

**SYSTEMATIC STUDY ON THE EXTRACTION OF ANTIOXIDANTS FROM
*PINHÃO (ARAUCARIA ANGUSTIFOLIA (BERTOL.) KUNTZE) COAT***

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ABSTRACT

Food byproducts containing bioactive substances have attracted attention due to the possibility of adding values to residues of the food industry. In this work, the extraction of phenolic compounds from *pinhão* seed coats (*Araucaria angustifolia* (Bertol.) Kuntze) using a Central Composite Rotatable Design was applied to obtain prediction models for the extract volume yield, total phenolic content, total phenolic acids and total flavonoids. Principal Component Analysis and Hierarchical Cluster Analysis were implemented showing an evident poor effect of the temperature on phenolic compounds extraction, which is in accordance with the prediction model obtained by the experimental design for total phenolic acids. Volume yield presented a high positive correlation with extraction temperature, followed by solvent composition. Scanning Electron Microscopy showed that higher temperatures and lower ethanol percentages resulted in highly defibrillated *pinhão* coats that retained more extract after the extraction process, leading to lower volume yield percentages.

Keywords: *Araucaria angustifolia* (Bertol.) Kuntze; Central composite rotatable design; Principal component analysis; Phenolic compounds; Solid's microstructure.

1. Introduction

Araucaria angustifolia (Bertol.) Kuntze is a subtropical species known popularly as Araucaria and can be found in mountain climate throughout southern Brazil, northeastern Argentina and eastern Paraguay. The araucaria seed, named *pinhão* (or pine fruit), is a seasonal product and has great nutritional value (Branco et al., 2016).

Pinhão flour can be regarded as a new technological option in terms of raw material utilization and as a nutritional source for possible formulations of food products including gluten-free breads as well as a microencapsulating agent (Peralta et al., 2016). The residual coat (external tegument or shell) is rich in phenolic compounds (Branco et al., 2015; Cordenunsi et al., 2004; Daudt, Back, Cardozo, Marczak, & Kulkamp-Guerreiro, 2015; Sant'Anna, Sfoglia, Mercali, Corrêa, & Brandelli, 2016; Sprada et al., 2014) and is usually discharged after being removed from the seeds.

The antioxidant activity is the most reported biological action for *A. angustifolia* and *pinhão* coats are a potential residue to be used as a source of antioxidants. Considering their effectiveness in the dietary system and health promoting products, the demand of these natural compounds is increasing (Belwal, Bhatt, Rawal, & Pande, 2017). Although several studies have indicated *pinhão* antioxidant activity, such as in methanol extracts of cooked and raw *pinhão* seeds by Cordenunsi et al. (2004), and 70% ethanol in water extracts by Koehnlein et al. (2012); in water extracts from *pinhão* bracts, which are undeveloped seeds by Souza et al. (2014) and in methanol extract from *pinhão* coats by Sprada et al. (2014), a more complete study of the extraction conditions on the phenolic composition of *pinhão* coat extracts is still not reported in the literature.

The Ultra-Turrax assisted extraction, or homogenizer assisted extraction, has been applied in the extraction of antioxidant compounds from plant matrices such as banana peels (Pereira, Molina, Arruda, & Pastore, 2016) and red salvia (*Salvia splendens*)

(Bilgin, Sahin, Dramur, & Sevgili, 2013) and should be considered to extract the *pinhão* coat antioxidants. In this method, the high shear rate applied promotes the rupture of the plant material in a few seconds, and consequently, the release of compounds in the extraction solvent (Pereira et al., 2016). Several authors discuss the effect of solid matrix swelling as a function of the presence of water in the extraction solvent, which may lead to an increase in the extraction yield since the diffusion of the compounds in the solid presents reduced resistance (Aguilera, 2005; Cravotto et al., 2008), however the influence of shear, such as promoted by the Ultra-Turrax, has not yet been considered in this process.

In order to obtain efficient extraction of antioxidants, factors such as the extraction temperature, time and solvent composition may be considered and the use of experimental designs allows the user to identify optimal conditions for a selected response, while minimizing the number of required experiments (Belwal et al., 2017; Karami et al., 2015). The central composite rotational design (CCRD) can be implemented to analyze the relationship between the independent variables that are relevant to the process and the dependent variable or responses (Alfaro, Biluca, Marquetti, Tonial, & de Souza, 2014). Another useful mathematical tool that can uncover the relationships between observations and variables as well as between the variables themselves is the principal component analysis (PCA). It is a multivariate data analysis whose main objective is to represent a large set of data through limited multivariate data, called principal components (PCs). Thus, it is possible to reduce the dimensionality of a data set while preserving the maximum information. PCA may reveal groups of observations, trends, and outliers (Boeing et al., 2014).

Different concentrations of bioactive compounds from *Pinhão* coats residue are achieved depending on the extraction conditions. In the present work the extraction of

phenolic compounds from the *pinhão* coats by Ultra-Turrax Extraction was investigated by means of a central composite rotatable design (CCRD). The effects of the solid percentage (*pinhão* coats) in relation to solvent mixture (ethanol and water), the ethanol concentration in relation to water in the solvent and the extraction temperature on extract's volume yield, total phenolic content, total phenolic acids and total flavonoids were evaluated. A principal component analysis was also implemented to classify the obtained experimental data. Furthermore, the microstructure of the residual *pinhão* coats after the extraction procedure was evaluated by Scanning Electron Microscopy.

2. MATERIALS AND METHODS

2.1. Materials

The seeds of *Araucaria angustifolia* (*pinhão* seeds) were acquired from the local market in Campo Mourão, Paraná State, Brazil in June 2015. Acetonitrile (99.9%, Fisher Scientific, HPLC grade) was used in the chromatographic analyses. Formic acid was purchased from Panreac Química S.L.U. (Barcelona, Spain). Phenolic standards were purchased from Extrasynthèse (Genay, France). Water was purified in a Milli-Q water system (TGI Pure Water Systems, USA).

2.2. Preparation of *Pinhão* Seed Coats

Initially, *pinhão* seeds were cooked in boiling water (526 g.L⁻¹), for 2 hours. Then, the coats were separated from the seeds and dried during 24 hours at 40 °C in an oven with forced air convection (Cienlab). Finally, the dried coats were crushed in a knife mill (Solab Científica) and sieved (0.037-0.074 mm).

2.3. Extraction of Antioxidant Compounds from *Pinhão* Coats

The antioxidant compounds were extracted from *pinhão* coats according to the experimental conditions determined by a Central Composite Rotatable Design (CCRD) generated by the *software* Statistica 7.1 (StatSoft Incorporation, Tulsa, OK, EUA, 2006) with two replicates at the central point. The dependent variables (X_1 , X_2 e X_3) were as follows:

X_1 = solid percentage (*pinhão* coats) in relation to solvent mixture (ethanol and water) (S , % w.v⁻¹)

X_2 = ethanol concentration in relation to water in the solvent (E , %v.v⁻¹);

X_3 = extraction temperature (T , °C);

The analytical range was determined based on preliminary experiments and literature reports. Special attention was given to the factor X_1 , ensuring sufficient fluidity for the mixture during stirring of the extraction mixture. For the variable related to the ratio of solvents (X_2), the limits were based on the work of Wong et al. (2015). For temperature levels (X_3), the values were kept below the ethanol boiling point.

The responses evaluated from the proposed experimental design were:

Y_1 = Extract volume yield (VY, %v.v⁻¹);

Y_2 = Total phenolic content (TPC, mg.g⁻¹_{dry extract});

Y_3 = Total phenolic acids (TPA, mg.g⁻¹_{dry extract});

Y_4 = Total flavonoids (TF, mg.g⁻¹_{dry extract});

To proceed with the extraction, previously prepared *pinhão* coats were weighed in a borosilicate jacketed vessel (100 mL). The vessel was then connected to a thermostatic bath with water circulation (Quimis, Diadema - SP, Brazil) with temperature set according to the experimental run. Then were added 50 mL of the solvent mixture (V_s ,

ethanol and water), with composition related to the experimental point. The homogenization was promoted at 12,000 rpm with an Ultra-Turrax® (IKA-T25, Staufen, Germany) dispersing device, for 15 min. Finally, the extracts were vacuum filtered (Whatman® quantitative filter paper, Grade 44) and stored in a freezer at -18 °C.

2.4. Extract Characterization

2.4.1. Determination of Volume Yield of the Extracts

To determine the volume yield (extract retention in the solid matrix after the extraction procedure), the extracts were filtered on a Büchner funnel of 90 mm diameter with quantitative paper filter, under vacuum (-500 mmHg, Primatec, Itu, SP, Brazil) for 15 min. The volume of collected extract after filtration (V_E , mL) was determined using a graduated test tube, of 50 mL, previously calibrated and the volume yield was calculated based on the initial solvent volume added to the vessel for extraction (V_S , mL) by Equation (1).

$$VY (\%v.v^{-1}) = \frac{V_E}{V_S} \cdot 100 \quad \text{Equation (1)}$$

2.4.2. Phenolic Compound Analyses

First, the extracts were submitted to ethanol evaporation in an evaporator and then freeze-dried (Liotop, L101). The lyophilized extracts were re-dissolved in ethanol:water 80:20 (v.v⁻¹), and the phenolic profile was determined by high-performance liquid chromatography couple to a diode-array detection (280 and 370 nm as reference wavelengths) and electrospray ionization mass spectrometry HPLC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA). A Waters Spherisorb S3 ODS-2C18 (3 µm, 4.6 mm × 150 mm, Waters, Milford, MA, USA) column thermostatted at 35 °C was used to achieve chromatographic separation, using a

gradient elution using as solvents 0.1% formic acid in water (A) and acetonitrile (B). as previously described by Bessada, Barreira, Barros, Ferreira, & Oliveira (2016). The MS detection was performed in negative ion mode, using an LTQ XL linear ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source. Quantitative analysis was performed using calibration curves, obtained for each available phenolic standard constructed based on the UV signal. For the phenolic compounds identified in the absence of an available commercial standard, quantification was performed based on the calibration curve of the most similar available standard. Results were expressed as $\text{mg.g}_{\text{dryextract}}^{-1}$.

2.5. Determination of the Experimental Models

A second order polynomial expression was adjusted for each dependent variable (responses) as described in Equation (2), where b_0 is the intercept; b_1 , b_2 and b_3 are the regression coefficients for linear effects; b_{11} , b_{22} and b_{33} are the regression coefficients for the quadratic effects; b_{12} , b_{13} and b_{23} are the regression coefficients for the interaction effects; and x_1 , x_2 e x_3 are the codified values for independent variables.

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3$$

Equation (2)

The significance of the individual effects was assessed by ANOVA (95%) in order to adjust the experimental models for all evaluated responses. Then the overall fit of the model containing the significant coefficients was evaluated by the coefficient of determination (R^2), the adjusted coefficient of determination (R_{adj}^2) and ANOVA (95%

and partition of the residual sum of squares in pure error and lack of fit). Contour plots were obtained for the validated models for the responses evaluated with the third independent variables fixed at the central point.

2.6. Principal Component Analysis and Hierarchical Cluster Analysis

To explore the relation between the phenolic composition of the extracts obtained in each the experimental condition, Principal Component Analysis and Hierarchical Cluster Analysis (HCA) were performed using MATLAB R2008b (MathWorks Inc., Natick, MA, USA). For PCA and HCA, the results obtained for phenolic compounds, TPC, TPA, TF and VY were placed in columns and the experimental runs were used as the rows. Before analysis, each column was mean centered and divided by its variance, resulting in a scaled matrix. The first principal components with eigenvalues higher than 1.0 were used to evaluate the samples distribution in the new projection space.

2.7. Scanning Electron Microscopy (SEM)

The *pinhão* coats, before and after extraction process (run n° 11, 12,13, 14 and 15), were analyzed by Scanning Electron Microscopy (SEM, EVO MA 15, Carl Zeiss, Oberkochen, Germany) to evaluate the effect of the extraction conditions on the solid matrix and correlate it with the extract retention. For this purpose, the dried residual coats obtained after the extraction procedure were covered with gold in sputter (Quorum, Q150R ES, United Kingdom) previously to the analysis (200x magnification at 20 kV).

3. Results and Discussions

3.1. Phenolic Composition of *Pinhão* Coats Extracts

The phenolic profiles of the extracts of *pinhão* coats were obtained by HPLC-DAD-ESI/MS. The peak identification was based on the comparison with the data obtained for commercial standards, when available, or based on literature data previously published, and fragmentation patterns. The MSⁿ fragmentation provided key structural information and allowed the identification of thirteen compounds, among them, nine procyanidins (catechin and epicatechin derivatives), two phenolic acids (protocatechuic acid and ferulic acid hexoside), and two flavonoids (one flavonol - quercetin-3-*O*-glucoside and one flavanone-eriodictyol-*O*-hexoside). All the mentioned compounds were previously identified in *pinhão* coats and cooking water (De Freitas et al., 2018). The phenolic compounds identified and their respective quantification in the extracts, obtained for the experimental design are summarized in **Tables 1** and **2**, respectively. An exemplificative phenolic profile (run 12) of the compounds tentatively identified in *pinhão* is present in Supplementary material (**Figure S1**). Flavonoids accounted for 89-92% of the phenolic composition, being B-type (epi)catechin dimer and (+)-catechin the most abundant compounds. Meanwhile phenolic acids were present in 7-9% of the phenolic composition, being protocatechuic acid the most predominant molecule.

3.2. Experimental Design

Experimental conditions used in the Central Composite Rotatable Design (CCRD) as well as the experimental results for the four evaluated responses of *pinhão* coats extraction are presented in **Table 3**.

Good fits were achieved and most of the variability of responses was explained by the models as can be observed by R^2 and R^2_{adj} for all models (**Table S1, Supplementary material**). The lack of fit test was used to verify the adequacy of the fit and results showed that for TPA response the model could adequately fit the experimental data. On the other hand, lack of fit was significant for TPC, TF and VY models. However,

conclusions obtained from the TPA model analysis can be safely extended to TPC and TF because there was a high correlation of TPA with TPC ($r = 0.96$) and with TF ($r = 0.95$).

The contour plot obtained is presented in **Figure 1** (regression coefficients and the respective p-value (t-test) of the model for TPA of the extracts are given in **Table S2, Supplementary material**). The effects of solid percentage (X_1) and ethanol concentration in relation to water in the solvent (X_2) affected significantly ($p < 0.05$) TPA. On the other hand, temperature (X_3) was not significant to TPA results ($p > 0.05$), however, this effect, as well as the interaction between X_2 and X_3 were kept in models due to the better results obtained for models significance (R^2 , R^2_{adj} and lack of fit). In **Figure 1**, it can be noted that the experimental region studied in the present work resulted in a minimum point meaning that higher TPA results can be found in the region of higher ethanol percentages and higher solid content.

3.3. Principal Component Analysis and Hierarchical Cluster Analysis

The results presented in **Table 2**, together with the responses evaluated by the experimental design (TPA, TPC, TF and VY from **Table 3**) were submitted to a principal component analysis (PCA) and the results are presented in **Figure 2a**. In **Figure 2b**, the dendrogram of Hierarchical Cluster Analysis of *pinhão* coat extracts is presented.

PCA is a useful statistical technique which has found application in reduction of the original variables (13 phenolic compounds, TPA, TF, TPC and VY) in a smaller number of underlying variables (principal component - PC). The PC always describes the statistical relationship that accounts for the greatest amount of sample variation and the following PCs successively explain smaller parts of the original variance. This

means that correlated variables are explained by the same PC and less correlated variables by different PC (Wong & Chye, 2009).

With two PC, representing 83.84% of results total variability, it was possible to describe how the experimental conditions contributed to the phenolic compounds extraction from *pinhão* coats. It can be observed that experiment runs number 7, 8 and 12 presented the higher amounts of the compounds identified in the extracts. These experiments present in common the solid percentage in the range from 8.75 to 12.50 %w.v⁻¹ and the ethanol concentration in the range from 85 to 96.93 %v.v⁻¹. Also, it can be noted that the central point repetitions from the experimental design (runs 15 to 17) are grouped together in the same quadrant. A cluster composed by the runs 7, 8 and 12 can be observed, being catechin (C6) the major compound present in those samples, followed by a B-type (epi)catechin dimer (C4) and (-)-epicatechin (C9). Another cluster, composed by runs 3, 4 and 14 was identified, also with catechin as the major compound. The difference between both clusters is the concentration of those compounds, that are higher for the 7, 8 and 12 runs cluster.

Experiment runs located in the opposite direction from the vectors that indicate the phenolic compounds (C1 to C13, as well as TPC, TPA and TF) present poorer composition relative to such compounds. Another point to be noted is the relation between temperature extraction conditions (T vector) and the phenolic compounds vectors (C1 to C13, TPA, TF and TPC) that indicates a poor effect of this experimental condition on these results. This is in accordance with the prediction model obtained by the experimental design for TPA.

The volume yield vector (VY) presents a high positive correlation with temperature extraction conditions, followed by ethanol percentage in the solvent (E). The correlation with solids content is negative since VY and S vectors point to opposite sides. This

effect was then investigated on the morphological changes produced on *pinhão* coats during the extraction process by SEM.

HCA calculates the distances (or correlation) between all samples using a defined metric such as Euclidean and is the most common approach in which clusters are formed sequentially. The most similar objects are first grouped, and these initial groups are merged according to their similarities (Patras et al., 2011). It can be observed in **Figure 2b** that the most similar samples are clustered, such as the group of replicates (runs 15, 16 and 17) and the experiment runs that presented the richest TPA, TPC, TF, C6 and C4 composition (runs 7, 8 and 12), confirming the result obtained by the PCA. The group composed by runs 3, 4 and 14 present the second highest concentration of C4 and C6 phenolic compounds (B-type (epi)catechin dimer and (+)-catechin, respectively). The third group in order of concentration from C4 and C6 compounds is the one composed by runs 5, 6 and 10. HCA was able to demonstrate that different bioactive concentrations may be found by changing extraction conditions.

3.4. Residual Coats Morphology After the Extraction Process

In **Figure 3** the micrographs of *pinhão* coats solid matrix, before (**Figure 3A**) and after extraction (**Figure 3B–3E**) are presented. The samples obtained after extraction were collected from experimental points in which the solid content remained constant ($X_1 = 8.75 \text{ g.100 mL}^{-1}$), varying only in percentage of ethanol and temperature of extraction. Analyzing **Figures 3B, 3C and 3F** one may see a visible defibrillation of the solid matrix for decreasing ethanol percentage allowing higher extract retention at the end of the extraction process. This observation corroborates with the volume yield results obtained for the experimental design because the higher ethanol percentage did not allow the fibers defibrillate, decreasing the liquid retention between them at the end of the extraction process. The effect of temperature may be observed comparing the

images of **Figures 3D, 3E and 3F**. For these images, the percentage of ethanol was maintained fixed at 67.50%, however the temperature was raised from 21.5°C to 63.5°C, and with this temperature increase the defibrillation process started, leading to a higher extract retention during the filtration process. These results are in accordance with the correlations observed in the PCA analysis.

The defibrillation process of lignocellulosics materials is studied in utilization of these materials as reinforcement in polymer composites, for example (Ten & Vermerris, 2013). Bulota & Budtova (2015) utilized the Ultra-Turrax for defibrillation of linseed for utilization as reinforcement material for laminated poly (lactic acid). In the present work the homogenization with Ultra-Turrax was applied to the extraction process of antioxidants compounds from the *pinhão* coats, resulting in a defibrillated material, which presents application potential as reinforcement.

4. Conclusion

The prediction models obtained for the central composite rotatable design (CCRD) four responses of the extraction of phenolic compounds from *pinhão* seed coats (*Araucaria angustifolia* (Bertol.) Kuntze) presented significant lack of fit ($p < 0.05$) (extract's volume yield, total phenolic content and total flavonoids) with exception of Total phenolic acids model. Positive and significant effects ($p < 0.05$) were detected for *pinhão* coats solid percentage in the extraction medium and ethanol concentration in relation to water in the solvent. In order to evaluate the other responses, the principal component analysis was implemented and results showed an evident poor effect from temperature conditions on phenolic compounds extraction and also in the experimental design for total phenolic acids. However, temperature presented a high positive correlation with the extracted volume yield. The ethanol percentage in the solvent also presented a significant correlation ($p < 0.05$) with volume yield and both effects

(temperature and ethanol percentage) were visually confirmed with the morphological changes (scanning electron microscopy) produced on *pinhão* coats microstructure during the extraction process. In this sense, to obtain extracts rich in the identified phenolic compounds, a low temperature may be used, higher amounts of ethanol in the solvent, as well as higher solids content during the extraction process. Furthermore, catechin-rich extracts may be obtained with the proper selection of the extraction conditions.

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6. References

- Aguilera, J. M. (2005). Why food micro structure? *Journal of Food Engineering*, 67(1–2), 3–11. <https://doi.org/10.1016/j.jfoodeng.2004.05.050>
- Alfaro, A. T., Biluca, F. C., Marquetti, C., Tonial, I. B., & de Souza, N. E. (2014). African catfish (*Clarias gariepinus*) skin gelatin: Extraction optimization and

- physical-chemical properties. *Food Research International*, 65(PC), 416–422.
<https://doi.org/10.1016/j.foodres.2014.05.070>
- Belwal, T., Bhatt, I. D., Rawal, R. S., & Pande, V. (2017). Microwave-assisted extraction (MAE) conditions using polynomial design for improving antioxidant phytochemicals in *Berberis asiatica* Roxb. ex DC. leaves. *Industrial Crops and Products*, 95, 393–403. <https://doi.org/10.1016/j.indcrop.2016.10.049>
- Bessada, S. M. F., Barreira, J. C. M., Barros, L., Ferreira, I. C. F. R., & Oliveira, M. B. P. P. (2016). Phenolic profile and antioxidant activity of *Coleostephus myconis* (L.) Rchb.f.: An underexploited and highly disseminated species. *Industrial Crops and Products*, 89, 45–51. <https://doi.org/10.1016/j.indcrop.2016.04.065>
- Bilgin, M., Sahin, S., Dramur, M. U., & Sevgili, L. M. (2013). Obtaining Scarlet Sage (*Salvia Coccinea*) Extract Through Homogenizer- And Ultrasound-Assisted Extraction Methods. *Chemical Engineering Communications*, 200(9), 1197–1209. <https://doi.org/10.1080/00986445.2012.742434>
- Boeing, J. S., Barizão, É. O., e Silva, B. C., Montanher, P. F., de Cinque Almeida, V., & Visentainer, J. V. (2014). Evaluation of solvent effect on the extraction of phenolic compounds and antioxidant capacities from the berries: application of principal component analysis. *Chemistry Central Journal*, 8(1), 48. <https://doi.org/10.1186/s13065-014-0048-1>
- Branco, C. D. S., Lima, É. D. De, Rodrigues, T. S., Scheffel, T. B., Scola, G., Laurino, C. C. F. C., ... Salvador, M. (2015). Mitochondria and redox homoeostasis as chemotherapeutic targets of *Araucaria angustifolia* (Bert.) O. Kuntze in human larynx HEP-2 cancer cells. *Chemico-Biological Interactions*, 231(July 2016), 108–118. <https://doi.org/10.1016/j.cbi.2015.03.005>

- Branco, C. S., Rodrigues, T. S., Lima, É. D., Calloni, C., Scola, G., & Salvador, M. (2016). Chemical Constituents and Biological Activities of *Araucaria angustifolia* (Bertol.) O. Kuntze: A Review. *Organic and Inorganic Chemistry*, 2(1:8), 1–10. <https://doi.org/10.21767/2472-1123.100008>
- Bulota, M., & Budtova, T. (2015). Highly porous and light-weight flax/PLA composites. *Industrial Crops and Products*, 74, 132–138. <https://doi.org/10.1016/j.indcrop.2015.04.045>
- Cordenunsi, B. R., De Menezes, E. W., Genovese, M. I., Colli, C., Gonçalves De Souza, A., & Lajolo, F. M. (2004). Chemical composition and glycemic index of Brazilian pine (*Araucaria angustifolia*) seeds. *Journal of Agricultural and Food Chemistry*, 52(11), 3412–3416. <https://doi.org/10.1021/jf034814l>
- Cravotto, G., Boffa, L., Mantegna, S., Perego, P., Avogadro, M., & Cintas, P. (2008). Improved extraction of vegetable oils under high-intensity ultrasound and/or microwaves. *Ultrasonics Sonochemistry*, 15(5), 898–902. <https://doi.org/10.1016/j.ultsonch.2007.10.009>
- Daudt, R. M., Back, P. I., Cardozo, N. S. M., Marczak, L. D. F., & Kulkamp-Guerreiro, I. C. (2015). Pinhão starch and coat extract as new natural cosmetic ingredients: Topical formulation stability and sensory analysis. *Carbohydrate Polymers*, 134, 573–580. <https://doi.org/10.1016/j.carbpol.2015.08.038>
- De Freitas, T. B., Santos, C. H. K., da Silva, M. V., Shirai, M. A., Dias, M. I., Barros, L., ... Leimann, F. V. (2018). Antioxidants extraction from Pinhão (*Araucaria angustifolia* (Bertol.) Kuntze) coats and application to zein films. *Food Packaging and Shelf Life*, 15, 28–34. <https://doi.org/10.1016/j.fpsl.2017.10.006>
- Karami, Z., Emam-Djomeh, Z., Mirzaee, H. A., Khomeiri, M., Mahoonak, A. S., &

- Aydani, E. (2015). Optimization of microwave assisted extraction (MAE) and soxhlet extraction of phenolic compound from licorice root. *Journal of Food Science and Technology*, 52(6), 3242–53. <https://doi.org/10.1007/s13197-014-1384-9>
- Koehnlein, A., E., Elise Santos Carvajal, A., Marcela Koehnlein, E., Coelho-Moreira, J. D. S., Dorneles Inácio, F., Castoldi, R., ... Peralta, R. M. (2012). Antioxidant activities and phenolic compounds of raw and cooked Brazilian pinhão (*Araucaria angustifolia*) seeds. *African Journal of Food Science*, 6(21), 512–518. <https://doi.org/10.5897/AJFS12.128>
- Patras, A., Brunton, N. P., Downey, G., Rawson, A., Warriner, K., & Gernigon, G. (2011). Application of principal component and hierarchical cluster analysis to classify fruits and vegetables commonly consumed in Ireland based on in vitro antioxidant activity. *Journal of Food Composition and Analysis*, 24(2), 250–256. <https://doi.org/10.1016/j.jfca.2010.09.012>
- Peralta, R. M., Koehnlein, E. A., Oliveira, R. F., Correa, V. G., Corrêa, R. C. G., Bertonha, L., ... Ferreira, I. C. F. R. (2016). Biological activities and chemical constituents of *Araucaria angustifolia*: An effort to recover a species threatened by extinction. *Trends in Food Science and Technology*, 54, 85–93. <https://doi.org/10.1016/j.tifs.2016.05.013>
- Pereira, G. A., Molina, G., Arruda, H. S., & Pastore, G. M. (2016). Optimizing the Homogenizer-Assisted Extraction (HAE) of Total Phenolic Compounds from Banana Peel. *Journal of Food Process Engineering*. <https://doi.org/10.1111/jfpe.12438>
- Sant’Anna, V., Sfoggia, N. M., Mercali, G. D., Corrêa, A. P. F., & Brandelli, A. (2016).

- Effect of cooking on polyphenols and antioxidant activity of *Araucaria angustifolia* seed coat and evaluation of phytochemical and microbiological stability over storage. *International Journal of Food Science and Technology*, 51(8), 1932–1936.
<https://doi.org/10.1111/ijfs.13170>
- Souza, M., Branco, C. S., Sene, J., Dallagnol, R., Agostini, F., Moura, S., & Salvador, M. (2014). Antioxidant and antigenotoxic activities of the brazilian pine *Araucaria angustifolia* (Bert.) O. Kuntze. *Antioxidants*, 3(1), 24–37.
<https://doi.org/10.3390/antiox3010024>
- Sprada, G., Da Mota, T., Arantes, A. B., Sacchetti, G., Spagnoletti, A., Ziosi, P., ... Manfredini, S. (2014). Antioxidant Activity of Cosmetic Formulations Based on Novel Extracts from Seeds of Brazilian *Araucaria angustifolia* (Bertoll) Kuntze Characterization, HPTLC, ORAC, PCL. *Journal of Cosmetics Dermatological Sciences and Applications Journal of Cosmetics*, 4(4), 190–202.
<https://doi.org/10.4236/jcdsa.2014.43027>
- Ten, E., & Vermerris, W. (2013). Functionalized polymers from lignocellulosic biomass: State of the art. *Polymers*, 5(2), 600–642.
<https://doi.org/10.3390/polym5020600>
- Wong, J. Y., & Chye, F. Y. (2009). Antioxidant properties of selected tropical wild edible mushrooms. *Journal of Food Composition and Analysis*, 22, 269–277.
<https://doi.org/10.1016/j.jfca.2008.11.021>
- Wong, W. H., Lee, W. X., Ramanan, R. N., Tee, L. H., Kong, K. W., Galanakis, C. M., ... Prasad, K. N. (2015). Two level half factorial design for the extraction of phenolics, flavonoids and antioxidants recovery from palm kernel by-product. *Industrial Crops and Products*, 63, 238–248.

<https://doi.org/10.1016/j.indcrop.2014.09.049>

7. Conflicts of interest

Authors declare no conflicts of interest.

TABLES

Table 1. Retention time (t_R), wavelengths of maximum absorption in the visible region (λ_{\max}), mass spectral data and tentative identification of the phenolic compounds present in *pinhão* coats extracts.

Peak	Rt (min)	λ_{\max} (nm)	[M-H] ⁻ (m/z)	MS2 (m/z)	Tentative identification
1	5.01	279	593	575(28), 549(3), 467(36), 441(57), 425(100), 423(31), 407(11), 305(18), 289(8)	Prodelphinidin B3
2	5.47	280	577	451(23), 425(100), 407(22), 289(12), 287(10)	B-type (epi)catechin dimer
3	5.74	260/sh292	153	109(100)	Protocatechuic acid
4	6.31	280	577	451(16), 425(100), 407(19), 289(8), 287(7)	B-type (epi)catechin dimer
5	6.67	314	355	193(100)	Ferulic acid hexoside
6	7.21	280	289	245(100), 203(50), 187(10), 161(9), 137(3)	(+)-Catechin
7	7.78	280	577	451(24), 425(100), 407(22), 289(11), 287(10)	B-type (epi)catechin dimer
8	7.9	280	577	451(18), 425(100), 407(22), 289(9), 287(7)	B-type (epi)catechin dimer
9	9.89	280	289	245(100), 203(35), 187(6), 161(8), 137(3)	(-)-Epicatechin
10	11.35	279	865	739(78), 713(47), 695(100), 577(62), 575(42), 425(12), 407(9), 289(6), 287(11)	B-type (epi)catechin trimer
11	12.02	281	865	739(78), 713(56), 695(100), 577(46), 575(82), 425(11), 407(9), 289(6), 287(9)	B-type (epi)catechin trimer
12	16.49	283/sh324	449	287(100)	Eriodictyol- <i>O</i> -hexoside
13	19.31	350	463	301(100)	Quercetin-3- <i>O</i> -glucoside

Table 2. Quantification of phenolic compounds (mg.g⁻¹) in *pinhão* coats extracts (mean ± SD).

Code for peaks	Compounds	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Run 9	Run 10	Run 11
C1	Prodelphinidin B3 ¹	1.60±0.05	1.36±0.01	2.23±0.02	2.28±0.02	1.62±0.07	2.40±0.02	3.87±0.09	3.20±0.05	1.90±0.06	2.08±0.08	2.30±0.01
C2	B-type (epi)catechin dimer ¹	1.86±0.03	2.20±0.01	3.0±0.1	3.33±0.03	2.07±0.04	2.43±0.02	6.8±0.1	5.22±0.08	2.6±0.1	3.10±0.09	2.90±0.02
C3	Protocatechuic acid ²	1.46±0.01	1.52±0.01	2.19±0.02	2.24±0.09	1.39±0.01	1.47±0.04	4.3±0.2	3.28±0.05	1.75±0.01	1.88±0.07	1.62±0.01
C4	B-type (epi)catechin dimer ¹	2.72±0.09	3.10±0.01	5.28±0.07	5.8±0.2	3.11±0.01	4.07±0.01	13.3±0.3	9.87±0.06	4.7±0.1	4.99±0.04	4.27±0.08
C5	Ferulic acid hexoside ³	0.48±0.01	0.45±0.01	0.72±0.03	0.72±0.01	0.51±0.01	0.63±0.01	1.52±0.04	0.99±0.07	0.61±0.01	0.60±0.01	0.53±0.01
C6	(+)-Catechin ¹	2.78±0.03	2.16±0.02	5.8±0.2	6.07±0.02	2.6±0.1	3.08±0.04	13.1±0.4	9.9±0.2	3.40±0.02	4.00±0.07	2.55±0.09
C7	B-type (epi)catechin dimer ¹	1.81±0.05	1.40±0.04	2.49±0.05	3.47±0.01	1.84±0.05	2.24±0.01	6.25±0.05	4.99±0.01	2.49±0.08	2.56±0.05	2.08±0.08
C8	B-type (epi)catechin dimer ¹	1.91±0.06	1.90±0.07	3.35±0.03	4.46±0.03	2.9±0.2	2.35±0.02	7.19±0.01	6.85±0.05	3.1±0.1	2.81±0.04	2.06±0.03
C9	(-)-Epicatechin ¹	2.03±0.06	1.62±0.04	3.54±0.03	4.70±0.03	2.02±0.02	2.08±0.08	8.1±0.2	6.37±0.03	1.91±0.01	2.28±0.01	1.12±0.07
C10	B-type (epi)catechin trimer ¹	1.3±0.2	1.08±0.02	1.8±0.1	2.01±0.04	1.51±0.03	1.38±0.09	2.4±0.1	2.03±0.09	0.9±0.1	1.64±0.01	0.85±0.02
C11	B-type (epi)catechin trimer ¹	1.62±0.03	1.29±0.03	2.16±0.08	2.36±0.01	1.94±0.05	1.37±0.03	3.25±0.04	2.60±0.09	1.21±0.01	1.85±0.02	1.04±0.03
C12	Eriodictyol-O-hexoside ⁴	0.0039±0.0004	tr	0.16±0.05	0.12±0.01	tr	tr	0.68±0.05	0.29±0.01	tr	0.15±0.01	tr
C13	Quercetin-3-O-glucoside ⁵	0.919±0.003	0.95±0.01	0.97±0.01	0.97±0.01	1.00±0.01	0.93±0.01	1.03±0.01	0.98±0.01	0.95±0.01	0.95±0.01	0.96±0.01
Code for peaks	Compounds	Run 12	Run 13	Run 14	Run 15	Run 16	Run 17					
C1	Prodelphinidin B3 ¹	3.11±0.08	1.96±0.01	2.56±0.06	1.93±0.02	2.11±0.03	1.87±0.03					
C2	B-type (epi)catechin dimer ¹	4.04±0.07	2.52±0.07	4.03±0.03	2.69±0.05	2.75±0.03	2.87±0.03					
C3	Protocatechuic acid ²	5.06±0.05	1.73±0.05	2.1±0.1	1.72±0.04	1.71±0.01	1.58±0.02					
C4	B-type (epi)catechin dimer ¹	7.6±0.1	4.64±0.05	6.55±0.08	4.48±0.03	5.17±0.04	4.68±0.01					
C5	Ferulic acid hexoside ³	1.33±0.01	0.61±0.01	0.71±0.02	0.61±0.01	0.67±0.02	0.56±0.02					
C6	(+)-Catechin ¹	11.6±0.3	2.91±0.05	5.33±0.08	3.52±0.08	3.64±0.06	2.91±0.05					
C7	B-type (epi)catechin dimer ¹	6.7±0.2	1.90±0.08	3.6±0.1	2.13±0.05	2.52±0.02	2.19±0.05					
C8	B-type (epi)catechin dimer ¹	5.6±0.1	2.38±0.08	3.53±0.07	2.70±0.02	2.81±0.05	2.53±0.04					
C9	(-)-Epicatechin ¹	7.48±0.01	1.89±0.06	2.71±0.02	2.10±0.01	1.89±0.08	1.93±0.07					
C10	B-type (epi)catechin trimer ¹	2.35±0.04	1.08±0.03	1.2±0.1	1.13±0.02	1.00±0.04	1.29±0.08					
C11	B-type (epi)catechin trimer ¹	3.73±0.07	1.31±0.06	1.74±0.06	0.93±0.02	1.06±0.02	1.25±0.04					
C12	Eriodictyol-O-hexoside ⁴	1.01±0.01	tr	0.30±0.08	tr	0.134±0.001	tr					
C13	Quercetin-3-O-glucoside ⁵	1.00±0.01	0.89±0.01	0.95±0.01	0.91±0.01	0.95±0.01	0.91±0.01					

tr- traces. Standard calibration curves: 1- catechin ($y = 84950x - 23200$; $R^2 = 1$); 2- protocatechuic acid ($y = 214168x + 27102$; $R^2 = 0.9999$); 3- ferulic acid ($y = 633126x - 185462$; $R^2 = 0.999$); 4- naringenin ($y = 18433x + 78903$; $R^2 = 0.9998$); 5- quercetin-3-O-glucoside ($y = 34843x - 160173$; $R^2 = 0.9998$).

Table 3. Coded levels (and real values in parentheses) for the experimental design (S- solid percentage in relation to extraction solvent; E- ethanol concentration on the extraction solvent; T- extraction temperature) and the obtained responses: Y₁ – extract volume yield (VY %), Y₂ – total phenolic content (TPC mg.g⁻¹ dry extract), Y₃ – total phenolic acids (TPA mg.g⁻¹ dry extract) and Y₄ – total flavonoids (TF mg.g⁻¹ dry extract).

Run n°	Coded Levels (Real Values)			Responses			
	X ₁ S (%w.v ⁻¹)	X ₂ E (%v.v ⁻¹)	X ₃ T (°C)	Y ₁ VY (%v.v ⁻¹)	Y ₂ TPC (mg.g ⁻¹)	Y ₃ TPA (mg.g ⁻¹)	Y ₄ TF (mg.g ⁻¹)
1	-1 (5.00)	-1 (50.00)	-1 (30.00)	50.00	20.5±0.2	1.94±0.02	18.6±0.2
2	-1 (5.00)	-1 (50.00)	1 (55.00)	58.00	19.0±0.2	1.97±0.02	17.1±0.2
3	-1 (5.00)	1 (85.00)	-1 (30.00)	74.00	33.7±0.7	2.91±0.05	30.8±0.7
4	-1 (5.00)	1 (85.00)	1 (55.00)	78.00	38.5±0.4	2.97±0.08	35.6±0.3
5	1 (12.50)	-1 (50.00)	-1 (30.00)	6.00	22.47±0.08	1.90±0.02	20.6±0.1
6	1 (12.50)	-1 (50.00)	1 (55.00)	38.00	24.4±0.1	2.10±0.05	22.34±0.09
7	1 (12.50)	1 (85.00)	-1 (30.00)	54.00	71.8±0.3	5.9±0.1	66.0±0.2
8	1 (12.50)	1 (85.00)	1 (55.00)	44.00	56.6±0.3	4.3±0.1	52.3±0.2
9	-1.68 (2.44)	0 (67.50)	0 (42.50)	80.00	25.5±0.3	2.37±0.01	23.1±0.3
10	1.68 (15.06)	0 (67.50)	0 (42.50)	38.00	28.9±0.4	2.48±0.08	26.4±0.4
11	0 (8.75)	-1.68 (38.07)	0 (42.50)	44.00	22.3±0.1	2.16±0.01	20.1±0.1
12	0 (8.75)	1.68 (96.93)	0 (42.50)	62.00	60.7±0.8	6.38±0.05	54.3±0.8
13	0 (8.75)	0 (67.50)	-1.68 (21.48)	62.00	23.8±0.4	2.34±0.06	21.5±0.4
14	0 (8.75)	0 (67.50)	1.68 (63.52)	68.00	35.2±0.3	2.8±0.1	32.4±0.1
15	0 (8.75)	0 (67.50)	0 (42.50)	68.00	26.5±0.2	2.38±0.02	24.1±0.2
16	0 (8.75)	0 (67.50)	0 (42.50)	72.00	25.33±0.03	2.33 ±0.04	23.07 ±0.07
17	0 (8.75)	0 (67.50)	0 (42.50)	72.00	24.2±0.1	2.14 ±0.05	22.06 ±0.05

Total Phenolic acids quantification took into consideration peaks: C3 and C5; Total flavonoids quantification took into consideration peaks: C1, C2, C4, C6, C7, C8, C9, C10, C11, C12, C13, C14, C15, C16, and C17.

FIGURE CAPTIONS

Figure 1. Contour plot for the effects of ethanol concentration on the extraction solvent (E, %v.v⁻¹) and of the solid percentage in relation to extraction solvent (S, %m.v⁻¹) at a constant temperature (42.5 °C) on total phenolic acids (mg.g⁻¹) of *pinhão* coat extracts.

Figure 2. (a) Score plot obtained from Principal Component Analysis of phenolic composition, TPC, TPA, TF and VY of the *pinhão* coat extracts; (b) dendrogram of hierarchical cluster analysis of *pinhão* coat extracts.

Figure 3. SEM images of *pinhão* coats before extraction (a) and after the extraction process referring to the experimental points: 11 where $X_2 = 38.07\%$ and $X_3 = 42.50^\circ\text{C}$ (b); 12 where $X_2 = 96.93\%$ and $X_3 = 42.50^\circ\text{C}$ (c); 13 where $X_2 = 67.50\%$ and $X_3 = 21.48^\circ\text{C}$ (d); 14 where $X_2 = 67.50\%$ and $X_3 = 63.52^\circ\text{C}$ (e); 15 where $X_2 = 67.50\%$ and $X_3 = 42.50^\circ\text{C}$ (f). 200x magnification.