 10^{-7}$ M. As it is seen, the results obtained by SWV technique using PolyLut/GC and PolyKae/GC electrodes are in well agreement with that obtained by AAS.

4. Conclusions and future developments

The applicability of PolyLut/GC and/or PolyKae/GC electrodes for the determination of Cu(II) ions with the LOD of 0.6×10^{-11} and 0.3×10^{-11} M, respectively was demonstrated in this study. Effective complexation of Cu(II) ions with flavonoids was observed. The PolyKae/GC electrode showed higher sensitivity to Cu(II) ions in comparison with other electrochemical Cu(II) ion detection systems reported up to date. Evaluation of presented data let to some future improvements of proposed analytical systems, e.g. by reduction of determination period. This is possible because here evaluated analytical systems posses high sensitivity and it is not necessary to work in the equilibrium time domain, therefore shorter Cu(II) pre-concentration periods could have been applied. Future our work in this direction will be related to the investigation of complexation ability of PolyLut/GC and/or PolyKae/GC with some biological compounds including cholesterol and to the application of this complexation ability for bioanalytical purposes.

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