ORIGINAL ARTICLE

Voltammetric determination of polyphenolic content as rosmarinic acid equivalent in tea samples using pencil graphite electrodes

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Abstract The quasi-reversible, diffusion controlled behavior of rosmarinic acid (RA) on a disposable pencil graphite electrode (PGE) was established by cyclic voltammetry. Using the anodic oxidation peak presented by RA on the PGE a differential pulse voltammetric (DPV) method was developed for the quantitative determination of RA. The linear range was $10^{-8} - 10^{-5}$ M RA and the detection and quantification limits were 7.93×10^{-9} M and 2.64×10^{-8} M RA, respectively. The applicability of the developed method was tested by recovery studies and by the assessment of the total polyphenolic contents (TPC$_{DPV}$) of green, white and black Turkish teas, which were found to be 40.74, 30.04 and 23.97 mg rosmarinic acid equivalent/g dry tea, respectively. These results were in good agreement with those obtained by the Folin-Ciocalteu method. The developed method is a sensitive and cheap tool for the rapid and precise evaluation of TPC$_{DPV}$ of tea samples.

Highlights
- Voltammetric investigation of rosmarinic acid on a cheap, disposable pencil graphite electrode (PGE)
- Rapid, simple and sensitive voltammetric method on PGE for rosmarinic acid quantification
- Cheap tool for the rapid assay of the total polyphenolic content of teas (mg RAE/g tea)
- Precision of the developed voltammetric method comparable to that of Folin-Ciocalteu method

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Introduction

Rosmarinic acid (RA), a naturally occurring polyphenolic compound, is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid that along with chlorogenic acid is found in a variety of plants (Petersens 2013). The major source of RA is the rosemary, but it can be found in many plants as culinary or medicinal herbs (Sonmezdag et al. 2016; Loganayaki et al. 2013). It is classified as phenolic acid, one of the major plant phenolics, that act as antioxidants by trapping free radicals. This polyphenol has a wide range of applications from food flavoring and preservatives to cosmetics and pharmaceutics (Petersens 2013). RA also presents anti-inflammatory (Swarup et al. 2007), antibacterial, antioxidant (Pérez-Tortosa et al. 2012), anti-carcinogenic (Osakabe et al. 2004), anti-mutagenic (Furtado et al. 2008) and antiviral properties (Astani et al. 2012). RA is also studied for its effects against the Alzheimer's disease (Alkam et al. 2007), for the improvement of cognitive performance and for the reduction of the severity of kidney diseases (Bulgakov et al. 2012).

For these reasons, the determination of RA is very important and several methods have been developed for this purpose. The common methods used for the determination of RA are high performance liquid chromatography (HPLC) (Hong and Kim 2010) and spectrophotometry (Öztürk et al. 2010). Other methods described in the literature are based on Fourier transform infrared (FT-IR) spectroscopy (Saltas et al. 2013), capillary electrophoresis (Başkan et al. 2007) or gas chromatography–mass

spectrometry (GC-MS) (Razboršek 2011). Many laboratories determine the total polyphenolic content (TPC) in different matrices using the Folin-Ciocalteu method (Shanker and Debnath 2015) which is based on their chemical reducing capacity relative to gallic acid as standard, in most cases (Singleton et al. 1999). Despite the fact that it is one of the standardized methods largely employed for TPC evaluation in leaf or instant tea, it suffers from the interference of other compounds such as sugars, aromatic amines, sulfur dioxide, ascorbic acid, some inorganic ions, etc. (Prior et al. 2005). The chromatographic methods are expensive, laborious and require skilled personnel and high amounts of reagents. On the other hand, electrochemical methods of analysis are simpler, cheaper, more rapid and can be used either in turbid or colored solutions. Some biosensors using redox enzymes like peroxidase and laccase or/and tyrosinase have been developed for the evaluation of the TPC total polyphenolic content expressed as rosmarinic acid equivalents (Brondani et al. 2011; Diaconu et al. 2011; Litescu et al. 2010; Eremia et al. 2013) in different samples such as pharmaceuticals, olive oil, tea or plant extracts. RA was preferred as reference in these determinations, being redox recycled between the redox enzyme (enzymatic oxidation) and the electrode (electrochemical reduction).

de Souza et al. 2013 and Wang et al. 2013 described the electrochemical oxidation of RA at an electrochemically pretreated glassy carbon electrode, in phosphate buffer solution of pH around 7. Under these experimental conditions RA undergoes a reversible two step oxidation, each step involving two electrons and two protons.

One of the problems encountered in the voltammetric analysis is related to the fouling of the solid electrode surface during the measurements due to the adsorption of matrix or electrode reaction products and therefore an electrode surface cleaning step is required before each new voltammetric recording. This inconvenient can be avoided by using disposable electrodes like the well-known screen printed electrodes and the pencil graphite electrode (PGE), the last one having the advantages of a good mechanical resistance and of being cheaper and readily commercially available. Despite the fact that the PGE was used for the voltammetric analysis of different types of compounds (David et al. 2013, 2015a, 2015b) but there are only two papers related to its use for the analysis of polyphenolic acids (e.g. caffeic acid) and the determination of TPC (Buratti et al. 2008; David et al. 2015a). Regarding the cyclic voltammetric behavior of RA on a PGE there is only an abstract of a poster presented by Uçar et al. at the 10th International Electrochemistry Meeting, Konya-Turkey, 2013.

Tea is the most popular beverage used all over the world either for simply pleasure or due to its health benefits which originate in the polyphenols contained in the dry material. Recently, a method was described for the polyphenolic content of refrigerated green tea beverage (Dhaouadi et al. 2016). Green, white and black teas are obtained by different procedures from *Camellia sinensis*, but they vary by the oxidation degree of the leaves compounds. Despite the fact that catechins represent the main polyphenols in these types of teas it is known that the tea leaves also contain phenolic acids which have an important contribution to the teas total antioxidant capacity (Dias et al. 2014). The composition of white, green and black teas found all over the world depends on factors like cultivation region and harvesting time (Dias et al. 2014; Lee et al. 2014) whereas the composition and antioxidant activity of tea infusions are influenced by their preparation methodologies (Lantano et al. 2015).

The present work describes, for the first time, the development of a differential pulse voltammetric (DPV) method using a bare pencil graphite electrode (PGE) for the simple, rapid, reliable and inexpensive determination of RA. Because it was shown that teas global antioxidant activity is correlated with their total polyphenolic content (TPC), regardless of their polyphenols profile and without taking into consideration the contribution of each antioxidant polyphenol (Lee et al. 2014; David et al. 2015a), the developed method was applied to the assessment of the TPC expressed as mg rosmarinic acid equivalents (mg RAE)/g dry tea in different herbal teas. Despite the fact that gallic acid is the polyphenolic acid commonly used as reference for the TPC expression, in the present work RA was selected due to the fact that in the experimental conditions optimized in this study, RA presents a simpler voltammetric behavior, with only one quasireversible well-defined oxidation peak, in comparison with gallic acid and catechins which exhibit two irreversible oxidation peaks. On the other hand, the RA oxidation peak is situated in the same potential range with the oxidation peak of gallic acid and catechins (Novak et al. 2009).

Materials and methods

Materials

The employed reagents were purchased from Sigma–Aldrich: rosmarinic acid (RA) (96 %), methanol and ethanol (≥99.5 %, ACS reagent), H_2SO_4 (95.0–98.0 % ACS reagent), acetic acid (≥99.7 %, ACS reagent), NaOH (pellets), $Na_2HPO_4 \cdot 2\,H_2O$ and KH_2PO_4 p.a. ACS and Merck: Folin Ciocalteu reagent, Na_2CO_3 anhydrous p.a.

A 2 M H_2SO_4 solution was used to obtain the 0.1 M H_2SO_4 supporting electrolyte in the voltammetric cell. 0.1 M acetate buffer solution (ABS) pH 4.03 and 0.1 M phosphate buffer

solution (PBS) pH 6.50 were also tested as supporting electrolytes. The stock solution of 10^{-3} M RA was daily prepared by dissolving accurately weighted 0.0036 g of RA in 1 mL ethanol and dilution with deionized water to the mark of a dark 10 mL volumetric flask. Between uses, the stock solution was stored in the refrigerator. Working solutions of RA employed in voltammetric measurements had concentrations from 10^{-8} to 5×10^{-4} M and were every time freshly prepared from the stock solution of RA by diluting either with the proper buffer solution or by adding the appropriate volume of 2 M H_2SO_4 solution and diluting with deionized water.

Preparation of tea infusion

The analyzed tea samples were: green tea (Çaykur ORGANIC Zumrut Yesil Cay 125 g); black tea (Çaykur ORGANIC Rize Cay 400 g) and white tea (bought from Istanbul free market). For the preparation of tea infusions the procedures existing in the literature (Buratti et al. 2008) were adapted: 1 g of tea leaves was extracted for 20 min into 25 mL freshly boiled water. After cooling, the infusion was filtered through Blue Ribbon Quantitative Whatman filter paper. After proper dilutions, the total phenolic content (TPC) of the freshly prepared infusions was analyzed in parallel by differential pulse voltammetry (DPV) and spectrophotometry (Folin-Ciocalteu procedure) (FC) (David et al. 2015a).

Methods

Electrochemical measurements

Voltammetric measurements were performed with an electrochemical system (potentiostat/galvanostat) Autolab PGSTAT 12. The voltammetric cell used for recording the voltammograms consisted of a working electrode, an Ag/AgCl (3 M KCl) reference electrode and a Pt wire as auxiliary electrode. The tested working electrodes were: pencil-graphite electrode (PGE) with a surface area of 15.86 mm^2 for 0.5 mm diameter and 1.0 cm height, glassy carbon electrode (GCE) having a surface area of 7 mm^2 (3 mm diameter) and platinum electrode (Pt) with a surface area of 3.14 mm^2 (1 mm diameter).

In order to ensure a reproducible electrode surface, the solid electrodes (GCE and Pt) were cleaned with alumina powder, rinsed with distilled water and dried, before each measurement.

The PGE was realized using a Rotring pencil as holder and 3 cm long 0.5 mm HB graphite pencil leads. The graphite lead was always inserted into the holder with the cut edge inside and 1.5 cm of the lead was left outside to be introduced in the analyzed solution. Every time, a constant part of 1 cm of the lead was introduced in the solution. A new lead was used for each measurement. The electrical contact was assured by

soldering a metal wire to the metallic part of the Rotring pencil (David et al. 2013, 2015a, 2015b).

Cyclic voltammograms (CV) were recorded in the potential range from –500 mV to +1200 mV at 100 mV·s^{-1} scan rate if not stated otherwise.

Differential pulse voltammetric (DPV) recordings were carried out in the potential range 300 to 800 mV under following parameters: step potential 4.95 mV, modulation amplitude 25 mV, modulation time 0.05 s and interval time 0.1 s. All the experiments were performed at 25.0 ± 0.2 °C.

The standard addition method was applied for recovery studies and determination of total polyphenolic content (TPC$_{DPV}$) of tea samples using DPV on PGE. DP voltammograms were recorded for 100 fold diluted tea sample using 0.1 M H_2SO_4 as supporting electrolyte. Then, three successive additions of 0.1 mL of 1×10^{-4} M RA solution were done to each of the analyzed tea sample solutions. DP voltammograms were also recorded after each addition and the corresponding peak currents were measured and used to calculate the percentage recoveries and the TPC$_{DPV}$ expressed as mg RAE/g dry tea.

Spectrophotometric measurements

Measurements were made on a Jasco V-530 UV–vis spectrophotometer (Japan) with quartz cuvettes of 1.00 cm path length.

Total polyphenolic content (TPC$_{FC}$) of the tea extract was determined in accordance with the Folin-Ciocalteu (FC) method with minor modifications and using RA as standard (Singleton et al. 1999). Results were expressed as mg RAE/g dry tea. For this purpose, different aliquotes (0.1–0.5 mL) of 100 μg·mL^{-1} RA methanolic stock solution were added into a series of 5 mL volumetric flasks. Into each flask, 2.5 mL volume of freshly prepared FC reagent (diluted 1:10 with deionized water) was also added. After 8 min, 2 mL of sodium carbonate (7.8 % w/v) was added to each mixture, thoroughly mixed and diluted with deionised water to the mark of the volumetric flask. The thus obtained solutions were left in the dark for 60 min, with intermittent shaking. In order to obtain the calibration graph the absorbance of each mixture was measured at 760 nm against a blank.

For the tea samples analyses, a volume of 0.1 mL of tenfold diluted tea infusion was added in a 5 mL volumetric flask and the general procedure was followed. The TPC$_{FC}$ was calculated using the calibration curve. Each measurement was done in triplicate and the results were given as the average of the obtained data.

Throughout the entire study repeated measurements were performed and percentage relative standard deviations (RSD %) were calculated. The recovery was determined on the tea samples. Two well known statistical tests

were applied in order to emphasize the reliability of the proposed method results.

Results and discussion

Cyclic voltammetric study. Establishing the optimum working parameters

Influence of the working electrode

The voltammetric behavior of an analyte often depends on the nature of the electrode surface material. Therefore, a Pt electrode, a GCE and a PGE respectively were employed as working electrodes to record cyclic voltammograms of RA in 0.1 M H_2SO_4 (Fig. 1). The best shaped and the highest peaks were obtained on PGE when compared with GCE and Pt electrode, respectively. The sensitivities of the RA response on the Pt electrode, GCE and PGE were 1592, 2286 and 4152 $\mu A \cdot mm^{-2} \cdot M^{-1}$, respectively. This observation suggests a more sensitive determinations of RA on PGE, thus leading to the selection of PGE as working electrode for further voltammetric studies of RA.

Influence of the supporting electrolyte

It is well known that the nature of the used supporting electrolyte can have a significant influence on the shape of the voltammograms. Therefore, 0.1 M H_2SO_4, ABS pH 4.03 and PBS pH 6.50 were employed to investigate the voltammetric behavior of RA on PGE. Comparing the cyclic voltammograms recorded using the three mentioned supporting electrolytes with different pH values, one can observe (Fig. 2) that the potentials of both anodic and cathodic peaks of RA are shifted towards more positive potentials and the peak current intensities increase when the solution pH decreases. The best defined cyclic voltammogram (with high peak currents) for RA was
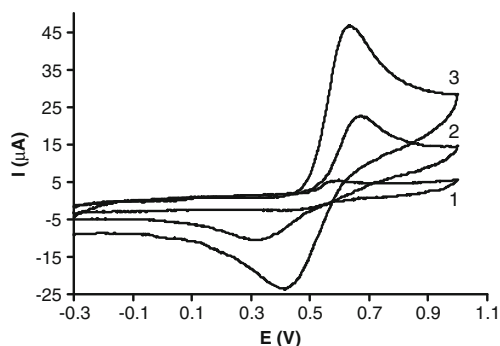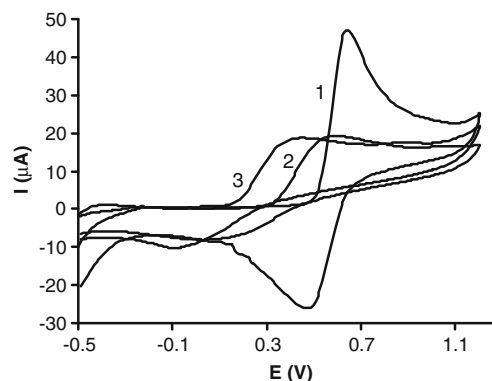
Fig. 2 Cyclic voltammograms for a 5×10^{-4} M RA solution recorded on a PGE in different supporting electrolytes 0.1 M H_2SO_4 (1), ABS pH 4.03 (2) and PBS pH 6.50 (3); scan rate 100 mV $\cdot$ s^{-1}

obtained in 0.1 M H_2SO_4 (Fig. 2). Consequently, for the subsequent voltammetric studies of RA, 0.1 M H_2SO_4 was used as supporting electrolyte.

Voltammetric behavior of RA

In order to evaluate if the electrode process is controlled by the reactant diffusion towards the PGE or by the reactant adsorption on the working electrode, cyclic voltammograms were recorded for RA at different scan rates (v), ranging from 10 to 1000 mV $\cdot$ s^{-1} (Fig. 3). For all scan rates, the cyclic voltammograms of RA on PGE present a pair of well-defined peaks with the anodic and cathodic peak potentials shifting with the potential scan rate. The value of the peak potential separation, $\Delta E_p = E_{pa} - E_{pc}$, increases with v and for all scan rates it is more than 59/n mV (where n is the number of exchanged electrons). The value of the I_{pa}/I_{pc} ratio (I_{pa} is anodic peak current and I_{pc} is cathodic peak current) is higher than 1 indicating a quasi-reversible behavior of RA on the PGE in 0.1 M
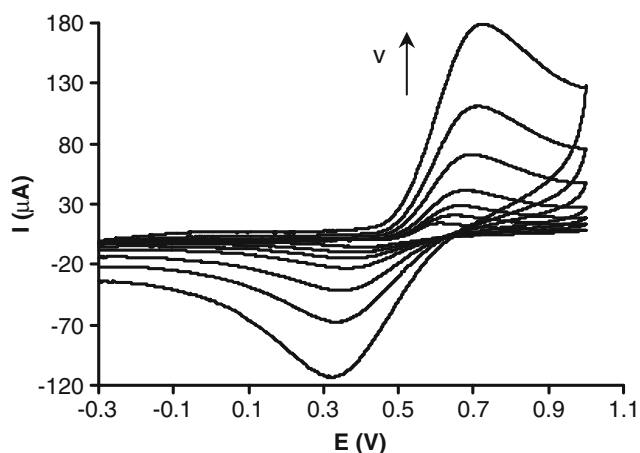
Fig. 1 Cyclic voltammograms for a 5×10^{-4} M RA in 0.1 M H_2SO_4 solution recorded on Pt (1), GCE (2) and PGE (3); scan rate 100 mV $\cdot$ s^{-1}

Fig. 3 Cyclic voltammograms recorded for a 5×10^{-4} M RA on PGE at different scan rates (10; 25; 50; 100; 250; 500 and 1000 mV $\cdot$ s^{-1}). Supporting electrolyte: 0.1 M H_2SO_4 solution

H_2SO_4. The apparent heterogeneous electron transfer rate constant (k_s) was calculated in order to thoroughgoing study the electrochemical behavior of RA using the following equation (Laviron 1979):

$$\log k_s = \alpha \log(1-\alpha)$$
$$+ (1-\alpha)\log\alpha - \log\frac{RT}{nFv} - \frac{\alpha(1-\alpha)nF\Delta E_p}{2.3RT},$$

where α is the charge transfer coefficient, n is the number of electrons involved in the reaction, R is the universal gas constant, F is Faraday number and T is the temperature. α was estimated from the plot of the anodic and respectively cathodic peak potential versus decimal logarithm of scan rate, based on the slopes of the two straight lines and the obtained value is 0.46. Hence, k_s was calculated as 1.45×10^{-2} cm·s^{-1} ($n = 2$). This value of k_s indicates that the electron transfer process on the PGE surface is effectively promoted.

As it can be observed from the superposed cyclic voltammograms (Fig. 3) both the anodic and cathodic peak currents increased with the increase of the potential scan rate. The linear dependence $I_p = f(v^{1/2})$ expressed by the equations $I_{pa} = 3.3432 v^{1/2} - 3.9352$ ($R^2 = 0.9928$) and $I_{pc} = 3.0593 v^{1/2} - 6.0799$ ($R^2 = 0.9877$) and the slope values of the log $I_p = f(\log v)$ plots (log $I_{pa} = 0.5126$ log $v + 0.4457$ ($R^2 = 0.9910$); log $I_{pc} = 0.5599$ log $v + 0.2587$ ($R^2 = 0.9950$)) near to 0.5 for both the anodic and cathodic peaks, respectively, indicate that the electrochemical oxidation of RA on PGE is diffusion controlled (Brett and Oliveira-Brett 1993).

Quantitative analysis

Linear range and detection limit

DPV was used to establish the dependence of the anodic peak current and the RA concentration. Differential pulse voltammograms were recorded for different RA concentrations in the range 10^{-8} to 5×10^{-4} M. The obtained results showed that the peak currents depend linearly on RA concentration in the range $10^{-8} - 10^{-5}$ M according to the following equation: $I_{pa} = 597859 C_{RA} + 0.824$ ($R^2 = 0.9952$) (Fig. 4).

The detection limit (LD) and the quantification limit (LQ) were determined from the calibration data, as follows: LD $= 3 s_{C_{min}}/b = 7.93 \times 10^{-9}$ M, LQ $= 10 s_{C_{min}}/b = 2.64 \times 10^{-8}$ M, where: $s_{C_{min}}$ is the standard deviation of the smallest concentration from the calibration curve and b is the calibration curve slope (González and Herrador 2007).

It was observed that, with one exception (Wang et al. 2013) that the performance characteristics (i.e. linear range and detection limit) of the here described DPV method on PGE are better than most of those reported in the literature (Brondani et al. 2011).
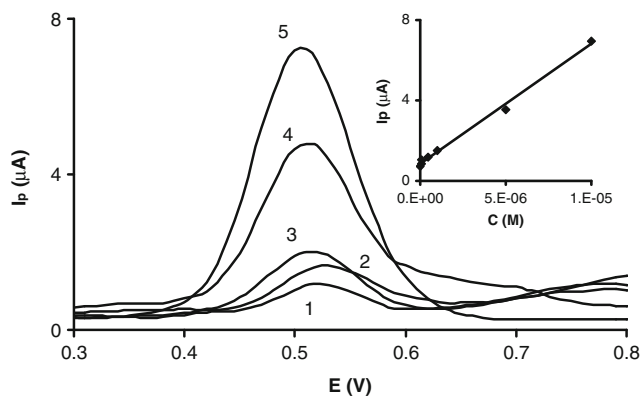

Fig. 4 Differential pulse voltammograms recorded on PGE for solutions with different concentrations of RA (1) 10^{-8} M; (2) 10^{-7} M; (3) 10^{-6} M; (4) 5×10^{-6} M and (5) 10^{-5} M; supporting electrolyte: 0.1 M H_2SO_4 and the corresponding calibration graph

Repeatability

The repeatability of the DPV method using a PGE expressed as percentage relative standard deviation (RSD %) was determined for 10 solutions of 10^{-6} M RA and the obtained value was 6.27 %.

Recovery studies and analytical applications

The applicability of the developed method on real samples was tested on three types of Turkish tea (green, white and black). Differential pulse voltammograms recorded for three replicate samples of each of the three previously mentioned teas presented a well-defined anodic peak situated at about 550–600 mV. DPV recordings on PGE in 0.1 M H_2SO_4 have shown that other polyphenolic acids (e.g. caffeic acid, chlorogenic acid, gallic acid) (Novak et al. 2009; David et al. 2015a) are oxidized at almost the same potential. Therefore the developed method can't be applied for the determination of RA in tea samples, but it was observed that the current intensity of this peak increased linearly with three successive additions of 0.1 mL stock solution of 10^{-4} M RA (Fig. 5). Thus, by applying the standard addition method,
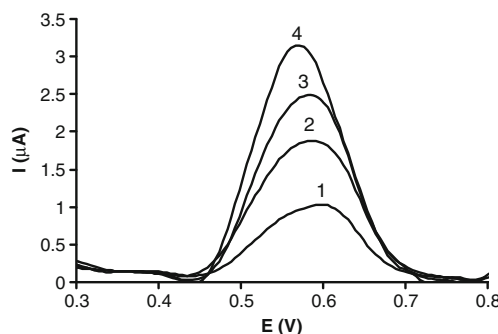

Fig. 5 Differential pulse voltammograms recorded on PGE for 10 mL black tea before (1) and after three successive additions of 0.1 mL of stock solution of 10^{-4} M RA (2–4); supporting electrolyte: 0.1 M H_2SO_4

Table 1 TPC_{DPV} (expressed as RA concentration) determination from green, white and black tea samples (3 sample replicates, 3 standard additions each), recovery values and comparison with TPC_{FC}

Tea sample	Sample replicate number	C_{RA}, mg RAE/g dry tea	$\overline{R} \pm SD$[a], %	$\overline{TPC}_{DPV} \pm SD$[b], mg RAE/g dry tea; RSD[c], %	$\overline{TPC}_{FC} \pm SD$[d], mg RAE/g dry tea; RSD, %	Relative error, er%[e]	F-value[f]	t value[g]
Green tea	1	43.36	100.40 ± 1.13	40.74 ± 2.31;	41.04 ± 1.82;	0.73	1.61	0.20
	2	39.00	101.71 ± 5.23	5.67	4.44			
	3	39.85	99.20 ± 1.72					
White tea	1	29.46	100.78 ± 2.08	30.04 ± 1.11;	30.09 ± 1.15;	0.17	1.07	0.05
	2	31.32	98.01 ± 4.00	3.68	3.81			
	3	29.35	98.62 ± 4.35					
Black tea	1	25.13	96.81 ± 5.75	23.97 ± 1.07;	24.76 ± 0.58;	3.19	3.40	1.19
	2	23.78	102.01 ± 4.34	4.48	2.32			
	3	23.01	100.22 ± 1.91					

[a] mean recovery ± standard deviation

[b] mean total polyphenolic content by differential pulse voltammetry ± standard deviation

[c] relative standard deviation

[d] mean total polyphenolic content by Folin Ciocalteu method ± standard deviation

[e] $er\% = ((TPC_{FC} - TPC_{DPV})/TPC_{FC}) \cdot 100$

[f] tabled F-value for $\nu_1 = 9$, $\nu_2 = 2$ at 95 % confidence level, two-sided is 4.26

[g] tabled t-value for 11 freedom degrees at 95 % confidence level, two-sided is 2.201

DPV on PGE can be used to determine the total polyphenolic content (TPC_{DPV}) of tea samples expressed as mg rosmarinic acid equivalent/g dry tea dry weight, i.e. mg RAE/g dry tea. Recoveries of RA added to the analyzed tea samples were also evaluated. The overall mean values were found to be 100.44, 99.14 and 99.70 % for green, white and black tea, respectively (Table 1).

For comparison, the spectrophotometric Folin-Ciocalteu method using RA as reference was also applied to evaluate the total polyphenolic content (TPC_{FC}) of the same Turkish green, black and white teas, respectively. The calibration curve described by the linear equation $A = 0.1108\ C_{RA} + 0.0181$ ($R^2 = 0.9828$) where C_{RA} is RA concentration, in $\mu g \cdot mL^{-1}$ and A is absorbance was used to estimate the TPC_{FC} of the analyzed samples. The results obtained by the above mentioned methods, expressed as mean ± standard deviation (SD) of three measurements were compared and the relative error values were calculated (JCGM 100 2008). For all three tea samples the obtained values (Table 1) show a very good consistency of the results.

For the obtained results using DPV and FC methods two statistical tests were also applied: the F test and *t* test (Table 1) (David et al. 2015a). In the case of F test, the calculated values are smaller than the theoretical ones (at 95 % confidence levels). This shows that the experimental data of the two mentioned methods have close precisions. When the *t* test was applied, for all three types of tea, the theoretical values are bigger than the calculated ones (at 95 % confidence levels). This means that the average values obtained by the two methods belong to the same values population. In conclusion, the outcome of the statistical comparison proved there are no significant differences between the performances of the proposed method and the reference one.

Conclusion

The present work describes a new sensitive DPV method for RA quantification based on RA oxidation on the disposable PGE. The method presented a linear range of three orders of magnitude, good repeatability and limits of quantification and detection among the lowest reported in the literature for the electrochemical detection of RA. The developed DPV method on PGE using RA as reference can be used as a sensitive and cheap method for the rapid and precise assessment of total polyphenolic content of tea samples, obtaining reliable results in accordance with those of the standardized Folin-Ciocalteu method.

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