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Development and validation of a voltammetric method for determination of total phenolic acids in cotton cultivars

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ABSTRACT

A highly sensitive and selective differential pulse voltammetric (DPV) method based on the oxidation of caffeic acid in a glassy carbon electrode is presented for determination of total phenolic acids (TPAs). Under optimized conditions (0.2 M phosphate buffer, pH 3, 50 mV pulse amplitude, 50 mV s^{-1} scan rate), the oxidation peak current (I_{pa}) of caffeic acid is linear ($I_{pa}(A) = -4.15\times10^{-8} + 0.97$ [Caffeic acid]) to caffeic acid concentration in the range from 1.0×10^{-7} to 1.0×10^{-6} M, with a correlation coefficient of 0.9985. The detection and quantitation limits obtained were 6.8×10^{-8} M and 1.0×10^{-7} M, respectively. The repeatability and intermediate precision of DPV method were acceptable (Relative Standard Deviation (RSD) <10%). The absence of the matrix effect on the determination of TPAs by DPV method was attested by F-test and *t*-test (95% confidence level). The method was successfully applied to the determination of TPAs in five cotton cultivars, with recoveries of 94–104% (RSD <2%).

1. Introduction

Since plants are devoid of mobility and immune system they have developed alternative defensive strategies, including the synthesis of secondary metabolites, to overcome stress constraints, adapt to the changing environment and survive to the attack of arthropod pests [1]. These secondary metabolites are synthesized during the normal development of plant tissues or in response to physical injury infection or to other stress and they are stored in specialized structures, such as glands, trichomes and vacuoles [2]. The various protective functions performed by secondary metabolites in plants (structure stabilizers, antioxidants, photoprotectors, signal transducers, antimicrobials) are related to the great diversity of chemical structures and the interactions (e.g. condensation and polymerization reactions, electrostatic interactions, absorption) of these molecules [1].

In cotton, several secondary metabolites, such as the phenolic compounds, have been associated with resistance to several species of arthropod pests [2]. The resistance of cotton to apple caterpillar, *Heliothis virescens* (LEPIDOPTERA: NOCTUIDAE), was assigned to the presence of terpenoid aldehydes in its leaves by Stipanovic et al. [3]. Other secondary metabolites such as tannins and gossypol were also associated with resistance of cotton to the apple caterpillar by Chan et al. [4] and Lukefahr et al. [5]. Alimukhamedov and Shvetsova [6] showed that aphid resistant cotton genotypes have a large amount of phenols. The resistance of cotton to thrips was associated with the presence of gossypol glands on the calyx of its flowers by Bourland and Benson [7]. A relationship between cotton resistance to whitefly and its production of phenols, tannins, and gossypol has been established by some authors [8]. Some studies showed that cotton genotypes with high levels of condensed tannins, phenolic acids and catechols are generally more resistant to mites [9–11]. Agrawal and Klein [12] have also demonstrated the involvement of gossypol glands on cotton resistance to mites and they also showed that these glands could be phenotypically induced by herbivory. In addition, some phenolic compounds such as gossypol, 3,4-dihydroxy benzoic acid, ferulic acid and caffeic acid were reported to be highly toxic to the nematode *Meloidogyne incognita* larvae and to the *Fusarium oxysporum*. In cotton breeding programs, the development of genotypes, which are resistant to these nematode and fungal diseases and can significantly limit cotton production, is identified as a breeding priority [13,14].

The determination of phenolic compounds in various matrices (fruits, vegetables, processed products) may be performed using several analytical methods, such as spectrophotometric, chromatographic, capillary electrophoretic, chemiluminescent and electrochemical [15]. Since most phenolic compounds are electrochemically active at moderate oxidation potentials, electrochemical methods of analysis are preferable due to the advantages of high sensitivity, simplicity, good stability, inexpensive and portable instrumentation and less interference from non-electroactive substances. Many publications concerning

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this topic can be found in the literature. Cyclic, differential pulse and square-wave voltammetry were proved to be excellent analytical tools to investigate the electrochemical behavior and characterize the antioxidant properties of some natural (flavonoids and phenolic acids) and synthetic (butylated hydroxyanisole (BHA), butylated hydroxitoluene (BHT), *tert*-butylhydroquinone (TBHQ)) phenolic compounds in fruits, fruit juices, tea, coffee, wine, seaweeds and synthetic samples, by using carbon, diamond and graphite electrodes [16–34]. Many electrochemical methods have been used in the determination of natural and synthetic phenolic compounds in different matrices. Stripping voltammetric methods based on mercury and carbon electrodes have been applied to the determination of nine flavonoids (in the free form and complexed with Cu^{2+} and Hg^{2+} metal ions) and one phenolic acid in juice, tea, drugs, soybean and biological fluids [35–40]. Cyclic voltammetric methods based on carbon and gold electrodes were successfully employed to flavonoids and phenolic acids determination in juice, wine, pharmaceutical and synthetic samples [17,22,27,29]. Differential pulse voltammetric (DPV) methods based on graphite and carbon electrodes were used to determine polyphenols in tea, wines, fruits, fruit juices and olive oils [16,41–44]. Isoflavones were determined in soybeans and soy-based foods by linear sweep voltammetric and amperometric methods using mercury and glassy carbon electrodes [45,46]. Square-wave voltammetric methods based on mercury, carbon and diamond electrodes have been applied to determination of rutin in pharmaceutical, biological fluid and tea samples [30,47], cathechin in tea [20], BHA and BHT in food [32] and TBHQ in soybean biodiesel samples [48].

However, to the best of our knowledge, electrochemical methods have not yet been applied for phenolics determination in cotton samples. Cotton is commercially cultivated in 78 countries [49] due to its importance in the textile industry (fibers) and in human and animal nutrition (seeds). Current worldwide production of cotton is about 25 million tons per year, with Brazil accounting for about nine million tons. The central-west region of Brazil accounts for 84% of Brazilian cotton production, in which the state of Mato Grosso is the largest producer. Insects are a major constraint to profitable cotton production and chemical insecticides are still the primary control method used in worldwide cotton production systems. This practice, however, presents a great risk to the environment and health and increases production costs. For this reason, in recent years researchers have been studying alternatives to control cotton pests, such as the development of cotton cultivars that are resistant to insect attack by using the information about secondary metabolites. This work describes a highly sensitive DPV method based on a glassy carbon electrode for the determination of total phenolic acids (TPAs) in cotton cultivars. Thus, different parameters were evaluated for their quantification and for the validation of the DPV method in cotton. Compared to other analytical techniques, the proposed method presents good selectivity, high sensitivity, rapid responses, low cost instrumentation and reduced sample size for the determination of TPAs. Moreover, the study presents new data about the total concentration of phenolic acids in Brazilian cotton cultivars, which have many applications in integrated pest management and breeding programs, contributing to the sustainability of cotton-based agricultural systems.

2. Experimental

2.1. Apparatus

Differential pulse voltammetric (DPV) measurements were carried out on a 797 Voltammetric Analyzer (VA) Computrace (Metrohm, Switzerland) with an electrochemical cell composed of a glassy carbon (GC) rotating disk ($\Phi = 2{,}0$ mm) as working electrode. Ag/AgCl (3 M KCl) electrode as reference electrode and a platinum wire as auxiliary electrode. DPV measurements were performed in the potential range of 0 V (initial potential, E_i) to 1.000 V (final potential, E_f) at the following settings: 50 mV pulse amplitude and 50 mV s^{-1} scan rate. The hydrogen ion potential (pH) of the solutions was determined using a 3030 pH-meter (Jenway, United Kingdom) with a DME-CV1 combination pH electrode (Digimed, Brazil). The 2840 D ultrasonic cleaner (Odontobrás, BRAZIL) was used for GC electrode cleaning and phenolic acids extraction in cotton leaves.

2.2. Chemicals and samples

Analytical grade reagents and ultrapure water (Milli-Q, Millipore, Billerica, United States) were used to prepare all solutions. Caffeic acid (98% purity), chlorogenic acid (95% purity), gallic acid (97% purity) and gentisic acid (95% purity) were purchased from Sigma-Aldrich (St Louis, United States) and used without further purification. Phenolic acid standard stock solutions (1.0×10^{-3} M) were prepared in 5 mL of ethanol (Sigma-Aldrich, St Louis, United States), diluted with water to 10 mL and then stored in dark bottles under freezing to prevent photodegradation. Phosphate buffers in the pH range of 3–7 were prepared using dibasic sodium phosphate, monobasic potassium phosphate and phosphoric acid (Sigma-Aldrich, St Louis, United States) and used as supporting electrolytes. Sodium hydroxide and hydrochloric acid (Sigma-Aldrich, St Louis, United States) were used for pH adjustment. Ethanol (Sigma-Aldrich, St Louis, United States) was used for phenolic acids extraction from cotton leaves. Alumina oxide powder < 10 μm (Sigma-Aldrich, St Louis, United States) and acetone (Sigma-Aldrich, St Louis, United States) were used for GC electrode cleaning. Nitric acid (Quimex, São Paulo, Brazil) was used for cleaning laboratory glassware. The cotton leaves employed were of the following cultivars: FM 966, BRS ARAÇÁ, BRS AROEIRA, BRS CEDRO and BRS 293 (Embrapa Arroz e Feijão, Brazil). Plants were grown under greenhouse conditions (temperature (T) = 24 ± 2 °C, relative humidity (RH) = 39%) and they were harvested at B1 growth stage.

2.3. Procedures

The laboratory glassware was kept in a 20% (by volume) nitric acid solution overnight. Afterwards, it was kept in an ultra pure water (Milli-Q, Millipore, Billerica, United States) bath overnight, rinsed thoroughly with ultra pure water and air-dried.

GC electrode cleaning was based on the following procedure: the GC electrode surface was manually polished with alumina suspension on polishing cloth for 1 min and rinsed with distilled water; The electrode was immersed in acetone and submitted to a ultrasonication bath for 4 min and rinsed with ultra pure water (Milli-Q, Millipore, Billerica, United States); the electrode was immersed in 0.2 M phosphate buffer, pH 3, and submitted to continuous potential cycling from 0 to 1.8 V at 50 mV s^{-1} for 3 min.

Experiments were performed with four phenolic acids (caffeic, chlorogenic, gallic and gentisic acids) at room temperature in three replicates.

The oxidation behavior of phenolic acids was studied by differential pulse voltammetry through additions of 50 μL of 1.0×10^{-3} M phenolic acid to the electrochemical cell containing 10 mL of 0.2 M phosphate buffer, pH 3.

In order to optimize the experimental conditions and to obtain the highest sensitivity for the differential pulse voltammetric (DPV) method, the influence of the pH, scan rate and pulse amplitude on the oxidation peak current (I_{pa}) of the phenolic acids was studied.

The influence of the pH on the peak current of phenolic acids by using differential pulse voltammetry was studied through additions of 100 μL of 1.0×10^{-3} M phenolic acid to the electrochemical cell containing 10 mL of 0.1–0.2 M phosphate buffers in the pH range of 3–7.

The study of the influence of pulse amplitude (range of 10–100 mV) and scan rate (rage of 5–50 mV s^{-1}) on the peak current of phenolic acids by using differential pulse voltammetry was accomplished through additions of 100 μL of 1.0×10^{-3} M phenolic acid to the electrochemical cell containing 10 mL of 0.2 M phosphate buffer, pH 3.

Calibration curves were obtained by using differential pulse voltammetry by successive additions of 1–10 μL of 1.0×10^{-3} M phenolic acid to the electrochemical cell containing 10 mL of 0.2 M phosphate buffer pH 3.

Eight parameters (linearity, linear range, limits of detection and quantitation, selectivity, repeatability, intermediary precision and recovery) were determined for the validation of DPV method in cotton using the guidelines of National Institute of Metrology, Quality and Technology (INMETRO) [50]. Linearity was evaluated using ten concentration levels of the four studied phenolic acids. The limit of detection (LD) was calculated from the expression $LD = 0 + t\ (n-1.1-\alpha).s$, where: t = student distribution for $n = 10$ independent sample blanks fortified at the lowest acceptable concentration measured for each phenolic acid; $\alpha = 95\%$ confident level and s = sample standard deviation of the fortified sample blank values. The limit of quantitation (LQ) was calculated from the lower end of the working range. The selectivity was evaluated by comparing the slopes of calibration curves obtained with a blank sample and a cotton sample (BRS Araçá) with caffeic acid standard additions. The repeatability (intra-day precision) of the DPV method was evaluated by the analysis of seven fortified blank samples with standard additions of caffeic, chlorogenic, gallic and gentisic acids at three concentrations levels (25%, 50% and 100%) over the working ranges. Repeatability was determined, considering the same analyst, equipment and laboratory and short timescale, through calculation of the relative standard deviation (RSD) at the corresponding concentration level. The intermediate precision (inter-day precision) of the DPV method was evaluated in the same way as repeatability, but the RSD was calculated considering experiments conducted in three different days. The trueness of the DPV method was assessed by recovery assays in which known amounts of caffeic acid (2×10^{-7} M; 5×10^{-7} M; 1×10^{-6} M) were added to BRS Araçá cotton sample.

The proposed method was applied to the determination of total phenolic acids (TPAs) in cotton leaves of five cultivars (FM 966, BRS ARAÇÁ, BRS AROEIRA, BRS CEDRO and BRS 293). Phenolic acids were extracted in triplicate from 100 mg sample of dried macerated cotton leaves by ultrasonication (20 min, room temperature) in 2.0 mL of 25% aqueous ethanol. The resulting extract was decanted and the supernatant was transferred to 2.0 mL microtubes and centrifuged for 10 min at 12,100 *g*. Then, the new supernatant was transferred to a volumetric flask and diluted to 10 mL with ultrapure water. After the extraction procedure, samples of cotton leaves were immediately analyzed. TPAs were determined in cotton leaves in triplicate at room temperature using the standard additions method by successive additions of 3 μL of 1.0×10^{-3} M caffeic acid to the electrochemical cell containing 10 mL of 0.2 M phosphate buffer, pH 3, and 50–200 μL of cotton sample.

2.4. Statistical analysis

Data were summarized with Microsoft Office Excel software (version 2007; Microsoft Corp, Redmond, WA, USA).

Cochran's test ($\alpha = 0.05$) was used to test the homogeneity of variances of linear regression data. F-test (Snedecor) and *t*-test were used ($\alpha = 0.05$) to evaluate the selectivity and the matrix effects of DPV method. F-test was used to evaluate the significance of variances of two sets of test samples (with or without the matrix). *t*-test was used to evaluate the significance of the mean difference of the two sample sets. Outliers were determined by using Grubb's test [51].

3. Results and discussion

3.1. Voltammetric behavior of four phenolic acids at a glassy carbon electrode

Fig. 1 shows the differential pulse voltammograms obtained for phenolic acids (5.0×10^{-6} M) in 10 mL of 0.2 M phosphate buffer, pH 3, at a glassy carbon electrode. Curve 1 represents the oxidation of the supporting electrolyte (0.2 M phosphate buffer pH 3). Curves 2–5 show the oxidation of gentisic, gallic, chlorogenic and caffeic acids in 0.2 M phosphate buffer, pH 3. All the studied phenolic acids presented one anodic peak at approximately 0.4 V that can be assigned to the oxidation of hydroxyl groups on the aromatic ring leading to the formation of *O*-quinone via semiquinone forms [17]. The voltammetric determination of phenolic acids is based on this anodic peak. The best results concerning oxidation peak currents of studied phenolic acids were obtained for caffeic acid. Thus, caffeic acid can be considered the most suitable standard for determination of total phenolic acids (TPAs) content in cotton samples.

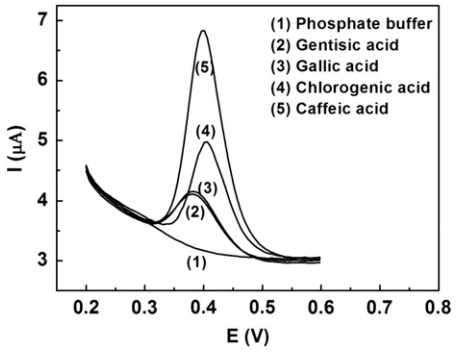

Fig. 1. Differential pulse voltammetric voltammograms obtained for phenolic acids in 10 mL of 0.2 M phosphate buffer, pH 3. (1) phosphate buffer 0.2 M. (2) 5.0×10^{-6} M gentisic acid. (3) 5.0×10^{-6} M gallic acid. (4) 5.0×10^{-6} M chlorogenic acid. (5) 5.0×10^{-6} M caffeic acid. Initial potential (E_i) = 0.2 V, final potential (E_f) = 0.8 V, pulse amplitude = 50 mV, scan rate = 50 mV s^{-1}, working electrode: glassy carbon, reference electrode: Ag/AgCl (KCl 3 M).

3.2. Influence of operational parameters

3.2.1. Influence of pH

The best results concerning signal enhancement and shape of oxidation peak currents of phenolic acids were obtained at pH 3. With decreasing pH of the solution, the oxidation peak currents of phenolic acids increase, sometimes slightly, up to pH 3 (Fig. 2). This phenomenon can be assigned to the increase of phenolic acid protonation degree and the resulting shift of the molecule charge to negative values. This is in accordance with that reported in the literature [25]. Hence, the pH 3 was chosen for further measurements.

3.2.2. Scan rate and pulse amplitude

The oxidation peak currents of phenolic acids decrease with increasing scan rate but they increase with increasing pulse amplitude. It was observed that these two parameters do not affect the phenolic acids oxidation peak currents for scan rate values above 25 mV s^{-1}

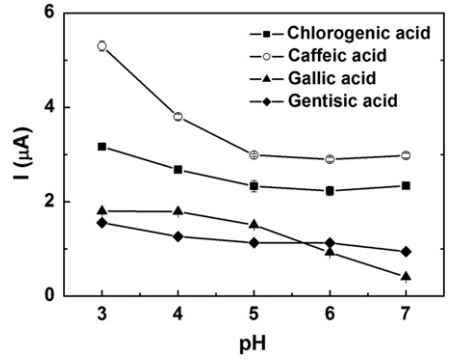

Fig. 2. Effect of the pH on the differential pulse voltammetric peak currents of 1.0×10^{-5} M of phenolic acids in 10 mL of 0.2 M phosphate buffer, pH 3. Initial potential (E_i) = 0.2 V; final potential (E_f) = 0.8 V, pulse amplitude = 50 mV, scan rate = 50 mV s^{-1}, working electrode: glassy carbon, reference electrode: Ag/AgCl (KCl 3 M).

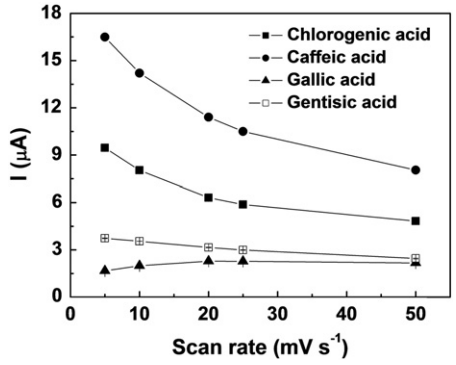

Fig. 3. Effect of the scan rate on the differential pulse voltammetric peak currents of 1.0×10^{-5} M of phenolic acids in 10 mL of 0.2 M phosphate buffer, pH 3. Initial potential (E_i) = 0.2 V, final potential (E_f) = 0.8 V, pulse amplitude = 50 mV, scan rate = 5–50 mV s^{-1}, working electrode: glassy carbon, reference electrode: Ag/AgCl (KCl 3 M).

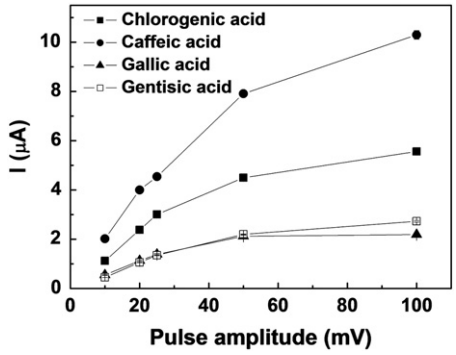

Fig. 4. Effect of pulse amplitude on the differential pulse voltammetric peak currents of 1.0×10^{-5} M of phenolic acids in 10 mL of 0.2 M phosphate buffer, pH 3. Initial potential (E_i) = 0.2 V, final potential (E_f) = 0.8 V, pulse amplitude = 10–100 mV, scan rate = 50 mV s^{-1}, working electrode: glassy carbon, reference electrode: Ag/AgCl (KCl 3 M).

and for pulse amplitude values above 50 mV (except for caffeic acid) (Figs. 3 and 4). The oxidation peak currents of phenolic acids decrease with increasing scan rate because the phenolic acids reach the electrode surface with a reaction rate that is not sufficient to provide their participation in the oxidation-reduction reaction and their electron exchange with electrode surface. However, the oxidation peak currents of phenolic acids increase with increasing pulse amplitude because the separation between the faradaic and capacitive currents, which is enhanced by the increase of the pulse amplitude, is more effective [52,53]. For further experiments, a 50 mV s^{-1} scan rate and 50 mV pulse amplitude were applied. The selection of these two parameters was made based on the best current sensitivity, resolution of the peaks and speed of analysis. According to theory, larger pulse amplitudes (above 100 mV) result in larger and broader peaks leading to a loss of resolution [52,53].

Table 2
Precision parameters for determination of phenolic acids by differential pulse voltammetry using glassy carbon electrode.

Parameters	Chlorogenic acid	Caffeic acid	Gallic acid	Gentisic acid
Repeatability (%) ($N^a=3$; $n^b=7$)	0.5–7.4	0.5–1.2	1.1–2.4	3.0–7.0
Intermediate precision (%) ($N^a=7$; $n^b=3$)	2.2–41	2.1–8.6	2.1–5.9	5.0–21

[a] Number of concentration levels.
[b] Number of replicates for each concentration.

3.3. Calibration curves and validation parameters

The overall performance of the differential pulse voltammetric (DPV) method is summarized in Tables 1–4. Under the optimized experimental conditions, a linear response of oxidation peak current as a function of concentration was observed for caffeic, chlorogenic, gallic and gentisic acids over the concentration ranges studied (Table 1). The detection limits (Table 1) were found to be in the range from 6.8×10^{-8} to 8.4×10^{-7} M, which are lower than previous reports [16,17]. Correlation coefficients (r) (Table 1) were higher than 0.99 for all studied phenolic acids, which attest to the linearity of the proposed method. Homocedastic results from Cochran's test (C calculated < C critical — 95% confidence level) (Table 1) are another evidence of the linearity of DPV method. The calculated repeatability and intermediate precision values were considered satisfactory (Table 2). The repeatability index was smaller than the intermediate index for all four phenolic acids, which indicates that the variation for measurements performed in different days was larger than the variation for independent experiments conducted within a day, as expected. It was also observed that a relationship between precision and the concentration of analyte: lower concentrations presented larger relative standard deviation than higher concentrations. In the evaluation of selectivity, the slopes obtained with the blank sample were similar to those obtained for BRS Araçá cotton sample with caffeic acid standard additions, attesting that there was no matrix interference in the assayed concentration range of 1×10^{-7} M to 1×10^{-6} M (Table 3). This result was supported by statistical analysis in which F-test and t-test (95% confidence level) were not significant (F calculated < F critical and t calculated < t critical). Thus, it can be concluded that the matrix does not have a significant effect on the precision of DPV method and also that there is no significant difference between the mean values of the two sample sets (i.e. blank

Table 1
Validation parameters for determination of phenolic acids by differential pulse voltammetry using glassy carbon electrode.

Parameters	Chlorogenic acid	Caffeic acid	Gallic acid	Gentisic acid
Curve equation	$I_{pa}(A)^d = -2.72\times10^{-7} + 0.51$ [chlorogenic acid]	$I_{pa}(A) = -4.15\times10^{-8} + 0.97$ [caffeic acid]	$I_{pa}(A) = -1.8\times10^{-7} + 0.22$ [gallic acid]	$I_{pa}(A) = -1.0\times10^{-8} + 0.23$ [gentisic acid]
Linearity range/working range (M)	5.0×10^{-7}–5.0×10^{-6}	1.0×10^{-7}–1.0×10^{-6}	1.0×10^{-6}–1.0×10^{-5}	1.5×10^{-6}–1.0×10^{-5}
Regression coefficient	0.9958 (p^e<0.0001)	0.9985 (p<0.0001)	0.9980 (p<0.0001)	0.9976 (p<0.0001)
Crochan's coefficient comparative test of the homogeneity of variances ($\alpha^a=0.05$; $N^b=10$; $n^c=3$)	C_{calc}[f]. (0.2262)<$C_{crit.}$[g] (0.4450)	$C_{calc.}$ (0.1301)<$C_{crit.}$ (0.5612)	$C_{calc.}$ (0.2262)<$C_{crit.}$ (0.4450)	$C_{calc.}$ (0.1170)<$C_{crit.}$ (0.4450)
Detection limit (M)	5.5×10^{-7}	6.8×10^{-8}	8.4×10^{-7}	3.3×10^{-7}
Quantitation limit (M)	1.0×10^{-6}	1.0×10^{-7}	1.0×10^{-6}	1.5×10^{-6}

[a] Confidence level.
[b] Number of concentration levels.
[c] Number of replicates for each concentration.
[d] Oxidation peak current.
[e] Probability.
[f] Calculated Crochan's coefficient.
[g] Critical value of Crochan's coefficient.

Table 3
Statistical evaluation of selectivity for determination of phenolic acids by differential pulse voltammetry using glassy carbon electrode.

Parameters	Blank sample	Cotton sample
Concentration range ($n=6$)	1×10^{-7} mol L^{-1}–1×10^{-6} mol L^{-1}	
Correlation coefficient	0.9985	0.9922
Intercept	3×10^{-7}	2×10^{-8}
Slope	1.336	1.142
F-test variances significance	F_{calc}[d] = 0.3254 F_{crit}[e] (α[a] = 0.05; N[b] = 6; n[c] = 3) = 5.05 $F_{calc.} < F_{crit.}$	
t-test	t_{calc}[f] = 0.0483	
Comparative test of slope	t_{crit}[g] (α = 0.05; N = 6; n = 3) = 2.036	

[a] Confidence level.
[b] Number of concentration levels.
[c] Number of replicates for each concentration.
[d] Calculated value of F-test.
[e] Critical value of F-test.
[f] Calculated value of *t*-test.
[g] Critical value of *t*-test.

Table 4
Accuracy parameter for determination of phenolic acids by differential pulse voltammetry using glassy carbon electrode.

Caffeic acid concentration in the recovery assay (M)	Recovery range (%) (n[a] = 3)	RSD[b] (%)
2×10^{-7}	98–103	2.3
5×10^{-7}	102–104	1.1
1×10^{-6}	94–97	1.6

[a] Number of replicates for each concentration.
[b] Relative standard deviation.

sample and BRS Araçá cotton sample with caffeic acid standard additions) in the concentration range investigated [51]. The results obtained for recovery tests (Table 4) were within 94–104% range (RSD<2.3%), suggesting that the accuracy of the proposed method was acceptable.

3.4. Analytical applications

3.4.1. Determination of TPAs in cotton samples

Fig. 5 shows the differential pulse voltammograms obtained for BRS Araçá cultivar in 10 mL of 0.2 M phosphate buffer, pH 3 with different caffeic acid concentrations. A well-defined peak at +0.41 V was observed with the addition of 100 μL of BRS Araçá aqueous

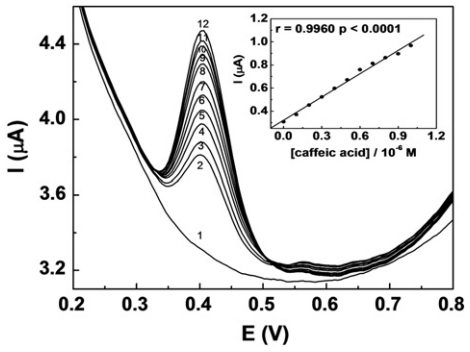

Fig. 5. Differential pulse voltammograms obtained for Araçá cotton sample (obtained by extraction with 25% aqueous ethanol) in 10 mL of 0.2 M phosphate buffer, pH 3 with ten standard additions of 1.0×10^{-3} M caffeic acid. (1) 10 mL of 0.2 M phosphate buffer pH 3. (2) 100 μL of Araçá sample. (3–12) successive standard additions of 1.0 μL of 1.0×10^{-3} M caffeic acid. Insert: The standard addition curve (correlation coefficient (r) = 0.9960; probability (p) < 1.0×10^{-4}). Initial potential (E_i) = 0.2 V, final potential (E_f) = 0.8 V, pulse amplitude = 50 mV, scan rate = 50 mV s^{-1}, working electrode: glassy carbon, reference electrode: Ag/AgCl (KCl 3 M).

Table 5
Determination of total phenolic acids (TPAs) in cotton cultivars.

Cotton cultivars	TPAs found (μg g^{-1})[a]
FM 966	73.0 ± 7.0
BRS 293	65.0 ± 2.8
BRS ARAÇÁ	59.1 ± 0.7
BRS CEDRO	98.8 ± 6.0
BRS AROEIRA	103.0 ± 3.0

Detection limit (LD) = 12.2 ng mL^{-1}.
[a] Mean values from three independent determinations of total phenolic acids.

ethanolic extract of leaves, which can be assigned to the oxidation of phenolic compounds. The other four studied cotton cultivars presented similar voltammograms. Table 5 presents the results obtained in the determination of TPAs concentration in five Brazilian cotton cultivars by the validated DPV method. All studied cultivars exhibited significant levels of phenolic acids with total concentrations ranging from 59.1 to 103 μg g^{-1}. These results are being reported for Brazilian cotton cultivars for the first time in the literature and are in agreement with those obtained for French cotton cultivars [54]. These results also support the hypothesis established by some authors [2–12] that phenolic acids are directly involved in plant resistance to insects and diseases. BRS Aroeira and BRS Cedro were the cotton cultivars that presented the highest concentration of TPAs, which makes them strong candidates to be used in integrated pest management and breeding programs.

4. Conclusions

A differential pulse voltammetric (DPV) method based on the oxidation of caffeic acid on a glassy carbon electrode was successfully applied to the determination of total phenolic acids (TPAs) in cotton leaves (extracted with aqueous ethanol) of five Brazilian cotton cultivars. The data obtained for cotton cultivars in the present work are important elements for future studies on integrated pest management and breeding programs, contributing to the sustainability of cotton-based agricultural systems. The DPV method represents an alternative tool for TPAs determination considering that it has the advantages of high speed and sensitivity (lower detection limits), low cost, easy operation and good selectivity when compared to other analytical methods. The main advantages of the proposed method over the existing electrochemical methodologies for determination of phenolic compounds are the following: a higher number of calculated validation parameters and its novel application in the agricultural and nutritional areas, providing data about phenolic acid concentrations in cotton cultivars, which are valuable information for future investigations upon using cotton in the textile industry and in human and animal nutrition.

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