Research Article

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Exploring the relationships between extraction conditions and anti-browning functionality of *Codium* sp. aqueous extract

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Abstract

BACKGROUND: *Codium tomentosum*, an edible green seaweed, shows significant potential as a natural anti-browning agent for fresh-cut apples, which often suffer from oxidation-induced browning that limits shelf-life. This study aimed to explore the effects of extraction conditions for *C. tomentosum* to maximize its anti-browning functionality, focusing on key extraction parameters and exploring the underlying inhibitory mechanisms.

RESULTS: Using Box–Behnken design, *C. tomentosum* was extracted under varying conditions of time (0–180 min), temperature (20–90 °C), and pH (3–10), followed by response surface methodology to analyze the effects. Higher extraction yields were achieved with extended extraction times (over 120 min) and pH levels between 6 and 10. Anti-browning effectiveness was primarily influenced by temperature, with optimal inhibition observed at temperatures above 60 °C. ^{1}H-n(i, ii)uclear magnetic resonance analysis further identified galactans as key components in the extracts, likely contributing to the anti-browning effect through their barrier-forming properties and potential interactions with browning-related enzymes.

CONCLUSION: The *C. tomentosum* extracts, even when subjected to intensified extraction conditions, demonstrated effective browning inhibition in fresh-cut apples, providing a promising, natural approach for shelf-life extension. This work highlights the potential of marine-based resources in food preservation and offers insights into optimizing extraction processes for food industry applications.
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Supporting information may be found in the online version of this article.

Keywords: seaweed extracts; *Codium tomentosum*; galactans; enzymatic browning; fresh-cut apples

INTRODUCTION

Marine macroalgae or seaweeds are known for the production of relevant bioactive compounds with antimicrobial and antioxidant properties, mostly produced as a defense response to the extreme conditions to which they are exposed in tidal zones.[1] *Codium tomentosum* is a green and edible seaweed that can be found in tidal zones of the Northeast Atlantic Ocean, from the British Isles to Azores and Cape Verde. Despite its wide distribution and biomass availability, published studies with *C. tomentosum* extracts or secondary metabolites are mainly limited to its potential for medical and biotechnological applications.[1,2] Recent studies have demonstrated the potential of *C. tomentosum* extracts in food science applications, particularly in the formulation of edible coatings and films.[3,4] Augusto *et al.*[4] found that fresh-cut 'Fuji' apples immersed in a *C. tomentosum* extract exhibited lower browning levels and reduced activities of polyphenol oxidase and peroxidase, likely due to the downregulation of

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browning-related genes. Additionally, *Codium* sp. extracts have shown potential antifungal activity. For example, Toledo *et al.*[5] reported a 36.60% inhibition of *Fusarium oxysporum* in an *in vivo* study with pear fruit. Moreover, Martins *et al.*[6] explored the conversion of *Codium* sp. extracts into value-added carbon materials, such as biochars, which can improve soil health and water retention. All three studies share a common approach: the extraction of *C. tomentosum* was performed using conventional solid–liquid extraction (SLE), a method known for its simplicity in design, operation, and scalability.[7]

SLE is a widely used method for isolating bioactive compounds from seaweeds.[8] The efficiency of this extraction process is influenced by several factors, including the solvent's characteristics, extraction temperature, and pH.[7,9] In studies by Toledo *et al.*[5] and Augusto *et al.*,[3] *Codium* sp. extractions were conducted using ethanol, water, or a 75/25 (v/v) water–ethanol mixture, with a seaweed-to-solvent ratio of 6.6% (w/v) and an extraction time of 4–6 h at room temperature.

Considering food safety and cost, hydroethanolic extractions are widely accepted for industrial applications in the extraction of natural products.[7] According to European Directive 2009/32/EC, ethanol is permitted as an extraction solvent, provided that its presence in the final product is minimal and poses no risk to human health (Commission Directive 2010/59/EU of 26 August 2010). Thus, despite ethanol's approved use, there is growing interest in exploring its total replacement with a 'cleaner solvent', such as water, especially when considering environmental concerns and energy consumption. The optimization of extraction parameters is critical to the production of extracts with maximum functionality, such as enhanced anti-browning and antifungal activities. Traditionally, optimization has been approached using a one-factor-at-a-time method, where only one variable is altered while keeping others constant. However, this approach is time-consuming and costly.[7,10,11]

Design of Experiments (DoE) offers a strategic approach to minimize the number of trials needed without compromising the quality of results, by simultaneously varying all factors under study.[11] For independent factors typically involved in extraction processes, statistical tools like response surface methodology (RSM) are valuable for assessing both individual effects and interactions among variables. The Box–Behnken design (BBD) is particularly effective for defining experimental conditions within a specified range.[12] BBD has been successfully applied in optimizing the extraction of phenolic compounds such as phlorotannins from *Fucus vesiculosus*,[8] as well as antioxidant compounds from *Grateloupia turuturu*[7] and *Ascophyllum nodosum*.[7,8,10] These studies have explored the impact of factors such as solvent-to-solid ratio, solvent concentration, extraction time, temperature, and pH on the desired outcomes. The use of BBD is useful in avoiding experiments performed under extreme conditions, for which unsatisfactory results may occur such as degradation of the compounds.[12] In this study, a second-order kinetic model was employed to complement the BBD approach by capturing the extraction kinetics of *C. tomentosum*. This model provides critical insights into the rates of solute diffusion and equilibrium states during extraction. Moreover, it enables the prediction of how variations in extraction parameters – time, temperature, and pH – affect key response variables, such as extraction yield and anti-browning functionality. By dynamically evaluating these variables, the model not only facilitates the identification of optimal extraction conditions but also is a valuable tool for understanding the underlying mechanisms.[12] Furthermore, by reducing experimental trials and minimizing resource usage, the model enhances the sustainability and scalability of the extraction process, which is vital for potential industrial applications.

This study builds on earlier work from Augusto *et al.*[3] and aims to develop an optimized aqueous extract of *C. tomentosum* with enhanced functionality for reducing browning in fresh-cut apples. Additionally, this research explores the effects of extraction parameters such as time, temperature, and pH on the efficiency of *C. tomentosum* extraction, which has not been extensively studied before. A key objective is to identify the primary compounds present in the extract using Fourier transform infrared spectroscopy–attenuated total reflection (FTIR-ATR) and ^{1}H-nuclear magnetic resonance (^{1}H-NMR) analysis. The findings from this study are expected to provide valuable insights for optimizing a marine-based anti-browning coating agent, with the potential to extend the shelf-life of fresh-cut fruits.

MATERIAL AND METHODS

Raw materials and reagents

Dried *C. tomentosum* (particle size with an average of 1.5 mm) was obtained from a Portuguese seaweed supplier (ALGAplus, Ílhavo). 'Fuji' apples were obtained from a local supplier in Torres Vedras, Portugal (Campotec SA) and stored at 4 °C before use. All reagents used for analytical procedures were analytical grade.

Extraction procedure

Following earlier works on the production of *C. tomentosum* extracts,[3] the process parameters, as well as the seaweed biomass-to-solvent ratio, were established in this work. Six sequential steps were employed to obtain the extract in a powder form: (i, ii) SLE with a seaweed biomass-to-water ratio of 6.6% (w/v), (iii) followed by centrifugation (2000 × *g*, 10 min, 4 °C) (5810R centrifuge, Eppendorf, Hamburg, Germany), (iv) filtration through a Büchner funnel (paper filter 20–25 µm), (v) freeze drying the filtrate at −80 °C (Telstar LyoQuest-85, Azbil Telstar Technologies SLU, Barcelona, Spain), and (vi) obtaining a dried extract. The extraction conditions investigated in this study, which are detailed in Table 1, include variation of time (0–180 min), temperature (20–80 °C), and pH (3–10). The solid-to-solvent ratio was kept constant due to the swelling properties of the dried seaweed; using a fixed ratio, it was possible to achieve adequate solvent penetration in the dried seaweed, allowing equilibrium to be reached between both interfaces. The extractions were performed in a glass vessel, 9 cm in height and 7.5 cm in diameter. To promote mixing, a propeller (3 cm diameter) was used at 1500 rpm to avoid external mass transfer effects. The extraction time excluded an initial 5-min period for the temperature and pH adjustments (with 1 mol L^{-1} HCl or 1 mol L^{-1} NaOH) to settle and become uniform throughout the mixture. Each extraction condition was studied in triplicate ($n = 3$), resulting in a total of 45 extracts. A flowchart indicating the extraction conditions and analyses performed on the extracts is given in Fig. 1.

Second-order model

The extraction kinetics was modeled as a second order-process.[13,14] The progress of extraction was followed by measuring absorbance at 260 nm (which gave maximum absorbance based on a wavelength scan between 200 and 800 nm) for 360 min. Aliquots of 200 µL were collected at 15-min intervals during the first 60 min, followed by collection at 30-min intervals until the end of the experiment. The absorbance was measured in a

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Table 1. Box–Behnken experimental design matrix, symbols, coded variables, and values for the considered independent variables (X_1: time in min; X_2: temperature in °C; X_3: pH)

Coded variables and variables values						
Run	X_1	X_2	X_3	Time	Temperature	pH
1	−1	−1	0	0	20	6.5
2	1	−1	0	180	20	6.5
3	−1	1	0	0	80	6.5
4	1	1	0	180	80	6.5
5	−1	0	−1	0	50	3.0
6	1	0	−1	180	50	3.0
7	−1	0	1	0	50	10.0
8	1	0	1	180	50	10.0
9	0	−1	−1	90	20	3.0
10	0	1	−1	90	80	3.0
11	0	−1	1	90	20	10.0
12	0	1	1	90	80	10.0
13[a]	0	0	0	90	50	6.5
14[a]	0	0	0	90	50	6.5
15[a]	0	0	0	90	50	6.5

[a] Central point.

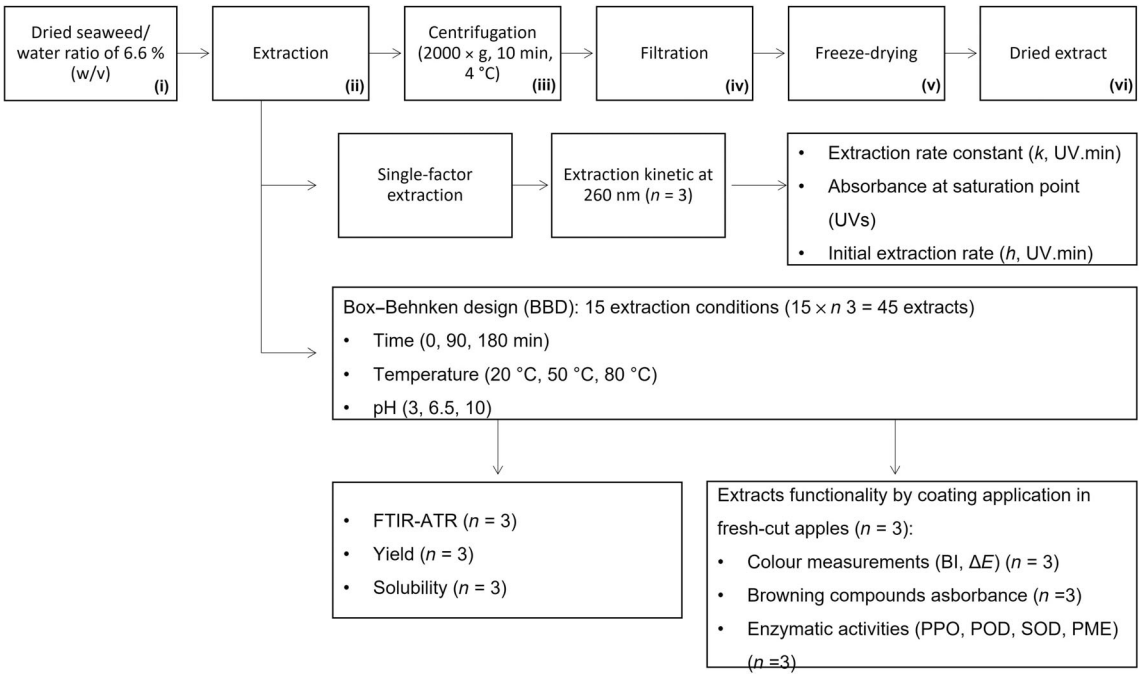


Figure 1. Flowchart of the experimental design. PPO, polyphenol oxidase; POD, peroxidase; SOD, superoxide dismutase; PME, pectin methylesterase.

96-well ultraviolet plate reader (Spectrophotometer Evolution 201, Thermo Scientific, Waltham, MA, USA).[15] The amount of liquid extract used for the analyses was kept to a minimum to avoid significant changes in the extraction volume, and filtered through a filter paper (20–25 µm) prior the absorbance measurements.

The linearized model presented by Nie *et al.*[14] (Eqn 1) was applied to determine the second-order extraction rate constant (k, UV.min), by measuring the extract absorbance at saturation (UV_s) and the initial extraction rate (h, UV.min) (Eqn 2).

$$\frac{t}{UV_t} = \frac{1}{k.UV_s^2} + \frac{t}{UV_s} \quad (1)$$

where UV_t is the extract absorbance (UV) at any time t (min). The plot of time (min) against t/UV gives a gradient of 1/UV and an intercept of $1/k.UV_s^2$, allowing the determination of k and absorbance at the saturation point. When t approaches 0, h can be defined as

$$h = k.UV_s^2 \quad (2)$$

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Extraction yield and solubility

Extraction yield was determined based on the weights of the dried seaweed taken initially and the weight of the dried extract obtained (expressed as grams of dried extract per 100 g dried seaweed). The extract solubility was determined as follows:[16,17] 50 mg dried extract (m) was dissolved in 10 mL deionized water and centrifuged at 10 000 × g for 10 min; the pellet collected was dried at 108 °C (m_1) and the solubility was calculated as follows:

$$\text{Solubility}\left(\text{g}\,100\,\text{g}^{-1}\text{ extract}\right) = 100 - [(m_1 \times 100)/m] \quad (3)$$

Fourier transform infrared spectroscopy–attenuated total reflection

The FTIR-ATR technique was used to evaluate the functional groups of the dried extracts and to detect possible differences between extraction conditions. The FTIR analysis was carried out using an Alpha-P FTIR-ATR spectrometer (Bruker Optik GmbH, Ettlingen, Germany) as described in Augusto *et al.*[4] Each sample was analyzed three times.

Extract functionality as anti-browning agent for fresh-cut apples

Guided by previous works, where a coating containing 0.5% (w/v) of *C. tomentosum* extract was used to extend the shelf-life of fresh-cut apples (n = 3), the functionality of the extracts produced was evaluated.

Fresh-cut organic 'Fuji' apples (*Malus domestica*) were cut into circular disks, 1.5 cm diameter, and on average weighing 2 g. Coating solutions were prepared using the extracts produced in the section 'Extraction procedure', above, with a final concentration of 0.5% (w/v). A total of 45 coating solutions were prepared (one solution from each extract replica). Maintaining a ratio of 6.6% (w/v) of apple biomass to the coating solution, six apple disks were dipped for 5 min in the coating solutions (n = 3). In the case of control samples, deionized water without any extract was used. To promote browning development, the coated samples were kept at room temperature and exposed constantly to light (laboratory light intensity) for 3 h. The functionality of the coating solutions was determined by measuring color and recording the absorbance at 440 nm to detect browning compounds and enzymatic activities. For each of these analyses, a total of three apple disks per coating solution were analyzed (n = 3).

Color measurements were made using a colorimeter (CR 400, Konica Minolta, Tokyo, Japan) and analyzed according to the CIE-Lab system. To avoid irregularities in readings, each sample was analyzed in five distinct areas, and the mean value was considered for color calculations (n = 3). Color variation (ΔE^*) was estimated between the control and an individual sample after 3 h incubation using the equation described by Lante *et al.*[18] Superficial browning index was determined as described in the work of Augusto *et al.*[3]

The absorbance of browning compounds (BC) was determined at 440 nm, adapting the procedure of Rasouli and Koushesh Saba[19] for smaller samples. Browning compounds were extracted from 1 g of apple slice and mixed with 7 mL deionized water. After 1 h incubation at room temperature, the mixture was centrifuged at 1000 × g for 5 min. From the supernatant, 2.5 mL was collected and mixed with 3.75 mL of 96% ethanol. The mixture was submitted to a second centrifugation and the supernatant was read at 440 nm in a microplate reader (Epoch 2, BioTek, Winooski, VT, USA). Three apple slices per treatment were evaluated (n = 3).

For polyphenol oxidase (PPO, EC 1.10.3.1) and peroxidase (POD, EC 1.11.1.7) determination, the Augusto *et al.*[3] protocol was adapted for smaller samples. Thus, 2 g of sample was homogenized with a twofold amount of extraction buffer containing 50 g L^{-1} polyvinylpyrrolidone (PVP) in 50 mmol L^{-1} sodium phosphate buffer (pH 7.0), following the remaining steps as described by the authors. Enzymatic reactions were performed in a multiwell plate, leading to adjustments in reaction for a total volume of 300 µL. Enzyme activities were expressed as U mg^{-1} protein. Protein was quantified by following Bradford's methodology.[20] A total of three samples per treatment were used for enzyme determinations (n = 3).

For superoxide dismutase (SOD, EC 1.15.1.1) determination, the protocol described by Augusto *et al.*[3] was followed. A 2 g sample was homogenized in 10 mL extraction buffer (0.1 mol L^{-1} sodium phosphate buffer (pH 7.5) containing 20 g L^{-1} PVP, 2 mmol L^{-1} dithiothreitol (DTT), and 0.1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA)). Specific activity, expressed as U g^{-1} fresh weight (FW), was measured by absorbance at 560 nm, by adding 150 µL of substrate mixture (50 mmol L^{-1} sodium phosphate buffer (pH 7.8), 13 mmol L^{-1} methionine, 75 µmol L^{-1} nitroblue tetrazolium (NBT), 10 µmol L^{-1} EDTA, and 2 µmol L^{-1} riboflavin) to 50 µL enzyme extract. After 10 min incubation under fluorescent light, absorbance was measured, and enzyme activity was determined by the 50% inhibition of NBT photoreduction.

For pectin methylesterase (PME, E.C. 3.1.1.11) activity determination, the extraction conditions were similar to those used for SOD but with a buffer containing 1.5 mol L^{-1} NaCl and 2.5% (w/v) PVP. PME activity was monitored spectrophotometrically at 35 °C (610 nm, 4 min) following the protocol described by Augusto *et al.*[3] The reaction mixture included 50 µL enzyme extract (pH 7.5), 15 µL 0.01% bromothymol blue in 0.003 mol L^{-1} sodium phosphate buffer (pH 7.5), and 235 µL substrate (5 g L^{-1} citrus pectin, pH 7.5). Results were expressed as U mg^{-1} protein.

Chemical screening by ^{1}H-NMR

A preliminary chemical screening of the aqueous extract from *C. tomentosum* was attained by ^{1}H-NMR spectroscopy on a Bruker AMX400 spectrometer (400.13 MHz; Bruker, Billerica, MA, USA). Samples (~30 mg) were dissolved in 0.5 mL deuterated water (Sigma-Aldrich, Steinheim, Germany) and the ^{1}H-NMR spectra were acquired with a water suppression pulse program (*zgcppr*, O1 4.79 ppm), at 25 °C. Chemical shifts (δ) are expressed in ppm and referenced to the residual water solvent signal (δ_H = 4.79).

Experimental design and statistical analysis

A three-level-three-factor BBD was applied to determine the best combination of extraction variables to produce *C. tomentosum* extract with efficacy to inhibit browning in fresh-cut apples. Three variables were considered: X_1, time (min); X_2, temperature (°C); and X_3, pH; each variable was coded at levels −1, 0, and 1 (Table 1). These levels were selected based on a series of preliminary experiments and previous literature that identified the most effective ranges for each factor. The temperature range was chosen based on results showing that, at temperatures above 80 °C, the extraction yield decreased by approximately 30% compared to room temperature. This decline was attributed to potential thermal degradation of key bioactive compounds. Thus, the upper limit for temperature was set at 80 °C to balance extraction efficiency and compound stability. The maximum extraction time was

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determined from assays that measured the extract's efficacy in preventing apple browning. It was observed that extending the extraction period beyond 180 min did not significantly enhance the anti-browning effects, indicating that longer times were unnecessary and inefficient. Therefore, 180 min was selected as the upper limit. For pH, a wide range of values was explored to ensure the extraction of compounds with diverse acidity characteristics. This strategy aimed to target both acidic and neutral components, which may contribute differently to the overall efficacy of the extract in inhibiting browning. The pH levels were thus set to cover a broad spectrum, allowing for a comprehensive evaluation of the extract's potential across varying pH conditions.[7] The model design included three replicates at the central point (experiments 13*z*, 14*z*, and 15*z*), which were randomly spread within the experiments, resulting in a total of 15 runs. The use of BBD allowed a reduction of 12 experiments, which in a full factorial design would have resulted in a total of 27 experiments.

A generalized second-order polynomial model was obtained and applied in the response surface analysis and explained by Eqn (4):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i \neq j=1}^{k} \beta_{ij} X_i X_j \quad (4)$$

where Y is the response variables: extraction yield, extracts solubility, browning compounds absorbance, enzymatic activities, and color parameters; β_0, β_i, β_{ii}, and β_{ij} are the intercept, linear, quadratic, and interactive coefficients of the regression, respectively; and X_i and X_j are the independent variables ($i \neq j$). The effects of the independent variables on the response variables were evaluated by the models.

Experimental design, RSM, and analysis of variance (ANOVA) were carried out in Minitab 20 (State College, PA, USA) and STATISTICA (version 10, StatSoft, Inc., Hamburg, Germany). Coefficient of determination (R^2) and the lack-of-fit test at a significance level of 5% were used to evaluate the model adequacy. The effects of independent variables and their interactions in the response variable were visualized by response surface contour plots.

RESULTS AND DISCUSSION

Kinetic model

To narrow the range of selected factors to be used in the BBD experiment, a preliminary single-factor experiment was performed. The single-factor experiment with time as the independent variable (see section 'Second-order model', above) resulted in a linear relationship between t/UV_t *versus* t that was fit to a second-order model (Supporting Information, Fig. S1). The successful fit ($R^2 = 0.950$) suggests monitoring the seaweed extraction through absorbance at 260 nm as an adequate indicator of extraction kinetics. Up to now, in studies where *Codium* sp. has been used, extraction variables like time and temperature have been fixed variables.[1,2,4,21,22] Augusto *et al.*[3] and Toledo *et al.*[5] fixed a 3-h and 4-h extraction at room temperature, respectively. Rodrigues *et al.*[22] set a 24-h extraction at 50 °C, and Silva *et al.*[2] selected an overnight extraction at room temperature. However, none of the previous studies were the extraction kinetics evaluated. This is the first study where the kinetics of extraction of *C. tomentosum* are being modelled. A kinetic model was obtained to determine the kinetic parameters, such as extraction rate constant (k), absorbance at saturation (UV_s), and initial extraction rate (h), as mentioned earlier in the section 'Second-order model' (see also Supporting Information, Fig. S1(A)).

The initial extraction rate (h) was 0.0594 units of absorbance (U_{abs}), and for every minute the extract absorbance (k) increased about 0.0035 U_{abs} until saturation at 4.11 U_{abs} (UV_s) was reached. The extract absorbance increased with time, revealing a good penetration of solvent into the dried seaweed. Contact between the seaweed and the solvent induced changes in the concentration gradient, leading to diffusion of solutes from the cells to the solvent until an equilibrium was reached between both interfaces. Nie *et al.*[14] reported a similar relationship between time and fucoxanthin extraction using the brown seaweed *Sargassum fusiforme*. Usually, after attaining extraction equilibrium, especially under long-term extractions, a degradation in the compounds can be observed.[7,14] To avoid degradation due to long extraction periods and to observe the sole effect of time on the extracts, the absorbance at 260 nm in Supporting Information, Fig. S1(B), where at 180 min the absorbance reaches a stable plateau, was selected for the subsequent experiments. These results also indicate that the external resistance as mass transfer phenomena may be neglected since the extraction reaches the stationary phase, which can be observed in Fig. S1(B).

Experimental outcomes

The influence of extraction conditions using an SLE methodology with *C. tomentosum* was studied following the scheme shown in Fig. 1. After extraction, the extracts were analyzed by FTIR-ATR, water solubility, yield, and the ability of the extracts to decrease superficial browning in fresh-cut apples. Table 1 summarizes the processing conditions applied in the extractions, and the corresponding experimental results obtained are presented in Supporting Information, Table S1. The experimental results were used for the RSM model construction to understand the effect of extraction conditions on the extract properties. Briefly, extraction yield values ranged between 61.51 ± 3.92 and 67.72 ± 3.56 g 100 g^{-1} of dried seaweed (Table S1), corresponding to extraction conditions of 0 min, 50 °C, and pH 3.0, and of 180 min, 80 °C, and pH 6.5, respectively. Extracts with an extended extraction period of 180 min, at 20 °C and pH 6.5 presented lower values of water solubility (63.00 ± 4.00 g 100 g^{-1}), in contrast to a solubility of 85.07 ± 5.80 g 100 g^{-1} determined in the extracts produced at 80 °C and pH 6.5. In relation to the experimental assays with fresh-cut apples (described in the section 'Extract functionality as anti-browning agent for fresh-cut apples', above), the observed extraction conditions with higher effectiveness to control browning were very distinct in each of the analyses. The lower browning compounds absorbance was determined in samples coated with an extract produced at 50 °C and pH 6.5 (0.095 ± 0.002 abs). Nevertheless, extracts that resulted from experiments at 180 min, 20 °C and pH 6.5 granted lower browning index (BI) and less color variation (ΔE) in fresh-cut samples (BI = 36.56 ± 6.95 and ΔE = 4.17 ± 2.83). The higher values of POD and PPO activities were determined in fresh-cut samples coated with extracts produced at 50 °C, pH 6.5 for 90 min (0.139 ± 0.024 U m^{-1} protein), and 180 min at 80 °C (pH 6.5) (0.054 ± 0.01 U mg^{-1} protein), respectively. High values of SOD (90.51 ± 10.15 U g^{-1} fresh weight) were observed in extracts produced at 80 °C, pH 6.5 (0 min). Lastly, the lower values of PME were determined in fresh-cut samples coated with a solution containing extracts that resulted from extraction with a duration of 90 min, 50 °C, and pH 6.5 (0.035 ± 0.01 U mg^{-1} protein).

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FTIR-ATR analysis

FTIR-ATR analysis was performed to identify the different functional groups present in the extracts and to establish possible interferences of the extraction conditions on the FTIR profile. A visual representation of the obtained FTIR spectra can be observed in Supporting Information, Fig. S2. FTIR spectra can be divided into three main regions: (1) 4000–2400 cm^{-1}; (2) 2400–1500 cm^{-1}; and (3) 1500–400 cm^{-1}. The main peaks observed in each region are described below.

Region 1: The association of a strong and broad band around 3526 cm^{-1} with a moderate peak around 1100 cm^{-1} suggest the presence of alcohol in all samples.[23] The medium bands observed at ~2929 and 2850 cm^{-1}, corresponding to —CH bond stretching typically found in CH_3 and CH_2 groups,[23] suggest the presence of aliphatic fragments or centers in the molecule. While these bands are characteristic of aliphatic components, phenolic compounds can also exhibit such features if they contain aliphatic side chains. Similar functional groups have been reported in ground samples of *C. fragile*, indicating that these bands may reflect a mixture of aliphatic and aromatic components in the sample.[24]

Region 2: The double-bound stretching of the functional group (C═O band) of amides can be observed at 1635 cm^{-1}. In the same spectral region, Fawzy[25] observed a similar peak in dried *C. vermilara* samples and Augusto *et al.*[4] in hydroethanolic extracts of *C. tomentosum*. However, it is also in this region that the unsaturated fatty acid C═O stretching occurs, suggesting the presence of fatty acids in the extract composition. In the species *C. tomentosum*, about 55% of fatty acids are unsaturated, namely 20:5 and 22:6 species, both long-chain polyunsaturated fatty acids.[21]

Region 3: Asymmetrical bending at 1406–1423 cm^{-1} and symmetrical bending at 1339–1374 cm^{-1} were observed, both common in methyl and methylene groups (CH_3 and CH_2).[26] A third peak in the region 1088 cm^{-1} can be assigned to the stretching vibration of sulfoxides (S═O) from the sulfate group (SO_4).[26] The presence of these two functional groups in the seaweed extracts strongly suggests the existence of sulfated polysaccharides in the composition of the extract.[24] These polysaccharides are components of the cell walls and are mostly linked to brown seaweeds,[24] albeit already described in green seaweeds, inclusively *Codium* species.[4,25] Further, considering the region between 1500 and 400 cm^{-1}, which corresponds to the carbohydrate region, the high-intensity bands that can be seen in the ATR spectra possibly reveal high carbohydrate content in the seaweed extracts, also identified by ^{1}H-NMR as detailed below in the section 'Effect of extraction variables'.[24]

FTIR analysis thus confirmed that, within the analyzed extracts, no apparent changes in the ATR spectra were observed, it being possible to identify amide, methyl, and sulfate groups as the main groups present in the seaweed extracts. This suggests that the primary functional groups present in the *C. tomentosum* extracts remained stable across the tested conditions. This stability may indicate that the core chemical structure of the extracted compounds is resistant to the tested variations in extraction parameters, implying that the functional groups detected were not significantly altered by temperature, time, or pH adjustments. Because the extraction was performed using water as the solvent, it is likely that the extracted compounds – mainly polysaccharides and some proteins – do not undergo significant structural modifications under the tested conditions.[7,34] Polysaccharides, especially sulfated galactans, are known to be relatively stable within the studied pH and temperature ranges, explaining the lack of spectral shifts.

Effect of extraction variables

BBD was utilized to investigate the effect of various extraction conditions, such as time, temperature, and pH, in the seaweed extract functionality to prevent browning development. The raw data respecting all the experiments made under the different extraction conditions are available in Supporting Information, Table S1. The contour plots were investigated and the *P*-values from ANOVA were used to validate the importance of individual factors and the interaction between factors (Supporting Information, Tables S2–S4). From the analyzed response variables (extraction yield, extract solubility, browning compounds absorbance, enzymatic activities, and color parameters) the models that showed the best-explained variances were chosen (higher R^2, with lack-of-fit *P*-value > 0.05). From the ANOVAs, the models that showed the best fit were extraction yield (Fig. 2 and Table S2), browning compounds absorbance at 440 nm (BC 440 nm; Fig. 3 and Table S3) and peroxidase activity (Fig. 4 and Table S4). The ANOVAs of the remaining response variables can be consulted in Tables S5–S10 (Supporting Information, Tables S5–S10). The experimental data were analyzed by multiple regression. The response and experimental variables were related by the following regression equation:

$$\begin{aligned}\text{Yield} = {} & 59.67 + 0.0095X_1 - 0.1009X_2 + 1.632X_3 \\ & - 0.000012{X_1}^2 + 0.000715{X_2}^2 - 0.1025{X_3}^2 \\ & + 0.00017X_1X_2 - 0.00092X_1X_3 + 0.00374X_2X_3\end{aligned} \quad (5)$$

$$\begin{aligned}\text{BC 440 nm} = {} & 0.10096 - 0.000041X_1 - 0.000068X_2 \\ & + 0.00091X_3 + 0.000000{X_1}^2 - 0.000000{X_2}^2 \\ & - 0.000104{X_3}^2 - 0.000000X_1X_2 + 0.000006X_1X_3 \\ & + 0.000000X_2X_3\end{aligned} \quad (6)$$

$$\begin{aligned}\text{POD} = {} & -0.0849 + 0.000039X_1 + 0.00348X_2 + 0.0229X_3 \\ & - 0.000002{X_1}^2 - 0.000037{X_2}^2 - 0.001912{X_3}^2 \\ & + 0.000003X_1X_2 + 0.000026X_1X_3 - 0.000040X_2X_3\end{aligned} \quad (7)$$

In the three response variables, a high correlation coefficient was observed (R^2 > 77%), indicating that the models were successful in fitting the experimental data. From the lack-of-fit analysis, the contribution of unknown factors to the response variable is not significant (*P* > 0.05) (Supporting Information, Tables S2–-S4). Despite the good fitness of models to the data, these models were not designed to integrate and predict extraction conditions for overall performance, it being necessary to discuss simultaneously each response variable. A separate model comprising the extraction yield and extracts efficacy is needed for a more accurate variables prediction.[27]

Effect of time

To study the effect of time on the response variables, three different extraction times resulted from the BBD: 0, 90, and 180 min. Time had a positive effect on the yield, suggesting that longer extraction periods allow higher compound recovery, as can be seen by the red-colored areas in Fig. 2(A,B).

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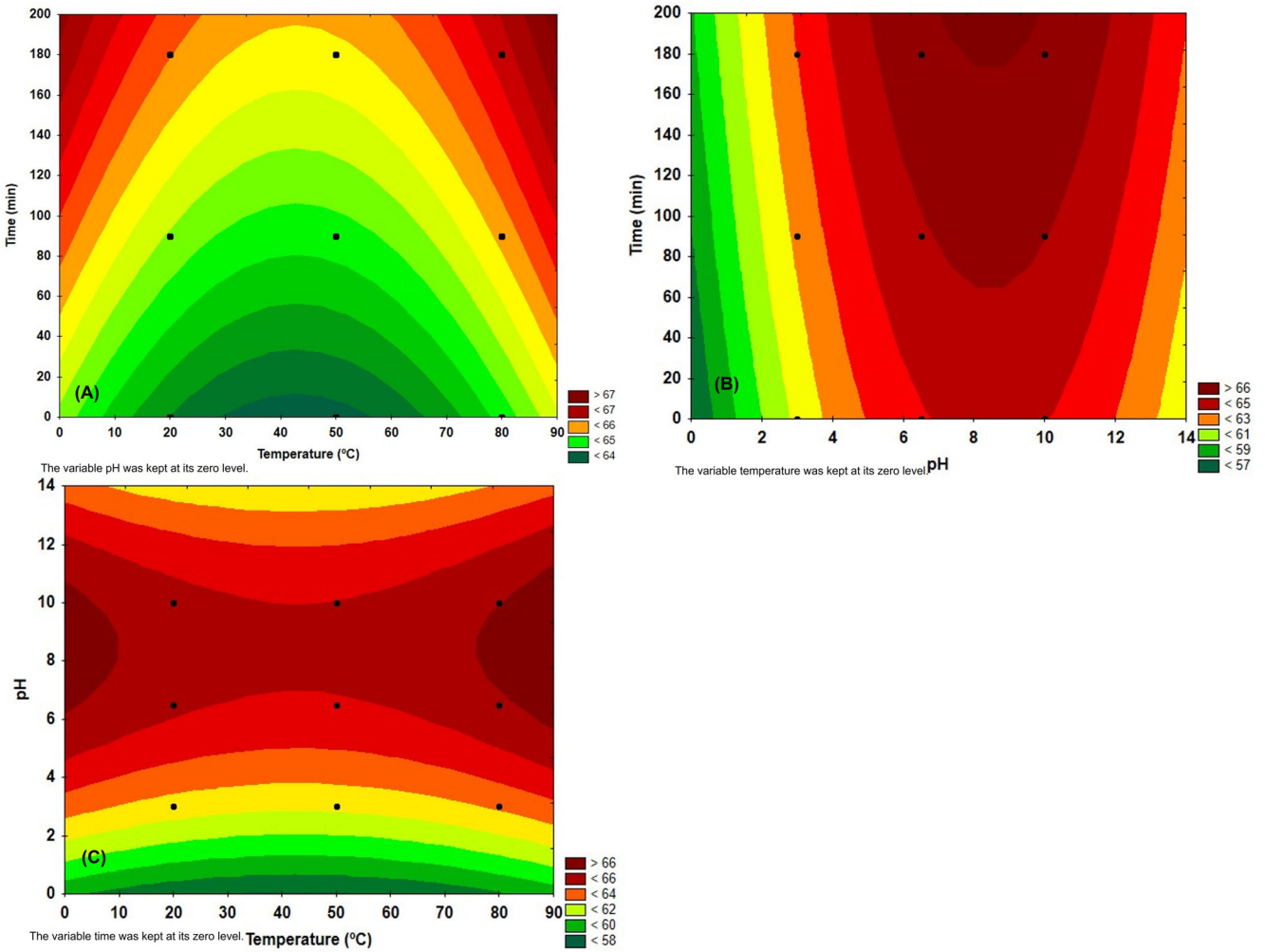


Figure 2. Contour plots showing combined effects of time, temperature, and pH on the extraction yield of *Codium tomentosum*.

The relevance of time on the extraction yield is evidenced by the ANOVA (Supporting Information, Table S2), where the effect of time is significant for the model ($P < 0.05$). It is usual to observe an increase in yield with time, since extended extraction periods enable solubilization of solutes, increasing the final extracted biomass,[7] although a compromise between long extraction periods, yield, and type of extracted compounds is essential. Recurrently, the degradation of the compounds is correlated with long extraction periods. The extraction of pectin from sunflower heads showed a positive correlation between time and yield;[28] however, with a longer time of extraction, the degradation of pectin occurred, derailing the use of extended periods; thus lower extraction yields but with high-quality pectin were preferred.

In the case of browning compounds absorbance (Fig. 3(A,B) and Supporting Information, Table S3) and peroxidase activity of coated fresh-cut apples (Fig. 4(A,B) and Supporting Information, Table S4), time played a relatively weaker effect on the efficacy of the extracts to decrease peroxidase activity and browning development on samples. The low impact of time on extract efficacy might suggest that compounds of interest are rapidly extracted with water at initial process stages and thus independent of time. Catarino *et al.*[8] observed a similar effect of time on total phlorotannin content from *Fucus vesiculosus* extractions. The authors observed an extraction equilibrium below 60 min, without significant variation with the extension of time, on phlorotannin recovery. Probably, in the present work, the compounds that are being extracted are mainly proteins and polysaccharides, which are easily extracted with water,[7] contributing largely to the recovered biomass but without impact on the extracts' functionality, as observed in the lack of significance of time on browning compounds absorbance and peroxidase activity.

Effect of temperature

As a result of BBD, the effect of temperature on the extraction yield and extract functionality was inspected at 20, 50, and 90 °C (Figs 2(A,C), 3(A,C), and 4(A,C)). To avoid high energetic costs that are associated with the use of extraction temperatures below room temperature,[7] 20 °C was chosen as the minimum temperature. For the same reason, and to avoid excessive solvent losses by evaporation, once at 100 °C the mixture was near to boiling point and 90 °C was chosen as the maximum temperature.

Contrary to expectation, in the aqueous extraction of *C. tomentosum* the temperature had a relatively small effect on the extraction yield, without significant impact on the model ($P > 0.05$) (Supporting Information, Table S2). As can be seen in Fig. 2(A,C), with increasing temperature values extraction yield rates are kept in the green areas of the figure, which correspond to extraction yield values lower than 65%, never reaching values

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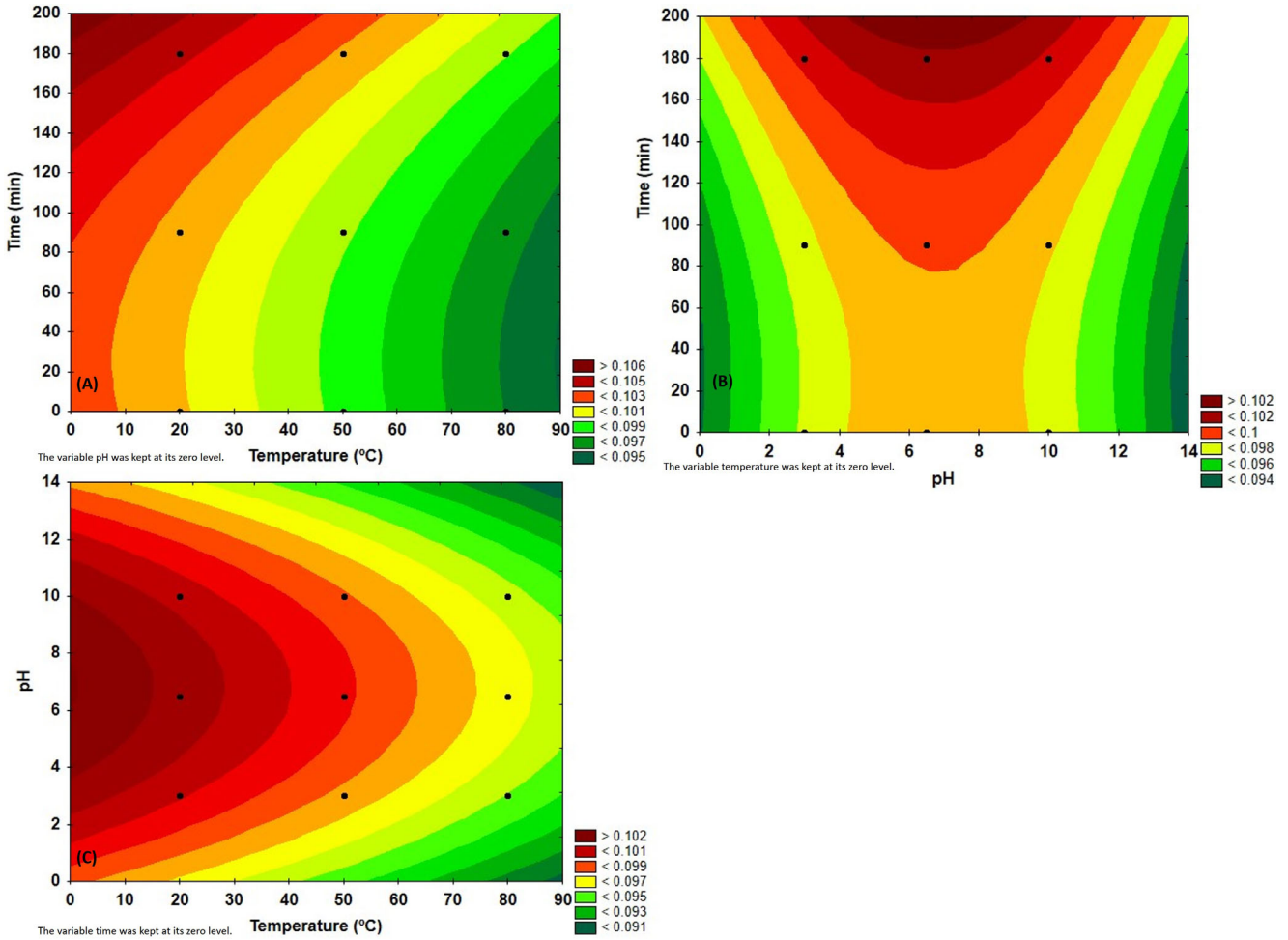


Figure 3. Contour plots showing combined effects of time, temperature, and pH on browning compounds absorbance at 440 nm.

of maximum extraction yield. Usually, increasing temperature generates higher mass transfer and high solvent diffusion rates, improving the recovery of biomass,[27] although in the present work this effect was not observed. In a single-factor experiment, the yield of phlorotannin extraction from *F. vesiculosus* was affected by temperature, but when integrated into a more complex experimental design, which included solvent concentration and solid-to-liquid ratio variations, the temperature had no significant effect on phlorotannin extraction yield.[8]

Interestingly, Fig. 3(A,C) suggests an effect of temperature on the extract efficacy to decrease browning in fresh-cut apples. From observation of the figures, lower values of browning compounds absorbance are obtained using extracts produced at higher temperatures, namely above 60 °C (greenish area of figures). The impact of temperature on the model is significant ($P < 0.05$; Supporting Information, Table S3), revealing the importance of this variable to obtain extracts with anti-browning efficacy. Peroxidase activity and fresh-cut apple browning being related,[3] a similar effect on peroxidase activity was expected. The temperature was also demonstrated to influence the extract ability to decrease POD activity in fresh-cut apples, but with a quadratic effect (X_2^2) on POD activity (Supporting Information, Table S4, and Fig. 4(A,C)). Even though temperature had no significant influence on the yield of extraction, interestingly it influenced extract efficacy. The observed shifts in extract efficacy with temperature may be justified by changes in compound solubility, especially organic compounds, that in normal conditions would not be extracted with water at low temperatures.[7] With higher temperatures, mass transfer phenomena become facilitated, increasing compounds' solubility and promoting their collection in the final extract. This observation also suggests that compounds that are being extracted are thermoresistant once their efficacy is not compromised by the extraction temperature. Linking type of solvent, FTIR-ATR results (refer to the section 'Experimental outcomes', above), previous results[3] and temperature resistance, these results reinforce opinion concerning the extracts' composition and their high content of polysaccharides, which are commonly extracted at high temperatures.[27]

Effect of pH

Studies on the effect of pH on seaweed extracts are scarce, the majority of works being focused on other extraction parameters like time, temperature, solid-to-liquid ratio, and solvent concentration.[7] Due to the possible composition of the *C. tomentosum* extract, in the present work pH was considered a relevant factor. The presence of polysaccharides in the extract could lead to a significant extract behavior when submitted to different pH conditions.

Here, pH was adjusted at three different levels: 3, 6.5, and 10 (Table 1). Considering the effect of pH on yield (Fig. 2B,C),

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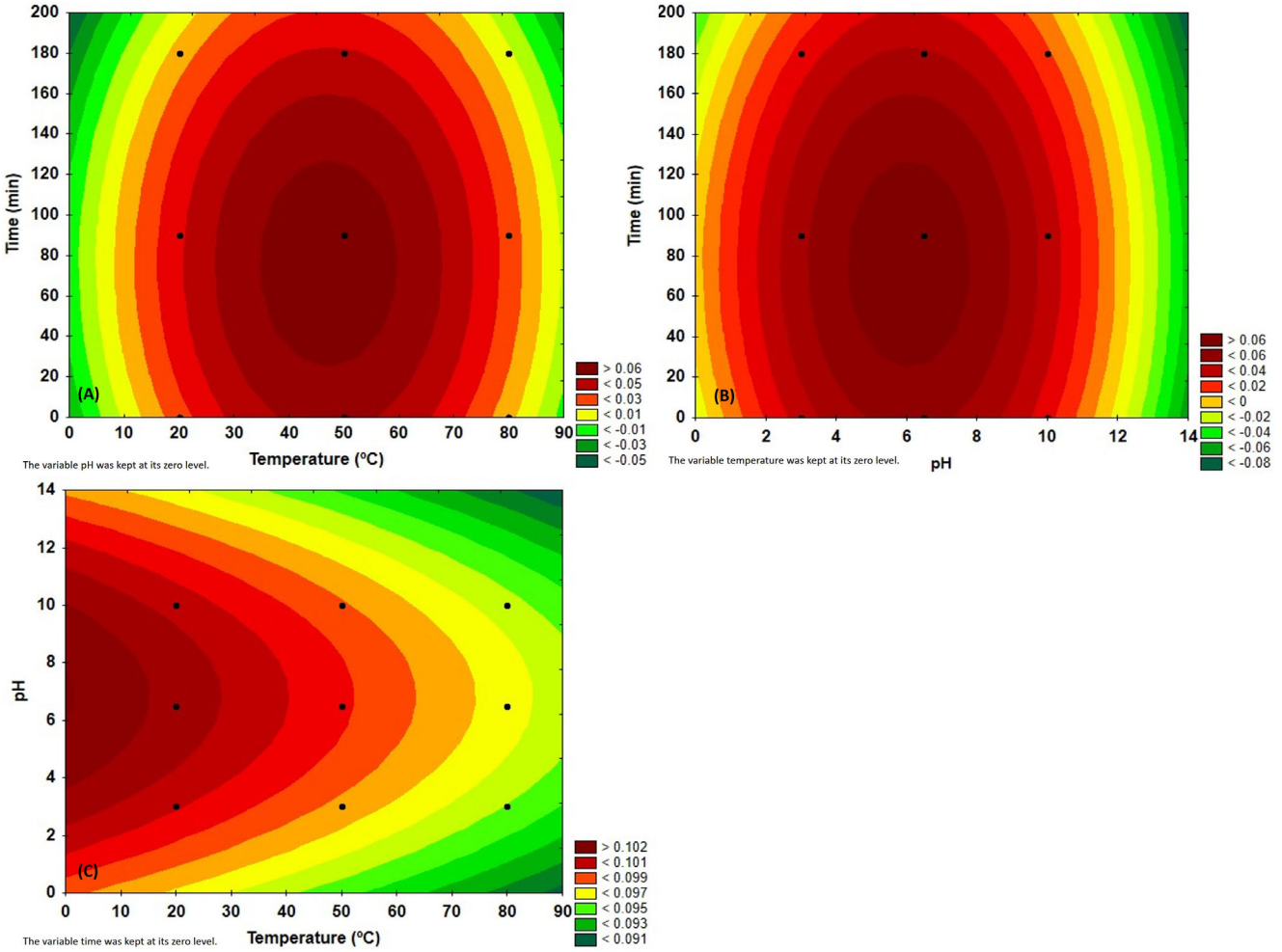


Figure 4. Contour plots showing combined effects of time, temperature, and pH on peroxidase activity.

which is characterized by a quadratic effect ($P < 0.05$; Supporting Information, Table S2), the strongest effect was observed between pH 6 and 10, corresponding to the higher values of the extraction yield of about 67%, which can be justified by protonation and deprotonation phenomena, increasing the yield of extraction. Félix *et al.*[7] observed a linear increase in yield with pH and temperature, suggesting that under alkaline conditions the hydrolysis of cell wall components is more effective, leading to the extraction of more compounds. The effect of pH in extract functionality can be seen in Figs 3(B,C) and 4(B,C). Although without a significant effect, the results suggest that over the pH range of 6–10 the extracts presented a low efficacy to prevent browning in fresh-cut apples, because the obtained values of absorbance are situated in the reddish area of the figure (Fig. 3), corresponding to higher values of browning compounds absorbance. A similar response to pH variations was observed in peroxidase results (Fig. 4B,C). However, in peroxidase, the interaction between pH and time suggests a much more intensified response in the efficacy of the extract to decrease peroxidase activity. Observing Fig. 4(B), it is possible to note that extractions performed during 90 min at 6.5 pH resulted in extracts with less capability to reduce POD activity, these conditions being those that allowed higher values enzymatic activity – 96% more POD activity than the lower POD activity determined at 90 min, 80 °C, and pH 3 (Supporting Information, Table S1). Despite the observed tendency, a single-factor study employing a wider range of pH, especially under alkaline conditions, may provide a more assertive answer about the effect of pH on extract efficacy.

Metabolite evaluation through ^{1}H-NMR

The ^{1}H-NMR spectra of the *C. tomentosum* aqueous extract (Fig. 5) revealed a predominance of carbohydrates and amino acids/proteins. Resonances between 3.01–4.19 ppm and 0.98–3.77 ppm were consistent with proton signals typical of these compound classes, respectively, aligning with observations from aqueous fractions of other green macro- and microalgae species.[29–32] This finding is further supported by the FTIR-ATR results (section 'FTIR-ATR analysis', above), which indicated the presence of complex polysaccharides. In the expanded spectra, less intense signals were observed in the aromatic region (6.05–8.48 ppm), indicating that phenolic and other aromatic compounds are present in minor amounts. This observation is consistent with the FTIR-ATR results, which show bands characteristic of phenolic compounds. Nevertheless, aromatic amino acids (e.g., tyrosine and phenylalanine) may be present along with aromatic nucleotide bases (5.89–6.88 ppm). Clearly, amino compounds together with carbohydrates are the main components of *C. tomentosum* water extract studied here. However, due to considerable overlap in the carbohydrate and amino acid regions it is difficult to assign specific metabolites only with ^{1}H-NMR data.[29,32]

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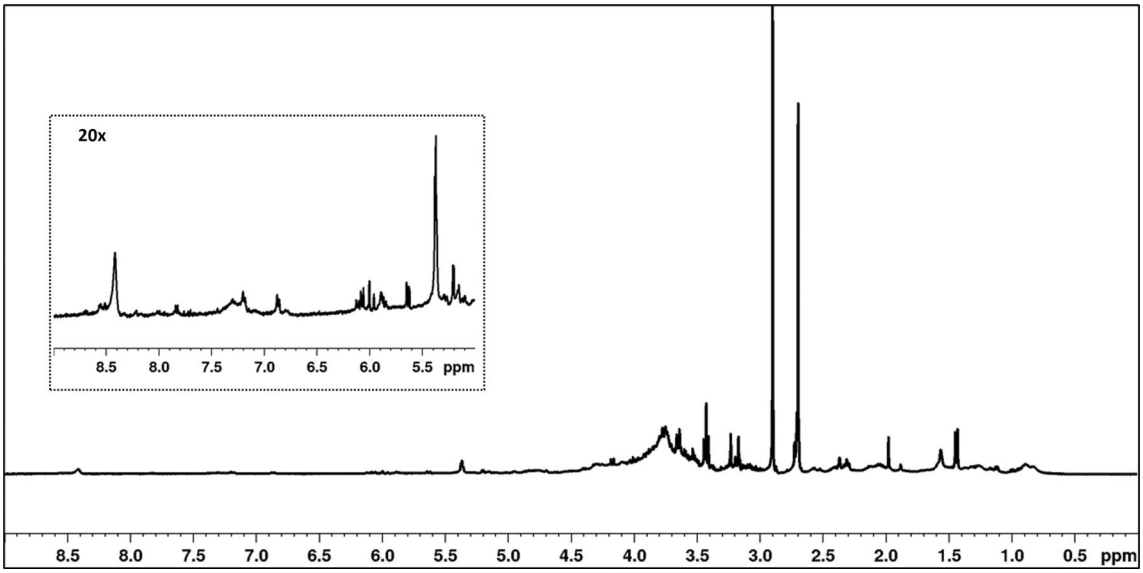


Figure 5. ^{1}H-NMR (400 MHz, D_2O with water suppression) spectrum of *Codium tomentosum* water extract.

Signals in the aliphatic region (0.80–2.90 ppm) are commonly attributed to methyl, methylene, and methine protons of lipophilic compounds such as fatty acids, pigments, and sterols, which are not expected to be present in aqueous extracts. Nevertheless, —CH_3 and —CH_2 groups of amino acids resonate between 0.98 and 3.07 ppm, while —CH_2 of organic acids such as glutarate, succinate, and citrate resonate at 2.16–2.19 ppm.[30]

Sulfated galactans are another group of metabolites biosynthesized by green seaweeds. Using one- and two-dimensional NMR tools, Farias *et al.*[33] have identified in *C. isthmocladum* a complex sulfated galactan with different structural components. The main variations come from different positions of glycosidic linkages (3- and 6-linked units), from different sulfation sites (positions 4 and/or 6), and from the presence of pyruvate groups involved in cyclic ketals with the positions *O*-3 and *O*-4 of the *β*-D-galactose located at nonreducing ends. According to these authors, the proton signals between 4.4 and 5.0 ppm in the spectra of the sulfated galactan contained a mixture of signals of H-1 from the *β*-anomers of galactopyranoses and of H-4 from the sugar rings, these exhibiting a down-field shift (~0.6 ppm) due to sulfation. Besides these signals, the remaining protons of galactose between 3.71 and 4.16 ppm are in accordance with the signals observed in *C. tomentosum* spectra, suggesting the presence of galactans in the water extract. Supporting Information, Table S11, lists the main classes of compounds tentatively assigned by ^{1}H-NMR, according to their characteristic chemical shift ranges. Given that galactans are well known for their gelling, thickening, and emulsifying properties,[33] their presence in the *C. tomentosum* extract likely plays a significant role in the observed anti-browning effects on coated apples. The aqueous extraction method used in this study support the recovery of highly polar compounds,[7] such as carbohydrates (including sulfated galactans) and amino acids/proteins, while less polar phenolic compounds are extracted in much lower amounts. Although phenolic compounds are known for their antioxidant properties,[7] their lower extraction yield in water under the tested experimental conditions suggests that the observed anti-browning effects are primarily mediated by the more abundant polysaccharides. These macromolecules can form a physical barrier on the apple's surface, limiting exposure to oxygen and moisture, which are key factors in enzymatic browning reactions. By restricting oxygen access, galactans help inhibit the activity of enzymes like polyphenol oxidase, which catalyze the browning process. Moreover, galactans may influence multiple reaction mechanisms beyond acting as a physical barrier. Their antioxidant properties can neutralize reactive oxygen species,[33] reducing oxidative stress and potentially impacting the gene expression of browning-related enzymes, supporting previous findings that demonstrated a correlation between the presence of *C. tomentosum* extract and the downregulation of enzymes associated with browning.[3] There is also the possibility that galactans interact with cellular pathways, modulating the expression of genes involved in browning and enhancing the fruit's natural defense mechanisms. Additionally, the antimicrobial activity of galactans can contribute to preserving the freshness and quality of the coated apples by inhibiting the growth of spoilage organisms, as observed in the study of Augusto *et al.*[35] To confirm the presence and specific structural characteristics of galactans in the *C. tomentosum* extract, further analysis, such as liquid chromatography–tandem mass spectrometry, is planned. This comprehensive approach will provide a deeper understanding of the bioactive compounds responsible for the extract's beneficial effects on fruit preservation.

CONCLUSIONS

The aqueous extraction of *C. tomentosum*, a green edible seaweed with proven efficacy in reducing browning in fresh-cut apples, was characterized by assessing the effects of time, temperature, and pH on extraction yield, solubility, FTIR-ATR spectra, and extract functionality. The results demonstrated that extraction yield is primarily influenced by time and pH, with longer extraction periods and neutral to alkaline pH resulting in higher recovery. In terms of extract functionality, browning compounds absorbance and peroxidase activity were significantly affected by temperature variations. Fresh-cut apples coated with extracts produced at temperatures above 60 °C exhibited a lower browning index, suggesting the benefit of applying extraction conditions above room temperature. Chemical analysis of the extracts

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via ^{1}H-NMR revealed a predominance of carbohydrates and amino acids/proteins, with phenolic compounds present in minor amounts. The presence of galactans as the major component suggests that these polysaccharides contribute to the anti-browning effects through multiple mechanisms. These include forming a protective barrier against oxygen and moisture, which inhibits enzymatic browning, and potentially interacting with cellular processes regulating the activity of browning-related enzymes. Overall, these findings offer valuable insights into optimizing the extraction conditions for *C. tomentosum* to maximize its functional properties while minimizing costs with extraction optimization. This research highlights the potential of *C. tomentosum* extracts as a natural and effective anti-browning agent for extending the shelf-life of fresh-cut fruits and vegetables.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Ana Augusto: conceptualization, methodology, investigation, writing – original draft. Juliana R Dias: investigation, methodology. Alice Martins: investigation. Helena Gaspar: investigation. Maria Oruna-Concha: methodology, resources. Nuno Alves: resources. Geoffrey Mitchell: supervision, writing – review and editing. Susana FJ Silva: supervision, conceptualization, methodology, writing – review and editing, funding acquisition. Keshavan Niranjan: supervision, conceptualization, methodology, writing – review and editing.

DATA AVAILABILITY STATEMENT

Data are available upon reasonable request from the corresponding author.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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