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Electrochemical evaluation and adsorptive stripping voltammetric determination of capsaicin or dihydrocapsaicin on a disposable pencil graphite electrode

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ABSTRACT

Capsaicin and dihydrocapsaicin are the two most abundant capsaicinoids in peppers, which are responsible for about 90% of the spiciness. A detailed study of the electrochemical properties of these compounds at pencil graphite (PG) electrode was carried out in the pH range 1.0–12.0 in aqueous solutions. The compounds underwent irreversible oxidation at PG electrode, which was an adsorption-controlled process with two protons and two electrons. The voltammetric data indicated that their oxidation proceeded *via* an ECE mechanism. Using the square-wave adsorptive stripping voltammetry with accumulation at a fixed potential of −0.1 V for 120 s, both of them yielded a well-defined voltammetric response at +0.31 V (vs. Ag/AgCl) in Britton-Robinson buffer, pH 9.0. Capsaicin and dihydrocapsaicin could be determined with detection limits of 1.12 ng mL^{-1} (3.7×10^{-9} M) and 0.28 ng mL^{-1} (9.1×10^{-10} M), respectively. The practical applicability of this methodology was tested in commercial Turkish pepper products. The concentration of total capsaicinoids was determined using capsaicin as standard.

1. Introduction

Hot or spicy peppers and chilies, herbaceous plant belonging to the genus *Capsicum* (family: Solanaceae), are the most popular food additives that are widely utilised in many parts of the world [1]. In the southeastern Anatolian region of Turkey peppers represent a culinary tradition and cultural identity.

The spicy pungent taste of most varieties of hot peppers is due to a group of closely related lipophilic alkaloids called capsaicinoids. The common structure of capsaicinoids is composed of a vanillylamide and a hydrophobic C_9–C_{11} branched-chain fatty acids with the degree of unsaturation. Capsaicin (8-methyl-*N*-vanillyl-*trans*-6-nonenamide) and dihydrocapsaicin (8-methyl-*N*-vanillylnonanamide) are the two most active components of capsaicinoids, and their molecules differ only in the saturation of the acyl group. The capsaicin comprises 33–59% of the natural capsaicinoids found in peppers, followed by dihydrocapsaicin (30–51%), located mainly within the white ribs (palcenta) and seeds of the fruit. These two compounds are also about twice as potent to the spiciness and nerves as the minor capsaicinoids nordihydrocapsaicin (7–15%) and the reminder (less than 5%), which is homodihydrocapsaicin and homocapsaicin. The concentration of capsaicinoids in fresh red pepper varieties, especially in paprika, ranges from 0.001 to 0.01%, and in strong chili varietes the concentration ranges from 0.1 to <1% by weight [1–4].

In addition to food additive uses in our diet, the other biological properties and medical applications of capsaicin make this compound very popular. It is currently available as various topical pharmaceutical forms (ointments, high-dose dermal patches, creams, large bandages) for a number of diverse clinical conditions, such as relief the pain of peripheral neuropathy, symptomatic treatment of arthritis, muscle and joint pains, other types of neurogenic pains such as Herpes zoster. It is used in the treatment of obesity and ulcer in the forms of oral herbal supplements. It is also the active ingredient in riot control and personal defense pepper spray chemical agents. Furthermore, it was demonstrated that capsaicin induces apoptosis in various cancer cells. However, studies are available showing that capsaicin increases 2–5 fold the incidence of stomach, pharynx, esophagus and larynx cancer in countries where capsaicin is highly consumed [4–6]. Because of the increasing demand by consumers for hot and spicy foods and also the increasing use in medicine and pharmacy, it has become important to establish a sensitive, accurate and simple technique for detection of capsaicin and its analogues in food products and pharmaceuticals.

Total capsaicinoid content of heat-producing chemicals is one of the main parameters that determine its commercial quality, and it is directly related to the heat (pungency) of the level of a pepper.

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This level has been measured in Scoville heat units (SHU) by an organoleptic method (Scoville Organoleptic Test) [7]. In addition to traditional Scoville method, several analytical techniques for determining capsaicin and/or the related capsaicinoids in food, pharmaceutical and clinical samples were described using high-performance liquid chromatography (HPLC) equipped with ultraviolet, diode-array, fluorescence, electrochemical or mass/tandem mass spectrometric detection [8–21], gas chomatography–mass spectrometry [22,23], capillary electrophoresis [24], micellar electrokinetic capillary chromatography [25], colorimetry [26], spectrophotometry [27], chemometry based on spectrophotometry [28] or thin layer chromatography-in situ densitometry [29]. Pungency is also measured by a method which uses HPLC technique. This method yields results, not in Scoville units, but in American Spice Trade Association (ASTA) pungency units [30]. Nowadays, HPLC method has replaced Scoville method because of being more reproducible and accurate [31].

Of the above mentioned methods, the most recent and widely used are gas and high-performance liquid chromatography coupled to more selective mass spectrophotometry, which offer sufficient sensitivity and high resolution, however they suffer from disadvantages such as complicated procedure, long response time and requirement of expensive instruments and/or tedious derivation steps to increase the volatility of capsaicinoids.

From the electroanalytical point of view, literature survey revealed only three voltammetric studies made on capsaicin so far. All of them are quite new and involve the use of modified electrodes. The first investigation performed by Compton et al. [32] was concerned with studying the electroactivity and determination of capsaicin using adsorptive stripping voltammetry at carbon nanotube-based electrochemical sensors. Its applicability was evaluated in a variety of hot pepper sauces. Previous work of our research group was demonstrated the effectiveness of boron doped diamond electrode in presence of sodium dodecylsulfate for the electrochemical oxidation of capsaicin. Adsorptive stripping voltammetry was proposed for its determination in two samples of commercial pepper products [33]. Last attempt was made to study the electrooxidation of capsaicin at a carbon paste electrode modified with amino-functionalized mesoporous silica. Adsorptive stripping voltammetry was applied to determine capsaicin in hot pepper samples [34]. On the other hand, to the best of our knowledge, no literature data were found on the electrochemical behaviors of dihydrocapsaicin and other capsaicinoids.

The single-use disposable electrodes may overcome the regeneration drawback of the solid electrodes. In this manner, pencil leads can be seen as important electrode materials which present high electrochemical reactivity, good mechanical stability, low cost, low technology, eases of renewal, and other interesting qualities [35,36].

In continuation to our earlier reports [37–40] on the electroanalytical application of pencil leads, and with the limited electrochemical data on capsaicinoids, the present study is intended to develop a new rapid and sensitive electroanalytical procedure for two members of capsaicinoid family, capsaicin or dihydrocapsaicin, at a pencil graphite electrode using adsorptive stripping voltammetry that could be adopted in two different commercial pepper products, such as flakes of Turkish red pepper and Urfa crushed red pepper (also known as Isot pepper).

2. Experimental

2.1. Caution

It should be used gloves and mask when handling of very strong irritant agents, capsaicin and dihydrocapsaicin, and their concentrated solutions.

2.2. Apparatus

Cyclic voltammetric (CV), linear sweep voltammetric (LSV) and square-wave adsorptive stripping voltammetric (SW-AdSV) measurements were performed by μAutolab type III electrochemical analyzer with GPES 4.9 Version conversion software (EcoChemie, The Netherlands). For SW voltammograms the raw data were treated using the Savicky and Golay filter and a moving average baseline correction (peak width=0.01 V) of the GPES software. Electrochemical experiments were carried out by a conventional three-electrode configuration. Pencil graphite (PG) electrode was used as working electrode. A platinum wire and an Ag/AgCl electrode (3 M NaCl) (Model RE-1, BAS, USA) were used as auxiliary and reference electrode, respectively. The pH values of solutions were measured using a WTW inoLab pH 720 m with a combined electrode (glass-reference electrodes).

PG electrodes were Tombo (Japan) leads with a total length of 60 mm and a diameter of 0.5 mm, which were purchased from a local bookstore. A Rotring pencil Model T 0.5 (Germany) was used as a holder for PG electrode. Electrical contact with the PG electrode was obtained by soldering a metallic wire to the metallic part of the holder. A total of 10 mm of lead was immersed in solution per measurement. The electrochemical pretreatment of PG was performed by applying a potential of +1.40 V for 30 s in aqueous media containing the blank supporting electrolyte without stirring, in order to increase the effective surface area of the electrode and to introduce the oxygen containing functional groups on the electrode surface by the oxidation of graphite layers [41].

2.3. Chemicals

All chemicals were of analytical-reagent grade and used as received without any further purification. The stock solutions ($1\ \mathrm{mg\ mL^{-1}}$) of capsaicin and dihydrocapsaicin (Sigma), which are the two standards of capsaicinoids commercially available, were prepared in ethanol due to their poor solubility in water. The stock solutions were stored in the dark and under refrigeration when not in use. Working solutions of these compounds were prepared daily by diluting the stock solution with a selected supporting electrolyte. Four different supporting electrolytes, namely perchloric acid (0.1 M), acetate buffer (0.1 M, pH 4.8), Britton-Robinson buffer (BR, 0.1 M, pH 9), and phosphate buffer (0.1 M, pH 2.5, 7.4 and 12.0) solutions were used. The content of organic solvent in the supporting electrolyte did not exceed 1% (v/v) for SW-AdSV measurements, which did not influence electrochemical determination of these capsaicinoids. All other chemicals were of analytical-reagent grade and used as received without any further purification. Aqueous solutions were prepared with deionised water further purified via a Milli-Q unit (Millipore).

2.4. Analytical procedure

The general procedure adopted for obtaining adsorptive stripping voltammograms was as follows: The required aliquot of the capsaicinoid working solutions was placed in a voltammetric cell containing BR buffer as a selected supporting electrolyte (unless otherwise stated) at a desired pH (e.g. pH 9.0). The previously treated electrode was placed in the cell, and the solution was stirred at 200 rpm at a chosen accumulation potential (e.g. –0.1 V) throughout the selected accumulation period (e.g. 120 s). Following the pre-concentration period, the stirring was stopped, and after a 10 s had elapsed, a sweep was carried out towards positive potentials over the range +0.1 to +1.1 V (for acidic media) and –0.1 to +1.1 V (for alkaline media) using the SWV technique. Each measurement was performed using a new pencil surface in a three-electrode-system in a 10-mL one-compartment voltammetric cell, at a laboratory temperature (20 ± 5 °C).

The best instrumental parameters for SWV which was used for investigating the determination of capsaicin and dihydrocapsaicin were as follows: frequency, 125 Hz; pulse amplitude, 20 mV; scan increment, 8 mV.

2.5. *Preparation of the commercial food products*

One of the analyzed samples was red pepper flakes, also known as crushed red pepper, which is a condiment consisting of dried and crushed (as opposed to ground) red chili peppers. They were harvested from southeastern regions of Turkey, especially from the city of Maraş (the main chili pepper plantation area). The other one was Urfa pepper (Isot pepper), technically a red pepper, which is a dried Turkish chili pepper of the type *Capsicum annuum* cultivated in the Urfa region of Turkey. The peppers go through a two-part process, where they are sun-dried during the day and wrapped tightly at night. The night process is called 'sweating', and works to infuse the dried flesh with the remaining moisture of the pepper. The result is an appearance ranging from deep purple to a dark, purplish black. Isot pepper is less spicy than many other chili peppers provides a more lasting build of heat.

Commercial flake samples in polyethylene bags were purchased from a local retail supermarket in Van-Turkey. Samples were taken from five package of the same label. In order to prepare a real sample matrix, the samples were submitted to an extraction procedure prior to electrochemical analysis. The extraction procedure was adapted from that of Compton et al. [32] with a slight modification. An accurately weighed portion of pepper flakes (about 0.2 g of sample) was transferred into a volumetric flask and dissolved in 12.5 mL ethanol. The flask was then placed in an ultrasonic bath and sonicated for 20 min, then stirred for 120 min using a magnetic stirrer and centrifuged at 10,000 rpm for 20 min to remove any undissolved material. An aliquot volume of the clear supernatant liquor was added to BR buffer, pH 9.0 in the voltammetric cell and analyzed in the day of preparation according to the procedure developed for the pure electrolyte. Quantifications were performed by means of standard calibration method and the average of triplicate measurements.

3. Results and discussion

3.1. *Voltammetric characterisation on the pencil graphite electrode*

Initial experiments by means of CV and LSV were performed without an accumulation step (t_{acc}=0 s) to characterize the basic voltammetric behavior and properties of capsaicin and dihydrocapsaicin at the electrochemically treated PG electrode.

Fig. 1 shows the repetitive cyclic voltammograms for 50 μg mL^{-1} capsaicin (Fig. 1A and B) and dihydrocapsaicin (Fig. 1C and D) in 0.1 M perchloric acid (Fig. 1A and C) and BR buffer of pH 9.0 (Fig. 1B and D) solutions recorded at a scan rate of 100 mV s^{-1}. As it can be observed, the voltammetry of capsaicin and dihydrocapsaicin in both acidic and alkaline media is almost identical with regard to the shape of voltammograms and oxidation potentials.

In strongly acidic solution such as 0.1 M perchloric acid, the CV behavior of these capsaicinoids yielded two oxidation peaks, I′a (at +0.52 V for both compounds) and Ia (at +0.71 V for capsaicin and +0.72 V for dihydrocapsaicin), whereas at the cathodic scan single and well-defined reduction peak, I′c was obtained at +0.50 and 0.48 V for capsaicin and dihydrocapsaicin, respectively. Upon first scanning, the oxidation peak I′a was very small and Ia was

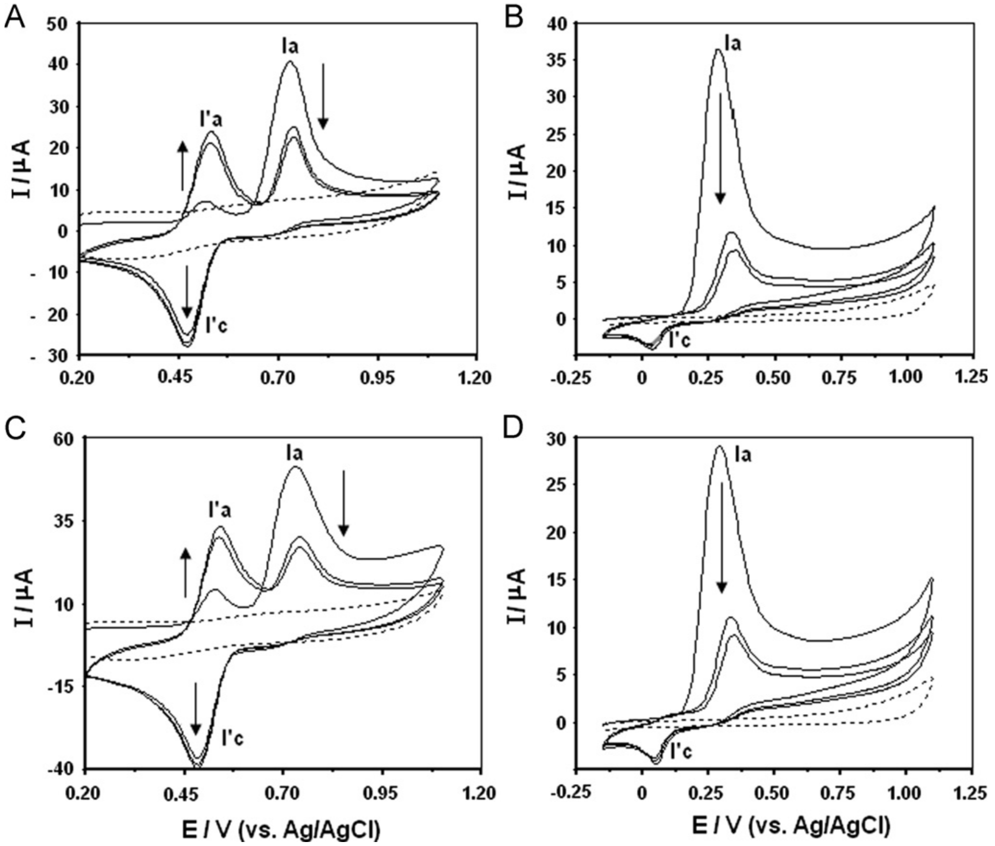

Fig. 1. The repetitive cyclic voltammograms of 50 μg mL^{-1} capsaicin (A) and (B) and dihydrocapsaicin (C) and (D) solutions in 0.1 M perchloric acid (A) and (C) and in BR buffer pH 9.0 (B) and (D). Scan rate, 100 mV s^{-1}. Dashed line shows background current.

much more developed and sharp. In the second scan, the magnitude of the peak Ia decreased gradually, while the intensity of I′a increased significantly. The peak height of the reduction peak remained nearly unchanged. An oxidation peak I′a corresponds to oxidation of the reduction products formed in peak I′c. The appearance of these closely spaced peaks located at lower positive potentials than the parent compound supported that a chemical reaction subsequent to the electron step occurs, giving rise to species more readily oxidized than capsaicin and dihydrocapsaicin. The observation at PG electrode is in almost accordance with those obtained in the previous investigations for capsaicin using carbon nanotube-based electrochemical sensors at the same pH value [32] and a carbon paste electrode modified with amino-functionalized mesoporous silica at pH 3.0 [34]. In our previous study at a boron-doped diamond (BDD) electrode [33], it was found that capsaicin was irreversibly oxidized in a single step at about +0.85 V corresponding to peak Ia under identical conditions (at pH 1.0). From our experimental results, the background current for PG electrode was higher than those for BDD electrode however its usage was proved to be more sensitive, yielding higher current densities (obtained by subtracting the background currents from the recorded currents). Furthermore, the process Ia took place at less positive potential than that obtained at BDD electrode, indicating that the oxidation process of the compound requires less overpotential at the PG surface, compared to the other type of carbon electrode material.

In alkaline solution such as BR buffer at pH 9.0, CV revealed the presence of single and well-defined oxidation peak, Ia (at +0.28 V for capsaicin and +0.29 V for dihydrocapsaicin), while a very small reduction peak, I′c was observed at +0.06 and 0.05 V for capsaicin and dihydrocapsaicin, respectively. On the second forward scan, the peak I′a almost disappeared at this pH. Further potential cycles at the same PG surface resulted in a remarkably decrease of the voltammetric response. This behavior may be partly attributed to the consumption of adsorbed these compounds on the PG electrode surface. It should be noted that the electrochemical behavior of capsaicin in the alkaline media was not explained in the previous reports [32,34]

The LSV curves for concentration of 50 μg mL^{-1} of these compounds carried out for the increasing scan rate values (ν) over the range 10–400 and 10–200 mV s^{-1} for 0.1 M perchloric acid and BR buffer pH 9.0, respectively, gave rise to an electrochemical response with increased anodic peak (Ia) current intensities (i_p). The results showed a linear increase with the scan rate which can be expressed by the following equations:

for capsaicin

$$i_p(\mu\text{A}) = 0.264\nu(\text{mV s}^{-1}) + 9.147, r = 0.997 (\text{in 0.1 M perchloric acid})$$

$$i_p(\mu\text{A}) = 0.155\nu(\text{mV s}^{-1}) + 4.799, r = 0.996 (\text{in BR buffer pH 9.0})$$

for dihydrocapsaicin

$$i_p(\mu\text{A}) = 0.234\nu(\text{mV s}^{-1}) + 9.732, r = 0.997 (\text{in 0.1 M perchloric acid})$$

$$i_p(\mu\text{A}) = 0.174\nu(\text{mV s}^{-1}) + 4.296, r = 0.995 (\text{in BR buffer pH 9.0})$$

This suggests that the electrode reaction at the PG electrode for both capsaicin and dihydrocapsaicin is controlled by the adsorption process in these media.

Their adsorption phenomenon o can be used as an effective pre-concentration step prior to the voltammetric quantification of the analytes. The AdSV response at PG electrode was examined using the SW excitation waveform due to intense sensitivity with high speed, and reducing problems with blocking of the electrode surface. As a consequence, various preliminary experiments were carried out in various supporting electrolytes at different pH values (1.0–12.0) in order to assess their impact on the monitored electroanalysis signal at PG electrode. After 30 s accumulation time for 10 μg mL^{-1} capsaicinoid solution at open-circuit mode, it was found that peak potential (E_p) of oxidation peak for both compounds by considering the peak Ia shifted negatively with the increase of solution pH (Fig. 2), indicating that their oxidation at the PG electrode is a pH-dependent reaction. In strongly acidic media (at pH 1.0 and 2.5) an additional peak (I′a) occurred at less positive potentials than the main oxidation peak (Ia) and it disappeared by operating at high pH. The plot of peak potential of peak Ia versus the solution pH value gave straight lines and corresponding equations:

$$E_p(V) = -0.052\ \text{pH} + 0.771, r = 0.997\ (\text{for capsaicin})$$
$$E_p(V) = -0.053\ \text{pH} + 0.782, r = 0.994\ (\text{for dihydrocapsaicin})$$

The slopes were found to be −52.0 and −53 mV/pH unit for capsaicin and dihydrocapsaicin, respectively, which are very close to that predicted by the Nernst equation for a redox process involving the transfer of an equal number of protons and electrons, where that number is likely equal to two. It demonstrates that the electrode process for both capsaicinoids is two-proton coupled two-electron transfer, which confirms the result obtained at carbon nanotube-based electrochemical sensors [32].

On the basis of the above results and bearing in mind the electrochemical behavior of *o*-methoxyphenol (guaiacol) and its derivatives at several electrodes [32,42–44], we may assume that

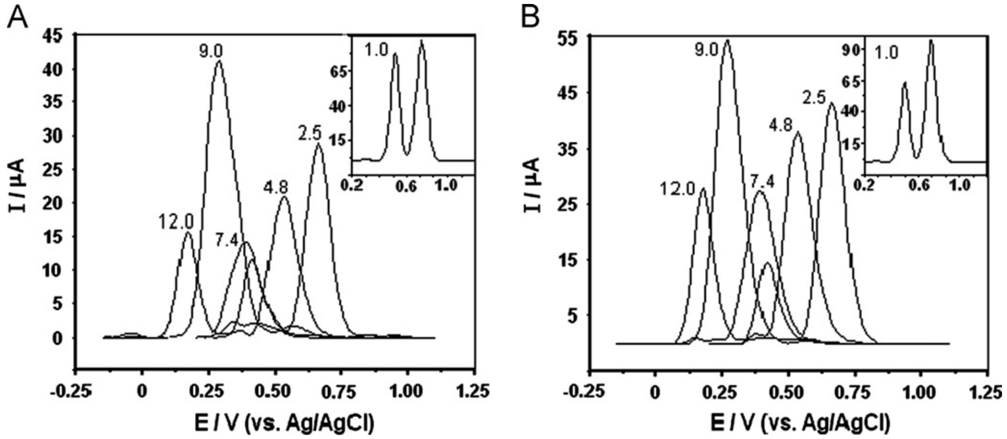

Fig. 2. SW stripping voltammograms of 10 μg mL^{-1} capsaicin (A) and dihydrocapsaicin (B) solutions at different pHs. Inset represents the SW stripping voltammograms at pH 1.0. Pre-concentration period, 30 s at open circuit condition; SWV parameters: frequency, 50 Hz; scan increment, 8 mV; pulse amplitude, 20 mV.

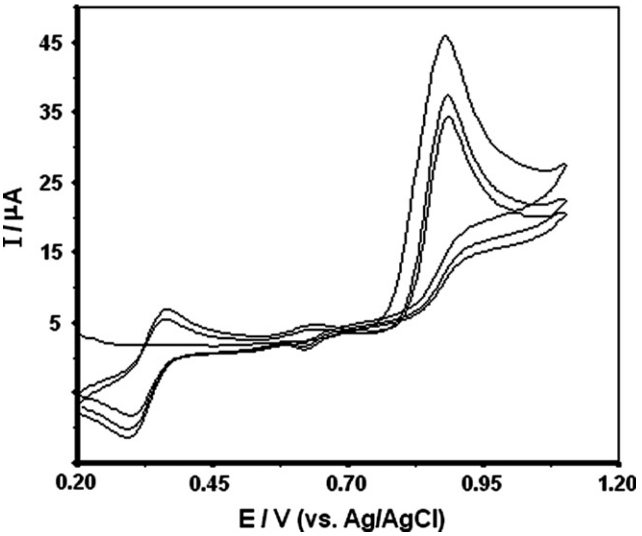

Fig. 3. The repetitive cyclic voltammograms of 50 μg mL^{-1} vanillin solutions in 0.1 M perchloric acid solution. Scan rate, 100 mV s^{-1}. Dashed line shows represent background current.

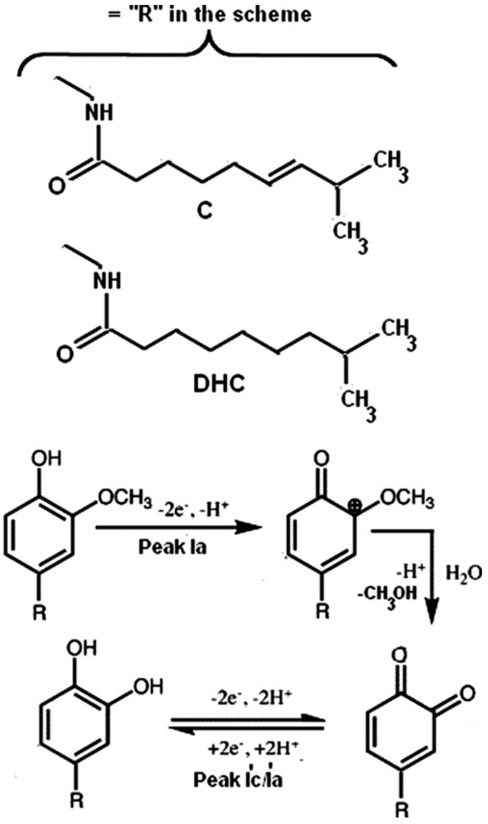

Fig. 4. The structure of capsaicin and dihydrocapsaicin and their reaction pathway in acidic and less alkaline solutions. *C*, capsaicin; DHC, dihydrocapsaicin.

the voltammetric response is attributed to the *o*-methoxyphenol moiety present in these molecules. More information on the mechanism of their oxidation was also provided by comparing their anodic behavior specifically with the oxidation of selected model compound, vanillin which also contains the same electroactive center. Both of these capsaicinoids differ from vanillin by having an amide moiety and hydrophobic side chain (9-carbon fatty acid) instead of aldehyde unit. The cyclic voltammograms of vanillin were performed by CV at PG electrode in 0.1 M perchloric acid at scan rate of 100 mV s^{-1} (Fig. 3). As can be seen from the figure, CV behavior of vanillin is similar to that observed for these two molecules, which confirms that *o*-methoxyphenol in the molecule is grouping responsible for the oxidation process. Our results also support the situation as that found in the previous study of capsaicin [32,34].

Keeping in mind the redox chemistry of *o*-methoxyphenol subunit within the capsaicinoid molecular structure [44], we may propose a similar oxidation mechanism for capsaicin and dihydrocapsaicin in acidic and alkaline medium. The pK_a value of *o*-methoxyphenol is reported to be pK_a=9.93 [43] Thus, at solution pH below about this pK_a predominant form is neutral molecule in the solution which undergoes a two-electron irreversible anodic oxidation leading to the formation of phenoxonium cation of capsaicinoids. Their hydrolysis which is the chemical follow-up reaction, by losing a proton and methanol produces *o*-benzoquinone unit in the structure of capsaicinoids, These compounds will be further reduced to the corresponding *o*-hydroxyphenol (catechol) part of capsaicinoids. Under the strong alkaline conditions (pH > pK_a), the hydroxyl of capsaicinoids turns to predominantly their anionic form (*o*-methoxyphenate anion), the oxidation of which is one-electron process to the phenoxy radical. One of the expected reactions of phenoxy radical is dimerization, which is further oxidized, probably to polymeric products. The second portion of the phenoxy radicals can undergo another pathway including several hydrolyses and oxidation steps ending with *o*-benzoquinone. To explain the observed voltammetry in acidic and less alkaline aqueous solutions using PG electrode, an $\mathrm{EC_{irrev}E}$ mechanism which is characteristic for the irreversible electrochemical oxidation of the *o*-methoxyphenol to form a quasi-reversible *o*-benzoquinone/catechol couple, could be proposed for capsaicinoids as depicted in Fig. 4.

The current response of capsaicin and dihydrocapsaicin peak decreased significantly with increasing pH from pH 1.0 to 2.5 and there was gradual variation over the pH range 2.5–7.4. Above pH 7.4 sharp increasing peak current was obtained up to pH 9 and then decreasing again significantly. Such behavior at PG electrode is in contrast to those obtained at carbon nanotube-based electrochemical sensors in the previous study [32], in which by raising the solution pH up to 12 a decrease in the peak height of capsaicin was observed. This demonstrates the importance of different molecular configurations (allotropes) of carbon material, which is probably involved in the electrode reaction. Although 0.1 M perchloric acid produced the largest peak currents, based on the superiority of the stripping voltammetric approach in terms of resolution, easiness of oxidation (lowering the oxidation potential), the lower background current as well as the minimization of surface fouling that occurred during the oxidation of phenolic product improving the stability, BR buffer (pH 9.0) was used throughout these studies.

As soon as it was described basic electrochemical behaviour of the both compounds measured on the surface of PG electrode, for developing a sensitive analytical methodology parameters influencing the their accumulation, such as accumulation time and potential were studied (data not presented). Voltammograms of 500 ng mL^{-1} capsaicin and dihydrocapsaicin in the BR buffer of pH 9 were recorded using SW potential-waveforms following preconcentration by adsorptive accumulation onto the PG electrode in the range 15–360 s at open-circuit condition. The response increased linearly up to 120 s and then leveled off, which probably indicates that saturation of the electrode area was reached. Thus, preconcentration time of 120 s was applied in the present analytical investigation. On the other hand, the dependence of the accumulation potential either at open-circuit condition or at a potential range from −0.2 to +0.3 V was studied with accumulation time of 120 s. A better developed peak current was achieved over the potential range of (−0.1 to +0.1 V), because of an increase

Table 1
Comparison of the efficiency of the pencil graphite electrode with literature modified electrodes for capsaicin determination.

Electrode	LOD (M)	Medium	Method	References
Carbon nanotube modified basal plane pyrolitic graphite electrode	3.1×10^{-7}	pH 1.0	AdSV after medium exchange	[32]
Carbon nanotube modified screen-printed electrode	4.5×10^{-7}	pH 1.0	AdSV after medium exchange	[32]
Boron-doped diamond electrode modified with sodium dodecylsulfate	3.4×10^{-8}	pH 1.0	SW-AdSV	[33]
Carbon paste electrode modified with amino-functionalized mesoporous silica	2×10^{-8}	pH 3.0	LS-AdSV	[34]
Pencil graphite electrode	3.7×10^{-9}	pH 9.0	SW-AdSV	Present work

of the accumulation rate due to the more favorable alignment of the molecules by the electric field at the electrode solution interface, however at more positive potentials the peak current decreased indicating that the reactant species were no longer strongly adsorbed. In addition, the stripping current obtained at open circuit voltage was nearly equal to the value obtained at +0.1 V. The maximum peak current was achieved at the potential of –0.1 V; hence, this value was chosen for the rest of the present analytical study.

Finally, optimization of pulse parameters (frequency f=25 to 140 Hz, scan increment ΔE_s=4 to 12 mV and pulse amplitude a=10 to 40 mV) on the peak current response was attempted for 500 ng mL^{-1} capsaicinoids in BR buffer, pH 9.0, following its preconcentration by adsorptive accumulation at E_{acc}=–0.1 V for 120 s. The scan rate in SWV is the result of the product of the frequency and scan increment. Therefore, the peak heights increased with the increase in the frequency and scan increment. At higher values than 125 Hz and 8 mV, a broading and a distortion in the voltammograms were observed. The analytical signal is also dependent on the pulse amplitude even if this parameter seems to be less important than the frequency. Peak heights increased linearly up to 20 mV. However with higher pulse amplitudes a broadening of the peak occurred. For all subsequent work the selected conditions were: f, 125 Hz; ΔE_s, 8 mV; and a, 20 mV.

3.2. Analytical applications

Having characterized the response of these capsaicinoids at the PG electrode, it was next performed a series of experiments adsorbing capsaicin and dihydrocapsaicin from solutions of differing their concentrations over the range 5–100 ng mL^{-1} (1.6×10^{-8} M–3.2×10^{-7} M) and 1–100 ng mL^{-1} (3.3×10^{-9} M–3.3×10^{-7} M), respectively, using BR buffer at pH 9.0, and optimized accumulation time of 120 s at –0.1 V. The stripping peak current at a potential of +0.31 V increased proportionally with capsaicinoid concentration according to the regression equations:

$$i_p(\mu A) = 0.070\,C\,(\text{ng mL}^{-1}) + 0.098\ (r = 0.997, n = 6)\ \text{(for capsaicin)}$$

$$i_p(\mu A) = 0.181C\,(\text{ng mL}^{-1}) + 1.561\ (r = 0.996, n = 6)\ \text{(for dihydrocapsaicin)}$$

where i_p is the adsorptive stripping peak current, C capsaicinoid concentration and r the correlation coefficient.

The sensitivity of the proposed method was evaluated both the limit of detection (LOD) and limit of quantification (LOQ) values. The LOD and LOQ were estimated using the following expressions:

$$\text{LOD} = 3s/m;\ \text{LOQ} = 10s/m$$

where s is the standard deviation of the peak current (three runs) of the lowest concentration of the linearity range (5 ng mL^{-1} for capsaicin and 1 ng mL^{-1} for dihydrocapsaicin) and m is the slope of the related calibration equation. LOD was achieved as 1.12 ng mL^{-1} (3.7×10^{-9} M) for capsaicin and 0.28 ng mL^{-1} (9.1×10^{-10} M) for dihydrocapsaicin. In the case of LOQ, the values were calculated as 3.73 ng mL^{-1} (1.2×10^{-8} M) for capsaicin and 0.93 ng mL^{-1} (3.0×10^{-9} M) for dihydrocapsaicin. As seen in Table 1, such remarkable enhancement for the sensitivity in terms of LOD of capsaicin, clearly demonstrates the superiority of this electroanalytical approach at disposable and minimal cost PG electrode (approximately 10 or 100 times higher) over the reported AdSV methods at modified electrodes [32–34].

The intra-day precision of the results was evaluated for nine independent determinations of 25 ng mL^{-1} for capsaicin at pH 9.0 following its accumulation onto the PG electrode for 120 s in a single day, yielding relative standard deviations (R.S.D.) of 7.1%. Further, inter-day precision was examined by measuring the stripping response for three consecutive days using the different working solutions of capsaicin and the R.S.D. was found to be 8.5%. The recovery results were found to be within the accepted limits for herbal products.

To study the stability of stock ethanolic solutions of these compounds, the samples were stored in the dark at 4 °C. Their concentration was monitored for a period of 15 day by SW-AdSV. It appeared to be stable and maintain its concentration up to end of this period, confirming the reported findings [45]. All aqueous working solutions (from the acid to alkaline region) were used making lower concentrations when needed by dilution of the stock solution and protected from light. While this potentially may be more time consuming, this will result in more reliable and reproducible data.

Next, the selectivity of the proposed method was evaluated on the PG electrode by the addition of various possible interfering substances such as some ions which are bound up with growth of plants and some phytochemicals, in a standard solution containing 1 μg mL^{-1} capsaicin at pH 9.0. For testing anions (Cl^- and NO_3^-) corresponding sodium salts were used. Cations were used as nitrates (Fe^{3+}, Cu^{2+}, Ca^{2+}, and Mg^{2+}) or chloride (Na^+ and K^+). Glucose and ascorbic acid were tested because they are commonly present in most plant tissues. Due to the structural similarities of *o*-methoxyphenol derivatives, the selectivity of the described method was also studied in presence of vanillin because capsaicin contains vanillylamide moiety. The tolerance limit was defined as the maximum concentration of foreign species that caused an error less than ±5% for the determination of capsaicin. The results showed that a 100-fold of glucose, Fe^{3+} or Cu^{2+}, and 1000-fold of Ca^{2+}, Mg^{2+}, K^+, Na^+, NO_3^- or Cl^- did not affect the accuracy of capsaicin determination at the sensitivity level of voltammetric measurements, although in case of some cations such as Fe^{3+}, Cu^{2+}, Ca^{2+} and Mg^{2+}, a precipitation was observed in solutions at pH 9.0. In addition, the metal ions and glucose are not or less extracted with ethanol in real sample analysis. The exposure of 100-fold of ascorbic acid resulted in peak widening because its oxidation peak overlapped with that of capsaicin (Fig. 5A-curve 1). As we know, interferent (ascorbic acid)-to-analyte (capsaicin) concentration ratio is much less in chili peppers than the above mentioned ratio. However, in the case of higher level concentration of ascorbic acid, medium exchange technique (transfer voltammetry) could be used, where the experiment was held after the pre-concentration step, and the electrode was then transferred into the blank electrolyte solution to proceed the stripping step (Fig. 5A-curve 2). These experiments

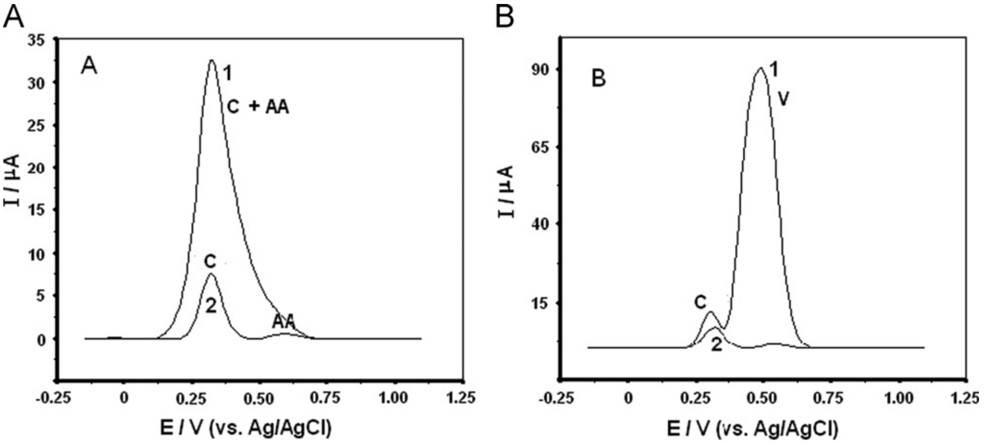

Fig. 5. SW stripping voltammograms of 100 μg mL^{-1} ascorbic acid (A) and 100 μg mL^{-1} vanillin (B) in presence of 1 μg mL^{-1} capsaicin without medium exchange (curve 1) and after medium exchange (curve 2). Medium, BR buffer pH 9.0; Pre-concentration period, 120 s at a fixed potential of −0.1 V; SWV parameters: frequency, 125 Hz; scan increment, 8 mV; pulse amplitude, 20 mV. *C*, capsaicin; AA, ascorbic acid; *V*, vanillin.

demonstrated clearly that after medium exchange which leaves behind ascorbic acid in the accumulation solution because of its poor adsorption ability on PG surface, this interferent did not contribute to the response. Although medium exchange experiments resulted in a peak that was about 50% of the height of the original peak, such sensitivity was still better than that of previous voltammetric methods. Under the conditions assayed the vanillin had individual oxidation potential at approximately +0.54 V, however the presence of high concentration level of vanillin (e.g. its 100-fold excess) can affect the capsaicin determination at the sensitivity level of voltammetric measurements (Fig. 5B-curve 1). In this case, applying a medium exchange technique was able to improve the selectivity of the measurement (Fig. 5B-curve 2). The stronger adsorption of capsaicin onto the the electrode surface could be attributed to the hydrophobic group (branched-chain fatty acid) in its molecule. On the other hand, bearing in mind that the perfect choice of the optimal accumulation potential decreases the possibility of the adsorption of many interfering electroactive compounds, which are slightly adsorbed at the electrode surface, low accumulation times could be used to eliminate vanillin and/or other chemically related compounds. However, the detection of capsaicin in the presence of other capsaicinoids is complicated by the fact that all compounds of this class are remarkably similar, which differ only in the number of the CH_2 group and the saturation degree in side chain moiety as compared to each other. The above experimental results obtained for dihydrocapsaicin with a saturation in side chain confirmed that the capsaicin could not be determined individually when dihydrocapsaicin was present.

It is noteworthy to underline that in the reported studies [32] and [33], the evaluation of not only the interference of other foreign species which present in real samples in general, but also the other capsaicinoids as the key interferents in particular have not been carried out. Therefore, voltammetric signals observed in real samples were assigned only to the oxidation of capsaicin. In other previous report [34], although the results of selectivity study were not shown, it was concluded that other types of capsaicinoids severely interfered with the determination of capsaicin.

The applicability of the proposed methodology in real samples was tested in two different samples of commercial Turkish pepper flakes. For the sample preparation, the influence of some variables such as nature and volume of the solvent, and the time of magnetic stirring on the extraction from dried sample matrix was tested. Bearing in mind that capsaicinoids are not water soluble and the solvents such as methanol, ethanol, acetone and ethyl acetate have given similar results for extracting the capsaicinoids [13], in the presented study ethanol was preferred as the suitable medium for extraction since it is an ecologically friendly solvent. To evaluate the effect of the ratio of mass/volume of the solvent, extractions were performed with different volumes of solvent (10–50 mL) for 0.2 g of sample. Since there were no significant differences when the volume of the extraction solvent was varied, it was decided to work with a volume of 12.5 mL. The time of extraction with ethanol was optimized by magnetic stirring (after employing sonication for 20 min) the sample in contact with the organic solvent during periods of time in 15–160 min range. A clear increase of the recovery was obtained with the increase of the extraction time in dried samples of peppers. Practically constant stripping currents were obtained from 120 min, and consequently, it was used as the extraction time.

Following the sample preparation, the voltammetric procedure under the experimental conditions described in Section 2.4, was carried out. Typical voltammograms of two pepper samples are shown in Fig. 6 (dashed line). Although the dominant component is capsaicin in most of the hot varieties of peppers, the peak potential shifted towards more positive value (~+0.36 V) than that obtained pure capsaicin solution (~+0.31 V), which is understandable because this peak in pepper samples solution is the result of the combined effects of dihydrocapsaicin together with other naturally occuring capsaicinoids in minor concentrations which may have slightly different oxidation potentials. On the other hand, the intensity of observed oxidation peak at +0.36 V is associated with their synergistic effect (i.e. as the sum of their peak intensities).

As observed in Fig. 6, this peak height increases as adding capsaicin standard solution which indicates that total capsaicinoid level can be quantified from the standard addition of capsaicin. To check the validity of the proposed method, the spike/recovery experiments were performed. To do so, the appropriate amount of standard capsaicin was added to the matrices of commercial pepper flakes and the voltammetric responses were evaluated. The recoveries of the compound in the pepper samples were examined at least three times and the determined with the result of 91.1–96.3% (Table 2).

By expressing the total capsaicinoid content as capsaicin equivalent, it was found the concentrations of 27.58 and 13.73 ng mL^{-1} of capsaicinoid in the measurement cell for the sample of red and Isot pepper flakes, respectively. Taking into account the successive dilutions of the samples, it was calculated that its amounts present in the original extract are 9.20 μg mL^{-1} (red pepper) and 4.58 μg mL^{-1} (Isot pepper), which correspond to 575.1 μg (red pepper) and 286.3 μg (Isot pepper) capsaicinoid per gram of dry sample. Capsaicinoid content can, therefore, be

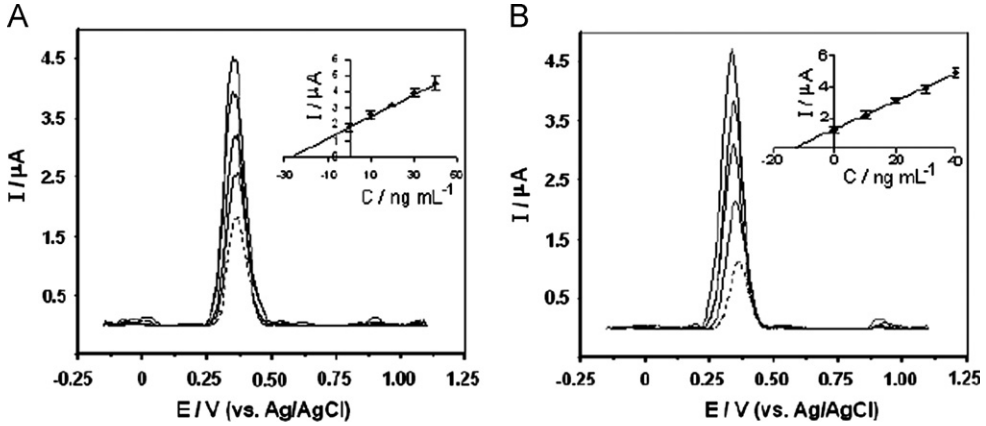

Fig. 6. SW stripping voltammograms for additions of 10, 20, 30 and 40 ng mL^{-1} capsaicin on diluted samples of red (A) and Isot (B) pepper flakes. Inset: corresponding standard addition plots for quantitation of capsaicin. Dashed line shows the signal of diluted sample of pepper flakes before the additions of capsaicin. Other operating conditions as indicated in Fig. 5.

Table 2
Results of the recovery analysis of capsaicin in the extract of pepper samples.

Capsaicin added (ng mL^{-1})	Level determined[a](ng mL^{-1})	Recovery (%) ± RSD (%)
Red pepper flakes		
0	27.58	
20	45.79	91.1 ± 5.1
30	56.18	95.3 ± 4.7
Isot pepper flakes		
0	13.73	
10	22.08	93.1 ± 6.3
20	32.49	96.3 ± 5.3

[a] Values reported are the average of three independent analysis of each spiked sample.

converted to Scoville units (SHU) with the following equation [2]:

$$\text{SHU} = \mu g/g(\text{ppm}) \times 16.$$

The values of 9259 and 4609 SHU (moderately pungent) were found for the samples of red and Isot pepper flakes, respectively. These results are in a good agreement with the common knowledge of pepper consumers regarding the pungency of red and Isot pepper flakes with the values in the range 6000–10000 and 4000–8000 SHU, respectively. However, it is difficult to compare the total capsaicinoid content from different pepper samples because it may vary in a given variety of peppers depending on several environmental factors such as soil, watering, light, temperature, stress, region, season, harvest period, drying methods, packaging, storage conditions, etc.

4. Conclusions

In this paper we have discussed a PG electrode combined with SW-AdSV that allows to a sensitive analysis of two capsaicinoid analogoues (capsaicin or dihydrocapsaicin) from the aqueous solutions, which may find application in industry sector. Compared to those obtained with the modified electrodes [32–34], the remarkable analytical sensitivity of the activated PG electrode even after the medium exchange technique, together with its simplicity and being low cost which enables its use without applying any series of chemical modification steps for electrode preparation, makes this electrode very promising for capsaicinoid determination. Because electrode reaction of capsaicinoid molecule was followed by solution chemical reactions which can produce products that adsorb on the electrode surface, gradual loss of electrode activity may occur in its determination during repeated measurements with the same electrode. However, benefitting from its single-use and disposable advantages, using PG electrode would eliminate the fouling problem with self-passivating electrode reactions. Pencil lead is the cheapest material on the one hand, the activation process based on the anodization of the PG electrode did not require using any extra chemicals on the other hand. Thus the further advantage of PG electrode is being much more economical than the modified electrodes which are usually expensive to make.

While extracting capsaicinoids from a chili pepper is straightforward, some potentially interfering electroactive compounds are simultaneously coextracted from the pepper. As a result, selectivity can frequently be a problem with complex samples (vegetable matrices). To obviate this problem for the samples in which an interfering compound with similar redox potential is weakly or not adsorbed, according to our preliminary tests, the pre-concentration/medium exchange/voltammetry protocol (analogous to solid phase extraction methods) could advantageously be used without any need for sample pre-treatment.

Voltammetric procedure has the disadvantage of individual and/or simultaneous determination of capsaicin and other capsaicinoids in case of analyzing natural sources (vegetables and plants) or food products. However, taking into account that total capsaicinoid content of pepper determines its commercial quality, electrochemical measurements using less complicated apparatus and inexpensive material compared with chromatographic techniques, could be suitable as a quick control test for global amount of capsaicinoids and pungency level of real samples. Besides, the capsaicin or dihydrocapsaicin oxidation at a PG electrode could be used for the more sensitive and individual detection of these compounds after chromatographic separation, i.e. by employing a monolithic column [14].

On the other hand, since the interference of other capsaicinoids is not a case of samples with one analyte (e.g. capsaicin) present, the proposed voltammetric method could also be useful in pharmaceutical applications such as topical ointment and cream.

In addition to using for their practical applications, in keeping with limited data available, the presented study of the voltammetric behavior of capsaicin and dihydrocapsaicin by using a PG electrode could help, at least in part, to gain in understanding the biological redox processes (antioxidant and antiradical activities, and chemoprotective properties) not only of the capsaicinoids but also of some other polyphenolic compounds.

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