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Electrochemical Behaviour of Some Chlorogenic Acids and Their Characterization in Coffee by Square-Wave Voltammetry

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The electrochemical behaviour of six chlorogenic acids (CGAs) isomers: three caffeoylquinic acids, CQAs (5-CQA, 4-CQA, 3-CQA) and three dicaffeoylquinic acids, diCQAs (3,4-diCQA, 3,5-diCQA, 4,5-CQA) was studied by SWV and compared with the electrochemical behaviour of caffeic acid (CFA), precursor of investigated CGAs. The study reveals that electrochemical behaviour of investigated CGAs strongly dependent on their chemical structure and electronic properties, particularly on electron-donating effect of –OH and –CH=CH- groups, and a strong electron-withdrawing effect of ester (-COOR) group presented in their structure. SWV measurements show that electrochemical oxidation/reduction of CGAs at GCE is reversible, pH-dependent, two electron-two proton process. The oxidation/reduction processes occurred on the catechol moiety (*ortho*-dihydroxyl groups) in the structure of CGAs molecules. The oxidation product(s) relatively strongly adsorb on the GCE surface especially at higher concentration of CGAs. The electrochemical behaviour of 5-CQA was investigate more in detail. It was observed that anodic peak current of 5-CQA show maximum in solution of pH 7, due to the maximum concentration of 5-CQA$^-$ anion at this pH value. The electrode reaction proceeded in solution of 5-CQAs is diffusion controlled process. The anodic peak current of 5-CQA shows linear relationship with the concentration in the range of 5-50 μmol L^{-1}, with low LOD (7.7×10^{-7} mol L^{-1}). SWV of coffee samples were also carried out, and the results show that electrochemical behaviour of coffee samples is very similar to that of investigated CGAs. Therefore, SWV was used for characterization of CGAs in coffee. It was shown that SWV is a very sensitive and selective method for determination of total CGAs content in coffee.

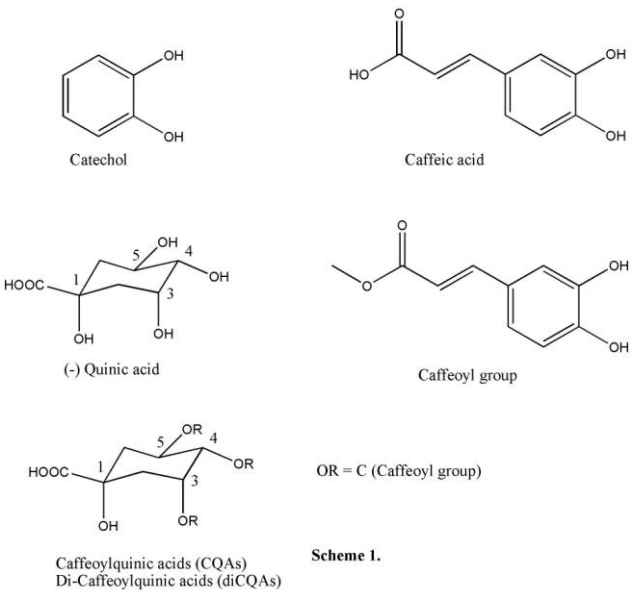
Keywords: Electrochemical behaviour, Chlorogenic acids, Square-wave voltammetry, Characterization, Coffee

1. INTRODUCTION

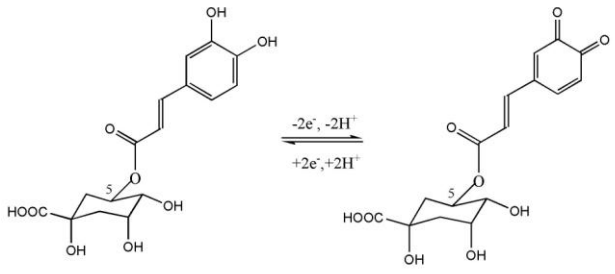
Chlorogenic acids (CGAs) are group of polyphenolic compounds widely distributed in different plant materials including many foods and beverages [1]. Chemically, CGAs are a family of different

esters (mono-, di-, tri- and mixed esters) formed between (-)-quinic acid and certain *trans*-cinnamic acids, most commonly caffeic, ferulic and *p*-coumaric acids. They can be subdivided into more subgroups according to the type of ester substituent (e.g., caffeoylquinic acids, CQAs; feruloylquinic acids, FQAs; *p*-coumaroylquinic acids, *p*-CoQAs, etc.) and the number of ester substituent (monoacylquinic acids, diacylquinic acids, or triacylquinic acids) [2].

A wide range of biological, pharmacological and physiological activities of CGAs including antioxidant, anti-inflammatory, antitumor and antibacterial activities have been reported [3,4]. CGAs are one of the most important dietary polyphenols in the human diet and coffee is considered to be the major source of dietary CGAs intake in the humans. Coffee contain a number of different CGAs, for example in green coffee bean about 70 isomers were detected, while in the roasted bean increases to over 200 derivates, as was reported by Kuhnert group [2] from tandem mass spectrometry (MS) experiments. But, according to many published HPLC analysis [5-8] nine main CGAs prevailed in most of investigated coffee samples: three isomers of caffeolquinic acids (CQAs), three isomers of dicaffeolquinic acids (diCQAs) and three isomers of feruloylquinic acids (FQAs). All other CGAs are presented in minor or very minor amount. CQAs were the most abundant CGAs in coffee and 5-*O*-caffeolquinic acid (5-CQA) always dominated in the content (see Scheme 1. and Table 1, -chemical structures and nomenclature of CQAs, diCQAs, their precursor caffeic and quinic acid, caffeoyl group and catechol).



Scheme 1.



Scheme 2.

Table 1. Structural pattern of electroanalyzed chlorogenic acids (CGAs)

No.	Name	Abbreviation	R^3	R^4	R^5
1	3-*O*-Caffeoylquinic acid	3-CQA	C	H	H
2	4-*O*-Caffeoylquinic acid	4-CQA	H	C	H
3	5-*O*-Caffeoylquinic acid	5-CQA	H	H	C
4	3,4-Di-*O*-caffeoylquinic acid	3,4-diCQA	C	C	H
5	3,5-Di-*O*-caffeoylquinic acid	3,5-diCQA	C	H	C
6	4,5-Di-*O*-caffeoylquinic acid	4,5-diCQA	H	C	C

In many papers have been reported that biological activities of polyphenolic compounds were in very close connection with their electrochemical properties [9-11]. Thus, it seems that the processes which proceeded during the antiradical/antioxidant activity of polyphenols are very similar or the same, as processes observed during the electrochemical oxidation/reduction of these compounds. Therefore, to understand the mechanism of antioxidant/antiradical activity of CGAs their electrochemical behaviour must be investigated.

Electrochemical behaviour of CGAs was rarely investigated and in the literature only reports on electrochemistry of chlorogenic acid were found (in these investigations term chlorogenic acid probably means 5-CQA -name for chlorogenic acid according to IUPAC nomenclature [2], see Scheme 1, although in the literature 5-CQA is still often called 3-CQA, pre-IUPAC numbering). The results of investigations of Namazian & Zare [12], Yardim [13], and Ziyatdinova et al. [14], show that electrochemical oxidation of chlorogenic acid is probably reversible process, but still leave some doubts regarding the mechanism of electrochemical oxidation of chlorogenic acid. Electrochemical behaviour of other CGAs including 3-CQA, 4-CQA and three isomers of diCQAs (major CGAs presented in coffee, see Scheme 1.) were not investigated until now according to the literature data.

Different electroanalytical methods were used for characterization (determination) of total CGAs content in coffee samples, mostly by different electrochemical sensors or biosensors using SWV [15-17], and DPV [18] as methods of analysis. Adsorptive transfer stripping square-wave voltammetry (AdTSSWV) on boron-doped diamond electrode [13,19] and differential pulse voltammetry (DPV) [14] on multi-walled carbon nanotube-modified glassy carbon electrode (MWCNT-GCE) were also used for determination of total CGAs content in coffee. All these methods were shown as very good for quantification of total CGAs in coffee, but in all these papers it is not quite correct assignation of oxidation peak observed in voltammetry of coffee samples, which peak authors used for quantification. All authors assigned this peak only to oxidation of chlorogenic acid (probably 5-CQA), but it seems that oxidation process in coffee is more complex. Coffee contained different CGAs and therefore oxidation peak in coffee samples is composed of electrochemical oxidation of different CGAs, mostly from oxidation of CQAs and diCQAs (the main CGAs in coffee), as we will show in this study.

Therefore, the aims of this study were: (i) to investigate the electrochemical behaviour of six main chlorogenic acids contained in coffee (3-CQA, 4-CQA, 5-CQA, 3,4-diCQ, 3,5-diCQA and 4,5-

diCQA); (ii) to investigate the influence of chemical structure of these compounds on their electrochemical behaviour; (iii) to propose the possible mechanism of oxidative/reductive behaviour of 5-CQA, prevailed CGA in coffee (iv) to use the results of electrochemical behaviour of investigated CGAs (including 5-CQA as a standard) to developed SWV as a sensitive and selective electrochemical method for characterization (determination) of total CGAs content in different brands of coffee samples.

2. EXPERIMENTAL

2.1. Chemicals

Some chlorogenic acids, caffeoylquinic acids: 3-CQA (neochlorogenic acid), 4-CQA (cryptochlorogenic acid) and 5-CQA (chlorogenic acid), see Scheme 1, Table 1, and caffeic acid (CFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chlorogenic acids, dicaffeolyquinic acids: 3,5-diCQA (isochlorogenic acid A), 3,4-diCQA (isochlorogenic acid B) and 4,5-diCQA (isochlorogenic acid C), see Scheme 1, Table 1, were obtained from Chengdu Biopurify Phytochemicals Ltd. (Chengdu Sichuan, China). Methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). Sodium dihydrogen phosphate and disodium hydrogen phosphate (for preparation of supporting electrolytes, 0.1 mol L^{-1} phosphate buffer solutions, PBS, of different pH) were obtained from Kemika (Zagreb, Croatia). Alumina powder of 0.05 and 0.3 μm was purchased from Buehler (USA).

Stock solutions of all chlorogenic acids were prepared in methanol (HPLC grade) and stored at 4 ^{o}C. Working solutions of CGAs of different concentration and pH for SWV measurements were prepared by dilution of the appropriate quantity of stock solution in supporting electrolyte 0.1 mol L^{-1} PBS. Supporting electrolyte solutions (PBS of different pH) were prepared using analytical grade phosphate salts and purified water obtained from a Milipore Milli-Q purification system (conductivity $\leq$ 0.1 μS cm^{-1}).

2.2. Coffee samples

Ten different coffee samples (brands) widely consumed in Croatia, were chosen for investigation as follows: (i) two brands of green coffee beans (*Coffee Arabica cv*. Rio Minas harvested in Brazil and *Coffee Robusta cv*. Cherry harvested in India); (ii) two brands of roasted coffee beans produced by roasting of above mentioned *C. Arabica* and *C. Robusta* samples at 150-155 ^{o}C for 18 min; (iii) two brands of ground coffees (Franck Quatemala and Flatscher Olimpia, declared as 100 % Arabica coffee); (iv) four famous instant coffees brands (Nescafé Classic, Nescafé Espresso, Jacobs Monarch and Jacobs Intense). Green and roasted coffee beans were obtained by one of the leading coffee manufacturer company in Croatia, while ground and instant coffees samples were purchased from local supermarkets.

The coffee samples were submitted to an extraction (brewing) procedure prior to electrochemical analysis. Bean coffee samples were ground in a standard domestic coffee-grinder prior to coffee brews preparation, while all other coffee samples were used as purchased. To prepare the coffee extracts, 2 g of coffee was added to 200 mL of Milli-Q hot water of 80 ^{o}C in a 400 mL beaker, and stirred with magnetic stirrer for 3 min. After extraction the coffee brews were filtered, first through the filter paper and then through 0.45 μm polytetrafluoroethylene (PTFE) filters to remove any sediment. Such prepared coffee samples were used for determination of total CGAs content in coffee by SWV method.

2.3. Square-wave voltammetry (SWV)

SWV measurements were performed using an μAutolab potentiostat/galvanostat running with GPES software (Eco Chemie, Utrecht, Netherlands). Measurements were carried out in a standard three-electrode electrochemical cell (Metrohm, Switzerland). Glassy carbon electrode (GCE) of 3 mm diameter (model MF-2012, Bioanalytical Systems, USA) was the working electrode, Pt-wire electrode was the counter electrode and Ag/AgCl (3 mol L^{-1} KCl) electrode was reference electrode (both electrodes made by Metrohm, Switzerland).

The experimental conditions for SWV measurements were: pulse amplitude of 50 mV, frequency of 25-150 Hz and potential increment of 2 mV, corresponding to an effective scan rate of 50-300 mV s^{-1}.

Before each measurement, the surface of GC working electrode was carefully polished with 0.3-0.05 μm alumina powder and then thoroughly rinsed with Milli-Q water. After mechanical polishing, the GC electrode was cleaned electrochemically by cyclic voltammetry (in a potential range -0.2 to 1.0 V, with scan rate of 50 mV s^{-1}) in supporting electrolyte (0.1 mol L^{-1} PBS) until a steady-state cyclic voltammograms were obtained. This procedure ensured very reproducible experimental SWV results. All measurements were done (at least) in triplicate, at room temperature (298 K).

All the SWV voltammograms were analysed using two softwares: GPES software (Eco Chemie, Utrecht, Netherlands) and ECDSOFT software [20] to get the electrochemical parameters. There was not significantly difference in results obtained using two different software. The drawing of all Figures and statistical analysis of numerical results presented in Table 2 were done with OriginPro 7.5 (OriginLab Corporation, Northampton, USA) software programme. Chemical structures were drawing using ChemDraw® Ultra, version 8.0.3 software (CambridgeSoft Corp., Cambridge, USA).

3. RESULTS AND DISCUSSION

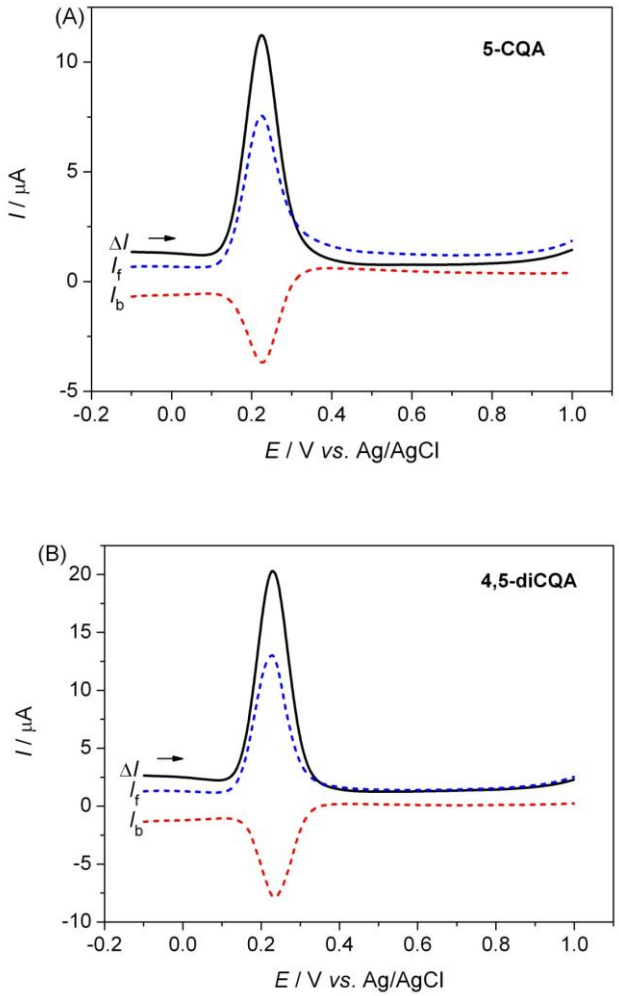
3.1. Electrochemical behaviour of CGAs

The electrochemical behaviour of some CGAs, three CQAs (5-CQA, 4-CQA and 3-CQA) and three diCQAs (3,4-diCQA, 3,5-diCQA and 4,5-diCQA), as the main CGAs presented in coffee, were investigated at GCE in different experimental conditions using SWV, over a wide pH range between

3.0 and 8.0 in 0.1 mol L^{-1} phosphate buffer solutions (PBS) as a supporting electrolyte. In addition, electrochemical study of caffeic acid (CFA) was also carried out in order to identify the redox active centres of investigated CQAs and diCQAs, because CFA is precursor of all these compounds.

3.1.1. Influence of chemical structure

Figure 1 shows square-wave voltamograms (SWVs) of $3x10^{-5}$ mol L^{-1} solutions of 5-CQA, 4,5-CQA and CFA in pH 7 0.1 mol L^{-1} PBS, where voltammograms of 5-CQA and 4,5-diCQA represented the electrochemical behaviour of other CQAs and diCQAs, respectively. All SWVs show one redox pair, i.e. one oxidation and one reduction peak. A sharp oxidation peaks at 0.223 V (5-CQA), 0.229 V (4,5-diCQA) and 0.191 V (CFA), and a sharp reduction peaks at 0.225 V (5-CQA), 0.233 V (4,5-diCQA) and 0.191 V (CFA) were observed. The facts that the forward, I_f (oxidative) and backward, I_b (reductive) components of the net current response (ΔI) of all SWVs are very well developed, and the oxidation/reduction peaks occurred at the same (or practically the same) potential values indicates that electrode reactions proceeded on GCE are reversible [21]. This was confirmed by reversing the scan direction. If the starting potential is 0.8 V and the scan direction is negative, the forward component is reductive current, but the net response at pH 7 is almost identical to the one shown in Fig. 1.



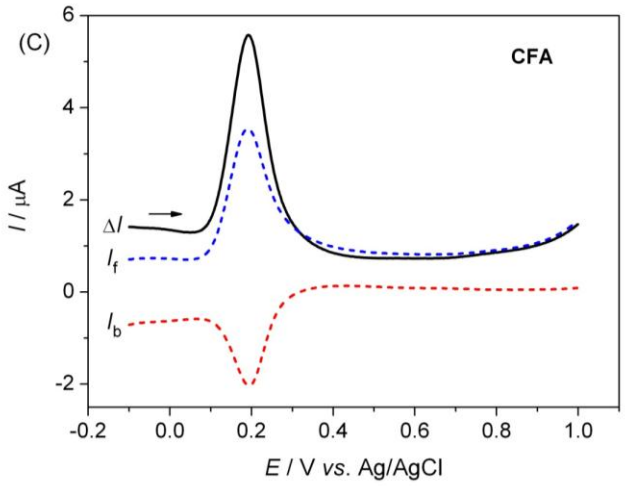


Figure 1. SWVs of $3x10^{-5}$ mol L^{-1} solution of 5-CQA (A), 4,5-diCQA (B) and CFA (C) in 0.1 mol L^{-1} PBS pH 7.0. The net current response (ΔI) and its forward (I_f) and backward (I_b) components are shown. The experimental conditions for SWV measurements are: frequency 50 Hz, pulse amplitude 50 mV, potential increment 2 mV.

Considering the structures of investigated compounds (Scheme 1.), their SWV response were compared with SWV of compounds with similar chemical structure. All investigate compounds have in structure one or two catechol moiety (group) with two -OH groups in the *ortho*-position. From the literature data it is well known that catechol and some polyphenols (like e.g. flavonoids catechin, quercetin and rutin) with catechol moiety in their structure undergo reversible oxidation to *ortho*-quinone structure by a transfer of two electrons and two protons during anodic polarization. During the reverse scan, i.e. cathodic polarization, *ortho*-quinone structure was electrochemically reversible reduced by a two-electron-two-proton mechanism back to a catechol structure [22-26]. It seems that electrochemical oxidation/reduction of 5-CQA and 4,5-diCQA (and other their isomers) proceeded *via* the same (or very similar) mechanism as that of above mentioned flavonoids, and were mainly determined by the presence of catechol moiety in the structure of investigated molecules (Scheme 1.). This is in accordance with earlier published investigations of electrochemical behaviour of chlorogenic acid (5-CQA) [12,13], where also two electron-two proton oxidation/reduction mechanism of catechol moiety were proposed.

Figure 2 shows the SWV net current responses (ΔI) of $3x10^{-5}$ mol L^{-1} solutions of all investigated CGAs in 0.1 mol L^{-1} PBS pH 7. It is interesting to notice the shift of net peak potential (E_p) and net peak current (I_p) with structure of investigated CGAs, E_p shifts to the more positive direction in the order: CFA (0.191 V), CQAs (0.223 V), diCQAs (0.229 V), while I_p values increases from CFA up to 4,5-diCQAs (see Fig. 2).

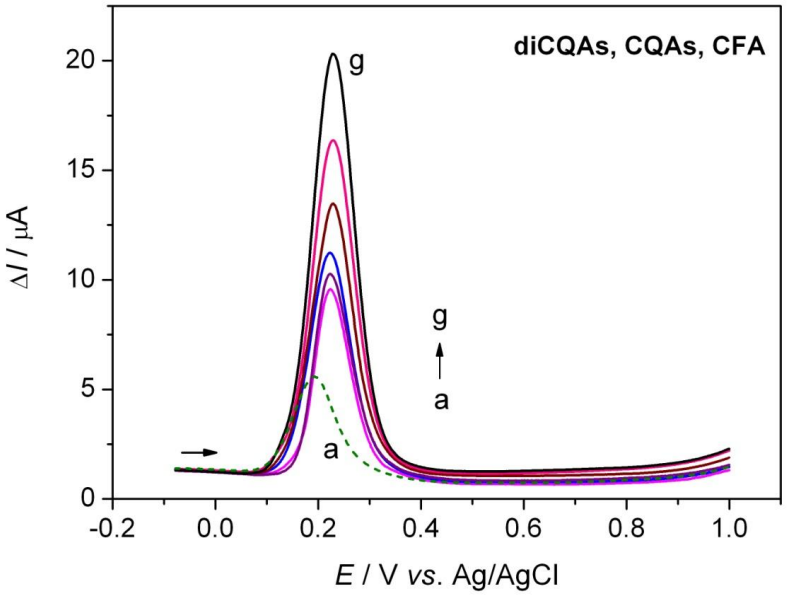


Figure 2. SWVs (net current response, Δ*I*) of $3x10^{-5}$ mol L^{-1} solution of CFA (a), 4-CQA (b), 3-CQA (c), 5-CQA (d), 3,4-diCQA (e), 3,5-diCQA (f) and 4,5-diCQA (g), in 0.1 mol L^{-1} PBS pH 7.0. The experimental conditions for SWV are as in Fig. 1.

There is some contrariness in the literature regarding the difference in the oxidation potentials of caffeic acid (CFA) and chlorogenic acid, CGA (5-CQA). Thus, Born et al. [9] measured the same value of oxidation potential for CFA and CGA, Ziyatdinova et al. [14] reported that CFA has more positive oxidation potential then CGA, while some other authors reported lower oxidation potential for CFA in comparison to CGA [13,27,28], but all authors don't get any explanation for such behaviour.

SWV measurements carried out in this investigation (Figs. 1. and 2.) clearly show that mono- and di-esters of CFA with quinic acid (CQAs and diCQAs) have something different electrochemical properties in comparison to CFA, i.e. more positive oxidation potentials (E_p) and significantly higher peak current values (I_p). Obviously, esterification of CFA with quinic acid (QA) influenced on the electrochemical properties of CQAs and diCQAs isomers. More positive oxidation potential and higher peak currents of CQAs and diCQAs relative to CFA can be explained by the difference in the electrochemical reactivity of these isomers due to different chemical structure (see Scheme 1.). In attempt to explain such behaviour we try to use quantum chemistry approach.

In many papers it was reported, based on quantum chemistry calculations and electrochemical measurements, a strong relationship between chemical structures, electronic and electrochemical properties of different molecules. Thus, a strong linear correlation between redox potentials and HOMO and LUMO energy of molecular orbitals of different molecules was observed [29-31]. According to the molecular orbital theory, the energy of highest occupied molecular orbital(s) (E_{HOMO}) represents the ability of HOMO orbital(s) to donate electron(s): the higher the E_{HOMO} energies, the easier it is for HOMO to donate electrons, what means the lowest oxidation potential value. The LUMO orbital(s) (the lowest unoccupied molecular orbitals) as an electron acceptor represents the ability to obtain an electron(s): the lower the E_{LUMO} energies, the easier it is for LUMO to accept

electrons, what means the lowest reduction potential value. The smaller the energy gap of LUMO and HOMO is, the easier it is for HOMO to donate electrons to LUMO orbitals.

If we want to compare the E_{HOMO} values of investigated molecules with their electrochemical properties observed in our paper, only E_{HOMO} for 3-CQA and CFA were found in the literature. E_{HOMO} value for 3-CQA (-6.246 eV) was reported by Mishra et al [32], while E_{HOMO} values calculated for CFA were: -5.71167 eV, reported by Riahi et al. [29] and -5.60 eV reported by Cornrad and Lapouge [33]. From these E_{HOMO} values, in accordance with molecular orbital theory and results of above mentioned papers [29-33], it could be concluded that 3-CQA must have higher oxidation potential then CFA. Such conclusion was confirmed by our SWV measurements (see Fig. 2). Unfortunately, there were was not in the literature the E_{HOMO} values for other isomers of CQAs and diCQAs, and therefore it is not possible to explain more in detail their electrochemical behaviour by molecular theory approach.

But, additionally another way to explain differences in electrochemical behaviour of investigated CGAs and CFA it is possible. Electronic properties and the differences in the oxidation potentials of CFA and CGAs derivates from different structure and different electronic properties of these molecules, which was determined by the Raman spectroscopy [32,34,35]. Electronic, and consequently electrochemical properties of CFA and CGAs molecules, were mainly determined by the presence of catechol moiety with two -OH groups in the *ortho* position. In addition, functional groups in the side chain attached to the catechol moiety of these molecules (see Scheme 1.), with different electronic properties, must also influence on electronic properties and electrochemical behaviour of CFA and CGAs molecules. It is well known (from organic chemistry) that carboxyl (-COOH) and ester groups (-COOR) are an electron-withdrawing group (EWG) that draws electron density from neighboring atoms (e.g. benzene ring) towards itself, while -OH groups and substituents with double bond (e.g. vinyl -CH=CH- group) act as electron donating groups (EDG), i.e. donates some of its electron density to the conjugated system (e.g. to the benzene ring). Both of these groups, EWG and EDG, can act by resonance or inductive effects. It can be seen from Scheme 1 that CFA molecule consist of catechol structure on which acrylic functional group is attached. Acrylic group consist of vinyl (-CH=CH-) group connected directly to the aromatic ring and carboxyl (-COOH) group connected to the vinyl group. Since in the structure of CFA the carboxyl group is not directly bound to the aromatic ring, the negative inductive effect of this group weakens along the chain, and the electron donating effect of double bond (vinyl group) prevailed in the side chain. The electron donating effect of -CH=CH-COOH group on the benzene ring was observed not only for CFA, but also for other hydroxycinnamic acids [36]. This electron donating effect of -CH=CH-COOH group increases the electron density on the benzene ring and lowering the oxidation potential of CFA in comparison to oxidation potential of catechol (see Scheme 1.), which don't have such side chain [22,37-39].

In the case of CGAs (see Scheme 1.) it seems that a strong electron-withdrawing effect of ester group -COOR and influence of R substituent (quinoyl moiety with cyclohexane ring of different electronic properties then phenyl ring [32,34]), prevailed over the electron donating effect of double bound. This was confirmed by Raman spectroscopy of 3-CQA and computational molecular electrostatic potential (MEP) mapping of electrostatic potential at the surface of 3-CQA [32]. The MEP plot of 3-CQA reveals that negative electrostatic potential regions (regions with high electron density)

are partially moved from catechol moiety to the ester group what confirm the strong electron-withdrawing effect of -COOR group. Consequently, electron density on the catechol moiety is decreased, electron transfer from -OH groups is more difficult, and anodic oxidation potential of 3-CQA (and other CQAs isomers) shifts to more positive value in comparison to that of CFA. In the case of diCQAs with two CFA moieties and two esters groups in the structure (Scheme 1.), it seems logically that the above mentioned strong electron-withdrawing effect of two ester groups is more pronounced and therefore oxidation potential of diCQAs is moved to the more positive value in comparison to that of CQAs. It is interesting to note that position of esterification of CFA with QA don't influence on the values of oxidation potentials of diCQAs and CQAs (e.g. 3-CQA, 4-CQA and 5-CQA have the same value of E_p). The value of oxidation peak current (I_p) depend on the position of esterification (see Fig. 2). In connection with above mentioned explanation of electronic effect of different functional groups on the oxidation potentials, higher oxidation potential corresponds to higher peak current. In other words, if electron transfer is more difficult, higher current is needed for oxidation of molecule. Therefore, peak currents (I_p) increases in the order: CFA, CQA, diCQAs (see Fig. 2.).

3.1.2. Effect of successive scans

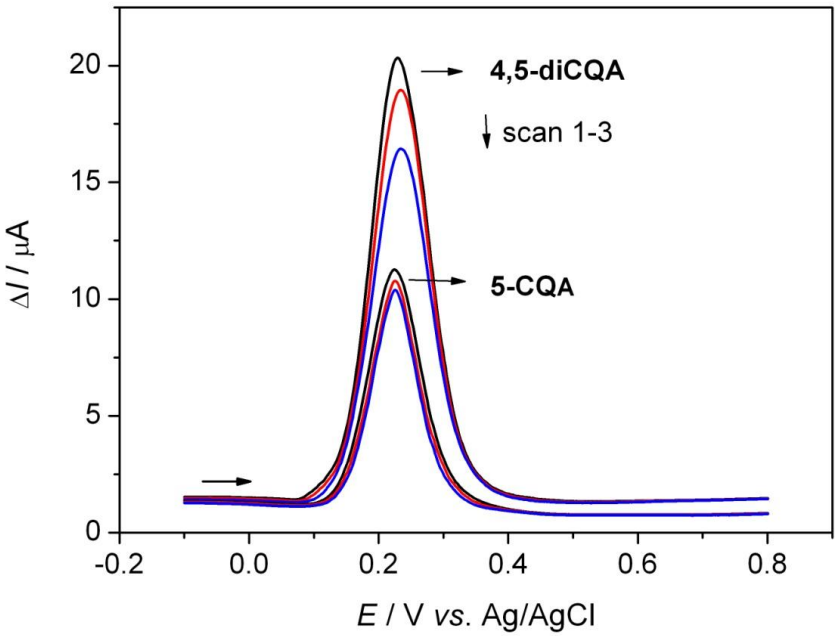


Figure 3. SWVs (net current response, ΔI) of $3x10^{-5}$ mol L^{-1} solution of 5-CQA and 4,5-diCQA in 0.1 mol L^{-1} PBS pH 7.0. Successive anodic scans (first, second and third) are shown. The experimental conditions for SWV are as in Fig. 1.

The effect of successive polarization (scanning) in the same solution, without cleaning the GCE surface between the scanning, on the shape of SWVs was investigated. Figure 3 shows this effect,

representing the behaviour of all investigated CGAs. It is evidently that during the second and third scan the current of oxidation peaks decreases. Electro-oxidation of CGAs on the GCE surface probably formed an adsorption monolayer of oxidation product(s). Further oxidation of CGAs molecules (in the second and third scan) diffusing from the bulk solution towards the electrode surface is more difficult, because occurs through the layer of adsorbed product(s). Therefore, the peak current decreases during successive scans. Such adsorption behaviour was observed for all investigated CGAs. But, adsorption is more pronounced in solutions of diCQAs, where the oxidation peaks potentials were moved in the more positive direction with scanning.

3.1.3. Effect of solution pH

The electrochemical behaviour of 5-CQA (as representative of other investigated CGAs) in 0.1 mol L^{-1} PBS of pH range 3-8, was investigated. Figure 4 shows that the oxidation peak current of 5-CQA increased rapidly varying pH from 3 to 7, and reaching a maximum at this pH value. At a higher pH than 7, the peak current decreased. Such behaviour can be explained as follow.

Chlorogenic acid (5-CQA) contained in the structure three centres (positions) for possible deprotonation (dissociation) (see Scheme 1, Table 1). These positions are carboxyl group on the cyclohexane ring, and two -OH groups on the catechol moiety. Therefore, three macroscopic acid dissociation constants of 5-CQA were reported in the literature. According to Maegawa et al. [40] the pK_a values for 5-CQA were: pK_1=3.50, pK_2=8.42; pK_3= 11.0. These three pK values were used for drawing distribution diagram of 5-CQA species in $3x10^{-5}$ mol L^{-1} of 5-CQA solution with ionic strength I=0.1, using appropriate software [41]. From distribution diagram (not shown here) it is evidently that in solutions of pH range 3-7, neutral molecules of 5-CQA and 5-CQA^- anion are presented. At pH 7, 5-CQA^- anion prevailed in the bulk solution (reaching concentration of about 95 % of all species contained). In solution of pH 8, 5-CQA^- and 5-CQA^{2-} anions are contained. 5-CQA^- anion is formed by dissociation of carboxyl group (deprotonation of one proton, H^+) in the cyclohexane ring of the structure of 5-CQA (see Scheme 2). Since phenolates anions were described as being more easily oxidized than neutral phenol molecules [23], it seems logically that oxidation current of 5-CQA^- anion reached its maximum at pH 7. According to the distribution diagram, with decreasing the pH value of solution (from 7 to 3), the concentration of 5-CQA^- anion decreases and consequently decrease the peak current (see Fig. 4). Maximum of oxidation current for CGA (5-CQA) at pH 7 was reported also by Santos et al. [18]. Similar result, i.e. maximum of oxidation current at pH 7 were also reported for some other phenolic compounds with similar chemical structure: caffeic acid [42] and catechin [23].

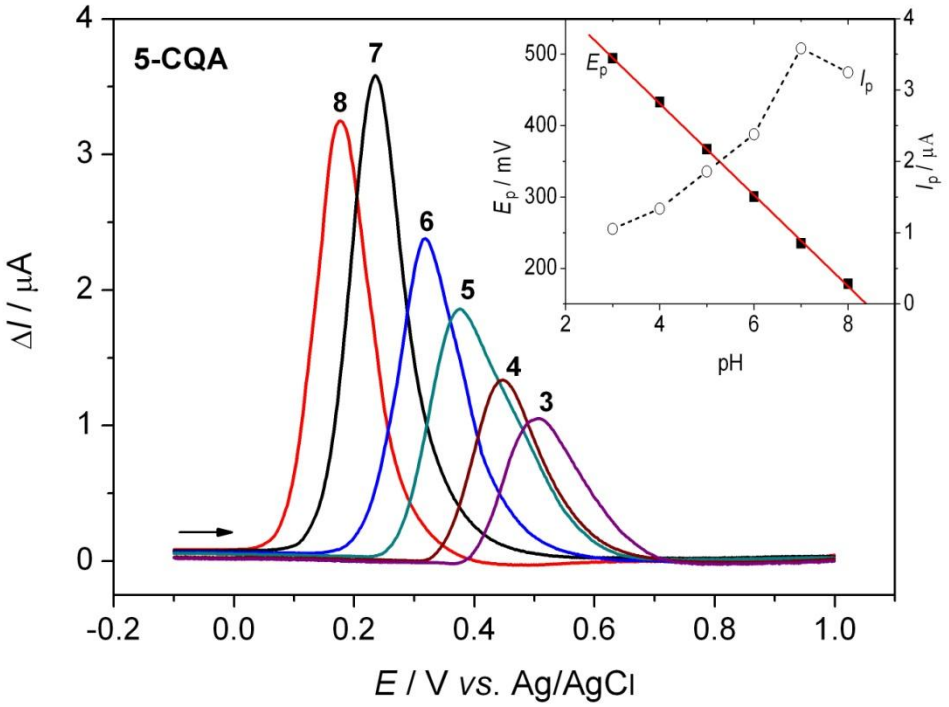


Figure 4. SWVs (net current response, Δ*I*, baseline corrected) of 1x10^{-5} mol L^{-1} solution of 5-CQA in 0.1 mol L^{-1} PBS of different pH. Inset: Plots of E_p and I_p *vs*. pH. The experimental conditions for SWV are as in Fig. 1.

From SWV measurements it is evidently (Fig. 4) that oxidation is pH-depended process. The oxidation peak potential shifted linearly to less positive values until pH 8 (inset in Fig. 4), with increasing pH, showing that deprotonation is involved (see above explanation for distribution of species). The slope of d(*E*)/d(*pH*) line is -64 mV per pH unit , (close to theoretical value of 59 mV/pH unit) showed that oxidation/reduction of 5-CQA at GCE involves the same number of electrons and protons [43]. DPV measurements of all CGAs investigated in this paper, including 5-CQA, were also carried out (the results will be published separately). Considering the DPV results, the width at half height of anodic peak, $W_{1/2}$~60 mV, for 3x10^{5} mol L^{-1} pH 7 solution of 5-CQA was observed, corresponding to the transfer of two electrons [43]. Therefore, the oxidation mechanism of 5-CQA involves the transfer of two electrons and two protons, and the current of the oxidation peaks has a maximum at pH 7.

3.1.4. Effect of frequency (effective scan rate)

The kinetics of electrode reaction(s) in 3x10^{-5} mol L^{-1} solution of 5-CQA pH 7 was investigated by the variation of square-wave frequency (effective scan rate) in the frequency range 25-150 Hz (potential increment 2 mV; effective scan rate 50-300 mV s^{-1}). It is evidently from Figure 5 that the SWV net current response (shape of SWVs) does not change significantly under the influence of

increased frequency. The net peak current (I_p) increases with increasing of frequency and is a linear function of square root of frequency (see inset in Fig. 5), while net peak potential (E_p) move to more positive values, but not more than 16 mV within the frequency range from 25 Hz to 150 Hz. According to the diagnostic criteria of SWV [21] these both facts indicates that reversible diffusion controlled charge transfer reaction proceeded on GCE in solution of 5-CQA, in this frequency range.

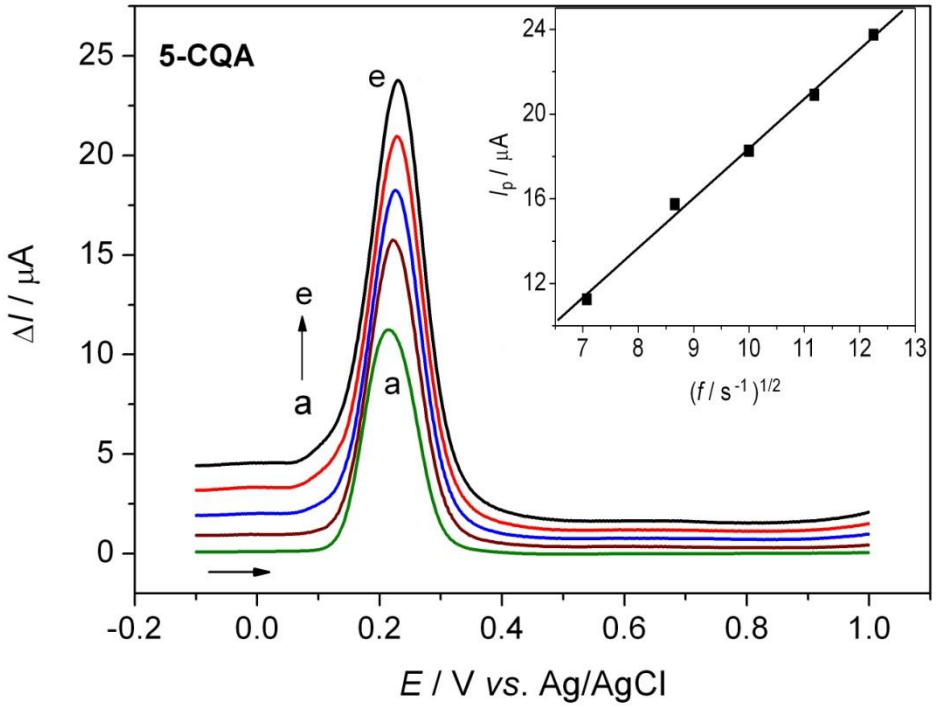


Figure 5. SWVs (net current response, Δ*I*, baseline corrected) of $3x10^{-5}$ mol L^{-1} solution of 5-CQA in 0.1 mol L^{-1} PBS of pH 7.0. Frequency: (a) 50, (b) 75, (c) 100, (d) 125 and (e) 150 Hz. Inset: Plot of I_p *vs*. square root of the frequency. All other experimental conditions for SWV are as in Fig. 1

3.1.5. Effect of concentration

Under analytical conditions determined in previously SWV measurements as optimal (solution pH 7, frequency 50 Hz, potential range -0.1 to 1.0 V), the effect of concentration of 5-CQA (as a standard for determination of total CGAs content in coffee) on the SWV responses were performed. Figure 6 shows SWVs obtained by successive additions of 5-CQA into the PBS in the 5-50 μmol L^{-1} concentration range. The peak current of anodic oxidation peak, at a potential 0.223 V, increased linearly with the 5-CQA concentration. (Fig. 6, inset). At higher concentration (c> 50 μmol L^{-1}) this relationship is not more linear (the peak current going to decrease), due to the strong adsorption of oxidation product(s). The calibration plot in the linear concentration range (5-50 μmol L^{-1}) can be represented by the equation: I_p (μA) = 0.20326+ 0.37483 x, where I_p is the net peak current and x is the concentration of 5-CQA species expressed in μmol L^{-1} (correlation coefficient, r= 0.99985; p<0.0001).

The sensitivity of SWV method was determined based on the values of the limit of detection and limit of quantification. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated from the parameters obtained from calibration curve using the formulas: $LOD = 3\ s_a/b$ and $LOQ = 10\ s_a/b$, where s_a is the standard deviation of the *y*-intercept of the regression line and *b* is the slope of the calibration curve [44]. The calculated LOD and LOQ values of 5-CQA were found to be 7.7 x 10^{-7} mol L^{-1} and 2.5 x 10^{-6} mol L^{-1}, respectively. Our LOD value obtained from SWV measurements is in accordance with results of other authors which also used SWV for quantification of chlorogenic acids content in coffee. Thus, Fernandes et al. [15] reported LOD of 9.15 x 10^{-7} mol L^{-1}; Carvalho et al. [16] calculated LOD of 8.0 x 10^{-7} mol L-1, while Moccelini et al. [17] obtained values of 8.02-8.52 x 10^{-7} mol L^{-1} as LOD for determination of CGAs content in coffee samples.

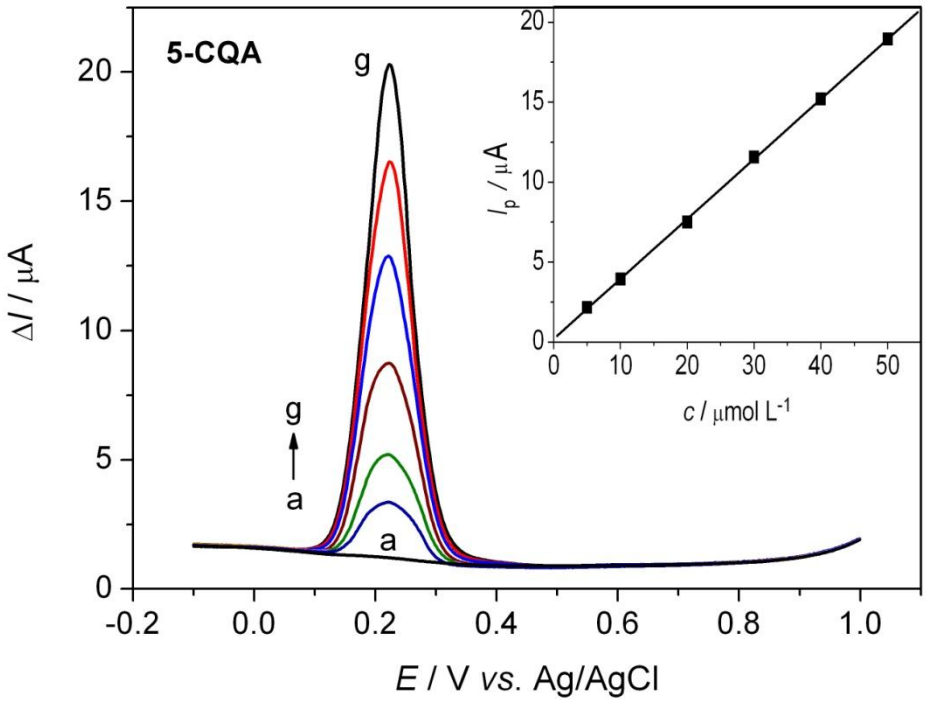


Figure 6. SWVs (net current response, Δ*I*) of 5-CQA solutions of different concentration: 0 (a), 5x10^{-6} (b), 1x10^{-5} (c), 2x10^{-5} (d), 3x10^{-5} (e), 4x10^{-5} (f), and 5x10^{-5} mol L^{-1} (g), in PBS of pH 7.0. Inset: calibration plot of 5-CQA. The experimental conditions for SWV are as in Fig. 1

3.1.6. Oxidation/reduction mechanism of CGAs

In order to understand the redox mechanism of investigated CGAs, their electrochemical behaviour was compared with the electrochemical behaviour of caffeic acid, which is precursor for all CGAs investigated. Considering all the SWV data presented (and literature data to), it can be concluded that electrochemical behaviour of investigated CGAs is strongly influenced by their chemical structure. Dicaffeoylquinic acids (diCQAs) have the highest oxidation potential and highest oxidation currents, CQAs have lower oxidation potential and lower oxidation current then diCQAS,

while CFA have the lowest redox potential and the lowest peak current. These facts strongly suggest that esterification influenced the electrochemical behaviour of investigated CGAs.

The proposed electrochemical oxidation/reduction mechanism of 5-CQA and 5-CQA$^-$ anion (and probably of other mono-esters, 4-CQA and 3-CQA) occurs by reversible $2e^-$-$2H^+$ mechanism, involving catechol moiety, i.e. oxidation of two -OH groups in the *ortho*-position in the structure of CQAs to *ortho*-quinone structure (see Scheme 2.). This process is pH-dependent process and maximum of peak current is observed at pH 7, at pH value where deprotonated anion species prevailed in the bulk solution. The oxidation product(s) adsorbed on the GCE surface and this adsorption is more pronounced if concentration of CQAs is higher. During the reverse scan, i.e. cathodic polarization, *ortho*-quinone structure was electrochemically reversible reduced by a two-electron-two-proton mechanism back to a catechol structure (see Scheme 2.).

In the case of di-esters, i.e. diCQAs (3,4-diCQA, 3,5-di-CQA and 4,-di-CQA), molecules have two catechol moieties in the structure (see Scheme 1), but show only one anodic and one cathodic peak, similar to mono-esters This suggests that oxidation potential of each catechol groups have the same or very close value, and the peaks becoming indistinguishable. Considering the SWV results, the probable oxidation reaction occurs *via* two reversible $2e^-$-$2H^+$ mechanism. In other words, oxidation occurs simultaneously (or successive) on both catechol groups in the structure of diCQAs (see Scheme 1). Similar oxidation mechanism was proposed for rosmarinic acid [37], which molecule also has two catechol moieties in the structure. Because total oxidation reaction involves four electrons, the net peak currents of diCQAs are significantly higher than that of mono-esters CQAs (see Fig. 2). Also, adsorption processes on the GCE were in the case of diCQAs more pronounced than that of CQAs.

3.2. Characterization of CGAs in coffee

Taking into account the above presented results of electrochemical behaviour of investigated CGAs and the advantages of SWV as electroanalytical method (great speed of analysis, low consumption of electroactive species, low LOD) SWV was applied for characterization (identification and quantification) of CGAs in different coffee samples. SWVs of coffee samples were recorded in order to investigate the analytical potential of the electrochemical oxidation of CGAs species present in coffee in determining the total CGAs content.

3.2.1. Electrochemical behaviour of coffee samples

The same experimental conditions (frequency, pulse amplitude, potential increment, etc.) and analytical methodology determined as optimal in the study of CGAs were used for investigation of electrochemical behaviour of coffee samples and determination of total CGAs content in coffees.

Square-wave voltammogram for *Coffee Arabica*, roasted bean extract diluted 50-fold in 0.1 mol L^{-1} PBS pH 7 (Fig.7) represent electrochemical behaviour of all other coffee samples. The net peak potential (E_p) was observed at 0.225 V, very close to the value of oxidation peak of 5-CQA (E_p=0.223 V, see Figs. 1 and 2). As can be seen in Fig. 7, the forward (oxidative) and backward

(reductive) components of the net current response are well developed. Anodic to cathodic peak separation (ΔE_p) is 26 mV, and the ratio of anodic to cathodic peak currents, I_f / I_b is -1.7. According to SWV criteria of the reversibility [21], all these results suggest that electrode reaction(s) proceeding on the GCE in the coffee samples are probably very close to the reversible reactions. Very similar SWVs were observed in all other coffee samples. The net peak potentials (E_p) of investigated coffee samples lie between 0.223 V and 0.229 V, i.e. in the range between oxidation potentials of CQAs (0.223 V) and diCQAs (0.229 V, see Fig. 2).

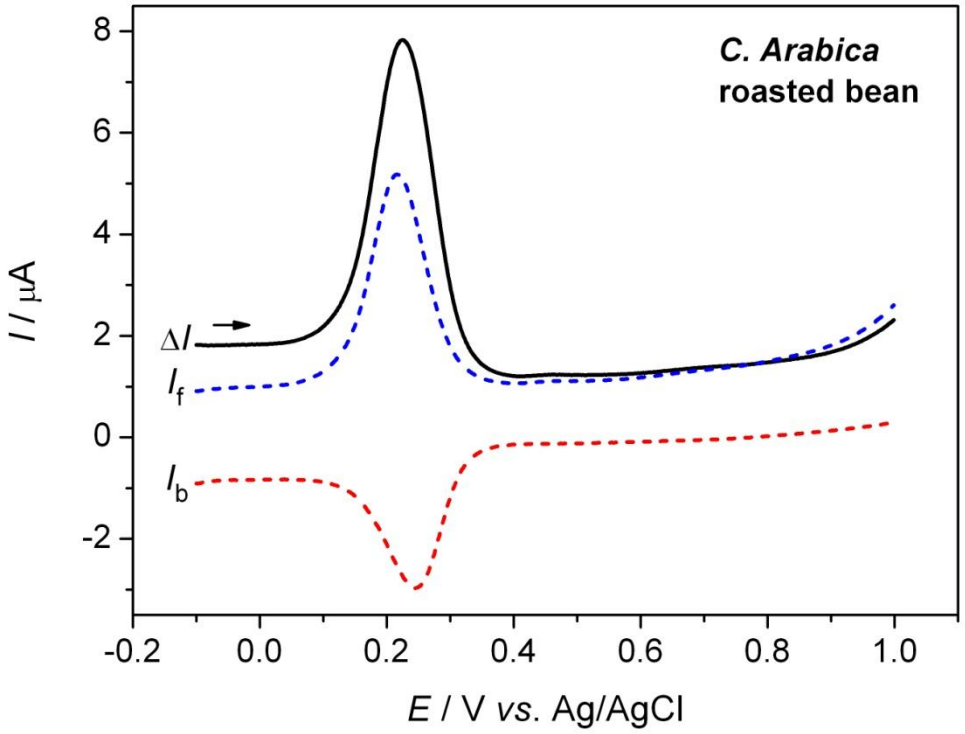


Figure 7. SW voltammogram of *C. Arabica* roasted bean extract (diluted 50-fold) in 0.1 mol L^{-1} PBS pH 7.0. The net current response (ΔI) and its forward (I_f) and backward (I_b) components are shown. The experimental conditions for SWV are as in Fig. 1.

Other parameters essential for analytical methodology, such as influence of pH of PBS, influence of successive scans, were tested in Nescafé Classic extract (diagrams not shown here). It was observed that the net peak potentials (E_p) of Nescafé Classic coffee extract depended on pH of PBS. E_p shifted linearly to less positive values from pH 4 to pH 8 with the slope of d(E)/d(pH) -60.6 mV/pH unit, showed that the oxidation/reduction processes proceeded on GCE in coffee samples are the same or very similar as that observed in model solutions of CQAs and diCQAs (see explanation in Section 3.1.6). That means that electrochemical oxidation of CGAs contained in coffee samples involves two electron-two proton mechanisms, i.e. oxidation of catechol moiety (moieties) in the structure of CGAs. The maximum of net peak current (I_p) in Nescafé coffee extract was observed in PBS of pH 7, similar as in the solution of 5-CQA (see Section 3.1.3). Therefore PBS of pH 7 was chosen for quantification of total CGAs content in all coffee samples.

Adsorption processes on GCE in extract of Nescafé Classic coffee was observed. The peak current decreases during successive SWV scans (second and third scan), while the net peak potentials move to more positive values. Such behaviour is similar to adsorption processes observed with CQAs and diCQAs solutions (see Section 3.1.2.).

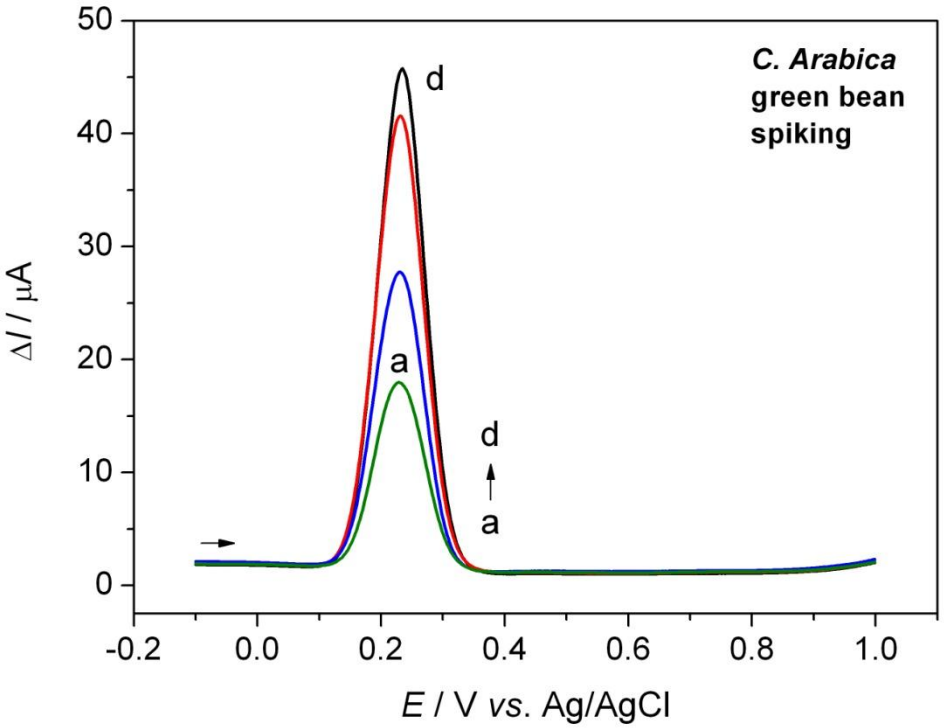


Figure 8. SWVs (net current response, Δ*I*) of *C. Arabica* green bean extract (diluted 50-fold), in 0.1 mol L^{-1} PBS pH 7.0 (a). Spiked samples: (b) *C. Arabica* + 1x10^{-5} mol L^{-1} of 5-CQA, (c) *C. Arabica* + 1x10^{-5} mol L^{-1} of 3,5-diCQA and (d) *C. Arabica* + 1x10^{-5} mol L^{-1} of 4,5-diCQA. The experimental conditions for SWV are as in Fig. 1.

Generally speaking, electrochemical behaviour and electrochemical properties of all investigated coffee samples (e.g. oxidation potentials, pH dependence, oxidation/reduction mechanism, etc.) are the same or very similar to the electrochemical behaviour and electrochemical properties of CQAs and diCQAs. Therefore, the anodic oxidation peak recorded in all coffee samples could be ascribed to the oxidation of mixture of the different CQAs (5-CQA, 4-CQA and 3-CQA) and diCQA (3,4-diCQA, 3,5-diCQA and 4,5-diCQA) contained in all investigated coffee samples. All these compounds were present as the major CGAs in coffees, as were reported by many of HPLC analysis of different coffees [5-8]. In addition, we also performed HPLC analysis of coffee samples investigated in this paper by SWV (HPLC results will be published separately), and found that six above mentioned CQAs and diCQAs represented ca. 90 % of all CGAs found in coffees; and 5-CQA was prevailed CGA in all coffee samples. Other CGAs found in coffee by HPLC were feruloylquinic acids, FQAs, but FQAs were not detected by SWV. Therefore, the sum of CQAs and diCQAs content (from SWV

anodic peak) can represent total CGAs content in coffee samples, and 5-CQA can be used as standard to express this content.

To confirm such assignation of anodic oxidation peak, coffee samples were spiked with 1×10^{-5} mol L^{-1} of all investigated CGAs. Figure 8 represent observed behaviour. *Coffee Arabica*, green bean extract (diluted 50-fold) was spiked with 1×10^{-5} mol L^{-1} solution of 5-CQA, 3,4-diCQA and 4,5-diCQA, in the separate SWV experiments. It can be seen that spiking of 5-CQA in *C. Arabica* green extract significantly increases the peak current of *C. Arabica* green bean extract and don't change the peak potential (0.229 V). Spiking of 3.4-diCQA and 4,5-diCQA increases peak current of *C. Arabica* green bean extract more then 5-CQA (in accordance with experiments shown in Fig. 2) and moves peak potential 2-6 mV to the positive direction. This is probably due to a strong adsorption of diCQAs oxidation product(s) on GCE, as was shown in Section 3.1.2. The experiments with spiking undoubtedly confirm that anodic oxidation peak observed in coffee samples by SWV experiments can be ascribed to the electrochemical oxidation of mixture of CQAs and diCQAs contained in each coffee sample.

3.2.2. Quantification of total CGAs content in coffee samples

Table 2. Total chlorogenic acids (CGAs) content in different brand of coffees determined by SWV and HPLC methods

Brand of coffee	CGAs (total)-SWV[a]	CGAs (total)-HPLC[b]
C. Arabica, Rio Minas, Brazil (green bean)	7631	7370
C. Arabica, Rio Minas, Brazil (roasted bean)	2730	2613
C. Robusta, Cherry, India (green bean)	9411	9112
C. Robusta, Cherry, India (roasted bean)	3186	2826
Flatscher Olimpia (ground coffee, 100 % Arabica)	4278	3932
Franck Quatemala (ground coffee, 100 % Arabica)	3914	3519
Nescafé Classic (instant coffee)	3566	3203
Nescafé Espresso (instant coffee)	3429	3185
Jacobs Monarch (instant coffee)	3447	3149
Jacobs Intense (instant coffee)	3796	3462

Results represent mean values of three independent measurements (n=3)
[a]- values determined by SWV, total CGAs content was expressed as 5-CQA equivalent (mg 5-CQAE/100 g of coffee)
[b]- values determined by HPLC, total CGAs content were sum of individual CGAs content and expressed as mg CGAs/100 g of coffee

The total CGAs content in coffee samples was calculated from the net peak current (I_p) values of SWVs, using calibration curve for 5-CQA as the standard (see Fig. 6 inset), and expressed as mg 5-CQA equivalents per 100 g of coffee (mg 5-CQAE/100 g of coffee, Table 2.). The results were mean values of three independent analyses. For comparison, the results of HPLC analysis (performed in separately experiments) were presented. In HPLC analysis the total CGAs content is the sum of content of all individual detected CGAs, and was expressed as mg CGAs/100 g of coffee.

It can been seen from Table 2 that SWV gave something higher values of total CGAs content than HPLC analysis. This can be due the different method of analysis and different method of expression of total CGAs content. But generally speaking, a good agreement between the results obtained by SWV and HPLC was obtained. The total CGAs content decreases in the order: green beans, ground coffees, instant coffees, roasted beans. Such results are in agreement with HPLC analysis reported, which shown significantly loss of CGAs content during roasting of green beans and processing of ground coffees [5,8].

If we compared our results of total CGAs content in coffees with results of other authors which measured CGAs content also by electrochemical methods [13,15-17], a reasonable agreement was observed, although more detailed comparison is not possible due to different coffee samples, different method of analysis and especially different method of preparation of coffee extracts.

4. CONCLUSIONS

In this work, the electrochemical behaviour of six important CGAs isomers: three CQAs (5-CQA, 4-CQA, 3-CQA) and three diCQAs (3,4-diCQA, 3,5-diCQA and 4,5-CQA) was investigated by SWV and compared with the electrochemical behaviour of CFA (precursor of investigated CGAs). The electrochemical behaviour of all chosen CGAs (except of 5-CQA) were investigated for the first time. The study reveals that electrochemical behaviour of investigated CGAs is strongly dependent on their chemical structure and electronic properties, particularly on electron-donating effect of -OH group and -CH=CH- group, and a strong electron-withdrawing effect of ester (-COOR) group presented in their structure.

SWV measurements show that electrochemical oxidation/reduction process of investigated CGAs at GCE is reversible, pH-dependent, two electron-two proton process. The oxidation/reduction processes occurred on the catechol moiety (*ortho*-dihydroxyl group) in the structure of CGAs molecules. The oxidation product(s) relatively strongly adsorb on the GCE surface, especially at higher concentration of CGAs.

The electrochemical behaviour of 5-CQA (as the major CGAs in coffee) was investigate more in detail. It was observed that anodic peak current of 5-CQA show maximum in solution of pH 7, due to the maximum concentration of 5-CQA$^-$ anion at this pH value. The kinetics of electrode reaction proceeded in solution of 5-CQAs at GCE is controlled by diffusion. The anodic peak current shows linear relationship with the concentration of 5-CQA in the concentration range of 5-50 μmol L^{-1}, with low LOD (7.7×10^{-7} mol L^{-1}).

Based on the SWV results of electrochemical behaviour of CGAs (particularly of 5-CQA), the SWV of coffee samples were performed. The SWV results show that electrochemical behaviour of coffee samples is very similar to that of investigated CGAs. Therefore, SWV was used for characterization of CGAs in coffee. It was shown that SWV is a very sensitive and selective method for determination of total CGAs content in coffee. A good agreement between the results obtained by SWV and HPLC analysis was observed.

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