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Determination of cercosporin (CER) phytotoxin isolated from infected peanut leaves by using adsorptive stripping square wave voltammetry

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Abstract

The adsorptive accumulation of cercosporin (CER) phytotoxin on glassy carbon (GC) electrodes from 1 M $HClO_4$ aqueous solutions is used to detect and quantify the phytotoxin isolated from infected leaf tissue collected from field-grown peanut in five locations in southern regions of the Provinces of Córdoba and San Luis, Argentina. The adsorptive stripping square wave voltammetry (ASSWV) is employed to perform the quantitative determination of CER in naturally infected extracts.

Thin layer chromatography (TLC) and HPLC were used to verify the presence of CER in extracts. Values of $R_f = 0.23$ and $R_f = 0.15$ were determined for CER by TLC when extracts were dissolved in ethyl acetate (EA) and acetone (Ac), respectively, while a retention time of 6.13 min (Ac) was determined by HPLC.

The recovery percentage of CER using ASSWV was determined by employing healthy peanut leaf tissue spiked with standard CER. The application of standard addition method was used to determine the recovery percentage as well as the CER contamination level in infected extracts. A recovery percentage of 93.27% was obtained with a relative standard deviation of 21.8% for a solution concentration of 1.04×10^{-6} mol dm^{-3} ($n = 11$). The limit of detection for a signal to noise ratio of 3:1 was about 6 ppb and the quantitation limit was about 50 ppb. The pondered relative standard deviation of 9.5% was calculated for the ASSWV method. Results found for CER contamination level in extracts through the electroanalytical method here proposed are in reasonable agreement with those values determined by using HPLC measurements.

Keywords: Cercosporin; Phytotoxins; Peanut; Square wave voltammetry; Adsorptive accumulation

1. Introduction

Since Kuyama and Tamura [1] first isolated cercosporin (CER) in 1957, production of CER has been found to occur in a large number of *Cercospora* species [2–6]. The CER chemical structure, a perylenequinone derivative (Fig. 1), was established independently by Lousberg et al. [7] and Yamazaki and Ogawa [8]. CER causes damaging leaf spot diseases on a wide range of economically important crops. Its phytotoxicity was widely studied and reviewed by Daub et al. [9–12]. The killing capability of CER is highly light dependent, acting as a photosensitising agent in host plants [9,11,13,14].

The electro-reduction of CER in 1 M $HClO_4$ + acetonitrile on a glassy carbon (GC) disk electrode has been studied in our laboratory [15]. Results showed that in this highly acidic non-aqueous medium the CER electro-reduction is diffusion controlled. Experimental results fitted fairly well the theoretical model proposed by Laviron for $2e^-$, $2H^+$ reactions. The thermodynamic and kinetics parameters of the redox reaction were obtained from a fitting procedure of experimental square wave voltammograms. A detection limit of 2.8×10^{-7} M for a signal to noise ratio of 2:1 was obtained from calibration curves generated from square wave voltammograms [15]. In addition, we also studied the adsorptive accumulation of CER on GC electrodes from acidic aqueous solutions of

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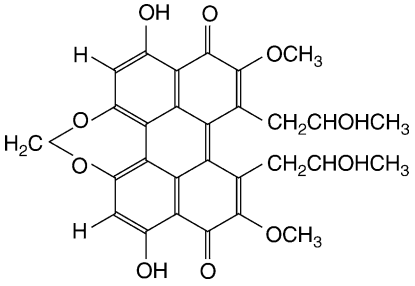

Fig. 1. Chemical structure of cercosporin.

pure commercial reagent by using square wave voltammetry (SWV) [16]. The combination of the "quasi-reversible maximum" and the "split SW peaks" methods [17–22] allowed performing a full thermodynamic and kinetics characterisation of the redox couple of CER adsorbed at GC electrodes. Besides, the quantitative determination of CER in solutions of the commercial reagent under these experimental conditions was carried out by SWV [16]. The detection limit determined experimentally for a signal to noise ratio of 2:1 was 3.7×10^{-8} M [16], about one order of magnitude lower than that obtained from the diffusion controlled reduction peak [15].

On the basis of these results, the adsorptive accumulation of CER on GC electrodes from acidic aqueous solutions is employed in this work to detect and quantify CER from infected leaf tissue collected from field-grown peanuts in five locations in southern regions of the Provinces of Córdoba and San Luis, Argentina, in the summers of the years 2002 and 2003. The adsorptive stripping square wave voltammetry (ASSWV) is used to perform the quantitative determination of CER in extracts of real samples for the first time. The results of the electroanalytical method are compared satisfactorily with those of HPLC measurements.

2. Experimental

2.1. Reagents and materials

The pure commercial CER (MW = 534.5 g mol^{-1}) was obtained from Sigma Chemical Company and was used as received. Acetonitrile (ACN), acetone (Ac), ethyl acetate (EA), hexane (Hx) and isopropanol (IsoOH) were Sintorgan, HPLC grade. Water was obtained from the Labconco Water-Pro Mobile system Model 90901-01 (HPLC grade water). Perchloric acid (Merck p.a.) and trifluoroacetic acid (Koch Light A. R.) were used as received. Silica gel plates (Silica gel 60, Merck) were used for performing thin-layer chromatography (TLC) measurements.

Infected leaves were obtained from field-grown peanuts in the southern region of the Province of San Luis as well as in four locations in the southern of the Province of Córdoba, Argentina, i.e., General Cabrera, General Deheza, Estación Sol de Mayo and Ucacha. They were collected during 2002–2003 summers.

Stock solutions of pure commercial CER were prepared in Ac. They were stored at 5 °C in the dark. Standard solutions for performing HPLC measurements were prepared daily in Ac from stock solutions. Solutions for carrying out electrochemical measurements were also prepared daily dissolving the evaporated residue (see below) in the minimum amount of Ac. Then, these solutions were added to 1 M $HClO_4$ aqueous solution medium.

Manipulation of all laboratory material was done using thin plastic gloves for security reasons. Experiments were performed at 20 ± 1 °C.

2.2. Extraction procedure and chromatographic measurements

The extraction of CER from the peanut leaves was carried out following a procedure previously described in literature [23]. Thus, infected leaf tissue was oven dried at 60 °C. Dried tissue was then ground in a blender and extracted (1:4, w/v) in EA for three days at −5 °C. Every day, solution of extracted material was separated from solid and new aliquots of pure solvent were added to solid residue. Then, solvent extracts were combined, filtered through Whatman No. 1 filter paper and evaporated to dryness in a rotary evaporator. The residue was redissolved in a known amount of EA or Ac for TLC experiments and in Ac for HPLC and electrochemical measurements, respectively. Samples of dried infected leaves in the range of 30–40 g were used for analysis.

TLC was first used to detect the presence of the phytotoxin in extracts obtained from dried peanut leaves. Extracts were spotted on TLC silica gel plates next to a CER standard sample as control. An Hx:IsoOH mixture (8:2) was used as the developing solvent. CER could be detected as a red spot, which fluoresced red under long wave UV light. Values of $R_f = 0.23$ and $R_f = 0.15$ were determined for EA and Ac extracts, respectively. The value of $R_f = 0.23$ is in reasonable agreement with the value reported in literature when TLC was performed in EA extracts [23].

The absence of CER in healthy leaves was checked by HPLC, which has a detection limit lower than TLC [23]. CER was also separated and identified by HPLC. Different CER standard solutions obtained from the commercial reagent were prepared by using Ac as the solvent. Aliquots of 20 μL were injected into the column using a syringe with a 26-gauge needle. The mobile phase consisted of ACN:H_2O (60:40) with 0.1% trifluoracetic acid at a flow rate of 0.3 mL min^{-1}. CER was detected by fluorescence at λ = 623 nm. The retention time (t_r) was 6.13 min. Interference level of leaf components was determined by HPLC injecting healthy leaf tissue extracts with known amounts of CER standard.

2.3. Apparatus and experimental measurements

The measuring system for the electrochemical techniques was composed by an EG&G PARC Model 273 potentiostat/galvanostat equipped with a PAR270 electrochemical analysis software. The square wave amplitude (ΔE_{SW}) and the staircase step height (ΔE_s) were 25 and 5 mV, respectively. The frequency was 40 Hz.

Electrochemical measurements were performed in a two-compartment Pyrex cell (effective volume: 7.2 mL) [24]. The working electrode was a GC disk (0.3 cm diameter). It was polished successively with wet alumina powder (0.3 and 0.05 μm, from Fischer), copiously rinsed with distilled water and sonicated in a water bath for 2 min. The polished electrode was further activated electrochemically in 1 M KOH (Merck p.a.) aqueous solution by a potential step of 1.2 V over 5 min according to a procedure previously described by Anjo et al. [25]. Its electrochemical area (A) was determined as previously reported [15]. The counter electrode was a platinum foil of large area (approx. 2 cm^2). Either an aqueous saturated calomel electrode (SCE), or a silver wire pseudo-reference, was used as reference electrode. They were fitted with a fine glass Luggin capillary containing a bridge solution identical to that of the sample being measured. In all cases, potentials are reported with respect to the SCE. Solutions were deareated by bubbling purified nitrogen for at least 10 min prior to the measurements.

HPLC experiments were carried out with a Modular Gilson chromatograph with a 306 pump and a fluorescence detector (Gilson, Model 121) with a fluorescence filter of 610–650 nm. A C18 reverse phase column (Phenomenex 150 mm × 4 mm) was employed.

The recovery percentage of CER from ASSWV measurements (see Section 3.2.1) was determined by employing healthy peanut leaf tissue spiked with standard CER. The same procedure employed to perform the extraction of CER from infected peanut leaves was used to healthy leaves. The evaporated residue was dissolved in the minimum amount of Ac.

The standard addition method was used to quantify CER in both, infected and healthy peanut leaf extracts. Very good straight lines were obtained for $I_{p,n}$ versus c^*_{CER} plots in all cases. The CER unknown concentration in extracts (infected and healthy) was calculated from the ratio between the intercept and the slope of the straight lines.

3. Results and discussion

3.1. HPLC measurements

From chromatograms of standard solutions calibration curves were constructed by using the areas under chromatographic peaks as a function of the CER analytical concentration (c^*_{CER}). Experimental points of the calibration curve were the average of at least three replicated measurements. The calibration curves were fitted by a second-order polynomial. Aliquots of the same infected peanut leaf extracts prepared for electrochemical measurements (see Section 3.2.2 below) were then injected, at least by duplicated, in the column. The CER concentration in extracts was calculated from the peak areas by using the proper calibration curve. Values of CER content corrected by loss in extraction procedure (17% average [23]) are shown in columns 4 (extract, mol dm^{-3}) and 5 (μg of CER/kg of infected leaf tissue) of the Table 1. Results obtained from HPLC method allow us to detect approximately 35 ppb of CER, including a loss factor during extraction of 17%.

3.2. Adsorptive stripping square wave voltammetry

As stated in Section 1, we have previously studied the adsorptive accumulation of CER using solutions prepared from the commercial reagent on glassy carbon electrodes in 1 M $HClO_4$ aqueous solutions. From these results, the quasi-reversible nature of the surface redox reaction could be clearly inferred by considering the characteristic of the forward (I_f), the reverse (I_r) and the net (I_n) currents obtained from SWV measurements [16].

The I_f, I_r, and I_n versus potential curves obtained from a given infected peanut leaf extract after the addition of a known amount of standard CER are shown (after blank

Table 1
Detectable levels of CER in different infected peanut leaf extracts obtained by both HPLC and ASSWV

Sample	Location	Sample weight (g)	$10^7\ c^*_{CER}$ [a,b] (mol dm^{-3})	CER content[c] (ppb)	$10^7\ c^*_{CER}$ [b,d] (mol dm^{-3})	CER content[e] (ppb)
1	San Luis	30.0008	8.1 ± 0.6	104	9.4 ± 0.9	121
2	General Cabrera	37.0142	9.4 ± 0.7	98	8.5 ± 0.5	88
3	General Deheza	42.9896	9.2 ± 0.9	83	9.6 ± 0.8	86
4	Estación Sol de Mayo	37.9857	7.8 ± 1.0	79	7.4 ± 1.1	75
5	Ucacha	42.5802	4.9 ± 0.8	44	4.8 ± 0.2	43

[a] Obtained by HPLC on extracts, corrected by loss during extraction (Section 3.1).
[b] Errors in c^*_{CER} are standard deviations.
[c] Obtained by HPLC on dried leaves, corrected by loss during extraction (μg CER/kg of dried leaves).
[d] Obtained by ASSWV and standard addition method on extracts, corrected by loss factors, both during the extraction procedure and electrochemical technique (Section 3.2.2).
[e] Obtained by ASSWV and standard addition method on dried leaves, corrected by loss factors, both during the extraction procedure and electrochemical technique (μg CER/kg of dried leaves) (Section 3.2.2).

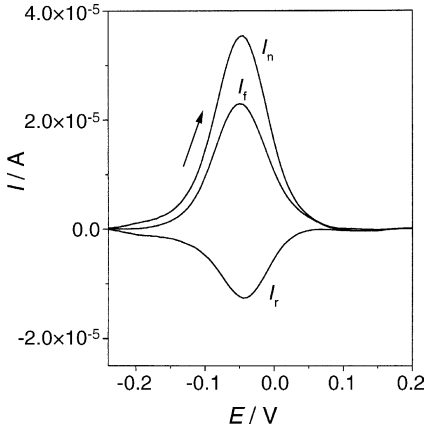

Fig. 2. Forward (I_f), reverse (I_r) and net (I_n) currents of a typical SW voltammogram obtained from a given infected peanut leaf extract (corrected by blank) after adittion of a known amount of standard CER ($c^*_{CER} = 6.75 \times 10^{-8}$ M). Working electrode: GC disk ($A = 0.089\ cm^2$), $\Delta E_{SW} = 25$ mV, $\Delta E_s = 5$ mV, $f = 40$ Hz. $E_{acc} = -0.70$ V, $t_{acc} = 20$ min. Arrow indicates the direction of the sweep.

correction) in Fig. 2. As demonstrated elsewhere, the voltammogram of the net currents obtained by SWV under proper conditions is the most useful analytical signal [26,27]. The combination of adsorptive accumulation with SWV provides an electroanalytical tool very valuable for performing trace analysis of compounds, which are both surface active and also electroactive [28]. Studies were then conducted to find the most favourable accumulation time (t_{acc}) as well as the optimum accumulation potential (E_{acc}) for performing the CER pre-concentration step at the electrode surface when the phytotoxin was obtained from infected peanut leaf extracts. These studies were carried out mixing the extract in 1 M $HClO_4$ aqueous solutions and adding a known amount of standard CER. The E_{acc} was found to be $E_{acc} = -0.7$ V versus SCE in agreement with results previously reported by us when the CER adsorptive accumulation at GC electrodes was studied from the pure commercial reagent [16]. On the other hand, from the net peak current ($I_{p,n}$) versus t_{acc} plots it was possible to infer that stationary currents could be obtained at $t_{acc} \geq 1200$ s. Under these conditions, the absence of interfering substances was checked. On the basis of these results, the quantitative determination of CER in both infected and healthy peanut leaf (spiked with standard CER) extracts was carried out on a GC electrode in unstirred 1 M $HClO_4$ aqueous solution after a $t_{acc} = 20$ min at $E_{acc} = -0.70$ V.

3.2.1. Recovery percentage from healthy peanut leaf extracts

A known aliquot of standard CER was added to the healthy natural leaf extracts. They were mixed in 1 M $HClO_4$ aqueous solution giving a final concentration of $c^*_{CER} = 1.04 \times 10^{-6}$ M for performing the ASSWV measurements and to determine the recovery percentage. The application of standard addition method allowed to obtain the corresponding $I_{p,n}$ versus c^*_{CER} curve. A very good linear relationship between $I_{p,n}$ versus c^*_{CER} was obtained in the range from 5.2×10^{-7} to 4.7×10^{-6} M at $f = 40$ Hz ($n = 11$) (correlation coefficient, $r = 0.9943$). Current data used in the regression analysis are the average of three replicated measurements for a given CER concentration. From the ratio between the intercept ($0.63 \pm 0.12\ \mu A$) and the slope ($(6.5 \pm 0.4) \times 10^5\ \mu A\ dm^3\ mol^{-1}$) of the $I_{p,n}$ versus c^*_{CER} plot, a value of $c^*_{CER} = (0.97 \pm 0.19) \times 10^{-6}$ M was determined. This value corresponds to a recovery percentage of $93.27 \pm 20.35\%$ with a R.S.D. of 21.8% for solution concentration of $1.04 \times 10^{-6}\ mol\ dm^{-3}$ ($n = 11$). Errors shown for CER concentration and for the intercept and the slope are standard deviations.

These results clearly show that the adsorptive accumulation of CER on GC electrodes from acidic aqueous solutions combined with SWV appears as a very useful analytical tool for the determination of CER in infected peanut leaf extracts.

3.2.2. Quantitative determination of CER in infected peanut leaves

The ASSWV was used to quantify CER in naturally infected peanut leaf extracts. Results obtained were compared with those determined by HPLC (see Section 3.1). The infected leaves were obtained from field-grown peanut leaves in the regions indicated in Section 2. All of them showed red spot disease. The extraction of the phytotoxin from contaminated leaves was performed as it was previously described in Section 2. The standard addition method was used to quantify CER in infected extracts. Good linear regressions were obtained from $I_{p,n}$ versus c^*_{CER} plots ($r = 0.9897$, 0.9993, 0.9981, 0.9971 and 0.9845 for extracts obtained from infected peanut leaves of San Luis, General Cabrera, General Deheza, Estación Sol de Mayo and Ucacha, respectively). Peak current data used in these calibration curves were the average of three replicated measurements for a given c^*_{CER} (six different concentration values were taken into account in the concentration range from 5×10^{-7} to $6 \times 10^{-6}\ mol\ dm^{-3}$). From the ratio between intercepts (2.51 ± 0.03, 1.12 ± 0.05, 0.67 ± 0.05, 0.46 ± 0.08 and $0.4 \pm 0.2\ \mu A$) and slopes ($(3.2 \pm 0.3) \times 10^6$, $(1.62 \pm 0.02) \times 10^6$, $(8.6 \pm 0.4) \times 10^5$, $(7.8 \pm 0.3) \times 10^5$ and $(10 \pm 1) \times 10^5\ \mu A\ dm^3\ mol^{-1}$) of the linear regressions for infected peanut leaf extracts from San Luis, General Cabrera, General Deheza, Estación Sol de Mayo and Ucacha, respectively), the CER contamination level was determined as shown in columns 6 and 7 of Table 1, along with the corresponding values determined by HPLC (columns 4 and 5 of Table 1), as explained in Section 3.1. Values indicated in columns 4 and 6 correspond to CER concentrations in extracts while those of columns 5 and 7 to CER content in dried peanut leaves, respectively. Values shown in Table 1 have been corrected by extraction loss (17% average [23]) in columns 4 and 5, and extraction loss plus ASSWV technique loss (6.73%) in columns 6 and 7. As it can be realised, all values reported are higher than the detection limits of the techniques. Pondered

percentual relative standard deviation [29] for ASSWV and HPLC are about 9.5 and 10.3%, respectively. The detection limit for a signal to noise ratio of 3:1 for the method proposed was approximately 6 ppb of CER, including loss factors during extraction (17%) and electrochemical technique (6.73%). This value could be compared favourably to the one determined by us by HPLC (about 35 ppb, see above) and some reported in the literature, also obtained by HPLC (1–10 ppm) [30,31] and about 4–40 ppb [23] with the usual inherent disadvantages, such as a strong commitment of time, labour and expense. Quantitation limit was about 50 ppb [29].

As it can be observed in Table 1, a good agreement is obtained between values of both, HPLC and electrochemical technique. A good linear correlation was found when the results obtained with ASSWV were plotted versus the results achieved with HPLC (slope = 1.1 ± 0.2; $r = 0.9381$). This good correlation is another check which allows the use of the ASSWV technique for the quantification of CER in real matrices.

Experimental results obtained demonstrate that ASSWV is a very useful technique to detect and quantify CER in infected peanut leaf extracts. It appears as a very valuable alternative with respect to chromatographic techniques, with the advantage that no separative procedure is necessary to be performed as a previous step to application of the electroanalytical method. Besides, instrumentation required in the methodology proposed in this work is less expensive than that necessary for performing chromatographic measurements.

4. Conclusions

It has been demonstrated for the first time that adsorptive stripping square wave voltammetry can be used to detect and quantify CER phytotoxin in naturally infected peanut leaves. This analytical methodology gives results which satisfactorily agree with those obtained by HPLC, such as almost identical pondered relative standard deviation, i.e. 9.5 and 10.3%, respectively, although ASSWV allows quantifying up to about 6 ppb of CER, which is five times lower than the HPLC value obtained by us. These results show that adsorptive stripping square wave voltammetry appears as a very promising alternative analytical technique for the determination of CER in real samples.

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