Electrochemistry as a screening method in determination of carotenoids in crustacean samples used in everyday diet

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Abstract

Electrochemistry of carotenoids has attracted a lot of interest because it provides an understanding of their oxidative properties. We report the application of electrochemistry in the analysis of carotenoids in crustacean samples. Voltammetry of microdroplets immobilized on paraffin impregnated graphite electrode in 0.1 mol dm^{-3} $HClO_4$ and KNO_3 aqueous electrolytes using square-wave voltammetry was applied. Previous studies have shown that carotenoids undergo complex oxidation process when characterized in aqueous media. In this research, the electrooxidation of carotenoid astaxanthin was confirmed. The obtained response allowed the development of an electroanalytical method with a limit of detection of 15.77 μmol dm^{-3}, limit of quantification of 47.8 μmol dm^{-3} and acceptable relative standard deviations for current (3.69%) and potential (0.41%). Extraction using DMSO and acetone was shown to be appropriate for voltammetric analysis. Astaxanthin content was determined electrochemically in shrimp and soft-shell crab samples (20.441 and 6.022 μg g^{-1}, respectively), yielding recoveries above 90%.

Keywords: astaxanthin; carotenoids; crustaceans; voltammetry; spectrophotometry

1. Introduction

Global commercial production is constantly increasing, so providing products that could have positive impact on human health is important. A lot of research has confirmed the importance of antioxidants in preserving health i.e. consummation of products rich in antioxidative compounds can provide a lot of positive impacts on human health such as anti-inflammatory and anticarcinogenic activity (Dauqan, Abdullah & Sani, 2011, Hamid, Aiyelaagbe, Usman, Ameen & Lawal, 2010, Cadenas and Packer, 2002, Pokorny, Yanishlieva & Gordon, 2001). With regard to the strict quality control of food that is present in the market, it is important to ensure an adequate nutrition during breeding and manufacturer should establish the preservation of nutritional value of the processed goods.

Carotenoids are a group of compounds that are firstly responsible for natural color of different products, e.g. plants, but also animals (fish meat, crustaceans and crabs). Color is a decisive criteria to consumers because it relates to nutritive value, healthiness, freshness and taste (García-Chavarría & Lara-Flores, 2013). Also, carotenoids have drawn a lot of attention in the last years because of their great antioxidative activity. Nowadays, astaxanthin is considered as one of the strongest antioxidants in the group of carotenoids (Liang, Tian, Yang, Zhang & Skibsted, 2009). Marine animals contain various carotenoids that show structural diversity (Page & Davies, 2006). Among the 750 reported carotenoids found in nature, more than 250 are of marine origin (Maoka, 2011). Astaxanthin is one of the widely and naturally distributed carotenoid in the aquaculture industry. It is mainly used as pigment in diet of marine organisms including shrimps, crabs and fish to provide desirable coloration, but it is also used as a vitamin source that is required for optimal nutritional value (Wade, Gabaudan & Glencross, 2015). However, carotenoids are extremely liposoluble molecules so reaction mechanism and further possibilities of this compounds are still not yet fully understood, especially when

looking on the cellular level. Further, carotenoids are highly degradable at higher temperatures, they are photosensitive and easily oxidized (Boon, McClements, Weiss & Decker, 2010), so extraction of this compound is somewhat demanding.

Until now, carotenoids were mostly analyzed using high-performance liquid chromatography (HPLC), thin layer chromatography (TLC) and spectrophotometry (Maoka 2011, Tolasa, Cakli & Ostermeyer, 2005, Yamada, Tanaka, Sameshima & Ito, 1990). Some difficulties regarding the time-consuming preparation of the sample and high cost of the reagents and analysis is a main drawback of this analytical methods. Electrochemical methods could provide cost-effective and fast-screening alternative. It is well-known that different electrodes, unmodified but specially modified, provide input and screening of different compounds, like anticancer drugs (Tahernejad-Javazmi, Shabani-Nooshabadi, & Karimi-Maleh, 2018), medicinal compounds (Shabani-Nooshabadi, Karimi-Maleh, & Tahernejad-Javazmi, 2019), antioxidative molecules (Tahernejad-Javazmi, Shabani-Nooshabadi, Karimi-Maleh, 2018, Tahernejad-Javazmi, Shabani-Nooshabadi, & Karimi-Maleh, 2019) etc. Until today, by only searching through the literature with keywords "electrochemistry" and "food", one can find up to 22 284 articles regarding this topic, so electrochemistry has shown to be a present and emerging technique in analysis of foodstuff. Accordingly, Ziyatdinova et al., 2012 have performed analysis of β-carotene in raw vegetables and berries in Triton X100 media on glassy carbon electrode (GCE). Also, some measurements of lipophilic vitamins were done on multi-walled carbon nanotube modified graphite electrodes (MWNT-GEs) (Ziyatdinova, Morozov & Budnikov, 2012). It should be noted that all of this studies have performed experiments in different organic solvents, such as chloroform and acetonitrile.

While searching through the literature, to the best of our knowledge, determination of carotenoids, specifically astaxanthin, in animal samples using electrochemistry was only conducted by the batch-injection analysis system on glassy carbon electrode (GCE) in mixed

solvent system with methanol:water and it was applied to salmon samples (Oliveira, Tormin, de O. Montes, Richter & Muñoz, 2016). In a previous research (Čižmek & Komorsky-Lovrić, 2019), we have studied electrochemical behaviour of carotenoid standards (β-carotene, lutein and astaxanthin) in purely aqueous acidic electrolyte using stripping voltammetry microprobe based on paraffin impregnated graphite electrode (PIGE). This electrode was already used as an abrasive sensor in the analysis of natural antioxidants like epigallocatechin gallate, myricetin (Komorsky-Lovrić & Novak Jovanović, 2016), capsaicinoids (Novak Jovanović, Čižmek & Komorsky Lovrić, 2016) etc. It was applied in analysis of different fruits (Komorsky-Lovrić & Novak, 2011) and even in the analysis of drugs (Komorsky-Lovrić, Galić & Penovski, 1999, Novak, Mlakar, Komorsky-Lovrić, 2013) and metals (Doménech-Carbó, Moya-Moreno & Doménech-Carbó, 2004). The main advantage of this electrode is unique active surface when comparing it with glassy carbon electrode. It can be used for quantification of a specific analyte because the amount of the sample volume is known and transferred to the entire electrode active surface.

In our pursuit to develop an electrochemical method that will enable determination of this highly lipophilic molecules in aqueous media and not in organic solvents, in this study the aim was to investigate another method named voltammetry of immobilized microdroplets applied to carotenoid standard astaxanthin dissolved in dimethyl sulfoxide. Lower limits of detection and quantification were obtained. The proposed electroanalytical method was applied for determination of carotenoids in crustaceans, shrimp and soft-shell crab samples that are available in food markets. Promising results were obtained with good reproducibility and applicability.

2. Material and methods

2.1. Reagents

Astaxanthin (analytical grade) was generously provided from Xi'an Lyphar Biotech Co., Ltd, China. Potassium nitrate (KNO_3) and buffer solutions 2.0-11.0 were from Kemika, Croatia and were analytical grade. Perchloric acid ($HClO_4$), acetone (p.a.) and dimethyl sulfoxide (DMSO, p.a.) were also from Kemika, Croatia. Water was deionised by Millipore Milli-Q system to the resistivity 18 MΩ cm. While using 0.1 mol dm^{-3} KNO_3 as a liquid electrolyte, solution was buffered to the particular pH-value.

2.2. Apparatus

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 (PIGE, diameter 5 mm, length 50 mm) as a working electrode (obtained from Professor Fritz Scholz from Ernst Moritz Arndt University Greifswald), Ag/AgCl (3 mol dm^{-3} KCl) electrode as a reference electrode and a platinum wire as a counter electrode were used. All potentials were expressed versus Ag/AgCl (3 mol dm^{-3} KCl) reference electrode.

Working electrode was mechanically cleaned before each experiment. 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. The cleanliness of the electrode was checked by recording a blank voltammogram. All experiments were performed in the dark by placing aluminum foil around the experimental cell at room temperature and each measurement was repeated six times. Single electrode was used for all the experiments.

2.3. Procedures

2.3.1. Square-wave voltammetry

The electrochemical technique square-wave voltammetry (SWV) was performed in two electrolyte solutions, 0.1 mol dm^{-3} $HClO_4$ and 0.1 mol dm^{-3} KNO_3. The solutions were degassed with high purity nitrogen for at least 20 minutes prior to all electrochemical measurements. A nitrogen blanket was maintained thereafter. Voltammetry of immobilized microdroplets (Scholz, Komorsky-Lovrić & Lovrić, 2000) was employed and it is based on three-phase system. Carotenoid standard astaxanthin has a maximum solubility of 50 g dm^{-3} in DMSO. Standard solutions were prepared by dissolving known weighs of astaxanthin in DMSO in different concentration range whereas for determination of carotenoids in crustacean samples, extraction methods were preceded. Extracts were then used as a one phase in experimental system. Briefly, volume of 5 μL of an analyte dissolved in DMSO was transferred onto the surface of PIGE. Attractive force between highly viscous DMSO and surface of the electrode, enabled contact between drop of an analyte and aqueous electrolyte solution. Less than 1 mm of the graphite rod of the working electrode was immersed in the electrolyte during the voltammetric measurements so that only the lower end of the electrode was in contact with the electrolyte solution, i.e. when droplet of DMSO-analyte was in first contact with electrolyte solution, voltammetric measurements were performed. The viscosity of water and DMSO enabled their contact and analysis. SWV on modified PIGE was performed using a potential step increment of 2 mV and a square-wave amplitude of 50 mV. The frequency varied from 10 to 1000 Hz.

2.3.2. Sample preparation and extraction of carotenoids

Shrimp and soft-shell crab samples were bought on the local market (Zagreb, Croatia) and kept in the refrigerator until used. Shrimp and soft-shell crab tissues were excised from the shell and each tissue was weighed and homogenized with Ultra-Turrax homogenizer (IKA, Germany). For extraction of carotenoids from crustacean samples, used methods (Michelon, de Matos de Borba, da Silva Rafael, Burkert & de Medeiros Burkert, 2012, Khanafari, Saberi, Azar, Vosooghi, Jamili & Sabbaghzadeh, 2007) were slightly modified and labeled as (1) and (2). Samples were weighed carefully so that approximate mass for each sample was 1.5 g. Using method (1), samples were homogenized, placed in Falcon tubes and 5 mL of dimethyl sulfoxide (DMSO, p.a.) was added. Tubes were sealed and placed in water bath (50°C). Samples were incubated (Innova 42 (New Brunswick, Canada)) for 30 minutes and mixed for 15 seconds every 10 minutes. After half an hour, samples were centrifuged for 5 min/4000 rpm (Centrifuge 5804R (Eppendorf), Germany). Supernatant was collected in volumetric flasks (25 mL) and remaining residue was re-extracted with 5 mL of acetone. This procedure was repeated until the supernatant was completely colourless. Each supernatant was collected after re-extraction procedures. Second method (2) is almost exactly the same as method (1) with exception of the last step, where collected supernatants were dried under nitrogen flow and residue was dissolved in DMSO.

2.3.3. Spectrophotometric measurements

Spectrophotometric analysis was performed for carotenoid standard and crustacean samples on the spectrophotometer Infinite M200 PRO plate reader. Considering that carotenoids absorb light between wavelengths from 450 to 500 nm, measurements were

conducted in a wider wavelength area, from 380 to 520 nm. Additionally, measurements were conducted on a specific wavelength for individual carotenoid astaxanthin on 480 nm. Carotenoid standard was dissolved in DMSO and absorbance of the used solvent was also measured. Additionally spectrophotometric method was employed in analysis of crustacean samples and obtained results were compared with voltammetric responses.

3. Results and discussion

3.1. Voltammetric analysis

3.1.1. Astaxanthin

Voltammetry of immobilized microdroplets was conducted on the three carotenoid standards, *β*-carotene, lutein and astaxanthin. Although solubility of *β*-carotene and lutein in DMSO is high, 30 mg dm^{-3} and 1000 mg dm^{-3} respectively, no electrochemical signal was obtained by applying this technique. However, astaxanthin has shown significant electrochemical response (Fig. 1(A)) which could be explained with its unique chemical structure. Astaxanthin is a part of xanthophyll group of carotenoids which are characterized with oxygen atoms and functional groups like hydroxy, epoxy, keto etc. at the end of the molecular structure. It consists of two terminal ring moiety connected with conjugated polyene chain. Also, this molecule has two asymmetric carbons located at the 3,3' positions of the β-ionone ring with hydroxyl group (-OH) at both ends of the molecule. This terminal groups are enabling specific layout of astaxanthin molecule through the double layer cell membrane (Yamashita, 2013). This is probably the main reason why astaxanthin was shown to be more electroactive then *β*-carotene and lutein in the form of a microdroplets, while a good dose-

response relationship was obtained for all three standards using stripping voltammetry microprobe, but in rather narrow concentration range (Čižmek & Komorsky-Lovrić, 2019).

Astaxanthin is highly soluble carotenoid in DMSO, and by applying SWV on astaxanthin microdroplets immobilized on the surface of PIGE, electrooxidation response was obtained. The measurements were conducted in 0.1 mol dm^{-3} $HClO_4$, as well as in 0.1 mol dm^{-3} KNO_3 which enabled change of the pH-values. Square-wave voltammogram of immobilized microdroplets of astaxanthin on the surface of PIGE while immersed in 0.1 mol dm^{-3} $HClO_4$ is shown in Figure 1(A). Voltammogram consists of peak 1 at the potential E_{P1} = -0.276 V *vs.* Ag/AgCl/3 mol dm^{-3} KCl. Well-defined reduction peak implies that the first electron transfer is reversible which is in a good correlation with the results obtained in our previous work on astaxanthin precipitate film (Čižmek & Komorsky-Lovrić, 2019). Peak potential was independent on the logarithm of frequency (10 Hz < f < 1000 Hz), so reversibility of this process was confirmed. Poorly developed second oxidation peak (P2) at the potential E_{P2} = -0.032 V is followed by third reversible response (P3) at the potential E_{P3} = 0.335 V. Peak P3 was also present when measuring response of the bare electrode, but it had increased when astaxanthin was present. This is in agreement with the results obtained for precipitate film of astaxanthin in our previous study (Čižmek & Komorsky-Lovrić, 2019) and it a result of using perchloric acid as an electrolyte in combination with PIGE. The increase of current of peak P3 in the presence of astaxanthin can be correlated with the fact that radical cation stability depends on interaction with electrode surface. In this reaction, with first electron transfer, allyl radical is formed and with the second electron transfer, diol is formed. Contrary to our previous results (Čižmek & Komorsky-Lovrić, 2019), where two small irreversible peaks at 0.470 V and 0.650 V can be noted, in this case only peak P4 at the potential E_{P4} = 0.529 V (when frequency is 100 Hz) can be seen, while peak P5 wasn't present. Furthermore, one broad and poorly defined peak P6 can be seen at the potential E_{P6} = 1.026 V. Potentials of peaks P4 and P6 were linearly dependent

on logarithm of frequency which confirms that this processes are irreversible (Mirčeski, Komorsky-Lovrić & Lovrić, 2007). Additionally, peak P6 was lost if f < 75 Hz because it is masked by the basic current. The same assumption can be applied for missing peak P5. Comparing to our previous results (Čižmek & Komorsky-Lovrić, 2019), in this study all of the astaxanthin oxidation net peak potentials were shifted for 50 mV to more positive values. The main reason for this is transfer of anion of the electrolyte from the water to the droplet of DMSO at PIGE surface which is necessary to keep the electroneutrality of the system. In other words, astaxanthin in the form of a microdroplet can be oxidized to radical cation and dication (P2 and P3) because DMSO contains low percentage of water, thus generated positive charges must be neutralized by transferring anions ClO_4^- from electrolyte solution to DMSO at PIGE surface. To perform this transfer, additional energy must be consume which is manifested with an increase of the net peak potentials. For the reduction of quinones at potential of -0.3 V (P1), transfer of cations from water molecules to DMSO molecules is necessary and for the oxidation of hydroquinones it is necessary for protons to return to water. Additional transfer of the OH^- anions (P4) is possible and they can react with astaxanthin cations, similar to the proposed reaction scheme for astaxanthin precipitate film (Čižmek & Komorsky-Lovrić, 2019). All of this processes are directly impacting final product potentials.

To investigate the sensitivity of this method and its application for the determination of astaxanthin microdroplets on PIGE and in real samples of crustaceans, the influence of astaxanthin concentration on the net peak current (at E_{P1} = -0.276 V) was studied. The dependence of the net peak currents on concentrations of astaxanthin microdroplets on PIGE is shown in Figure 1(B). Current response was obtained in the range 7.0 μmol dm^{-3} – 400.0 μmol dm^{-3} , but linearity of the response was obtained in the narrower range 50.0 μmol dm^{-3} – 400.0 μmol dm^{-3} with correlation coefficient r = 0.995. Comparing this results with the results previously obtained for astaxanthin precipitate film where linearity was obtained in the range

0.08 – 1.17 mmol dm^{-3} (Čižmek & Komorsky-Lovrić, 2019), it can be concluded that voltammetry of immobilized microdroplets is a more sensitive method. The accuracy of the method is expressed as a recovery for peak P1 and obtained value was 99.23±9.52 %. Relative standard deviation for maximum astaxanthin concentration in the form of a droplet expressed for current of peak P1 was 3.69% and for potential was 0.413% which implies non contaminated surface of the working electrode and high repeatability in identification of this oxidation peak. The limits of detection (LOD) and quantification (LOQ) for peak P1 were calculated from the parameters obtained from the calibration curve using the equations LOD=3 s_a/b and LOQ=10 s_a/b where s_a is the standard deviation of the y-intercept of the regression line and b is the slope of the calibration curve (Ribani, Collins & Bottoli, 2007). Limit of detection for astaxanthin in the form of a microdroplets for peak P1 was 15.77 μmol dm^{-3} while limit of quantification was 47.8 μmol dm^{-3}. Considering relatively low concentration of carotenoids that were expected in real samples, astaxanthin in the form of a microdroplet has shown to be a good standard for determination of total carotenoid content in the crustacean samples.

After conducting measurements in 0.1 mol dm^{-3} $HClO_4$ as a basic electrolyte, possibility to change electrolyte solution for better explanation of electrooxidation of astaxanthin in aqueous media was investigated. For that purpose, most common electrolyte, 0.1 mol dm^{-3} potassium nitrate (KNO_3) was used under optimal experimental conditions: f = 100 Hz, E_{SW} = 50 mV, ΔE = 2 mV while changing the pH-values. Square-wave responses for astaxanthin dissolved in DMSO ($c = 1.34 \cdot 10^{-3}$ mol dm^{-3}) were recorded on PIGE in different pH solutions, ranging from 1.0 to 9.0. The pH of the solution affects the voltammetric response of astaxanthin microdroplet, i.e. the oxidation current is strongly dependent on pH (data not shown). Between pH 1.0 and pH 5.0, responses for astaxanthin microdroplet are almost identical to those obtained for astaxanthin precipitate (Čižmek & Komorsky-Lovrić, 2019) and to the results obtained in 0.1 mol dm^{-3} $HClO_4$ (Fig. 1(A)). Net peak potentials for the first three responses (P1, P2 and

P3) shift towards more negative values as the value of pH increases (1 > pH > 5), while other three responses (P4, P5 and P6) are pH independent. Further, at pH 5.0 peak P2 couldn't be identified, while peak P6 was only present at the lowest pH 1.0. It should be noted, that although the shapes of obtained peaks were identical to those obtained in 0.1 mol dm^{-3} $HClO_4$, they were shifted to more negative values when the pH increase to pH 6.0 and pH 7.0. At the pH 8.0 and 9.0, peak P1 was poorly developed at the most negative peak potential (E_{P1} = -0.763 V), while peak P2 was more pronounced, i.e. higher value of current was obtained. Net peak potentials of peaks P1 and P2 were linearly dependent with changing the pH-values as depicted in Figure 2. The potential of the peak P1 is a linear function of pH over the pH range from 1 to 9, and follows the relationship: $-E_{P1}$ (V) = 0.243 + 0.058·pH, r = 0.999. Slope of the line, + 58 mV per pH unit, suggests that the electro-oxidation of carbonyl group in astaxanthin involves equal number of protons and electrons. This conclusion confirms our first premise from previous research about proposed oxidation reaction for astaxanthin in aqueous media (Čižmek & Komorsky-Lovrić, 2019). Net potential for peak P2 was also linearly dependent in pH-range from 1.0 to 9.0 and it is described with equation: $-E_{P1}$ (V) = 0.032 + 0.050·pH, r = 0,993. Slope of 50 mV/pH was similar to that obtained for peak P1 and implies that in the oxidation of hydroxyl groups in astaxanthin molecule, equal number of protons and electrons is participating as well: =Ċ-OH ↔ =C=O + e^- + H^+. However, it should be noted that net current for peak P2 was not dependent on the astaxanthin concentration. From the obtained results, first redox reaction for astaxanthin (peak P1) has a highest current response in acidic solution, pH 1.0 to pH 3.0 and with increasing the pH value of solution, current for peak P1 drastically decreases (i.e. net peak current has decreased 2.3 times). These results are in favor of the previously used electrolyte, 0.1 mol dm^{-3} $HClO_4$, in which the best voltammetric responses were obtained.

3.1.2. Extraction of astaxanthin from crustacean samples

Numerous articles are evaluating different extraction procedures of carotenoids from plant and animal samples (Ambati, Moi, Ravi & Aswathanarayana, 2014, Michelon, de Matos de Borba, da Silva Rafael, Burkert & de Medeiros Burkert, 2012, Khanafari, Saberi, Azar, Vosooghi, Jamili & Sabbaghzadeh, 2007). In this research two of the most used methods were implemented. The main objective was to obtain optimal extraction method which will enable characterization of carotenoids in crustacean samples by applying electrochemical techniques.

Two comparative voltammograms for the extract from shrimp sample obtained with methods 1 (a) and 2 (b) are shown in Figure 3. The SWV was used for analysis of immobilized precipitate film (curve a, obtained with method (1)) and of immobilized microdroplet (curve b, obtained with method (2)) on the surface of PIGE and immersed in 0.1 mol dm^{-3} $HClO_4$. The obtained net peak currents for sample b (extraction method 2) are about 5 times higher when compared to the currents obtained for sample a (i.e. extraction method 1). In addition, for the extract obtained with method 2 (curve b, Fig. 3) reversible peak P1 occurs at the potential E_{P1} = -0.281 V with the net peak current I_{P1} = 0.154 μA which was not recorded by conducting voltammetric measurement on the second sample a. Other present responses were: reversible peak P2 at the E_{P2} = -0.118 V with the net peak current I_{P2} = 0.787 μA and irreversible peak P4 at the potential E_{P4} = 0.491 V with the net peak current I_{P4} = 9.672 μA. The obtained responses are influenced by the applied method of analysis and it can be seen that the technique of immobilized microdroplets was more sensitive than voltammetry of precipitate film. The "pre-peak" P3 at ~ 0.35 V, corresponds to capacitive current as a result of charging the electrical double layer at the electrode surface. This is confirmed by measuring oxidation current on the bare PIGE in the aqueous electrolyte and it is also in agreement with previously obtained results for the carotenoid standards (Čižmek & Komorsky-Lovrić, 2019).

Samples of the soft-shell crab were also extracted using both extraction methods (1 and 2). For the extracts obtained with method 1, no significant voltammetric signal was seen at the potential E = 0.500 V (peak P4), while for the extracts obtained with method 2, similar responses to the extracts from the shrimp sample were obtained. These observations suggest that the method 2 is more favorable as optimal for relatively quick and simple extraction of carotenoids from crustaceans and was used for further investigations of the samples.

3.1.3. Analysis of crustacean samples

The method described was applied to the determination of astaxanthin in crustacean samples, which had been previously treated as described in Section 3.1.2. Square-wave voltammogram for microdroplets of extract from the shrimp sample (lat. *Nephrops norvegicus*) in 0.1 mol dm^{-3} $HClO_4$ is shown in Figure 4(A). Reversible peak P1 at the potential E_1 = -0.281 V can be observed which is in accordance with peak P1 obtained for microdroplets of standard astaxanthin (Fig 1(A)). This is a good indicator that extracted carotenoid from shrimp sample was astaxanthin. In favor to this assumption is presence of the other relevant oxidation peaks, peak P2 at E_2 = -0.118 V and the irreversible peak P4 at E_4 = 0.491 V.

Similar results were also obtained for soft-shell crab samples (lat. *Aristeidae*) as shown in Figure 4(B) i.e. one reversible peak P1 can be seen at E_1 = -0.273 V, peak P2 at E_2 = -0.106 V and one irreversible oxidation peak P4 at E_4 = 0.505 V. Further on, one poorly defined, broad peak P6 at E_6 = 0.993 V can be seen for soft-shell crab sample. All obtained peaks are in agreement with peak potentials obtained for astaxanthin microdroplets (chapter 3.1.1).

For each sample analysed here, the identification and quantification of main peak assigned to astaxanthin (peak 1 in SWV response of shrimp and soft-shell crab extracts at peak potential of -0.281 V and -0.273 V, respectively) was validated by standard addition method,

where different concentrations of astaxanthin were added to samples. As shown in Table 1, a presence of astaxanthin in crustacean samples is confirmed. Quantities of astaxanthin were found in low concentrations, 20.441 ± 0.008 μg g^{-1} for shrimp and 6.022 ± 0.002 μg g^{-1} for soft-shell crab sample. In this case, shrimp sample contained higher amount of astaxanthin relative to the soft-shell crab sample and higher than one recorded in the literature (Yamada, Tanaka, Sameshima & Ito, 1990). However, it should be noted that this tested samples were bought at the local market, so differences in carotenoid content are expected since the origin and feeding during breeding of this crustacean samples is unknown. The results obtained for standard addition protocol are listed in Table 2. The results were obtained by spiking samples with three different concentrations of astaxanthin solution in DMSO (9.4, 18.8 and 56.6 μmol dm^{-3}) which are expressed in Table 2 as mass ratio (*w/w*, μg g^{-1}). As can be seen from Table 2, recoveries ranged from 90.407 % to 107.498 %. The somewhat higher recovery is attributable to the matrix effect. Although relative standard deviations (RSD) are high, there are acceptable when determining analyte content in biological samples (RSD $\leq$ 20%) (Tiwari & Tiwari, 2010). Also, higher relative standard deviations are common in stationary electrochemical measurements when graphite electrodes are employed. It should be noted that PIG electrode was manually and electrochemically cleaned and polished before each measurement. Further on, before each run, electrochemical signal was recorded for bare electrode but also with used solvent, in this case, DMSO with RSD of 0.53 %. This implies that electrode surface was renewed, clean and non-contaminated. The results reveal sufficient accuracy, and therefore the possibility of using the proposed analytical method for astaxanthin determination in crustacean samples, but also in other marine organisms and food.

Extracts from the crustacean samples were further analyzed using UV-Vis spectrophotometry. It is known that absorption maximum is highly dependent on the used organic solvent and on the sensitivity of used spectrophotometer (Lichtenthaler, 1987).

Absorption maximum of astaxanthin dissolved in DMSO was obtained at 480 nm which is in accordance with the literature data when methanol was used as a solvent (Tokarz, Cisek, El-Ansari, Espie, Fekl & Barzda, 2014). Linearity range for astaxanthin was obtained from $2.5 \cdot 10^{-8}$ mol dm^{-3} to $1.02 \cdot 10^{-3}$ mol dm^{-3}. Absorbance for all samples was measured at wavelength λ = 480 nm considering astaxanthin was expected in the samples. However, it is important to emphasis that other carotenoids could also be present in this samples, such as β–carotene, zeaxanthin, violaxanthin, neoxanthin etc., which all have similar absorption maxima (Lichtenthaler & Buschmann, 2001). Accordingly, one can only assume total carotenoid content (TCC) in the crustacean samples using spectrophotometric method. TCC was also calculated from standard addition method correlating absorbance value (480 nm) and concentration of carotenoids and expressed as μg g^{-1} (Table 1). Obtained values were higher for the shrimp samples then for the soft-shell crab.

When comparing the results obtained with spectrophotometric and electrochemical methods (voltammetry of immobilized microdroplets) for the shrimp and soft-shell crab samples shown in Table 1, it can be seen that higher values were obtained using spectrophotometric method for both samples. This can be explained with non-selectivity of spectrophotometric method, i.e. using this method total carotenoid content in sample was determined. As mentioned before, it is possible that other carotenoids with similar absorption maxima were present in the samples and they contribute to total carotenoid content in the samples. In contrast, applying votammetric techniques, voltammetric responses for extracts from the crustacean samples, shrimp and soft-shell crab can be seen in Fig. 4 (A) and (B) which is in agreement with voltammetric response for standard astaxanthin (Fig. 1(A)). Thus, the amount of carotenoids expressed in Table 1 directly corresponds to the net current responses for peak P1 present in Fig. 4 (A) and (B) which is characterized as the main peak for electrochemical redox reaction of astaxanthin (Fig. 1(A)). Therefore, the amount of carotenoid

obtained by voltammetric measurement is lower than the amount obtained by spectrophotometric measurements i.e. astaxanthin is quantified in samples using voltammetry and all carotenoids present in the samples were spectrophotometrically measured at λ_{max} = 480 nm. In this case, voltammetric technique has shown to be a more selective method. However, one can see that higher discrepancies can be observed for soft-shell crab sample, i.e. 4 times lower concentration was found using voltammetry. This implies lower concentration of carotenoid astaxanthin in soft-shell crab sample, but spectrophotometry confirms presence of some other carotenoid in this sample. In shrimp sample, one can conclude that the main carotenoid responsible for its coloration is principally astaxanthin which is confirmed with both electrochemistry and spectrophotometry.

4. Conclusion

In this paper electrochemical characterization of natural antioxidative pigments, which are frequently used in food industry was performed. Optimized voltammetric technique has been developed for the characterization of carotenoids in crustacean samples (shrimp and soft-shell crab). Also, extraction method was optimized for electrochemical characterization of carotenoids in aqueous media which was not done before. Electrochemical oxidation of astaxanthin in aqueous media was further explained using voltammetry of immobilized microdroplets in two electrolytes, 0.1 mol dm^{-3} $HClO_4$ and 0.1 mol dm^{-3} KNO_3. Electrochemical process includes redox reaction of carbonyl groups at the end of the benzene rings in astaxanthin molecule, which is followed by oxidation of cation radicals, dications and hydroxyl groups at the end part of the molecule. Explanation of electrooxidation of astaxanthin has enabled its determination in real samples. Applied electrochemical method allowed quantification of a specific carotenoid in crustacean samples, while total carotenoid content was

425 determined using spectrophotometry. Developed electrochemical method has a significance
426 because of the use of aqueous electrolyte and has a potential as an alternative to other more
427 complicated and cost-effective methods of analysis which could be applied in a control of
428 industrial processes when developing a product or preserving its quality.
429
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431
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435 astaxanthin and lutein.
436
437 ***6. Conflict of interest***
438
439 Authors declare that there is no conflict of interest to report.
440
441 ***7. References***
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Figure captions

Figure 1. (A) Square-wave voltammograms of a bare electrode (– ·–) and of astaxanthin microdroplet (c = 1.34 · 10^{-3} mol dm^{-3}) immobilized on the surface of PIGE and immersed in 0.1 mol dm^{-3} $HClO_4$ electrolyte solution. The frequency is 100 Hz, pulse amplitude is 50 mV and the step potential is 2 mV. The net current (ΔI) and its forward (I_f) and backward (I_b) components are shown. **(B)** Dependence of the net peak current P1on concentration of immobilized astaxanthin microdroplet on PIGE surface. Inset: linear dependence of the net peak current P1 on concentration of astaxanthin.

Figure 2. Dependence of the net peak potentials P1 (●) and P2 (▲) of the SW voltammogram of astaxanthin microdroplet (c = 1.34 · 10^{-3} mol dm^{-3}) on pH of aqueous electrolyte, 0.1 mol dm^{-3} KNO_3. Frequency is 100 Hz, pulse amplitude 50 mV and step potential 2 mV.

Figure 3. Square-wave voltammograms of a bare electrode (– –) and of the extracts from shrimp samples obtained with extraction methods 1 (**a**) and 2 (**b**) immobilized on the surface of PIGE while immersed in 0.1 mol dm^{-3} $HClO_4$ solution. All experimental data are as in Fig. 1. The net current (ΔI) and obtained peaks are shown.

Figure 4. Square-wave voltammograms of a bare electrode (– ·–) and of extracts from **(A)** shrimp and **(B)** soft-shell crab samples in the form of microdroplets immobilized on the surface of PIGE and immersed in 0.1 mol dm^{-3} $HClO_4$. All experimental data are as in Fig. 1. The net current (ΔI) and its forward (I_f) and backward (I_b) components are shown.

605 **Fig 1.**

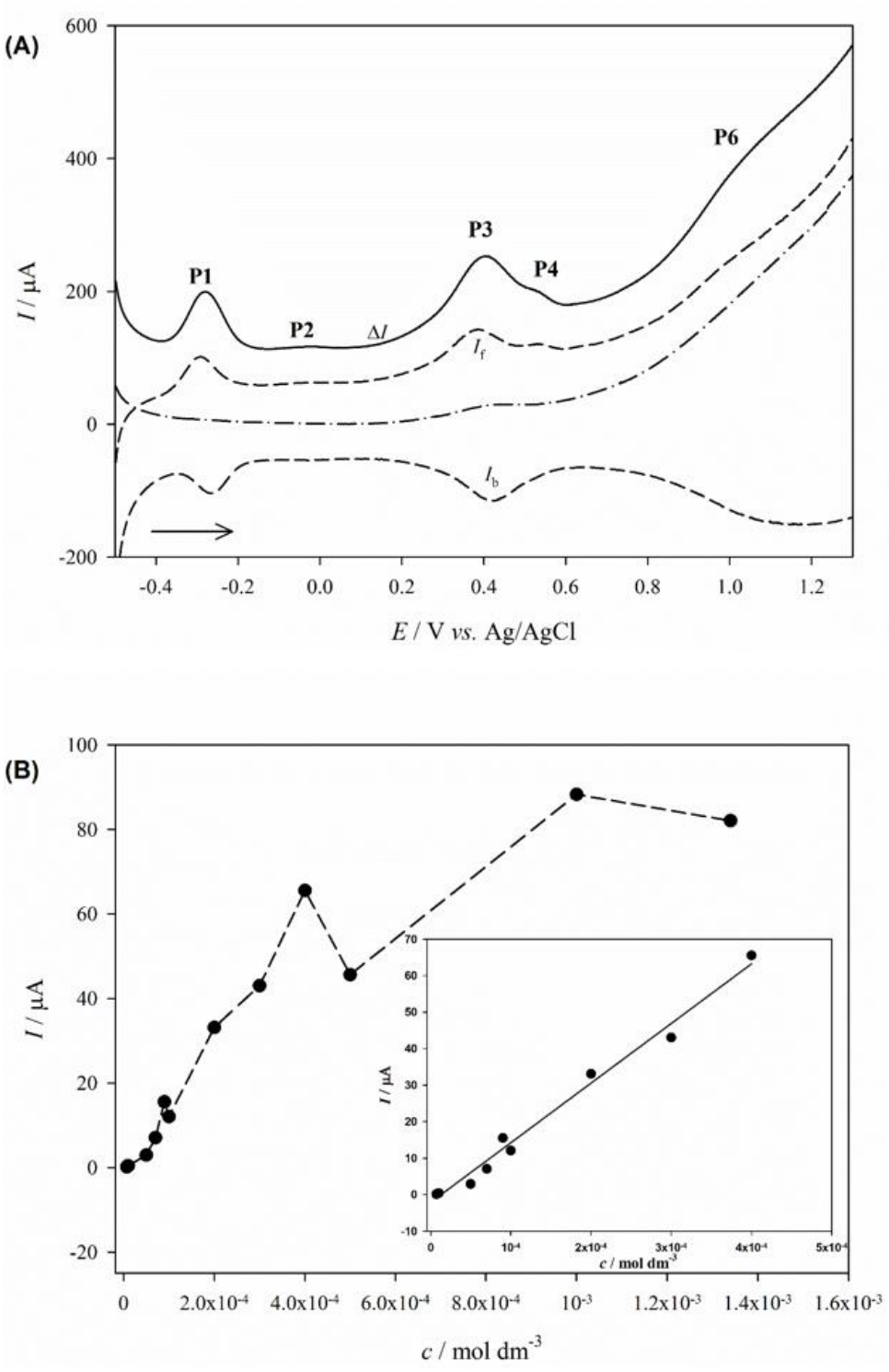

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Fig. 2.

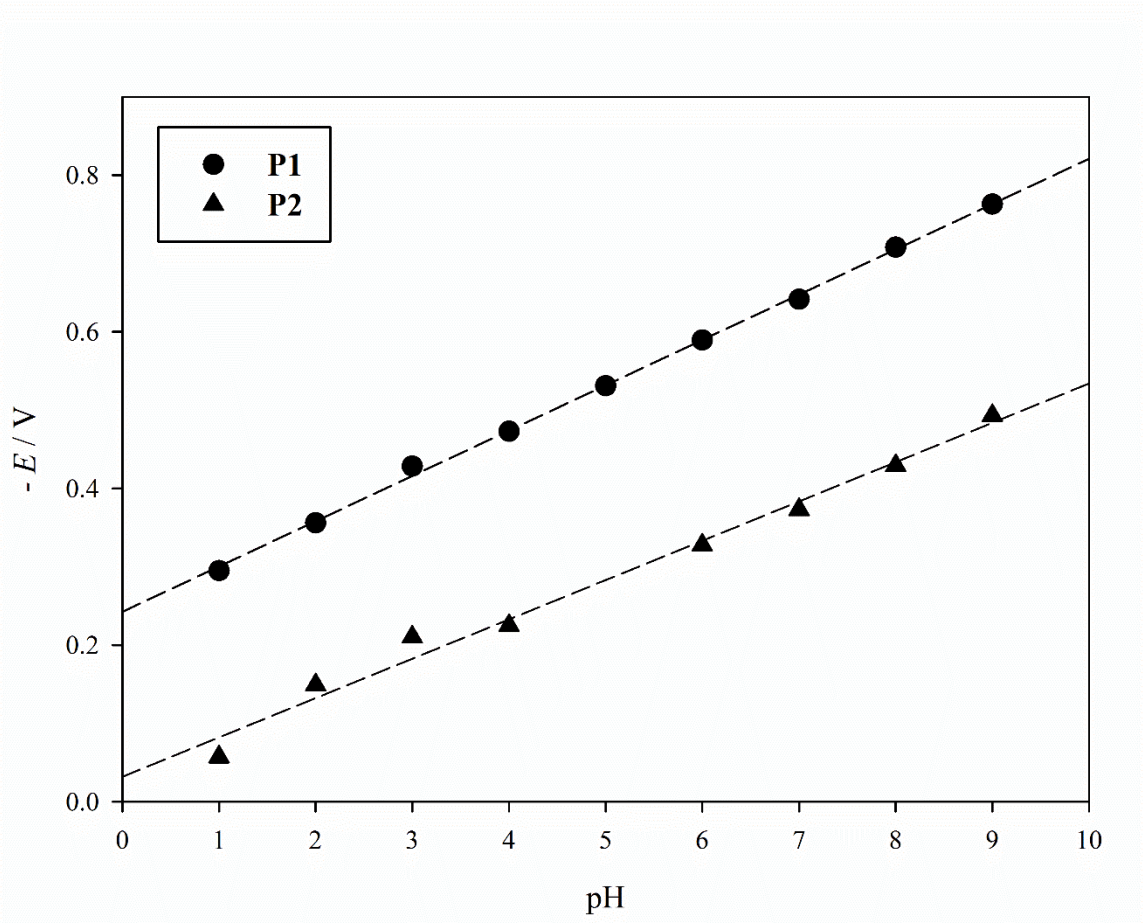
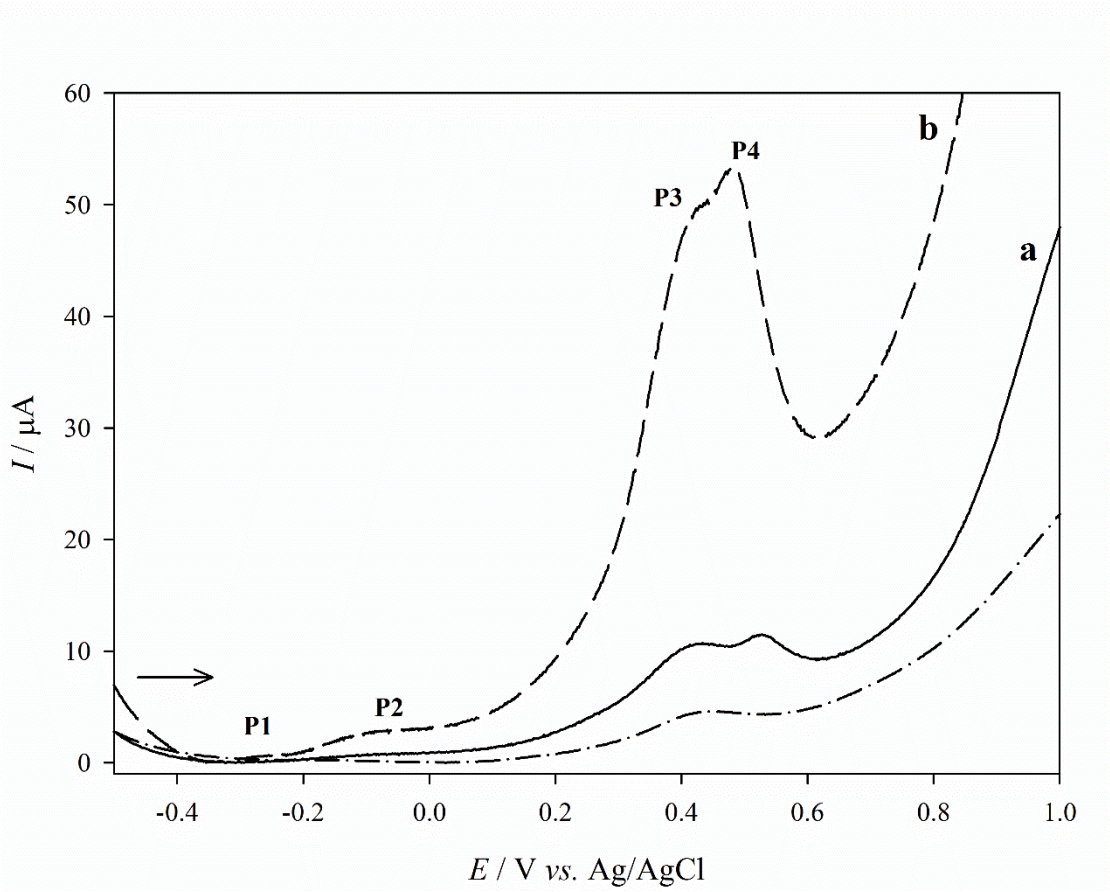


Fig 3.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a
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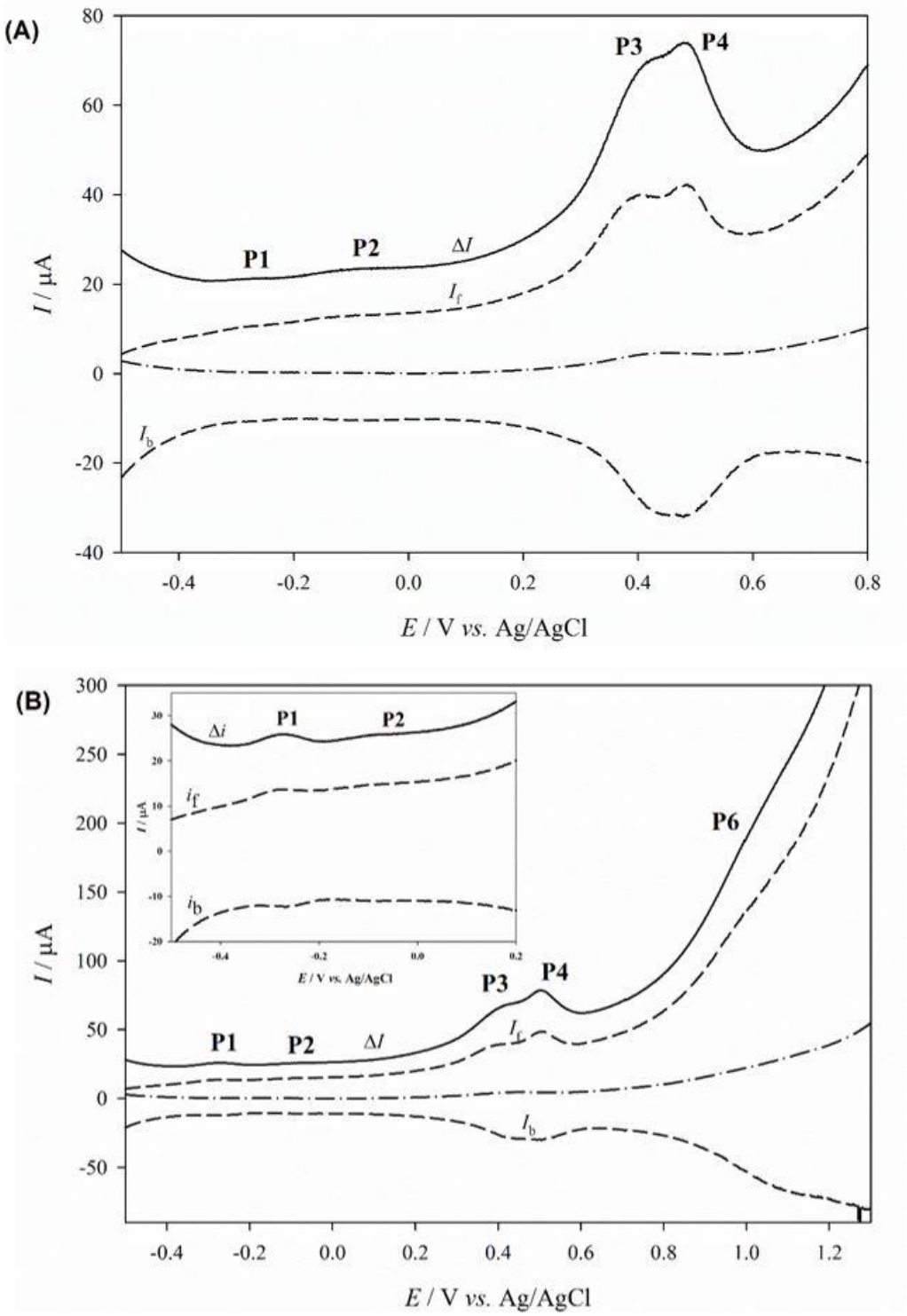
Fig. 4.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I / μA
E / V vs. Ag/AgCl

644 **Table 1.** Comparison of analytical results of astaxanthin in crustacean samples.

Sample	Voltammetric method		Spectrophotometric method	
	***c* (astaxanthin)[a] (μg g^{-1})**	**RSD (%)**	**TCC[b] / μg g^{-1} (λ = 480 nm)**	**RSD (%)**
Shrimp	20.441 ± 0.008	4.082	29.722 ± 0.021	7.031
Soft-shell crab	6.022 ± 0.002	7.014	25.156 ± 0.019	5.606

645 [a] n = 3.
646 [b] TCC – total carotenoid content in sample.

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650 **Table 2.** Recovery study of astaxanthin in crustacean samples using voltammetry of immobi-
651 lized microdroplets.

Sample	***c* (astaxanthin), μg g^{-1}**		Recovery, %[a]	RSD, %[b]
	Added	**Found[b]**		
Shrimp	14.208	14.751	103.821	3.513
	28.264	26.566	93.992	4.789
	85.298	85.097	99.765	3.679
Soft-shell crab	14.403	15.483	107.498	5.978
	28.652	25.903	90.407	4.673
	86.467	86.749	100.326	2.636

652 [a] (100 * Found/Added).
653 [b] n = 3.