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Determination of Ellagic Acid in Strawberries, Raspberries and Blackberries by Square-Wave Voltammetry

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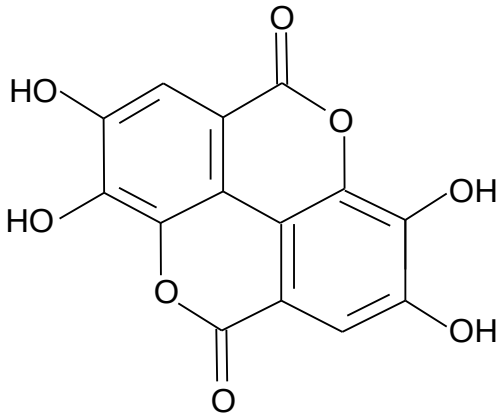
In square-wave voltammetry at pH 6.5 the response of ellagic acid appears at 0.278 V *vs*. Ag/AgCl. This is the basis of a new electroanalytical method described in this paper. The peak current is linearly proportional to the concentration of ellagic acid within the interval between 5×10^{-7} mol/L and 8×10^{-6} mol/L. LOD is 1.35×10^{-7} mol/L and LOQ is 4.51×10^{-7} mol/L. The concentrations of ellagic acid determined by this method in the samples of strawberries, raspberries and blackberries are 5.52, 40.06 and 37.60 mg / 100 g FW, respectively.

Keywords: Square-wave voltammetry, ellagic acid, strawberry, raspberry, blackberry

1. INTRODUCTION

Ellagic acid is a dimeric derivative of gallic acid (Scheme 1). It is found in numerous fruits and vegetables including strawberries, raspberries, pomegranates and walnuts. There, it is usually present in ellagitannin as esters of diphenic acid analogue with glucose [1]. The content of ellagic acid is preserved in the food products, such as jams and juices. Its daily intake is estimated to 90 mg [2]. Because of antioxidative and antiviral properties [3] it can be used as a food additive [4]. For its determination in foodstuff the high-performance liquid chromatography with diode array detector [4 - 6] or mass spectrometer [7 - 9] is used. The separation is achieved by RP C_{18} column. Limits of quantification are 50 ng/g in fruits [4] and 1.9 μg/L in wines [5]. Ellagic acid is electroactive and can be oxidized on graphite electrode [10 - 13] . In square-wave voltammetry, at pH 5.5, the potential of the first peak of oxidation is 0.42 V *vs*. Ag/AgCl [11]. Voltammetic techniques were used for electrochemical determination of free and total ellagic acid in some fruits and juices [11, 12]. In this communication the square-wave voltammetry of ellagic acid on glassy carbon electrode is analyzed in

more details. For quantitative analysis of ellagic acid in the presence of flavonols in strawberries, raspberries and blackberries extracts, the deconvolution procedure is applied.



Scheme 1. Structure of ellagic acid.

2. EXPERIMENTAL

2.1 Reagents

Ellagic acid (EA) (≥ 95 %) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A). Stock standard solution of ellagic acid (1.0×10^{-3} mol/L) was prepared from the dry pure substance in HPLC grade methanol (Merck, Darmstadt, Germany). The stock solution was protected from light with aluminium foil, kept in a refrigerator and used within one week. Working solutions were prepared daily by diluting the stock solution with a selected supporting electrolyte. All buffer solutions (pH 3-11) were from Kemika, Zagreb, Croatia, analytical grade. For the supporting electrolyte analytical grade 1 mol/L KNO_3 (Kemika, Zagreb, Croatia) was used. Purified water from a Millipore Milli-Q system was used throughout the study.

2.2 Instrumentation

All voltammetric measurements were carried out using the computer-controlled electrochemical system Autolab PGSTAT 30 (Eco-Chemie, Utrecht, Netherlands). Voltammetric curves of dissolved ellagic acid were recorded using a three-electrode system (Metrohm, Switzerland) with Ag/AgCl (3 mol/L KCl) electrode as a reference electrode and a platinum wire as a counter electrode. Glassy-carbon (GC) electrode of 3.0 mm diameter (MF-2012, Bioanalytical Systems, Inc., West Lafayette, Indiana, USA) and spectral-grade paraffin-impregnated graphite rod (PIGE) (diameter 5 mm, length 50 mm) were used as the working electrodes. Working electrodes (PIGE and GCE) were mechanically cleaned before each run. GC working electrode was polished with diamond spray (6 μm), rinsed with ethanol and deionised water. The circular surface of PIGE was rinsed with distilled

water, polished on a wet polishing cloth, rinsed again, dried with a fine-grade paper tissue and carefully polished on a dry, white paper sheet. The solutions were degassed with high-purity nitrogen prior to the electrochemical measurements. A nitrogen blanket was maintained thereafter. All experiments were performed at room temperature.

2.3 Sample preparation

Strawberries, red raspberries and blackberries were purchased from market stalls in Zagreb during summer of 2010. Samples were frozen and stored at -20 °C until analyzed.

Acid hydrolysis of the ellagitannins was performed as described by Oszmianski et al. [14]. Frozen samples were thawed and homogenized with a household blender. Approximately 2 g of each homogenate were accurately weighted and refluxed with 2 ml of 2 mol/L HCl in a water bath at 100 °C for one hour. After cooling, 2 ml of 2 mol/L NaOH and then 6 ml of methanol (HPLC grade) were added to the vial. Subsequently, the samples were immersed in an ultrasound bath (Bandelin, Sonorex, Germany) and subjected to ultrasound treatment at room temperature and constant frequency of 35 kHz during 20 min. Before analysis, extracts were filtered over cotton wool and then through a 0.45 μm syringe filter and adequately diluted directly in voltammetric cell.

3. RESULTS AND DISCUSSION

3.1 Voltammetry of ellagic acid

Figure 1 shows cyclic voltammograms of ellagic acid dissolved in 0.1 mol/L KNO_3.

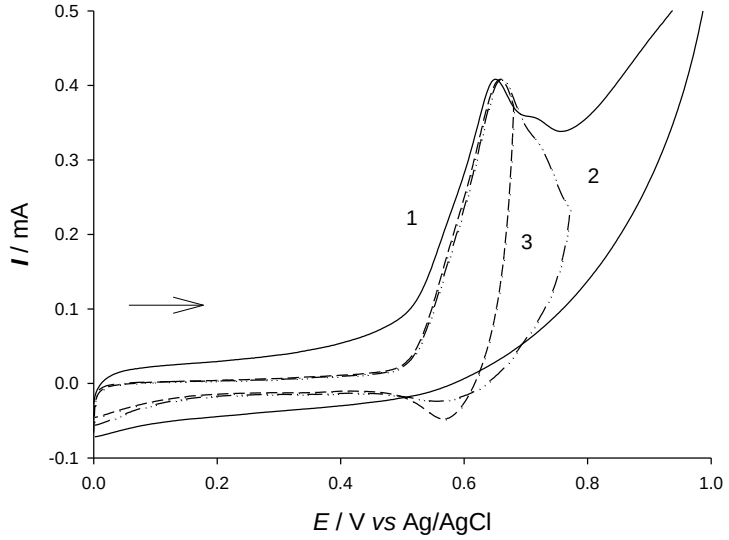
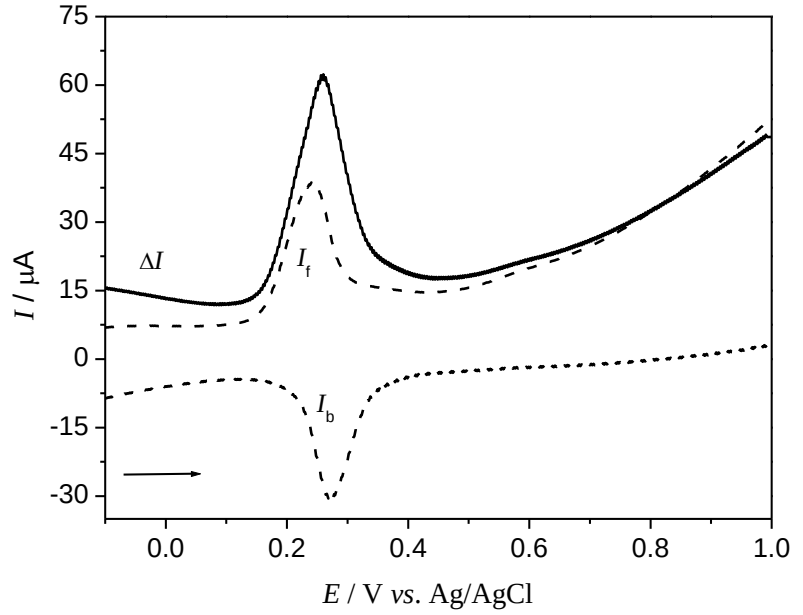
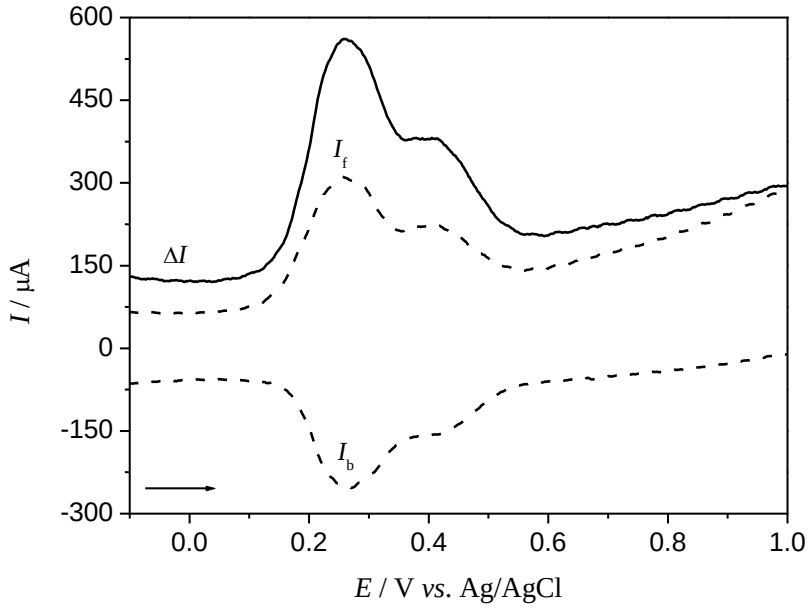


Figure 1. Cyclic voltammetry of 5×10^{-4} mol/L ellagic acid on the paraffin impregnated graphite electrode in 0.1 mol/L KNO_3 at pH 2. The scan rate is 25 mV/s and the reversing potential is 1 V (1), 0.772 V (2) and 0.680 V (3).

The first oxidation peak potential is 0.655 ± 0.005 V. It is followed by the shoulder at about 0.72 V and by the second oxidation process at higher potentials.



A



B

Figure 2. Square-wave voltammetry of 1×10^{-5} mol/L ellagic acid on glassy-carbon electrode in 0.1 mol/L KNO_3 at pH 7. E_{sw} = 50 mV, dE = 2 mV and f /s^{-1} = 100 (A) and 1000 (B).

If the potential scan is reversed at 1 V, no reduction peak can be noted, but if the reversing potential is 0.680 V the reduction current is in minimum at 0.565 V. This is similar to the response of gallic acid [15, 16]. The first, quasireversible oxidation / reduction process is ascribed to *ortho*-

hydroquinone groups, while the second, irreversible oxidation process is explained by the formation of polymer film on the electrode surface [17, 18].

Square-wave voltammograms of dissolved ellagic acid are shown in Figure 2. At the frequency of 100 Hz a single peak appears with the maximum at 0.260 V. The peak potentials of the forward and backward components are 0.250 V and 0.265 V, respectively. This shows that in square-wave voltammetry the influence of film is much smaller and the response appears reversible. If the frequency is increased to 1000 Hz, the voltammogram consists of two peaks with the maxima at 0.265 V and 0.410 V, respectively. The second peak appears as a shoulder at the frequency of 250 Hz and develops into a separate peak if the frequency is higher than 500 Hz. These two peaks can be explained by the assumption that the electrode reactions of two *ortho*-hydroquinone groups are not equally fast so that the response of slower reaction appears at higher potential than the response of the quicker one. At frequencies lower than 200 Hz these two responses merge into a single peak.

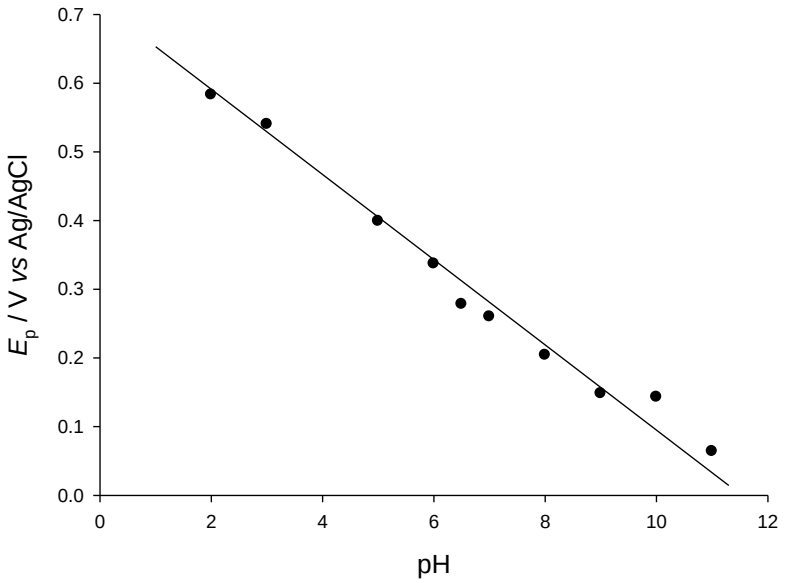


Figure 3. Square-wave voltammetry of ellagic acid on GCE. Dependence of peak potentials of the net response (circles) on pH of supporting electrolyte; f = 100 s^{-1} and all other data are as in Figure 2. The straight line is the first approximation: $E_p = 0.715 - 0.062 \times$ pH (V).

The dependence of peak potentials of the net response at f = 100 Hz on pH of supporting electrolyte is shown in Figure 3. This relationship can be approximated by the straight line: $E_p = 0.715 - 0.062 \times$ pH (V), which is in agreement with the properties of 1,2-benzo-hydroquinone [19] and gallic acid [20].

3.2 Square-wave voltammetry of berry extracts

SWV analysis of raspberry, strawberry and blackberry extracts was performed under the optimized experimental conditions of pH 6.5, pulse amplitude of 50 mV, frequency of 100 Hz and step

potential of 2 mV. Under the optimal experimental conditions, SW voltammogram of ellagic acid shows one oxidation peak with the maximum at 0.278 V.

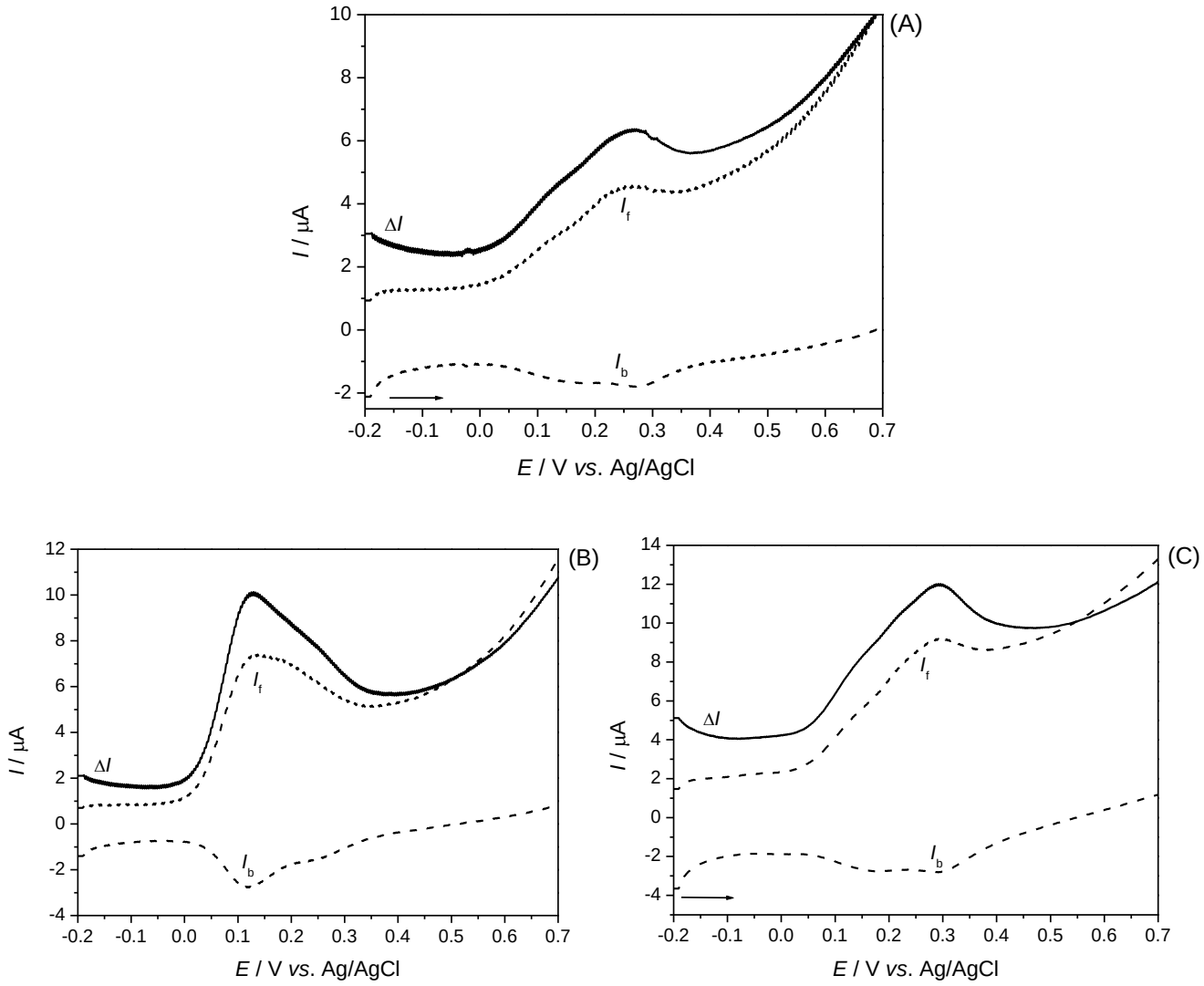


Figure 4. Square-wave voltammograms obtained for raspberry (A), blackberry (B) and strawberry (C) extracts. Experimental conditions: pH 6.5, pulse amplitude 50 mV, frequency 100 Hz and step potential 2 mV.

Fig. 4 shows voltammograms obtained from analyzed extracts. In these figures one can observe the presence of other current peaks produced by electroactive compounds that oxidize at potentials near to that of ellagic acid. These compounds interfere with the analytical signal of ellagic acid, complicating its determination in extracts. Thus, in order to analyze only the analytical signal attributed to oxidation of ellagic acid, the overlapped peaks were distinguished by deconvolution procedure. Prior to deconvolution, a baseline correction of voltammograms was performed allowing better deconvolution. Table 1 shows the oxidation potentials of deconvoluted peaks in SW voltammograms of fruit extracts.

Table 1. Oxidation potentials of deconvoluted peaks in SW voltammograms of ellagic acid and berry extracts. Experimental conditions: pH 6.5, pulse amplitude 50 mV, frequency 100 Hz and step potential 2 mV.

Sample	$E_{p,1}$ / V	$E_{p,2}$ / V	$E_{p,3}$ / V
Ellagic acid*	0.278		
Raspberry	0.124	0.251	
Blackberry	0.129	0.240	
Strawberry	0.128	0.196	0.286

(*)Oxidation potential was determined after the baseline correction, but deconvolution procedure wasn't necessary.

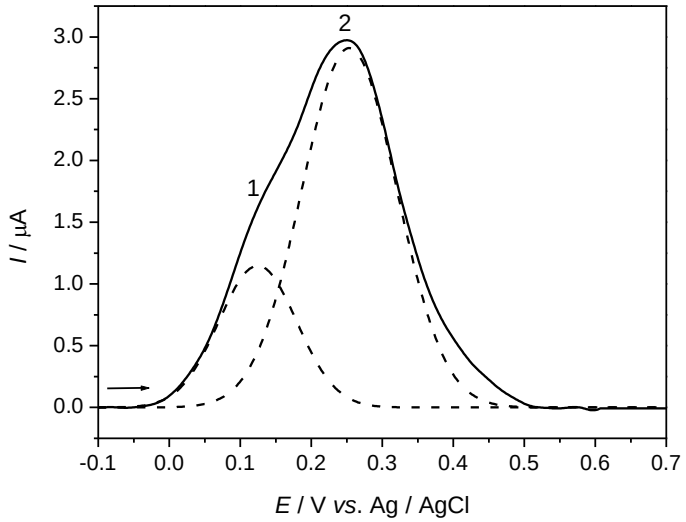


Figure 5. Deconvoluted SW voltammogram of raspberry extract. Experimental conditions are as in Fig. 4.

Deconvoluted voltammetric peaks of SWV response obtained for raspberry extract are shown in Figure 5. It can be observed that voltammetric response of raspberry extract can be decomposed into two oxidation peaks; peak 1 appears at 0.124 V and peak 2 at 0.251 V. Deconvolution of SWV signals obtained for blackberry extract also resulted in two peaks, with maxima at 0.129 V (peak 1) and 0.240 V (peak 2). Oxidation peak in potential range from 0.124 V to 0.129 V is probably due to oxidation of flavonols, primary quercetin [21, 22], which is the main flavonol in raspberries and blackberries [23 - 25]. The second oxidation peak in the SW voltammograms of raspberry and blackberry extracts closely corresponds to that of ellagic acid (see Table 1). Figure 4C shows square-wave voltammogram of strawberry extract. The response consists of a wave between 0.03 V and 0.2 V and a peak with a maximum at about 0.290 V. After deconvolution, three oxidation peaks were observed in SWV response of strawberry extract; peak 1 appears at 0.128 V and is due to oxidation of quercetin, peak 2 at 0.196 V and peak 3 at 0.286 V. Peak 1 and 3 are due to oxidation of quercetin and ellagic acid, respectively. The identification of peak 2 was based on literature data. Namely, strawberry contains approximately the same concentrations of flavonols quercetin and kaempferol [23, 26, 27]. Since

kaempferol oxidizes at more positive potentials than quercetin [28, 29], peak 2 in SWV response of strawberry extract is most probably associated with the oxidation of kaempferol. For each sample analysed here, the identification of peak assigned to ellagic acid (peak 2 in SWV response of raspberry and blackberry extract and peak 3 in SWV response of strawberry extract) was confirmed by spiking the standard component in the sample, which resulted in a marked increase in current for that peak. SWV response of raspberry extract due to addition of ellagic acid is shown in Figure 6.

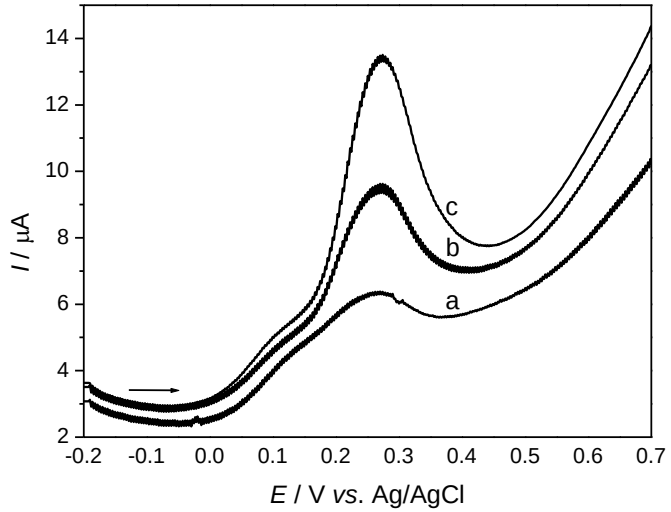


Figure 6. Square-wave voltammograms of raspberry extract diluted 100-fold (a), with 3 μmol/L (b) and 5 μmol/L (c) additions of EA made to the solution. All experimental conditions are as in Fig. 4.

3.3 Method validation

Calibration plot was constructed by plotting the peak current height, which was quantified after the baseline correction and peak deconvolution, against concentration of ellagic acid. A good linearity was found in the concentration range from $5 \cdot 10^{-7}$ mol/L to $8 \cdot 10^{-6}$ mol/L, which is represented by the linear equation:

$$I_p\ (\mu A) = 0.728 + 1.114 \times [EA]\ (\mu mol/L), \qquad R = 0.997.$$

The sensitivity of the SWV was determined based on the values obtained for determination and quantification limits. Under the given conditions, the calculated LOD and LOQ of EA were found to be $1.35 \cdot 10^{-7}$ mol/L and $4.51 \cdot 10^{-7}$ mol/L, respectively.

The method was also validated by standard addition method, where different concentrations of ellagic acid were added to samples. The results obtained for internal calibration protocol are listed in Table 2. The internal calibration slopes were used for determination of the recovery. Namely, the recovery was calculated as the ratio of the internal calibration slope to the external calibration slope. As can be seen from Table 2, the calibration slopes obtained using standard addition method were

significantly lower than that found using external calibration and recoveries ranged from 62.85 % to 83.35 %. The low recovery is attributable to the strong matrix effect. In order to overcome matrix effects the standard addition method was employed for quantification of ellagic acid in extracts of different berries.

Table 2. Validation of the method using standard addition protocol.

Sample	Internal calibration intercept ± S_a	Slope ± S_b	R	Recovery (%)*
Raspberry	2.548 ± 0.258	0.928 ± 0.087	0.991	83.35
Blackberry	3.560 ± 0.221	0.700 ± 0.049	0.993	62.85
Strawberry	3.411 ± 0.026	0.889 ± 0.014	0.999	79.84

3.4 Quantification of ellagic acid in real samples

The determination of ellagic acid in fruit extracts by square-wave voltammetry on GC electrode was carried out using standard addition protocol plus the deconvolution procedure and the results are listed in Table 3.

Table 3. Concentration of ellagic acid in berry extracts.

Sample	EA* (mg / 100 g FW)
Raspberry	40.06
Blackberry	37.60
Strawberry	5.52

*Mean of six determinations.

The ellagic acid content in raspberry and blackberry was 40.06 and 37.60 mg / 100 g of fresh weight, respectively. These contents are similar to those reported by Cuartero et al. [11], Anttonen et al. [24], de Ancos et al. [30], and Siriwoharn et al. [31]. Relatively low content of ellagic acid was found in strawberry (5.52 mg / 100 g of fresh weight). This result is in accordance with those reported by Williner et al. [32], who studied the content of ellagic acid in different strawberry cultivars by spectrophotometric method. According to these authors, ellagic acid contents in strawberry extracts estimated after hydrolysis ranged from 2.86 to 6.84 mg / 100 g of fresh weight. Similar contents of ellagic acid in strawberry were also reported by Jakobek et al. [23], who explored the ellagic acid content in strawberries with HPLC coupled with PDA detection.

4. CONCLUSIONS

These results show that electro-oxidation of ellagic acid on glassy-carbon electrode appears reversible in square-wave voltammetry. This technique can be used for quantitative determination of

ellagic acid in the presence of flavonols, such as quercetin and kaempferol. This is achieved by the standard addition method and the deconvolution procedure that is described in this paper.

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