

## Full Length Research Paper

# Profile, antioxidant potential, and applicability of phenolic compounds extracted from *Spirulina platensis*

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This work aimed at an investigation of the profile of free phenolic compounds (PC) of *Spirulina platensis* and assesses their antioxidant potential, applying them as a natural conservative in minimally processed apples. The phenolic extract showed 396  $\mu\text{g g}^{-1}$  gallic acid, 347  $\mu\text{g g}^{-1}$  of caffeic acid, 54  $\mu\text{g g}^{-1}$  salicylic acid and 3.5  $\mu\text{g g}^{-1}$  trans-1-cinmamic a total of 608  $\mu\text{g PC g}^{-1}$  of *S. platensis*. With the use of PC, it was possible to inhibit the radical DPPH over 180 min with  $\text{IC}_{50}$  of PC 202  $\mu\text{g mL}^{-1}$ . The inhibition of polyphenol oxidase and peroxidase of PC were 19.9 and 9.7%. In addition, verifying the constants  $K_m$  and  $V_{max}$ , it was concluded that inhibition of the peroxidase and polyphenol occurs in an uncompetitive manner. Application of crude extract of PC under minimally processed apples showed inhibition of browning by 40%. The general acceptance of apples was not affected by the addition of PC.

**Key words:** Apple, enzymatic browning, peroxidase, phenols, polyphenol oxidase.

## INTRODUCTION

The use of antioxidants in foods prevents the formation or propagation of free radicals resulting from the oxidation of various metabolic and environmental oxidative processes, thus reducing enzymatic browning and rancidity, which are the main oxidative processes occurring in these matrixes (Musa et al. 2013; Mai and Glomb, 2013). However, synthetic antioxidants are associated with a number of acute and chronic problems in humans (Hua-Bin et al., 2007), which has motivated the search for

natural compounds with similar effects for use in large-scale. In the search for natural products with antioxidant activity, the microalgae belonging to the genus *Spirulina* stands out, which is commercialized and studied for its nutritional potential attributed to its protein content, pro-vitamins, unsaturated fatty acids and phenolic compounds (Derner et al., 2006). The use of bioproducts derived from these microalgae is favored by the viability of its large-scale cultivation and by the facility to optimize

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**Abbreviations:** PC, Phenolic compounds; PPO, polyphenol oxidase; POD, peroxidase.

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the production of compounds of interest (Kepekçi et al., 2013).

Phenolic compounds are substances whose effectiveness as antioxidant agents is due to the ease of hydrogen atom donation to a free radical molecule (Giada and Mancini, 2006). The evaluation of antioxidant activity can be performed by various methods, always based on preventing or propagating the oxidation process. The most common is the DPPH free radical scavenging method (Noipa et al., 2011; Mishra et al., 2012). From the point of the food industry, preventing the oxidation process is a challenge to the safety of the product, especially concerning the new marketing methods, including the minimally processed products, in which the conventional heat treatment is not used for preserving the nutritional, functional and sensory characteristics of the product. In the case of plant products, the enzymes polyphenol oxidase and peroxidase are primarily responsible for the oxidative damage in fruits and vegetables, and their activation occurs when the tissue cells are ruptured during processing, while inactivation is done by the combination of heat treatment and conservatives in conventional products (Olivas et al., 2007). Because of the new food demands, such as minimally processed products, there is a need to seek innovative and effective ways to control oxidative enzymes in fruits. Given the benefits of phenolic compounds, they can be a promising alternative, but knowing its profile and its mechanisms of action is fundamental to recommend the use of these compounds.

This study aimed to investigate the profile of the free phenolic compounds from *Spirulina platensis*, and evaluate its antioxidant potential in the extract, aiming to apply them as conservative in minimally processed apples.

## MATERIALS AND METHODS

### Materials

*S. platensis* strain LEB-18 was isolated from Mangueira Lagoon (33° 30' 133" S; 53° 08' 593" W) and cultivated in a pilot plant located on the coast of this pond. Three tanks with surface area of 37.1 m<sup>2</sup> were lined with glass fiber, and covered by a greenhouse structure constructed from transparent polyethylene film. Cultures of *S. platensis* were stirred by rotating paddles at 18 rpm for 24 h during 387 days under natural light (Morais et al., 2009). The biomass was removed from the tanks, dried in an oven, ground in a Wiley mill, and stored in plastic containers under refrigeration until the extraction of phenolic compounds. The *S. platensis* LEB-18 has in its composition approximately 86% protein, 6.7% moisture and 3% lipids (Figueira et al., 2011). Apples (*Pirus malus*) varieties "Gala" were bought in a local market in the city of Rio Grande/RS, and kept under refrigeration (≈ 8°C). The reagents DPPH (95% purity), guaiacol, and the chromatographic patterns (98 to 99% purity) of gallic acid, trans-cinnamic acid, caffeic acid and salicylic acid were purchased from Sigma Chemicals (St. Louis, MO). Phenolic acids were dissolved in methanol from the stock solution, resulting in solutions of 100, 300 and 500 µg mL<sup>-1</sup> for both gallic and trans-cinnamic acids, caffeic acid, and salicylic acid, respectively.

### Determination of the free phenolic compounds (PC)

PC were extracted by adding 10 mL methanol to 3 g *S. platensis*, followed by horizontal shaking at 800 rpm for 1 h. The agitation was stopped for 15 min, after which 10 mL methanol was added and agitation was performed for 1 h. The methanol extract was filtered and evaporated on a rotary evaporator, and the residue was resuspended in distilled water and clarified with addition of 0.1 mol L<sup>-1</sup> Ba(OH)<sub>2</sub> and 5% ZnSO<sub>4</sub> and allowed to rest for 20 min. The clarified extract was centrifuged, filtered, and the volume made up to 25 mL with distilled water (Souza et al., 2010). The PC quantification was carried out by mixing 1 mL phenolic extract, 4.5 mL alkaline solution (Na<sub>2</sub>CO<sub>3</sub> 4%: CuSO<sub>4</sub> 2%: KNaC<sub>4</sub>H<sub>4</sub>O<sub>6</sub> 4% at a ratio of 100:1:1, and allowed to rest for 15 min at 40°C. Subsequently, 0.5 mL Folin-Ciocalteu reagent (1:2, v/v) was added, and allowed to stand for 10 min. The absorbance was measured at 750 nm and the concentrations were calculated by a standard curve of gallic acid (from 2 to 30 mg mL<sup>-1</sup>).

### Phenolic acids profile

The determination of phenolic acids in the *S. platensis* extract was performed by HPLC-UV, with LC-AT pump connected to the degasser DGU with an integrator CBM-20A, 7725i manual injector, UV-VIS detector 10AXL, and software Shimadzu LC-Solutions. Separation was performed on a reversed phase column C18 CLC-ODS (150 mm x 4.6 mm ID 5 µm). A sample volume of 20 µL was injected into the column and eluted with a constant flow rate of 1.0 mL min<sup>-1</sup>. Gradient elution was applied with "A" (water containing 1% acetic acid), "B" (methanol) and "C" (acetonitrile) as solvents, as follows: 0 min 31.5% B and 3.5% C; 3 min 38.7% B and 4.3% C; 10 min 53.1% B and 5.9% C; 12 min 31.5% B and 3.5% C, and total run time of 18 min. Each standard was assessed individually to check the retention times, confirmed by injection of three increasing concentrations to identify the compounds in the mixture. The linearity of the response was assessed using standard curves, with injections of five different concentrations, ranging from 2 to 37 µg mL<sup>-1</sup> for gallic acid, from 0.4 to 7 µg mL<sup>-1</sup> for trans-cinnamic acid, from 11 to 176 µg mL<sup>-1</sup> for caffeic acid and from 18 to 293 µg mL<sup>-1</sup> for salicylic acid. The coefficients of the standard curves were obtained with the aid of software.

### Antioxidant potential

The antioxidant potential of PC extracted from *S. platensis* was evaluated by spectrophotometry according to the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method. The reaction was carried out with 3 mL DPPH solution (5x10<sup>-5</sup> mol L<sup>-1</sup>), 1 mL phenolic extract (40 µg PC mL<sup>-1</sup>) and 2 mL methanol. The mixture was stirred vigorously and allowed to stand for 30, 60, 90, 120, 150 and 180 min in the dark at room temperature (25°C). The consumption of the radical was evaluated at 515 nm. For the control test (time zero), the absorbance was measured immediately after mixing. Specific inhibition (SI) was calculated by the Equations 1 and 2. The inhibitory concentration (IC 50) was obtained by the concentration (µg mL<sup>-1</sup>) of phenolic extract required to inhibit 50% of the initial DPPH radical.

$$\%I = [(U_{abs1} - U_{abs2}) / U_{abs1}] \cdot 100 \quad (1)$$

Where, U<sub>abs 1</sub> refers to the absorbance of the control, and U<sub>abs 2</sub> refers to the absorbance of the sample.

$$SI = \%I / PC \quad (2)$$

Where, I = inhibition; IE = Specific inhibition; PC = phenolic compounds.

**Table 1.** Wavelength ( $\lambda_{\max}$ ), retention time ( $t_R$ ), analytical curve, and correlation coefficient (R) of standard phenolic acids.

Standard	$t_R$ (min)	$\lambda_{\max}$ (nm)	Curve	R
Gallic acid	3.1	270	$y = 97058.88x - 129558.6$	0.9987
Caffeic acid	4.0	323	$y = 40102.72x + 35500.59$	0.9980
Salicylic acid	10.9	261	$y = 4411.899x - 8862.657$	0.9996
Trans-cinnamic acid	14.9	277	$y = 208924.5x - 16602.11$	0.9993

### Potential inhibition of oxidative enzymes

Polyphenol oxidase (PPO) and peroxidase (POD) present in the crude enzyme extract from 'Gala' apples were used. The latter was obtained by stirring the apple pulp with 20 mmol L<sup>-1</sup> phosphate buffer pH 6.0 (1:5, w/v) in a blender for 2 min. The homogenate was centrifuged at 3220 g for 10 min at 4°C, and then filtered and kept in this temperature (Oliveira et al., 2007). The enzymatic activity was determined by the reaction of 1 mL enzyme extract, 1.5 mL 20 mmol L<sup>-1</sup> phosphate buffer pH 6.0, 2 mL distilled water, and 0.5 mL substrate specific for each enzyme, as follows: 0.1 N catechol for PPO and 1% guaiacol for POD. In the POD reaction, 1 mL 0.08% H<sub>2</sub>O<sub>2</sub> was added. The reactions were carried out in a water bath at 30°C for 10 min. The protein of the extracts was determined by Lowry method (1951), in which a volume of 0.5 mL crude extract was mixed with 4 mL alkaline solution for 10 min. Then, 0.5 mL Folin reagent (1:2 v/v) was added and the mixture was allowed to stand for 30 min. A standard curve of albumin (from 0.05 to 0.42 mg mL<sup>-1</sup>) was used for protein quantification. The readings were performed at 425, 470 and 660 nm for PPO, POD, and protein extracts reactions, respectively. The results for the specific enzyme activity were expressed as Uabs.mg protein<sup>-1</sup> where 1 Uabs represents an increase of 0.001 absorbance unit per minute. To identify the inhibition mechanism of the PC, the Michaelis-Menten constant  $K_m$  and  $V_{\max}$  was determined in the presence and absence of phenolic extracts. The enzymatic reactions were carried out by varying the concentrations of phenolic extract (4, 20 and 40 µg mL<sup>-1</sup>) and the substrate (0 to 1.0 mL). In the control experiment, the phenolic extract was replaced by distilled water. The results were expressed in mg mL<sup>-1</sup> for  $K_m$  and Uabs min<sup>-1</sup> for  $V_{\max}$ .

### Application of phenolic compounds in minimally processed apples

The apples were washed with detergent and sanitized with sodium hypochlorite (200 ppm/5 min). Then, the fruits were peeled, their seeds were removed, and the pulp was cut into pieces of 1.0 × 1.0 cm, and 4 cm length. Two treatments were performed with and without submersion in the PC extract. Apple samples from both treatments were packed in PVC film-wrapped expanded polystyrene trays, and kept under refrigeration (≈ 8°C) for 8 days. The pH of the minimally processed apples was measured in potentiometer Hanna model 200 (AOAC, 2000). The titratable acidity was determined according to AOAC (Kepekçi et al., 2013) and expressed as percentage of malic acid. The weight loss was determined by weighing the packages with the samples and the results were expressed as percentage (g 100 g<sup>-1</sup>). The firmness of the samples was determined in a texture analyzer Stable Micro Systems (TA.XT Plus, England). The color of the samples was determined using a Minolta colorimeter (Chroma Meter CR400, Japan), by L, a\*, b\* system, and the browning (BI) index was calculated according to Fontes et al. (2008) as described in Equation 3:

$$BI = [100(x - 0.31)] / 0.172 \quad (3)$$

Where, BI = browning index;

$$x = (a + 1.75 L) / [(5.645 L) + a - (-3.02 b)]$$

### Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) and Tukey's test for comparison between means, using the Software Statistics 5.0.

## RESULTS AND DISCUSSION

### Phenolic acid profile

*S. platensis* is considered an excellent source of phenolic acids including caffeic acid, chlorogenic acid, salicylic acid, synaptic acid, and trans-cinnamic acid (Colla et al., 2007). However, there are no studies on the profile of these phenolic acids extracted from *S. platensis* cultivated under the experimental conditions similar to the present study. Table 1 shows the retention times for the respective wavelengths, analytical curve and the correlation coefficient for each phenolic acid studied. The analytical curves of all phenolic acids had correlation values that allowed reliable quantification of the samples in the linearity range determined by the instrument (Ribani et al., 2004). The profile of *S. platensis* phenolic extract is shown in the chromatogram of Figure 1, which exhibits the separation of four compounds under the established conditions. The concentrations in µg g<sup>-1</sup> (±CV) of the phenolic acids present in the *S. platensis* crude extract determined by HPLC-UV were 396±11.3, 347±7.6, 54±2.5, and 3.5±0.6 for galic acid, caffeic acid, salicylic acid, and trans-cinnamic acid, respectively, totaling 801 µg g<sup>-1</sup> free phenolic acids. The PC concentration determined by spectrophotometry was 608 µg PC g<sup>-1</sup> for *S. platensis*. This analysis was based on the reaction of the phenolic groups with Folin-Ciocalteu reagent, yielding a blue colored product, determined by spectrophotometry, whose value was 25% lower to that found by liquid chromatography (HPLC-UV), which enables the determination of each component without overlapping effects (Ribani et al., 2004).

Qualitative and quantitative differences in the same species can be due to the effect of abiotic and biotic variables, which are main determinant of the production of metabolic compounds with PC. In this context, Klejduš

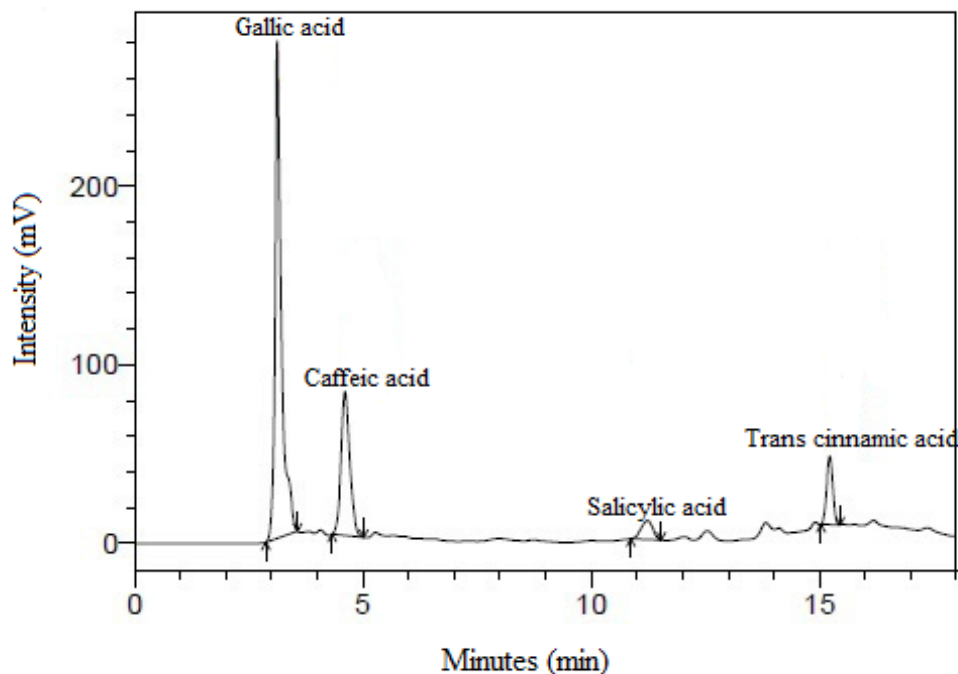


Figure 1. Chromatographic separation of phenolic acids by HPLC-UV.

et al. (2009) separated and identified phenols from microalgae by mass spectrometry, including *S. platensis*, which presented  $0.169 \mu\text{g g}^{-1}$  caffeic acid,  $0.072 \mu\text{g g}^{-1}$  chlorogenic acid,  $0.254 \mu\text{g g}^{-1}$  vanillic acid and  $2.23 \mu\text{g g}^{-1}$  p-hydroxybenzoic acid, which are lower values than those found in this study. This difference may be a consequence of the abiotic conditions.

### Antioxidant activity on DPPH

The method used to estimate the antioxidant activity of the *Spirulina* phenolic extract is based on the electron transfer from an antioxidant compound to a free radical, DPPH that, when it is reduced, loses its purple color (Duarte-Almeida et al., 2006). The procedure is fast and allows evaluating the ability to inhibit the propagation phase of the oxidation process. In the present study, the inhibition potential of the extract was studied over 180 min (Table 2) as specific inhibition (% inhibition  $\mu\text{g}^{-1}$  PC), demonstrating the ability to inhibit DPPH. This ability and stability are very promising to inhibit the enzymatic browning in the fruits. The  $\text{IC}_{50}$  of the PC estimated for the reduction of DPPH is  $202 \mu\text{g PC mL}^{-1}$  phenolic extract, which is similar to that reported by Mendiola et al. (2007), who found  $297 \mu\text{g mL}^{-1}$ , extracting PC from *S. platensis* by supercritical extraction. It should be emphasized that the lower the  $\text{IC}_{50}$  the larger the consumption of DPPH by a sample and the greater its antioxidant activity (Sousa et al., 2007; Morais et al., 2009).

Table 2. Specific inhibition of PC from *S. platensis* on DPPH.

Time (min)	Specific inhibition * (%CV**)
0	0.023 <sup>b</sup> (12.5)
30	0.241 <sup>a</sup> (6.1)
60	0.269 <sup>a</sup> (7.5)
90	0.348 <sup>a</sup> (4.9)
120	0.345 <sup>a</sup> (8.0)
150	0.305 <sup>a</sup> (4.5)
180	0.361 <sup>a</sup> (5.2)

Same letters in the column do not differ statistically ( $p < 0.05$ ); \*% inhibition /  $\mu\text{g PC}$ ; \*\* CV= coefficient of variation ( $n = 3$ ).

### Antioxidant activity on POD and PPO

The inhibitory effect of PC in catalyzed oxidation processes is still poorly investigated, once there are some phenolic compounds among the substrates of the oxidative enzymes. Thus, the enzyme activity in adverse situations results in oxidation and polymerization of derivatives to protect tissue from injury. However, some phenolic compounds may act as inhibitors of enzymatic browning (Oliveira et al., 2007). The POD and PPO from crude enzyme extract of 'Gala' apples were inhibited by 19.9 and 9.7%  $\mu\text{g}$  of PC  $\text{mL}^{-1}$ , respectively, in the presence of the phenolic acid extract from *S. platensis*. Colla et al. (2007) found inhibition values of 35% for POD

**Table 3.**  $K_m$  values ( $\text{mg mL}^{-1}$ ) and  $V_{\text{máx}}$  ( $\text{Uabs min}^{-1}$ ) of the enzymatic browning in the presence and absence of *S. platensis* phenolic extract.

PC ( $\mu\text{g mL}^{-1}$ )	Peroxidase		Polyphenoloxidase	
	$K_m$	$V_{\text{máx}}$	$K_m$	$V_{\text{máx}}$
Control	0.33	0.05	0.03	0.06
4	0.14	0.01	0.02	0.02
20	0.13	0.02	0.02	0.02
40	0.01	0.01	0.02	0.03

**Table 4.** Physicochemical characteristics of minimally processed apples, subjected to treatment with PC extracted from *S. platensis* and control.

Condition	Time (days)	Parameters				
		BI**	Firmness (N)	Weight loss (%)	Acidity*	pH
Control	0	68.27	5.01	0	0.2	4.3
	2	57.93	11.15	1.9	0.2	4.3
	4	58.39	4.56	3.6	0.2	4.4
	6	78.56	2.53	5.7	0.2	4.4
	8	70.18	5.95	7.4	0.2	4.3
With PC	0	48.39	14.18	0	0.2	4.3
	2	41.41	31.61	0.8	0.2	4.3
	4	46.70	24.39	4.4	0.2	4.3
	6	49.99	24.83	6.7	0.2	4.2
	8	52.18	26.04	8.8	0.1	4.2

\*expressed in % malic acid  $\text{g}^{-1}$  sample; \*\* BI.

extracted from potato at a *S. platensis* concentration of phenolic compounds of  $4.9 \text{ mg g}^{-1}$  dry biomass. The enzyme extract used in the test was not purified to better verify the potential of the PC extract to protect the apples against enzymatic browning if it would be applied in processing them. The Michaelis-Menten constant  $K_m$  and  $V_{\text{máx}}$  for POD and PPO in the presence of the *Spirulina* extracts are shown in Table 3 (Schnell and Maini, 2003). A decrease in the maximum velocity ( $V_{\text{máx}}$ ) and substrate consumption ( $K_m$ ) was observed with the addition of PC for both studied enzymes. This reduction is a characteristic of uncompetitive inhibition, in which the inhibitor interacts with the enzyme-substrate complex, preventing formation of the ternary enzyme-substrate-inhibitor complex to release the product (dark pigments).

Thus, the POD or PPO possibly binds to their preferred substrates and subsequently to the PC, forming the complex. The greater the affinity between the enzyme and its substrate, the faster is its inhibition, that is the formation of a complex with the compound added to the medium to inhibit enzymatic browning.

#### Application of PC in minimally processed 'Gala' apples

The use of synthetic antioxidants in foods such as sodium

bisulfite, butylated hydroxyanisole, butylated hydroxytoluene, among others, are restricted by their respective toxicities that depend on its concentration in the product, besides the negative effect on the texture, aroma and taste of the processed product (Teixeira and Monteiro, 2006; Machado and Toledo, 2006). *S. platensis* is considered a GRAS microorganism (generally accepted as safe) permitted as a food additive by the Food and Drug Administration (FDA). Thus, the PC extracted from these microalgae can be applied into minimally processed apples, with benefit of the possibility of the PC to act as antioxidant against the enzymatic browning, and possible synergistic effect on the product functionality. To illustrate the treatment effect with PC extracted from *S. platensis* on the characteristics of minimally processed apples, Table 4 shows the results for browning index, firmness, weight loss, titratable acidity and pH determined over 8 days of experiment. Although, the browning index increased over time for both samples, the apple samples treated with PC presented lower values than the untreated samples. The browning inhibition was on average 40% as compared to the control samples. The browning index during the experiment was variable because the enzymes that cause browning are unevenly distributed without homogeneity across the apple, since the whole pulp of

the apple samples were used in the experiment (Oliveira et al., 2007). The weight loss increased with storage time, being one of the characteristics that interfere with the acceptability of the product by the consumer as it affects the turgidity of the tissues (Chitarra and Chitarra, 2005). However, it may not be directly related to the enzymatic browning.

Organic acids in foods influence the acceptance of a particular product, modifying its sensory properties. For this reason, these compounds were determined, since they indicate the effect of other compounds altered during aging. The acidity values also varied from 0.1 to 0.2% malic acid, similar to the study carried out by Drake et al. (1993) with fruits of the variety 'Golden Delicious' stored at different CO<sub>2</sub> levels (0.18 to 0.19% malic acid). The pH parameter has great importance on oxidative browning of plant tissues, since its decrease may result in reducing the rate of enzymatic browning of a fruit besides being an indicator of microbial activity (Carvalho et al., 2005). The pH values ranged from 4.3 to 4.2, and were higher than those values found by Fontes et al. (2008) for 'Gala' apples treated with a preservative solution (ascorbic acid, citric acid, calcium chloride, sodium-chloride) in the order of pH 3.5. With respect to firmness, the samples treated with PC presented values 76% higher values than the control samples. The firmness loss mainly occurs due to the action of proteolytic and pectinolytic enzymes on the cell wall components (Belloso et al., 2007).

In the present study, the PC may have also inhibited the action of these enzymes. The variability of the results during the experiment may be caused by the formation of ligno-suberized boundary layer in the cutting zone of the fruits (Empis and Moldão-Martins, 2000). It has been demonstrated that the PC from natural sources such as that extracted from *S. platensis* is very promising to replace the conventional conservatives, aimed to prevent browning catalyzed by the oxidative enzymes and to preserve sensory properties improving the consumer acceptance of the products.

## Conclusion

The PC extracted from *S. platensis* exhibited antioxidant activity on both the synthetic DPPH radical and oxidative enzymes such as POD and PPO of apples. When applied to minimally processed apples, the PC was effective in reducing oxidative browning, besides preserving other acceptance characteristics of the product. The natural antioxidants tested could be an interesting alternative to minimize or delay the oxidative deterioration, allowing the extension of the induction period, thus contributing to strengthening the supply of functional compounds.

## Conflict of interests

The authors did not declare any conflict of interest.

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