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Quantitative determination of capsaicinoids in ground hot pepper samples using voltammetry of microparticles

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Applicability of standard addition method for determination of pungency in commercially available hot pepper samples using voltammetry of microparticles (VIM) is presented in this work. Analysis was performed on paraffin-impregnated graphite electrode (PIGE) using square-wave voltammetry (SWV) under optimal experimental conditions of pH 11, pulse amplitude of 50 mV, frequency of 150 Hz, and step potential of 2 mV. Concentrations of capsaicinoids in hot pepper samples were determined from internal calibration curves constructed using standard addition method (SAM) and good correlation was obtained between our results and those reported in literature (correlation factor r = 0.976). In addition to VIM, applicability of stripping voltammetry microprobe (SPV) method for electrochemical analysis of capsaicinoids was investigated. SWV responses obtained for capsaicin precipitate using SPV method were similar to those obtained using VIM. There was a good dose-response relationship between capsaicin concentrations precipitated on PIGE but in rather narrow concentration range.

Key words: capsaicinoids, hot peppers, standard addition method, voltammetry of microparticles, stripping voltammetry microprobe

INTRODUCTION

Hot peppers (*Capsicum spp.*) are among the oldest cultivated plants that are consumed worldwide mainly because of their unique pungent flavour and aroma. The pungency is caused by capsaicinoids, a group of alkaloids found exclusively in the fruits of hot pepper varieties. The most abundant capsaicinoids in hot peppers are capsaicin (8-methyl-N-vanillyl-*trans*-6-nonenamide) and dihydrocapsaicin (8-methyl-N-vanillylnonanamide), which are responsible for about 90% of the spiciness [1]. In addition to their importance as food additives, capsaicinoids were found to have beneficial effects on human health including chemopreventive and anticancerogenic effects, antioxidative activities, regulation of the energy metabolism, anti-inflammatory, analgesic and antimicrobial properties [2].

An accurate determination of the levels of various capsaicinoids has become important because of the increasing demand by consumers for spicy food and their increasing use in pharmaceuticals. The oldest method for determination of hot pepper pungency is Scoville organoleptic test [3] which is based on the tester's perception of pungency.

Additionally, more accurate and sensitive analytical methods have been developed, including high-performance liquid chromatography (HPLC) which is considered to be the most reliable and accurate method for quantification of individual capsaicinoids in hot peppers as well as for determination of their pungency [4]. However, chromatographic methods are generally complicated, time-consuming and require expensive instruments. Electrochemical techniques, particularly voltammetry, have appeared as a promising alternative due to low cost, miniaturization potential and rapid and simple analysis. There are several published papers dealing with the application of voltammetry for determination of hot pepper's pungency, but all of them describe the solution phase detection of capsaicinoids meaning that extraction of capsaicinoids is required prior to analysis [5-7].

In our latest work we developed a sensor for direct measurement of different pungency levels in hot peppers using voltammetry of immobilized microparticles (VIM) [8]. The proposed method exploits the current intensity of capsaicinoids in pepper samples in order to classify samples according to their pungency. Till date, voltammetry of microparticles has mostly been applied for qualitative electrochemical analysis of various solid compounds, e.g. minerals, alloys, and organic molecules [9, 10]. Also, it was used for the direct

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identification of powders of pharmacological substances [11] and for estimation of antioxidative activity in tea leaves [12].

In this work, we wanted to apply VIM for quantification of capsaicinoids in solid hot pepper samples. However, solid-state voltammetric quantification is not an easy task, mainly because there is no possibility to accurately determine the exact amount of sample attached on the electrode surface. To overcome this problem, a standard addition method (SAM) can be used [13]. Standard addition method in combination with VIM allows the determination of the mass fraction of a depositable analyte in a material on addition of known amounts of a standard material containing analyte of interest to a mixture of that material and a depositable reference compound. The method has been successfully applied for quantification of boron and zirconium in minerals and ceramic materials [13, 14] as well as antidepressant drugs in phytotherapeutic formulations [15]. The main drawback of SAM is the need for relatively high amounts of sample and standard.

Hence, another electroanalytical method, namely stripping voltammetry microprobe (SPV), has received our attention. SPV is a fully new approach in electroanalytical chemistry proposed by Gulppi et al. [16, 17]. This method allows electrochemical trace analysis in micro samples which is a great advantage for such measurements [17]. In this study we wanted to investigate possible application of SPV for electrochemical analysis of capsaicinoids.

EXPERIMENTAL

Reagents and samples

Capsaicin (≥ 95 %, from *Capsicum sp*.), indigo (95%) and sepiolite were purchased from Sigma, Germany. KNO_3 and buffer solution pH 11 (analytical grade) were from Kemika, Croatia. Ethanol (p. a., 96%) was from Gram-Mol, Croatia. Water was deionised by Millipore Milli-Q system to the resistivity ≥ 18 MΩ cm. Five ground hot pepper samples (Bhut jolokia, Red savina, Fatalli, Habanero orange and Carolina cayenne) were purchased from local stores in Croatia. The samples were stored at room temperature until the time of analysis. The sources of the tested samples are listed in the footnotes to the Table 1.

Instrumentation and procedures

Voltammetric measurements were carried out using the computer-controlled electrochemical system Autolab PGSTAT 30 (Eco-Chemie, Utrecht, Netherlands). A three-electrode system (Methrom, Switzerland) with paraffin-impregnated graphite rod (diameter 5 mm, length 50 mm) as the working electrode (PIGE), Ag/AgCl (3 M KCl) electrode as a reference electrode and a platinum wire as a counter electrode were used. All potentials were expressed *versus* Ag/AgCl (3 M KCl) reference electrode. Working electrode was mechanically cleaned before each run. The circular surface of PIGE was rinsed with ethanol and distilled water, polished on a wet polishing cloth, rinsed again, dried with a fine-grade paper tissue (P1200 grade) and carefully polished on a dry, white paper sheet.

Voltammetric measurements were performed in 0.1 mol L^{-1} KNO_3 within the potential range from -1.0 to +1.0 V. Optimal experimental conditions for determination of capsaicinoids in hot peppers are pH 11, potential step increment 2 mV, square wave amplitude 50 mV and square wave frequency 150 Hz, as reported in our previous paper [8]. The solutions were degassed with high purity nitrogen prior to all electrochemical measurements. A nitrogen blanket was maintained thereafter. All experiments were performed at room temperature. Unless otherwise stated, each voltammetric measurement was repeated six times.

Modified electrodes preparation

The 15 mg of ground pepper sample, 30 mg of indigo and 30 mg of sepiolite were accurately weighted, powdered in an agate mortar and then ultrasonicated for 5 min. Then, the mixture was spiked with different additions of pure capsaicin, ultrasonicated for 5 min, and powdered again on the agate mortar forming a finely distributed material. The surface of PIGE was contaminated with microparticles of sample by pressing it into a small pile of substance powder on a highly glazed ceramic tile and moving it with circular motion. The working electrode was immersed in the electrolyte only during the voltammetric measurements. Less than 1 mm of the graphite rod was immersed into the electrolyte in order to minimize the capacitive current.

Additional experiments were performed with precipitates of capsaicin (pure and extracted from hot peppers). Namely, a precipitate of capsaicin was formed onto the surface of PIGE by pippeting 5 μL of pure capsaicin solution in ethanol (0.05, 0.07, 0.1, 0.3, 0.4, 0.5 and 1 g L^{-1}) or hot pepper extract, and allowing the solvent to evaporate in air. The precipitate was then analysed by square-wave voltammetry at optimal experimental conditions. The extraction of capsaicinoids from hot pepper samples (200 mg) was performed ultrasonically with ethanol (12.5 mL) as extraction solvent for 20 min. Additional solutions were prepared by pipetting corresponding volume of our original extract and diluting it with ethanol (prepared concentrations were as follows: 8, 4, 2, 1, 0.5 g L^{-1}).

Description of the standard addition method

For quantification of capsaicinoids in solid hot pepper samples a standard addition method was used, as described by Doménech-Carbó et al. [9]. Briefly, in a standard addition experiment a homogenized mixture of mass m of sample (ground hot pepper) which contains an unknown mass m_X of the analyte X (capsaicin), with mass m_R of the reference compound R (indigo) was spiked with known amounts of standard compound (pure capsaicin), m_X*. The calibration curve was constructed by plotting the ratio between the peak currents of the stripping oxidation of pepper sample and indigo [$\Delta I_p(X)/\Delta I_p(R)$] *versus* mass ratio $m_X^*/\ m_R$: $\Delta I_p(X)/\Delta I_p(R) = \mathrm{K}m_X^*/m_R + \mathrm{K}(m/m_R)(m_X/m)$. The slope K of this straight line is the ratio of amperometric constants of capsaicin and indigo. The mass fraction of capsaicin in hot pepper sample (m_X/m) is estimated from the ordinate at the origin. Note that m/m_R is constant and known (m/m_R = 15 mg/30 mg). So, the mass fraction of capsaicin in hot pepper samples was estimated from the intercept multiplied by 2 and divided by the slope.

Calibration was performed by spiking a mixture of indigo + hot pepper + sepiolite (1:0.5:1) with increasing amounts of pure capsaicin. For construction of calibration curves, a minimum of five calibration standards were used.

RESULTS AND DISCUSSION

Quantification of capsaicinoids in hot peper powders

In our previous study [8], the electrochemical behaviour of capsaicin microparticles mechanically attached to a paraffin-impregnated graphite electrode was investigated using square-wave and cyclic voltammetry. It was found that the electrochemical oxidation of capsaicin on PIGE is a complex, pH dependent, irreversible process, and proceeds via the transfer of two electrons. Representative SW voltammogram of capsaicin-modified PIGE recorded under the optimum analytical conditions (pH = 11, f = 150 Hz, dE = 2 mV, E_{SW} = 50 mV) in 0.1 M KNO_3, consists of a single anodic peak at +0.256 V vs. Ag/AgCl (Fig. 1A). In addition, we have shown that voltammetry of microparticles can be used for estimation of pungency levels of hot peppers. However, quantitative determination of capsaicin in hot peppers using VIM as such was not possible, because the exact amount of sample deposited on the electrode surface is generally uneasy to control. To overcome this obstacle, Doménech-Carbó et al. [13] developed a methodology for quantification of solids with VIM using mixtures of the test material and an electroactive reference compound. Although the exact amount of each one of the compounds (reference compound and the test sample) deposited on the electrode surface is uncertain, their quotient must be equal to their known mass ratio in the original mixture and proportional to the ratio between their respective peak currents.

Selection of the reference compound is mainly dictated by these two conditions: (i) the stripping of reference compound must occur at potentials clearly separated from those at which the stripping of our analytical objective takes place, and (ii) there should be no effects or reactions between analyte of interest and the reference compound. In this study, indigo was tested as a reference compound, because its voltammetric properties in solid phase are relatively well-known [10]. As can be seen in Fig. 1B the SWV response of indigo at optimum experimental conditions consists of a well-defined peak P2 at -0.639 V and a poorly defined peak P3 at +0.137 V. The SWV of capsaicin gave one peak P1 at +0.258 V if the potential scan was initiated at -1.0 V in the positive direction (Fig. 1A). SWV response of capsaicin + indigo mixture consists of two well-separated peaks P2 at -0.588 V and P1 at +0.288 V corresponding to oxidation of indigo and capsaicin, respectively, as can be seen on Fig. 1C. Capsaicin peak is superimposed on the peak P3 of indigo (see Fig. 1B), and hence this peak was not seen on the SWV response of capsaicin + indigo mixture. The net peak current of the second peak of indigo is very low (order of magnitude 10^{-7} μA), so it is reasonable to assume that its contribution to peak related to capsaicin oxidation is negligible.

The background signal (a square-wave voltammogram recorded with no compound immobilized on PIGE surface) has been measured before each measurement and peak at ca. -0.1 V was always present which can be attributed to buffer solution. Repeatability tests were performed as a series of independent measurements on six freshly prepared electrodes modified with the mixture of capsaicin and indigo (1/1, w/w).

The peak potential was reproduced with a maximum deviation of ca. 6% and the relative standard deviation of peak current ratio was ca. 26%.

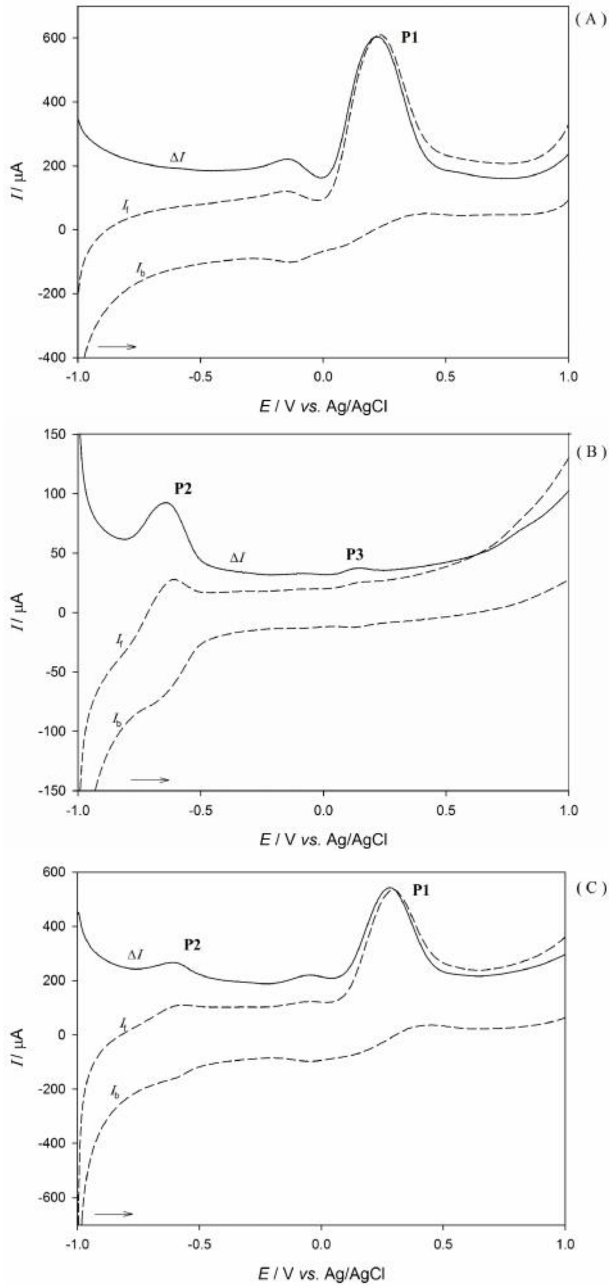

Fig. 1. Square-wave voltammetry of capsaicin (A), indigo (B) and mixture of capsaicin and indigo (1/1, *w/w*) (C) microparticles in 0.1 M KNO_3, pH 11. The pulse amplitude is 50 mV, the step potential is 2 mV and the frequency is 150 Hz. The net response (Δ*I*) and its forward (I_f) and backward (I_b) components are shown.

Relatively high standard deviation of the peak current ratio is due to non-uniformity in deposition of microparticles onto the PIGE surface. In a repeated repeatability test performed with reaction mixture diluted with sepiolite (capsaicin/indigo/sepiolite = 1/1/1), the standard deviation of peak current ratio decreased to 3.4%. Accordingly, further experiments were performed using a mixture of test sample and indigo with sepiolite.

Representative SW voltammograms obtained for a mixture of Habanero orange with indigo and sepiolite spiked with increasing amounts of pure capsaicin, together with the resulting plot of [Δ*I*p(X)/ Δ*I*p(R)] versus *(*m_X*/ m_R), is shown in Fig. 2.

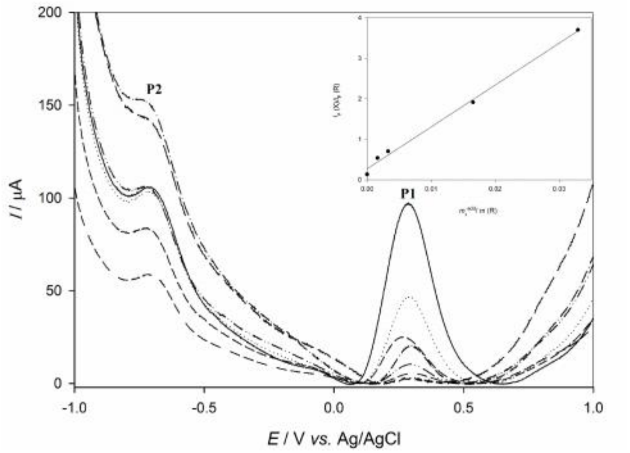

Fig. 2. SWV responses for different additions of standard compound in mixture of hot pepper Habanero orange recorded in 0.1 mol L^{-1} KNO_3 (pH 11). Additions are as follows: without standard addition (—— ——), 0.05 mg mg^{-1} (— · · —), 0.07 mg mg^{-1} (– – – – – –), 0.1 mg mg^{-1} (— · —), 0.3 mg mg^{-1} (- - -), 0.5 mg mg^{-1} (····) and 1 mg mg^{-1} (——). The peak P2 can be attributed to indigo, and peak P1 to capsaicinoids. Insert graph in figure: resulting plot of [ΔI_p(X)/ ΔI_p(R)] *versus* (m_X*/ m_R).

The SWV response of this mixture is characterised by two anodic peaks, peak P2 at -0.687 V and peak P1 at +0.292 V, corresponding to oxidation of indigo and capsaicin, respectively. Similar voltammetric behaviour was observed for all pepper samples in mixture with indigo and sepiolite. A slight shift of net peak potentials for pepper samples towards more positive values as compared with the peak potential for pure capsaicin (*E* = +0.258 V) is due to synergistic effects of all naturally occurring capsaicinoids in pepper samples (including dihydrocapsaicin and minor amounts of nordihydrocapsaicin, homocapsaicin, homodihydrocapsaicin, etc.), which have slightly different oxidation potentials. Accordingly, the intensity of peak P1 at +0.290 V is not associated exclusively with capsaicin but involves the record of all capsaicinoids (i.e. total capsaicinoids) in a particular sample. Moreover, in our previous study we have found a strong positive correlation between SWV current responses corresponding to capsaicinoids in pepper samples and the Scoville heat units reported in the literature indicating that SWV peak currents associated with oxidation of capsaicinoids are directly proportional to concentrations of capsaicinoids samples, i.e. the hot pepper's pungency.

Table 1. Data from calibration curves for different samples of chilli peppers obtained from SQWVs under the experimental conditions described in Fig. 1.

Chilli pepper	Slope	Origin ordinate	$m_X/m \cdot 100\%$	SHU
Bhut jolokia[a]	116.95	0.8233	1.410	$1\cdot10^6$
Red savina[b]	124.22	0.3661	0.590	$4.2\cdot10^5$
Fatalli[b]	103.71	0.2832	0.546	$3.9\cdot10^5$
Habanero orange[b]	103.50	0.2708	0.523	$3.7\cdot10^5$
Carolina cayenne[c]	179.19	0.2423	0.270	$1.9\cdot10^5$

[a] Harissa; [b] Volim Ljuto; [c] Začini Bušić

Quantification of capsaicinoids in commercially available ground hot pepper samples was performed by means of standard addition method. Calibration curves were obtained for pepper + indigo + sepiolite mixtures with $m_X{}^*/m_R$ ratios ranging from 0.0 to 0.03 mg/mg. Linear dependencies of $[\Delta I_p(X)/\Delta I_p(R)]$ *versus* ($m_X{}^*/\ m_R$) were observed in all cases with correlation coefficients in the range from 0.972 to 0.999 (see insert in Fig. 2). The results obtained with SAM for capsaicinoids content in pepper samples are listed in Table 1. Concentrations of capsaicinoids were found in the range from 0.27% to 1.41%, which is in accordance with results obtained with other analytical techniques such as HPLC [18, 19]. If the concentration of capsaicinoids in Bhut jolokia is ascribed to the pungency level of $1\cdot10^6$ SHU [20], the pungency of other peppers can be estimated from linear relationship between the concentration of capsaicinoids and the level of pungency [8,19]. These estimations are reported in the last column of Table 1. The values for Red savina and Fatalli are in agreement with the results from organoleptic method, but for the other two peppers the estimated levels are somewhat higher [20]. Deviations in results can be explained with different environmental conditions, technological processing of peppers in food industry and genetic factors. Environmental factors that could have impact on capsaicinoid content include water deficit [21], addition of mineral supplements [22] and nitrogen supply [23]. Furthermore, capsaicinoid content of peppers can also vary between different fruits within the same plant, even when harvested at the same time. Regarding the technological processing of peppers, drying, grinding and high temperatures could all have great influence on the capsaicinoid content. They can be a negative factor on the stability of capsaicinoids in certain varieties of chili peppers, so processing could affect the total capsaicinoid content. Also, it is not negligible to mention that capsaicinoids are mostly synthesized and accumulated in the placenta of peppers so total capsaicinoid content in pepper powders can vary because it is not known which part of the dried pepper is dehydrated in technological process [24, 25].

Voltammetry of precipitate-modified electrodes

The SW voltammetric response of 1 g L^{-1} pure capsaicin precipitate recorded under optimal experimental conditions (aqueous electrolyte of pH 11, f = 150 Hz) is shown in Fig. 3. The net SW response consists of a single oxidative peak at E = +0.254 V, similar to potential obtained for oxidation of capsaicin microparticles. The forward and backward components of the response indicate totally irreversible oxidation. These results are in accordance with the results for capsaicin microparticles (see Fig. 1A.). It can be concluded that both, capsaicin microparticles and precipitate, undergo the same oxidation mechanism.

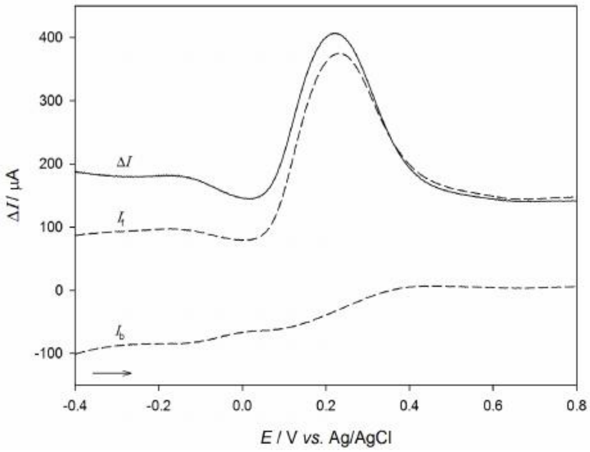

Fig. 3. Square-wave voltammograms corresponding to the oxidation of 1 g L^{-1} capsaicin precipitate immobilized at a paraffin impregnated graphite electrode (5 mm diameter) in homogeneous buffer solution, pH 11 containing 0.1 M KNO_3. The pulse amplitude is 50 mV and the step potential is 2 mV.

The SWV response of capsaicin precipitate attached on PIGE surface depends on the amount of capsaicin. Respective square-wave voltammograms recorded for different amounts of capsaicin attached on PIGE in the form of precipitate are shown in Fig 4. The net peak potential is linearly shifted in the positive direction by increasing the concentration of capsaicin precipitated on electrode surface (see insert plot of Fig. 4) i.e. higher energy is needed for electrooxidation.

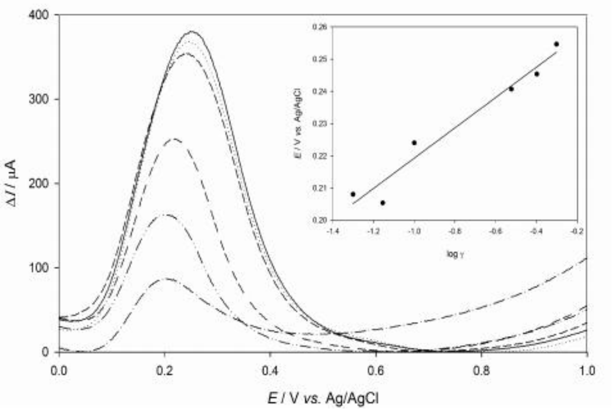

Fig. 4. SWV responses for different concentrations of capsaicin dissolved in ethanol, immobilized on electrode surface as a precipitate and recorded in 0.1 mol L^{-1} KNO_3 (pH 11). Concentrations are as follows: 0.05 g L^{-1} (− · −), 0.07 g L^{-1} (− ·· −), 0.1 g L^{-1} (− − − −), 0.3 g L^{-1} (——), 0.4 g L^{-1} (− − −), 0.5 g L^{-1} (······). Insert graph in figure: a plot of the potential against the logarithm of mass concentration of capsaicin.

This phenomenon could be explained on the basis of steric effects due to electroinactive hydrophobic parts of capsaicin molecule causing charge transfer hindrance. SWV current increases with increasing amount of capsaicin precipitated on electrode surface and the relationship between net peak current and concentration is linear with correlation coefficient $r = 0.935$. However, for the concentrations higher than 0.3 g L^{-1} voltammetric response starts to decline which can be assigned to saturation of electrode surface, respectively the hydrocarbon chain blocks the electron transfer leading to significant decrease in peak current and to the shift of their potential toward more positive values.

SWV responses for different concentrations of pepper Habanero orange are shown in Fig. 5.

If mass concentration of Habanero orange is 16 g L^{-1}, the voltammogram consists of a single irreversible oxidative peak at $E = +0.271$ V which is in accordance with electrochemical behaviour of pure capsaicin. With decreasing of mass concentration of capsaicinoids in pepper extract, a slight shift of potential to more negative values was observed. All net peak currents increase linearly with increasing mass concentration. However, at concentrations above 8 g L^{-1} net peak current reaches plateau due to saturation of electrode surface. Similar behaviour was observed for all tested hot pepper samples. These results suggests that net peak current could potentially be used for quantitative determination of capsaicinoids in real pepper samples.

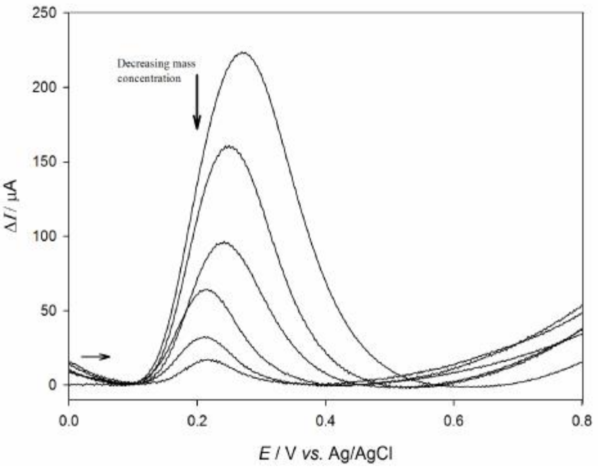

Fig. 5. Square-wave voltammograms ($f = 150$ Hz) for the oxidation of different amounts of pepper Habanero orange deposited in form of precipitates onto a 5.0 mm diameter paraffin impregnated graphite electrode and immersed in an aqueous 0.1 M KNO_3 solution

Fig. 6 shows SW voltammetric responses recorded for precipitates of different pepper samples (16 g L^{-1}). The highest signal intensity was observed for Bhut jolokia (ΔI = 266.87 μA), followed by Habanero orange (ΔI = 220.26 μA), Fatalli (ΔI = 165.53 μA), Carolina cayenne (ΔI = 129.96 μA) and Red savina (ΔI = 118.30 μA). It should be emphasized that this trend between samples completely corresponds to that obtained for microparticles of real pepper samples [8].

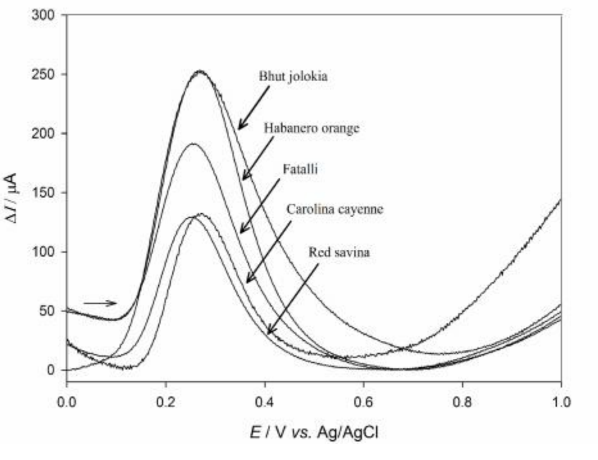

Fig. 6. Square-wave voltammograms ($f = 150$ Hz) for the oxidation of different pepper samples deposited in form of dry precipitate onto a 5.0 mm diameter paraffin impregnated graphite electrode and immersed in an aqueous 0.1 M KNO_3 solution

CONCLUSIONS

This work has investigated the possibility of application of standard addition method on methodology of voltammetry of immobilized microparticles for the quantification of total capsaicinoid content in real pepper samples. Also, preliminary investigation was done in context of stripping voltammetry microprobe (SPV), relatively new approach in electrochemical measurements. In each case, we have seen that the results are similar but with some distinct differences between mentioned methodologies. In the first case, with SAM method we successfully quantified pepper samples classified as very highly pungent. Variations in the results can be explained with time and place of the harvest, the stage of maturation, technological process for preservation of dry hot peppers, commercial packaging etc.

Experiments with dry precipitates gave us insight into the electrochemical process that takes place in microsamples, i.e. microvolumes. We have seen that voltammetry is consistent with our previous work [8] with minor differences which can be explained with molecule configuration. Application of stripping voltammetry microprobe showed high sensitivity with microsampling, thus providing a potential for the future determination of low amounts of analytes. However, additional measurements should be performed for complete explanation of mentioned processes.

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КОЛИЧЕСТВЕНО ОПРЕДЕЛЯНЕ НА КАПСАИСИНОИДИ В СМЕЛЕНИ ПРОБИ ОТ ЛЮТИ ЧУШКИ ЧРЕЗ ВОЛТАМЕТРИЯ НА МИКРОЧАСТИЦИ

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(Резюме)

Представен е стандартен метод на добавяне за определяне на остротата на вкуса на проби от лют пипер разпространен в търговската мрежа, използвайки волтамерия на микрочастици (VIM). Анализът се извършва на графитни електроди импрегнирани с парафин (PIGE), като се използва квадратно-вълнова волтаметрия (SWV) при оптимални експериментални условия: pH 11, амплитуда на импулса 50 mV, честота 150 Hz и стъпка на потенциала 2 mV. Концентрацията на капсаисиноиди в проби от лют пипер са определени с помощта на вътрешни калибрационни криви построени използвайки стандартния метод на добавяне (SAM). Получена е добра корелация между нашите резултати и тези, докладвани в литературата (корелационен фактор R = 0.976). В допълнение на VIM, е изследвана приложимостта на стрип волтаметрия на микропроби (SPV) за електрохимичен анализ на капсаисиноиди. Получените SWV резултати за капсаисинов пресипитат по метода SPV са аналогични на получените с помощта на VIM. Установено е ясно изразено съответствие между концентрациите от капсаисин отложен върху PIGE, но в много тясен диапазон от концентрации.