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Analytical Methods

Electroanalytical tools for antioxidant evaluation of red fruits dry extracts

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ABSTRACT

Red fruits are rich sources of antioxidant compounds with recognized health benefits. Since they are perishable, dried extracts emerge as more durable products and their quality control must include antioxidant capacity assays. In this study, the redox behavior of commercial dried products obtained from camu-camu, açai, acerola and cranberry red fruits was evaluated by electroanalytical approaches. The antioxidant potential was determined by 2,2-diphenyl-1-picrylhydrazyl free radical assay and the electrochemical index concept. The total phenol content was estimated by using a laccase based biosensor. A significant correlation was found between all methods and literature data. The voltammetric profile (cyclic, differential and square wave) obtained for each type of dried extract showed distinguishable features that were correlated with their main major markers, being also useful for identification purposes. The electrochemical methods were cheaper and more practical for evaluation of antioxidant properties and total phenol content in dried powders obtained from different red fruits.

1. Introduction

The consumption of natural antioxidants is associated with a delay in aging processes, as well as with the prevention of degenerative diseases. Therefore, the search for sources of dietary antioxidants (Escarpa, 2012; Oliveira-Neto et al., 2016) has focused on red fruits that contain large amounts of polyphenols and vitamins with antioxidant activity (Jakobek, Šeruga, Novak, & Medvidović-Kosanović, 2007).

The fruits are usually processed in many ways, in order to improve their stability, because they rapidly spoil and their production is far from the target consumers (Zotareli, Porciuncula, & Laurindo, 2012). One of the most efficient methods to extend the stability of natural products is to remove their water content, since dehydration lessens deleterious chemical and biochemical processes (Lüle & Koyuncu, 2015; Zotareli et al., 2012). However, the heating based drying methods can also provoke decomposition, mainly in the case of thermally unstable compounds, which can include many antioxidants can be enrolled (Fujita, Borges, Correia, Franco, & Genovese, 2013).

In order to preserve the chemical and biological properties of these products, some analytical procedures must be performed. Considering that many biological properties of red fruits products are due to their antioxidants compounds with radical scavenging activity, a number of analytical protocols have been established with the aim to evaluate the antioxidant capacity of these substances, which include spectrometric methods such as DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-Azinobis-3-ethylbenzo thiazoline-6-sulfonic acid, diammonium salt), ORAC (Oxygen radical absorbance capacity) assays, measure of total phenols by Folin-Ciocalteu method (Mishra, Ojha, & Chaudhury, 2012; Oliveira-Neto et al., 2016), chromatographic determination (Huber & Rupasinghe, 2009; McDonald, Prenzler, Antolovich, & Robards, 2001), and evaluation of redox behavior by means of electrochemical approaches that provide an antioxidant profile (Blasco, González, & Escarpa, 2004; González, Vidal, & Tzanov, 2009; Kilmartin, Zou, & Waterhouse, 2002; Makhotkina & Kilmartin, 2009; Rebelo, Rego, Ferreira, & Oliveira, 2013). In addition, is possible to calculate the electrochemical index value, which is a reliable measure associated with the antioxidant potential (Escarpa, 2012; Lino et al., 2013; Oliveira-Neto et al., 2016).

Also, selective biosensors have been developed for the quantification of specific compounds or for determination of major compounds with similar structures. The laccase, superoxide dismutase (SOD) and DNA based biosensors have been used for total phenol content and antioxidant potential determinations (Barroso et al., 2011; Campanella, Bonanni, Finotti, & Tomassetti,

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2004; Escarpa, 2012; Mello, Hernandez, Marrazza, Mascini, & Kubota, 2006). The laccase performs the biochemical oxidation followed by electrochemical reduction in a biosensor, which is detected by amperometric means (Garcia et al., 2015). While the DNA-Biosensor is based in protection DNA against oxidative damage, the lower the decrease of former anodic peaks, the higher the protection (Campanella et al., 2004). In turn, SOD are based on the selective enzymatic dismutation of $O^{\cdot -}$ into O_2 and H_2O_2 followed by sensitive amperometric detection (Mourad, Barsan, Dridi, Ben Ali, & Brett, 2016).

This study was performed with the aim to evaluate the electrochemical approaches as a tool for quality control and redox characterization of natural products. The antioxidant and redox profile of red fruits crude extracts were characterized by means of cyclic voltammetry (CV), square wave voltammetry (SWV) and differential pulse voltammetry (DPV). The antioxidant potential expressed by electrochemical index (EI) was compared to results obtained with the DPPH method. Finally, a Laccase based biosensor was employed in order to determine the total phenol content of natural products here studied.

2. Materials and methods

2.1. Reagents

Ethanol, DPPH and the other HPLC solvents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All electrolyte solutions were prepared by using analytical grade salts, which were diluted in double distilled Milli-Q water (conductivity $\leqslant 0.1\ \mu S\ cm^{-1}$) (Millipore S. A., Molsheim, France). The phenolic antioxidants, rutin, gallic acid, catechin, hesperidin, caffeic acid, peonidin-3-O-glucoside, quercetin were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Sample Stock Solution

The commercial dried products (powder) obtained from red fruits (RF) dried extracts (DEs), namely acerola (AC1, AC2 and AC2), açai (A1 e A2), camu-camu (CC1 and CC2) and cramberry (CR1 and CR2) were purchased from local pharmacies.

A suitable amount of each sample (50 mg) was weighed and then extracted with 5 mL of ethanol by sonication for 30 min, in order to reach 1% ethanol extract. The crude extracts were centrifuged at 1000 rpm, and 1 mL of the supernatant was diluted with 4 mL of a diluting solution prepared using 2 mL of ethanol and 2 mL of 0.1 M phosphate, pH 5.0, thus getting 0.2% RFDE solutions. The aliquots of 250 μL of this final solution were added to 2.5 mL of electrolyte, in order to reach 0.02% RF-DE for all voltammetric assays. In order to compare the antioxidant power of each class of RFDE a pool constituted by the same amount of each supplier (N = 2 or 4) was used.

2.3. Electroanalytical assays

Voltammetric experiments were carried out with a potentiostat/galvanostat μAutolab III® integrated to the GPES 4.9® software, Eco-Chemie, Utrecht, The Netherlands. The measurements were performed in a 5.0 mL one-compartment electrochemical cell, with a three-electrode system consisting of a carbon paste electrode, a Pt wire and the Ag/AgCl/KCl 3 M (both purchased from Lab solutions, São Paulo, Brazil), representing the working electrode, the counter electrode and the reference electrode, respectively. The experimental conditions for DPV were: pulse amplitude 50 mV, pulse width 0.5 s and scan rate 10 mV s^{-1}. The experimental conditions for SWV were: pulse amplitude 50 mV, were frequency 50 Hz and a potential increment of 2 mV, corresponding to an effective scan rate of 100 mV s^{-1}. The experimental conditions for cyclic voltammetry (CV) were: scan rate of 100 mV s^{-1} and scan range from 0 to 1 V. The DP voltammograms were background-subtracted and baseline-corrected, and then all data were analyzed and treated with the software Origin 8®.

All experiments were done at room temperature (21 ± 1 °C) in triplicate (n = 3) and the main electrolyte used was the 0.1 M phosphate buffer solution (PBS), pH 5.0.

2.3.1. Electrochemical index

The electrochemical index (*EI*) concept was first proposed by Escarpa group (Blasco, Rogerio, González, & Escarpa, 2005; Escarpa, 2012) taking into account to the main voltammetric parameters, peak potential (E_{pa}), and peak current (I_{pa}). Based on the fact that the lower the E_{pa} (thermodynamic parameter), the higher is the electron donor ability, and the higher the I_{pa} (kinetic parameter), the higher is the amount of electroactive species, *EI* was calculated using the following equation (Lino et al., 2013):

$$EI = \frac{I_{pa1}}{E_{pa1}} + \frac{I_{pa2}}{E_{pa2}} + \cdots + \frac{I_{pan}}{E_{pan}}$$

In which I_{pan} and E_{pan} correspond to current and potential value for each anodic peak, 1a, 2a ...na, observed in the DP voltammograms.

2.3.2. Laccase based biosensor (LBB)

A laccase crude extract, *Pycnoporus sanguineus* (CCT-4518), with 2019 $U.L^{-1}$ obtained as ascribed in previous work (Garcia et al., 2015) was used to prepare the optimized LBB. Briefly, 250 μL of enzymatic crude extract was mixed with 100 mg of graphite and left to dry at room temperature for 6 h. Then, 30 mg of mineral oil, the agglutinating agent was added and rigorously mixed in order to achieve a homogenous paste. This final paste was used to fill the cavity, that was 2 mm in diameter and 0.5 mm in depth within the electrode support, namely a Teflon cylinder with a central hole overpassed by brass wire, the electrical contact.

2.4. DPPH assay

The radical scavenging activity assays were performed using the stable DPPH· reagent, in accordance with very well established procedures (Oliveira-Neto et al., 2016). In summary, the blank control was composed by a mixture of and 2.7 mL of DPPH· ethanolic solution (0.1 mM) and 0.3 mL of ethanol, in which the final absorbance at = 517 nm was of *c.a.* A = 0.7. The ethanol was used in order to adjust the baseline (A = 0.000). Antioxidant activity was expressed as EC_{50}, representing the amount (L) of sample solution to produce 50% of decolorization of DPPH· relative to the blank control after five minutes of reaction.

The measurements were carried out by using a UV–vis spectrophotometer (Quimis, model Q-798U2VS, Brasil). All samples were analyzed in a 1 cm glassy cell length at room temperature.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). Comparisons among groups were made using ANOVA. Post hoc comparisons were performed using Tukey's comparison test. The significance level considered was 0.05.

3. Results and discussions

3.1. Voltammetric characterization and redox profile

The oxidation behavior of RF-DEs was investigated by CV, scan rate 100 mV s^{-1}, in 0.1 M PBS, pH = 5.0. CVs in 0.02% of RF-DEs were performed in the potential range 0 V to +1.2 V and the cyclic voltammetric profile was very distinctive, when comparing different sources of RFs (Fig. 1).

The cyclic voltammograms obtained for açai DE exhibits a reversible redox couple at $E_{p1/2} \cong 0.3$V and low defined anodic processes at higher potentials (Fig. 1A). The CC-DE (Fig. 1 IB) and AC-DEs (not shown) presented similar voltammetric behavior, that is akin to the expected for ascorbic acid, their main antioxidant compound, presenting an anodic peak 1a, at $E_{p1a} \cong 0.28$ V (Benjamin et al., 2014). In turn, the successive cyclic voltammograms obtained for CR-DEs presented a particular behavior. At the first scan, it can be observed, one well defined anodic process 1a, at $E_{p1a} \cong 0.28$ V, which is reduced on the reverse scan, thus exhibiting a reversibly behavior. On the other hand, a second large anodic peak, 2a, seems to be consequence of two or more merged oxidation processes with irreversible behavior, that start around 0.5 V. On successive scans, the peak current falls deeply for all DEs, which is consistent with electrode surface reactions leading to production of insulating film (Fig. 1). Indeed, the electrochemical oxidation products showed a strong passivation behavior.

Some features can be better observed by SWV, which presents higher sensitivity and is faster allowing lower electrode blockade, as well as, concomitant anodic and cathodic detection of reversible processes.

The SW voltammogram obtained for açai in pH 5.0 0.1 M PBS show three anodic processes, the first and second being clearly reversible (Fig. 1 IIA). On the other hand, the single anodic process observed for CC-DE (and also for acerola) that was irreversible in CV assays, exhibits a reversible behavior in SWV (Fig. 1 IIB). It can be explained by means of low time to adsorption of the insulating film at electrode surface, and also because the anodic and cathodic pulse are applied almost simultaneously, allowing the detection of both processes at same time. Indeed, the AC and CC-DEs showed higher peak fall in CV assays (Fig. 1), which is consistent with larger amount of electroactive species presenting strong adsorption and insulating behavior.

Also, the reversible redox pair 1a/1c observed for CR-DE in CV assays is better evidenced by SWV. Meanwhile, the second larger peak still remains undefined (Fig. 1 IIC).

The DPV assays were also performed in order to increase the resolution of anodic peaks and allow calculation of the electrochemical index (Lino & Gil, 2013) for antioxidant power determinations. Furthermore, this technique allows discounting the capacitive currents, hence improving the resolution and even allowing the detection of hidden peaks. Thus, the DPV was used to evaluate the influence of pH on the electrochemical oxidation behavior of RF DEs at glassy carbon electrode in 0.1 M acetate and phosphate buffers at pH range from 3.0 to 9.0. The Fig. 2 shows the corrected DP voltammograms, and the plots of E_{pa} and pH obtained for A2-DE. In all cases the slope was close to the theoretical value of 59 mV/pH unit, indicating the contribution of an equal number of electrons and protons in the redox process (Fig. 2B). The resolution and sensitivity was better at pH 5.0, therefore, all further assays were performed using this electrolyte.

In case of peak potential, 1a, it is consistent with "catechol like" phenolics. At the same time, the anodic peak 3a is at a potential expected for oxidation of an isolated phenolic compound (Blasco et al., 2005; Gil & Couto, 2013).

The DPV profile equalities for the same fruit even for different suppliers, as well as, the differences for different fruits, were also better evidenced in corrected DP voltammograms (Fig. 3).

Among the RFs, the AC-DEs purchased from different suppliers showed larger differences (Fig. 3A), which is in agreement with the amount of synthetic ascorbic acid added, as determined in previous work (Benjamin et al., 2014). On the other hand, the other RF-DEs exhibited patterns quite similar even for different suppliers (Fig. 3B). Also, the camu-camu that is another fruit very rich in ascorbic acid, some synthetic ascorbic acid is conventionally added in order to reinforce its natural content. In both, AC and CC samples, the anodic peak, 1a, at peak potential at E_{p1a} = +0.27 V (Fig. 3A) is consistent with ascorbic acid redox behavior and it was confirmed by HPLC assays in previous work (Ngai, Tan,

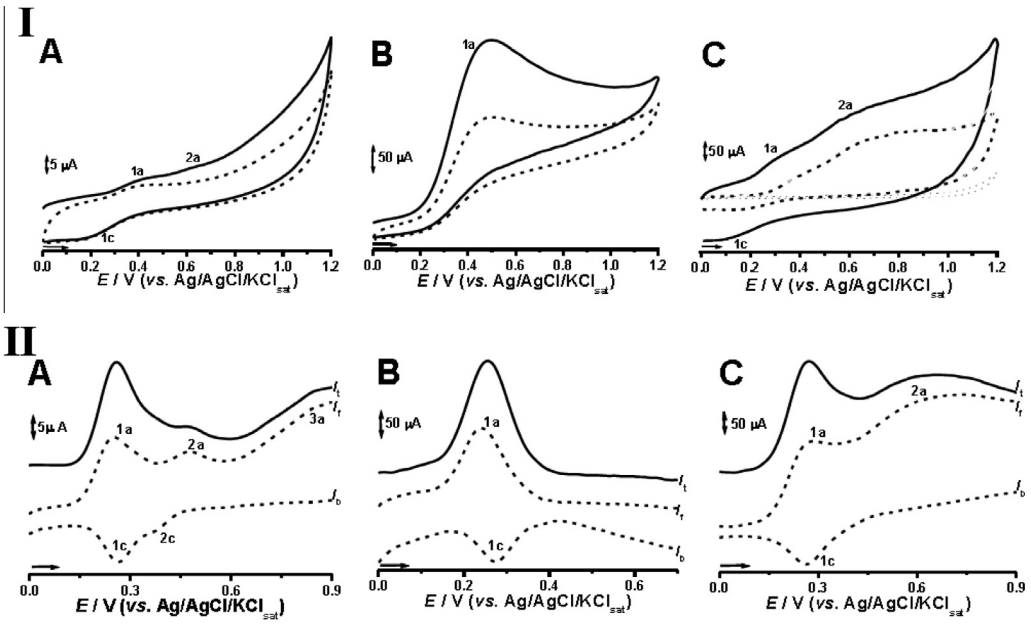

Fig. 1. CV (**I**) and SWV (**II**) profiles of 0.02% RF-DEs in 0.1 M PBS, pH 5.0 at glassy carbon electrode obtained for dried extracts of acerola (A), camu-camu (B) and cramberry (C).

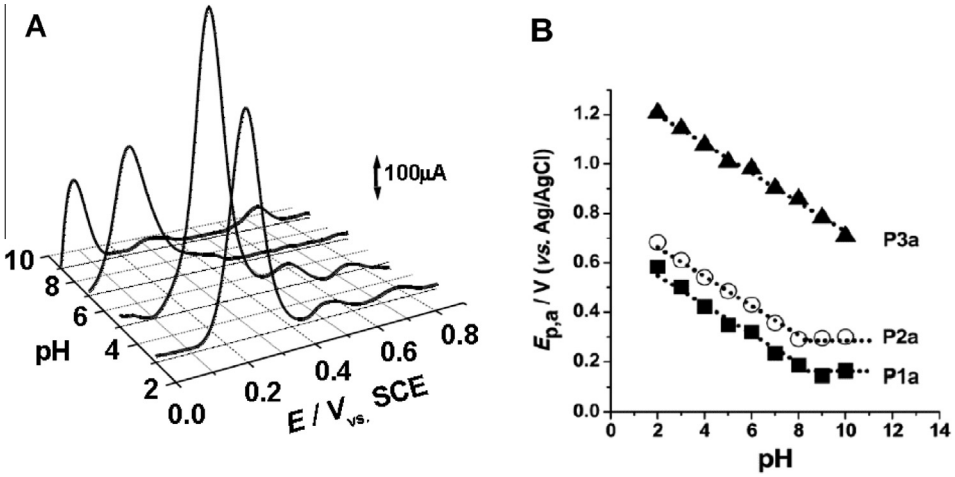

Fig. 2. (A) 3D plot of DP voltammograms, baseline corrected, in 0.02% A2-DE as a function of pH. (B) Plot of E_{p1a} (•), E_{p2a} (□) and E_{p3a} (◆) vs. pH.

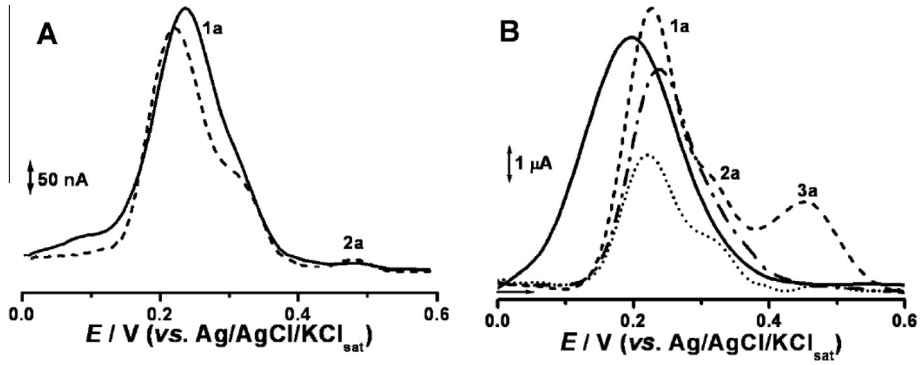

Fig. 3. DP voltammograms obtained for: A) different DE suppliers, 0.01% AC1-DE (—) and 0.02% AC2-DE (- - -), and B) different RF-DEs pool solutions, 0.02 % A-DE (- - -); 0.01% AC-DE (• • •); 0.01% CR-DE (- • -); 0.01% CC-DE (—), all in 0.1 M PBS, pH 5.0.

Zainal, Zawawi, & Zidan, 2008; Benjamin et al., 2014). Nevertheless, this procedure mischaracterizes the natural product and also overshadows the anodic peaks process expected for natural phenolic compounds. In fact, only in sample AC2 (Fig. 3A), in which no synthetic ascorbic acid was added, it was possible to observe anodic peaks related to other natural electroactive species (Benjamin et al., 2014).

3.2. Electrochemical index determinations

From the ratios obtained between peak currents and peak potentials of all anodic peaks of each RF-DE sample pools (Fig. 3B), a electrochemical index (EI) was calculated, as described in Section 2.4. The EI obtained for the different DE pool of samples (N = 2 or 4) was 6.9 ± 0.5 (A); 5.8 ± 1.7 (AC); 16 ± 0.3 (CC) and 14 ± 0.3 (CR) µA/V, respectively (Fig. 3A). The order of antioxidant potential was CC = CR > AC = A.

The EI is calculated by means of the peak current and peak potential, in which the first parameter would be directly proportional to the antioxidant power, while the second inversely. The first electrochemical parameter is related to the electron transfer kinetic and/or overall amount of reduced compounds, whereas the second has a thermodynamic nature that expresses the electron donor character ability of the electroactive species. Therefore, the EI provides a useful measure of the antioxidant power of products. In turn, the antioxidant power is usually correlated with phenolic compounds. Hence, the analysis of such products may be also evaluated by means of total phenol content.

3.3. Laccase based biosensor and total phenol content

One problem with the Folin-Ciocalteu method is that it lacks selectivity, being also used for protein analysis and ascorbic acid (Olgun, Ozyurt, Berker, Demirata, & Apak, 2014). The use of phenoloxidase based biosensor offer more exact results about phenol content, and laccase enzyme has proved to be one of the best options (Gil et al., 2008; Garcia et al., 2015; Rodríguez-Delgado et al., 2015).

The LBB was evaluated against 10 µM solutions of different natural phenolic antioxidants. The highest response was observed for the flavone rutin, a widespread polyphenol antioxidant in plant kingdom, the lowest for the non-phenolic antioxidant, ascorbic acid. Therefore, it is very reasonable to express the total phenol content of natural products by means of rutin equivalents. Hence, a calibration graph for rutin was constructed in order to get a linear equation and then apply it for further total phenol content estimations (Fig. 4).

The biosensor response against different RF DEs was evaluated, and the results obtained expressed in equivalents of rutin (N = 2–4) were: 0.5 ± 0.1 (A); 0.4 ± 0.1 (AC); 1.1 ± 0.2 (CC) and 1.5 ± 0.3 (CR) rutin Eq/µL, respectively (Fig. 4B). The total phenol content order was CC ⩾ CR > AC ⩾ A.

3.4. Radical scavenging DPPH assay

The spectrometric radical scavenging assay was also performed for pool samples, being that the highest is the antioxidant power/

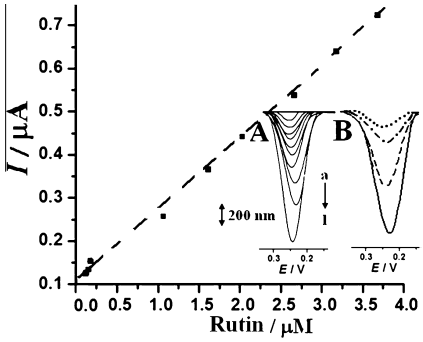

Fig. 4. Calibration Graphs obtained with LB at different concentrations of rutin standard solutions: a to l (0.01–3.5 μM). Inset. A) The related DP voltammograms for GA standard solutions, B) different RF-DEs pool solutions for 0.02% CC-DE (- - -); 0.02% AC-DE (• • •); 0.01% CR-DE (—) and 0.02% A-DE (- • -); in 0.1 M pH 5.0 PBS.

Table 1
Results obtained for dried extracts of açai (A), acerola (AC), camu-camu (CC) and cramberry (CR) pool samples in the EI, TP/LBB and DPPH assays. For each method, the results were analyzed using one-way ANOVA and significant differences between samples means were determined using the Tukey's post-hoc test. Data with same superscript letter over the values are not significantly different from each other ($P > 0.05$) and different letters denotes the significant differences between each other ($P < 0.05$).

Pool samples	EI (μA/V)	TP/LBB (rutin Eq/μL)	DPPH EC_{50} (μL)
A (N = 3)	6.9 ± 0.5^{a}	0.5 ± 1.0^{a}	18.0 ± 0.6^{a}
AC (N = 4)	5.8 ± 1.7^{a}	0.4 ± 1.0^{a}	6.00 ± 1.0^{b}
CC (N = 3)	16 ± 0.3^{b}	1.1 ± 0.2^{b}	1.60 ± 0.5^{c}
CR (N = 3)	14 ± 0.3^{b}	1.5 ± 0.3^{b}	2.00 ± 0.2^{c}

N = number of the samples analyzed.

content, the highest is the decolorization of DPPH· to the reduced form DPPH, and the lowest is the required aliquot of sample solution. Thus, the DPPH assay, when expressed by EC_{50} (Section 2.4) presents an inverse correlation. The values of EC_{50} (μL) (N = 2–4) determined by DPPH assay for red fruits were: 18.0 ± 0.6 (A); 6.00 ± 1.0 (AC); 1.60 ± 0.5 (CC); 2.00 ± 0.2 (CR) μL, respectively, showing the following antioxidant potential order: CC ⩾ CR > AC > A.

3.5. Comparison among three analytical methods

The electrochemical index and laccase based biosensor determinations showed the same order of antioxidant potential (CC = CR > AC = A). On the other hand, the order obtained with the DPPH assay was CC = CR > AC > A (Table 1). The results here obtained showed a correlation between total phenol content and antioxidant potential and a significant similarity of results between three analytical methods, pointing to the electrochemical approaches as a tool for quality control and redox characterization of natural products. The opposite response obtained in the DPPH assay for A-DE when compared to other RF-DE pool samples, can be explained by presence of interfering chromophores, *i.e.* anthocyanins in this colored RF-DE, which may leads to some deviation, when using spectrometric assay (Gomes, Ghica, Rodrigues, Gil, & Oliveira-Brett, 2016).

4. Conclusions

The electroanalytical and spectrometric approaches for total phenol content and antioxidant capacity determinations have exhibited good correlations. Moreover, the electrochemical index concept and laccase based biosensor proved to be rapid to use and appropriate tools to evaluate the AOC and TPC of dried extracts of red fruits, and may be applicable to other food sources rich in natural antioxidants.

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Further reading

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