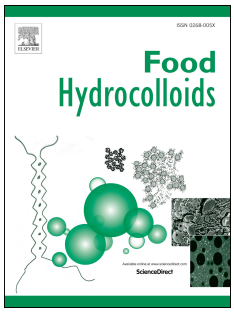

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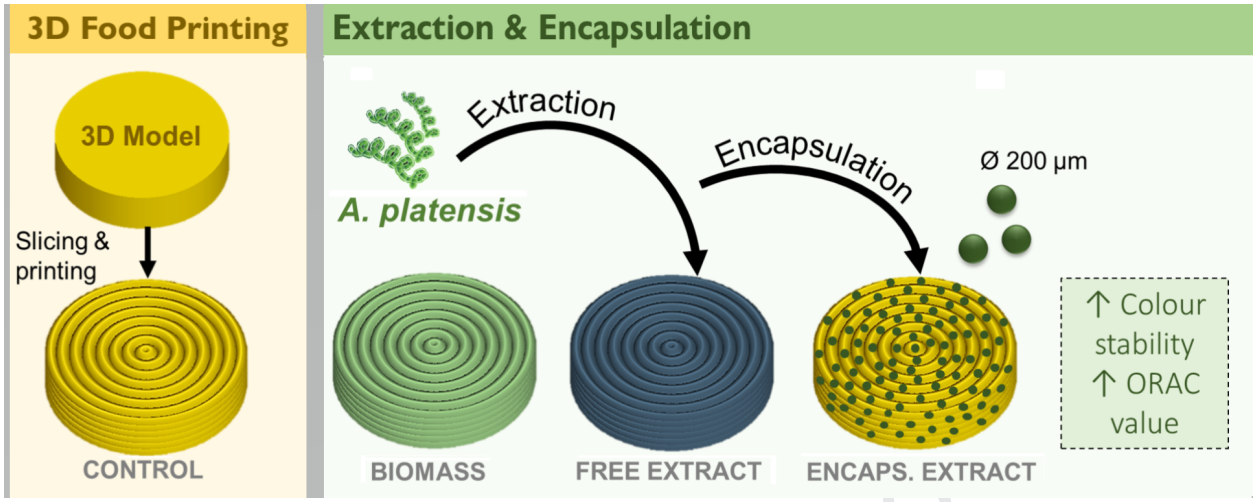

3D printed functional cookies fortified with *Arthrospira platensis*: evaluation of its antioxidant potential and physical-chemical characterisation

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Abstract

In the last few decades, consumers' growing attention to the close relationship between health and nutrition is emerging as a new trend, mostly regarding the incorporation of natural ingredients into food. Among those ingredients, microalgae are considered as innovative and promising compounds, rich in valuable nutrients and bioactive molecules. In the present work, 3D printed cookies were fortified with the microalga *Arthrospira platensis* aiming at developing a new functional food with antioxidant properties. *A. platensis* antioxidants were recovered using ultrasound-assisted extraction in hydroalcoholic solutions. Ethanol/water and biomass/solvent ratios were optimised through a Design of Experiments (DOE) approach, using the antioxidant activity (ORAC and ABTS) and total phenolic content (TPC) as response variables. The highest ORAC, ABTS and TPC values were observed in the extract obtained with 0 % ethanol and 2.0 % biomass; thus, this extract was chosen to be incorporated into a printable cookie dough. Three different incorporation approaches were followed: (1) dried biomass, (2) freeze-dried antioxidant extract and (3) antioxidant extract encapsulated into alginate microbeads to enhance the stability to heat, light, and oxygen during baking and further storage. All dough formulations presented shape fidelity with the 3D model. The cookies had aw values low enough to be microbiologically stable, and the texture remained constant after 30 days of storage. Moreover, the extract encapsulation promoted an improvement in the ORAC value and colour stability when compared to all other formulations, revealing the potential of *A. platensis* for the development of a functional 3D food-ink.

Keywords: *Arthrospira platensis*; Encapsulation; Food-ink; Functional food; 3D printing.

1. Introduction

Many nutrition concepts have changed during the past few decades, and the food industry has made a significant effort to follow them and adapt their products to these changes. Traditionally, the primary role of diet was to provide enough nutrients to meet metabolic requirements while giving consumers a feeling of satisfaction and well-being. Nowadays, however, it is established that beyond meeting nutritional needs, the diet may modulate various bodily functions and may play detrimental or beneficial roles in some diseases (Bigliardi & Galati, 2013; Roberfroid, 2000). In this regard, it is possible to observe an increasing consumer's health consciousness and demand for healthy foods - facts that are stimulating innovation and new product development in the food industry. This trend is also responsible for an ever-increasing worldwide interest in functional food, which also can be explained by the increasing cost of the health care and the steady boost of life expectancy (Betoret, Betoret, Vidal, & Fito, 2011; Lopez-Rubio, Gavara, & Lagaron, 2006; Plaza, Herrero, Cifuentes, & Ibánez, 2009; Sun, Zhou, Yan, Huang, & Lin-ya, 2018).

Functional food is a natural or processed food that contains known biologically-active compounds which, when in defined quantitative and qualitative amounts, provide a clinically proven and documented health benefit; and, hence, a useful tool for the prevention, management and treatment of diseases. There is a wide range of compounds that have already been incorporated into functional foods, with particular attention being given to ingredients from natural resources (Day, Seymour, Pitts, Konczak, & Lundin, 2009; Herrero, Martín-Álvarez, Senoráns, Cifuentes, & Ibánez, 2005).

Microalgae can be considered an innovative and promising food ingredient, rich in nutrients such as high-value proteins, long-chain polyunsaturated fatty acids, carotenoids, vitamins, minerals, and phenolic compounds, as well as other bioactive molecules (Gouveia, Marques, Sousa, Moura, & Bandarra, 2010). Among them, *Arthrospira platensis* is one of the main species exploited by the food and nutrition industries, being traditionally used as food by different cultures. This microorganism is a blue-green filamentous prokaryotic cyanobacterium well known for its unique composition, comprising not only up to 70 % of protein containing all the essential amino acids, but also polysaccharides, vitamin B12, C, E, and γ-linolenic acid (GLA). Furthermore, it is a source of potent antioxidants, such as carotenoids, polyphenols and phycobiliproteins -a group of photosynthetic pigments majority

represented by C-phycocyanin, which are related to numerous reported pharmacological properties; including anticancer, antidiabetes, hepatoprotective and anti-inflammatory (Czerwonka et al., 2018; Da Silva et al., 2019; Hu, Fan, Qi, & Zhang, 2019; Plaza et al., 2009; Soni, Sudhakar, & Rana, 2017).

The incorporation of microalgae biomass into traditional foods (e.g. breakfast cereals, bread, pasta, cookies, gelled desserts, and beverages), which are primarily consumed on a daily basis, has been researched and several products have already been launched in the market (Gouveia et al., 2010; Lafarga, 2019). In particular, cookies are considered a convenient dense snack food, offering a valuable supplementation vehicle for nutritional improvement as they are widely accepted and consumed by all age groups. There is a trend for research and innovation in this market segment, which promotes the inclusion of healthy ingredients into cookies, such as antioxidants, vitamins, minerals, proteins and fibers (Batista et al., 2017; Nogueira & Steel, 2018; Šaponjac et al., 2016).

Besides the change in consumer's attitudes towards a healthier diet, it is noteworthy that food ingredients and their nutritional needs vary among individuals, especially children, elderly and athletes (Tan, Toh, Wong, & Li, 2018). This context motivates a growing market for personalized healthy nutrition, which aims to tailor food and diets specifically based on an individual's health condition. In light of this, three dimensional (3D) food printing has gained increasing attention for its distinctive potential to create complex geometric structures, enabling mass customisation while having economic and environmental benefits. The main advantage of this emerging technology is being able to personalize food by tailoring nutrition in a novel multi-flavoured, coloured and textured structure, allowing the incorporation of a broad range of ingredients (Dankar, Haddarah, Omar, Sepulcre, & Pujolà, 2018; Liu et al., 2018a; Pérez, Nykvist, Brøgger, Larsena, & Falkeborg, 2019; Sun et al., 2018).

Considering the above mentioned, this study aimed at developing 3D printed functional cookies fortified with antioxidants extracted from *A. platensis*, to create a new functional food based on an innovative 3D food-ink. Due to the inherent instability of C-phycocyanin, carotenoids and other antioxidant compounds present in this microalga, the encapsulation of its extract in alginate microbeads was proposed as a way of improving the cookies stability to heat, light, and oxygen during the baking and further storage. Parameters such as colour, texture, water activity and antioxidant potential were investigated and compared with the freeze-dried extract and whole biomass incorporation into the cookie dough.

2. Materials and Methods

2.1 Materials

Arthrospira platensis biomass was obtained commercially in a specialized store (Braga, Portugal). Potassium phosphate dibasic and potassium di-hydrogen phosphate were purchased from Fisher Bioreagents (Pittsburgh, USA) and AppliChem (Darmstadt, Germany), respectively. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents and reagents used were of analytical grade.

2.2 Optimization of *A. platensis* antioxidants extraction

A. platensis antioxidants were recovered using ultrasound-assisted extraction in hydroalcoholic solutions. The influence of the ethanol/water and biomass/solvent ratios were assessed through a Design of Experiments (DoE) approach, using the antioxidant activity (ORAC and ABTS) and total phenolic content (TPC) as response variables. The lower and upper limits for the independent variables were based on previously reported conditions for extracting antioxidants from *Arthrospira* spp. (Oh et al. 2011; El-Baz et al. 2013; Syarina et al. 2015; Silva et al. 2017). Table 1 shows the coded variables and their real values for *A. platensis* antioxidant extraction. The obtained extracts were analysed as described in Section 2.3. The extract with higher antioxidant activity was freeze-dried for further encapsulation and incorporation into the cookie doughs.

Table 1. Full Factorial 2^k Design of Experiments for *A. platensis* antioxidants extraction, with two factors and two central points. Real values in parentheses.

Run	X_1 (Ethanol/Total solvent ratio)	X_2 (Microalgae mass/Volume of solvent)
1	1 (100 %)	1 (12 %)
2	1 (100 %)	-1 (2 %)
3	-1 (0 %)	1 (12 %)
4	-1 (0 %)	-1 (2 %)
5	0 (50 %)	0 (7 %)
6	0 (50 %)	0 (7 %)

163
164
165

166 2.3 Antioxidant activity and total phenolic content

167

168 The Oxygen Radical Absorbance Capacity (ORAC) of the extracts was performed in
169 96-well microplates, based on the method proposed by Ou et al. (2001) and further modified
170 by Dávalos et al. (2004). In brief, 20 µL of different concentrations of the extracts were
171 added to 120 µL of a 116.67 nmol.L^{-1} fluorescein solution prepared in 75 mmol.L^{-1}
172 phosphate buffer at pH 7.4. The mixture was incubated for 15 min at 37 °C and,
173 subsequently, 60 µL of 40 mmol.L-1 2,2´-azobis(2-methylpropionamidine)-dihydrochloride
174 (AAPH) were rapidly added using the automatic reagent injector of the plate reader (Biotek
175 Synergy H1). A blank (Fluorescein + AAPH) prepared with 20 µL of phosphate buffer
176 instead of the extracts was also analysed, and Trolox was used as standard. Fluorescence was
177 recorded every 5 min after AAPH addition (excitation wavelength 485 nm, emission
178 wavelength 520 nm) for 120 min. Results were calculated based on the differences in areas
179 under the fluorescein decay curve between the blank and the samples and were expressed as
180 µmol.L^{-1} of Trolox equivalents/g sample.

181 The spectrophotometric analysis of ABTS radical scavenging activity was conducted
182 according to the method of Re et al. (1999). Firstly, an ABTS solution was prepared by
183 mixing 7 mmol.L^{-1} ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
184 diammonium salt with 2.45 mmol.L^{-1} potassium persulfate, allowing this mixture to stand at
185 room temperature for 12–16 h in the dark. Subsequently, the ABTS solution was diluted with
186 Milli-Q water to obtain an absorbance of 0.70 ± 0.02 at 734 nm. In a 96-well microplate, 10
187 µL of the sample was added with 200 µL of the ABTS solution, and after 6 min of reaction,
188 the absorbance was measured at 734 nm. The scavenging capacity percentages (% RadScav)

were calculated using the Eq. 1. Results were expressed as Trolox Equivalent Antioxidant Capacity (TEAC) in mmol.L^{-1} of Trolox per g of *A. platensis* biomass (mmol.L^{-1} TEAC/g).

$$\% \text{ RadScav} = 1 - (\text{Abss} - \text{Absc}/\text{Absb}) * 100 \quad (1)$$

Where Abss, Absb and Absc are the absorbance of the sample, blank and negative control, respectively.

The total phenolic content (TPC) of the extracts was determined according to the method of Singleton et al. (1999), using the Folin–Ciocalteu reagent (FCR) and gallic acid as a standard. Initially, 0.5 mL of sample was mixed with 0.1 mL of Folin-Ciocalteu reagent and vigorously stirred. After 5 min, 0.5 mL of a 7.0 % sodium carbonate solution was added to alkalinize the medium, and the mixture was allowed to react for 1 h at room temperature. The absorbance was measured spectrophotometrically at a wavelength of 760 nm, and the results were expressed as mg of gallic acid equivalent (GAE) per g of *A. platensis* biomass (mg GAE/g).

2.4 Preparation of the cookie dough

Control cookies were prepared according to the formulation reported by Kim *et al.* (2019), using wheat flour, butter, powdered sugar, milk and xanthan gum. *A. platensis* incorporation was done by replacing an equivalent amount of wheat flour following three different approaches: (1) direct addition of 2.0 % whole *A. platensis* dried biomass, (2) incorporation of the freeze-dried antioxidant extract obtained from the same amount of biomass and (3) incorporation of an equivalent amount of antioxidant extract encapsulated into alginate microbeads. The dough formulations are presented in Table 2.

Table 2. Cookies dough formulations fortified with different *A. platensis* forms.

Ingredients	Control	Biomass	Free Extract	Encapsulated Extract
	(g/100g)	(g/100g)	(g/100g)	(g/100g)
Wheat flour	40	38	39.2	30
Butter	25	25	25	25
Powdered sugar	22	22	22	22
Milk	13	13	13	13
Xanthan gum	0.5	0.5	0.5	0.5
A. platensis	0	2	0.8*	10**

*Amount of freeze-dried extract present in 2.0 % of *A. platensis* biomass

**Incorporation of 10 % of *A. platensis* alginate microbeads with an amount of extract equal to the free extract formulation.

2.4.1 *A. platensis* extract encapsulation

Freeze-dried *A. platensis* extract was encapsulated within alginate microbeads through vibrational extrusion technique using the Büchi Encapsulator B-395 Pro® (Büchi Labortechnik AG, Flawil, Switzerland). Briefly, a 2.0 % (w/v) sodium alginate aqueous solution was prepared, and the freeze-dried extract (8% w/v) was added under stirring. The parameters selected were chosen based on the manufacturer's recommended conditions for air-flow configurations: inner nozzle size of 150 µm and outer nozzle of 600 µm, frequency 2000 Hz, electrode 1200 V, amplitude 2, airflow 0.8 mbar and flow rate of 1.5 mL/min.

The beads formed were collected into a 0.1 $mol.L^{-1}$ calcium chloride solution stirred at 500 rpm. After all the alginate solution was dispensed, the beads were left at a lower agitation rate (200 rpm) for 2 h to complete the hardening process. The resulting *A. platensis*-calcium alginate microbeads were retrieved by filtration using a 100-µm strainer and rinsed with distilled water. Before weighting for cookie dough incorporation, the water excess was removed off the microbeads with filter paper.

2.5 Dough characterization

241 2.5.1 Rheological analyses

242

243 Oscillatory dynamic measurements and creep-recovery tests were carried out in an
244 HR-1 rheometer (TA Instruments, USA) equipped with a stainless steel parallel plate
245 geometry (40 mm diameter, 1000 μm gap) within the linear viscoelasticity domain, in
246 duplicate. The samples were handled gently to avoid structural damage, and they were
247 allowed to rest for 3 min before analysis. Temperature-sweep profiles were performed in the
248 range of 25 °C-150 °C at 5 °C/min and 1 Hz. Complex modulus (G*) and tanδ were
249 evaluated.

250 Creep-recovery assays were carried out at 25 °C by applying constant stress (55 Pa)
251 for 360 s on the dough and allowing strain recovery for 600 s after load removal. The strain
252 was obtained as a function of time, and the data were represented by creep compliance:
253 $J(t)\ (Pa^{-1}) = \gamma/\sigma$, where γ and σ are the strain and constant shear stress during the creep
254 test, respectively. The creep compliance data of the dough samples were fitted with Burger's
255 model for creep and recovery stages (Eq. 2 and 3, respectively).

256

257 $$J(t)_c = J_0 + J_m\left(1 - exp\left(\frac{-t}{\lambda}\right)\right) + \frac{t}{\eta_0} \quad (2)$$

258

259 $$J(t)_r = J_{max} - J_0 - J_m\left(1 - exp\left(\frac{-t}{\lambda}\right)\right) \quad (3)$$

260

261 where J_0 (Pa−1), J_m (Pa−1), and J_{max} (Pa−1) represent the instantaneous, viscoelastic, and
262 maximum creep compliance values, respectively; t (s) and λ (s) are the phase and average
263 retardation time, respectively; and η_0 is the viscosity coefficient (Pa.s). The relative elastic
264 portion (%) was determined by the ratio between the equilibrium compliance and the
265 maximum compliance.

266

267 2.5.2 Texture measurement

268

269 The dough firmness was assessed by a uniaxial compression assay in a Texture
270 Analyser TA-HD plus Stable MicroSystem (Godalming, Surrey, UK). Cylindrical dough

samples (12 mm diameter, 10 mm height) were compressed at a speed of 1mm/sec, with trigger force of 5 g up to 90 % strain level. Results were expressed by the peak force in the force-time graph (N.s). Measurements were repeated five times for each formulation.

2.6 3D printing and post-processing

The cookies were produced using a 3D food printer (Focus, Byflow, Netherlands) equipped with a paste printing head and a 1.6 mm aperture nozzle. A cylinder shape (27.6 mm diameter and 6.72 mm height) was sliced (Slic3r software) to micro-extrude 6 layers, each one with 1.12 mm thickness, through a nozzle moving at a speed of 10 $mm.s^{-1}$. During printing, flow rate and Z-offset were adjusted to obtain the adequate dough weight and shape (diameter and thickness) according to the 3D model. Each formulation was printed at least in triplicate.

The printed cookies were baked at 150 °C for 25 min. Then, depending on the type of experiment, the cookies were analysed in triplicate (using three cookies) or in quintuplicate (using five cookies). Measurements of height and diameter were performed in three replicates, before and after post-processing, aiming to evaluate shape fidelity. This parameter was based on the differences between the cookies theoretical and measured dimensions, as described in Eq. 4. The effect of baking on the cookie's shape was determined as the percentual variation of the dimensions before and after baking (Eq. 5).

$$Shape\ fidelity\ (\%) = \frac{(Measured\ dimension * 100)}{Theoretical\ dimension} \quad (4)$$

$$Variation\ (\%) = \frac{[(Baked\ cookie\ dimension - Raw\ cookie\ dimension) * 100]}{Raw\ cookie\ dimension} \quad (5)$$

2.7 Cookies physical-chemical characterization

All cookies were analysed in terms of colour variation, water activity, texture and antioxidant potential, 24 h after baking and after 30 days of storage at room temperature, protected from light.

303 2.7.1 Colour analysis

304

305 The colour of cookies samples was measured using a colourimeter (CR-400; Konica
306 Minolta, Inc., Tokyo, Japan). The first colour measurement was acquired 24 h after baking to
307 ensure and appropriate cooling before the readings. The results were expressed in terms of
308 L^*, lightness (values from 0 to 100 %); a^*, redness to greenness (60 to −60, respectively); b^*,
309 yellowness to blueness (60 to −60, respectively), according to the CIELab system. The total
310 colour di□erence (ΔE^*) between sample cookies along storage time (30 days), as well as
311 between raw dough and cooked samples, was determined using L^*, a^* and b^* average values,
312 according to the equation 6. The measurements were conducted using a white standard (L^* =
313 93.90, a^* = 0.3158, b^*= 0.3321), under artificial fluorescent light at room temperature. Three
314 replicates were analysed for each formulation, with five measurement locations per cookie,
315 including the centre and its surrounding (Batista et al., 2017).

316

317 $$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{\frac{1}{2}} \quad (6)$$

318

319 2.7.2 Texture analysis

320

321 The cookies' texture was evaluated using a Texture Analyser TA-HDplus Stable
322 MicroSystem (Godalming, Surrey, UK) in penetration mode, with a 2 mm cylindrical
323 stainless probe, a target distance of 4 mm and speed test of 0.5 $mm.s^{-1}$. The resistance to
324 penetration (or hardness) was measured by the peak force in the force-time graph (N.s).
325 Measurements were repeated five times for each formulation sample (one measurement per
326 cookie).

327

328 2.7.3 Water activity determination

329

330 The cookies water activity (a_w) was determined using an Aqualab 4TE Water Activity
331 Meter (Meter Group, Inc., Pullman, USA) at 25 ± 0.5 °C. Measurements were repeated three
332 times for each formulation as a crushed powder.

333

334 2.7.4 Antioxidant activity

335

The antioxidant activity of the cookies was evaluated following the methods described in section 2.3 in triplicate; however, with results expressed per gram of cookie instead. For the extraction of cookies antioxidant fraction, aliquots of 0.5 g of the control, free and encapsulated extract cookies, previously milled with a mortar and pestle, were mixed with 2.5 mL of 75 mmol.L-1 phosphate buffer at pH 7.4 on a vortex for 1 min; and followed by centrifugation at 9,000 rpm for 10 min. This process was repeated twice, and supernatants were combined and filtered through a 0.45 µm syringe filter. Antioxidants from cookies containing *A. platensis* biomass were recovered by ultrasound-assisted extraction for 1 h, using 5 mL of 75 mmol.L-1 phosphate buffer at pH 7.4 as the solvent.

2.8 Statistical analysis

Statistical analysis of the experimental data was performed through the t-test or analysis of variance (one way ANOVA), followed by Tukey's Post Hoc test at a significance level of 95 % ($p < 0.05$), using the software GraphPad Prism 5.0. All results were presented as mean ± standard deviation. Design of Experiments (DoE) and its statistical analysis were performed using Statsoft Inc. Statistica™ (version 13).

3. Results and discussion

3.1 Optimization of *A. platensis* antioxidants extraction

For a practical application in the food industry, antioxidants should be first extracted; however, the extraction process efficiency may affect its availability (Wardhani, Vásquez, & Pandiella, 2010). Recently, ultrasonic-assisted extraction (UAE) has been widely employed for the recovery of target compounds from many natural products due to its facilitated mass transfer between immiscible phases, through super agitation at low frequency. The enhanced extraction obtained by ultrasounds is mostly attributed to the acoustic cavitation produced in the solvent by the passage of an ultrasound wave. Moreover, UAE also exerts a mechanical effect, allowing greater penetration of solvent into the cell wall, increasing the contact surface area between the solid and liquid phase. As a result, the solute quickly diffuses from the solid phase to the solvent, increasing bioactive recovery when compared to conventional methods

(Haque, Dutta, Thimmanagari, & Chiang, 2016; Kurd & Samavati, 2015; Liu, Wei, & Liao, 2013; Zou, Jia, Li, Wang, & Wu, 2013).

Various parameters play a significant role in optimizing the experimental conditions for the development of an extraction method. Extraction time, temperature and the solid-to-liquid ratio are generally considered to be the most critical factors that affect bioactive recovery. The choice of an extracting solvent is also a crucial step towards extraction optimization; different solvents will yield different extract amounts and composition. In the present study, water and ethanol were employed as extraction solvents for the microalga *A. platensis* considering food application safety (Chaiklahan et al., 2013; Zou et al., 2013).

The recovery of antioxidant compounds from *A. platensis* biomass was optimised through a Design of Experiments (DoE) approach, using a 2^k full factorial design with 2 factors. The effect of ethanol/water and biomass/solvent ratios on the antioxidant activity (ORAC and ABTS) and total phenolic content (TPC) were analysed as response variables (see Table 1). All the response curves exhibited an excellent fitting (r^2= 0.99) and statistical significance. The response surfaces after 30 min and 1 h of ultrasound treatment are represented in Fig. 1. It is possible to notice that the phenolic content increased proportionally with the amount of microalga. Additionally, increasing the treatment time to 1 h promoted a higher phenolic content, which was also higher *with increasing A. platensis* content. Nevertheless, the recovery of phenolic compounds was more efficient at 0 % ethanol independently of the time. The ABTS exhibited a similar trend; although in this case, the extraction time was crucial, being the antioxidant activity at 1 h more than 2.5-fold higher than at 30 min. The ORAC assay corroborated the ABTS results, pointing out that to obtain an extract with high antioxidant activity, the ultrasound treatment should be performed during 1 h with 2.0 % biomass and 0 % ethanol. After freeze-drying the liquid extract obtained in this condition, 0.4 g of dry antioxidant extract was obtained per gram of biomass. This extract was used for the cookie formulation experiments.

[Insert Fig. 1 here]

3.2 Cookie dough characterization

Cookies quality is influenced by several factors, such as the quality and amount of ingredients used, processing conditions and moulding of the dough, as well as baking and

cooling of the cookies. Among those factors, dough rheology is of considerable importance in cookies manufacture as it influences the dough machinability and the final sensorial characteristics. Doughs with extreme degrees of firmness or softness will not process satisfactorily on the dough forming equipment and will not yield adequate products (Manohar & Rao, 2002).

In this work, cookies were shaped through a 3D food printer, where the main physical properties involved can be divided into two categories: firstly the ones that affect the extruding process, which includes the flow behaviour and viscous modulus (*G''*) of the dough; and secondly, the factors which influence the ability to support the three dimensional structure of the printed products or to maintain its shape and structure, such as the elastic modulus (*G'*), gel strength, among others (Yang, Zhang, Prakash, & Liu, 2018).

Initially, cookies doughs were characterized through a creep-recovery test and texture analysis. During the creep-recovery assay, a stress is applied for a specific interval, it is then removed, and the recovery is monitored for another period. This property provides information about the ability of the sheared and micro-extruded food-ink to recover; therefore, the faster the recovery, the higher shape fidelity should be expected. Likewise, less strain during the test indicates a stronger ability of the material to maintain the shape and structure of printed products; which, however, will also require higher extrusion rates (Yang, Zhang, Fang, & Liu, 2019).

Figure 2a shows the creep–recovery curves expressed by a compliance variation (J) as a function of time, which is the ratio of the deformation γ to the applied stress τ. Dough deformation could be used to characterize its strength, which means the harder the dough, the higher the amount of energy required to achieve the same deformation when compared with a softer dough. Accordingly, if a material has a high compliance, it will present low rigidity and high deformation or strain; and, consequently, better extrudability (Ahmed, 2015). The cookie dough incorporated with encapsulated extract showed higher maximal strain or compliance with the applied stress, while the one prepared with microalgae biomass led to a more stiff dough (Table 3). The replacement of a small amount of flour by microalgae biomass resulted in the inclusion of a complex biological ingredient, rich in proteins and polysaccharides. These molecules have an important role in the water absorption process, which promote the increase of dough firmness (Bolanho et al., 2014; Gouveia, Batista, Miranda, Empis, & Raymundo, 2007; Gouveia et al., 2008). On the other hand, the encapsulated antioxidant extract dough led to an unstructured network due to the water

molecules present among the calcium-alginate microbeads, resulting in a reduction of its viscosity. Furthermore, the addition of the free antioxidant extract did not change the dough behaviour, matching with the one observed for the control cookies.

The creep and recovery stages were fitted to Burger's model (Eq. 2 and 3, respectively) and results are shown in Table 3. The instantaneous elastic compliance (J_{0C}), viscoelastic compliance (J_{1C}) and the steady viscosity (μ_0) corroborated with Figure 2a. Biomass cookie dough revealed high μ_0 and lower compliances, which means that the higher the steady-state viscosity, the higher the resistance to deformation. The opposite behaviour was observed for the encapsulated extract dough, which presented higher compliances and lower steady-state viscosity; therefore, less resistance to deformation.

Contrastingly, retardation time (λ) did not show the same behaviour, and only the biomass and the encapsulated extract doughs were significantly different ($p < 0.05$). However, this parameter agrees with the compliance results. The retardation time (λ) mainly indicates the time required for the sample strain to decay to the initial value at $1/e$, and the less stiff dough (encapsulated extract) showed higher (λ). The recovery phase exhibited similar behaviour, with elastic compliance (J_{0C}), viscoelastic compliance (J_{1C}) and the steady viscosity (μ_0) following the same tendency. More stiff doughs take longer to deform, and also to recover their structure. Nevertheless, despite the differences, the elastic portion of the dough did not differ significantly ($p < 0.05$) among all formulations.

[Insert Fig. 2 here]

456 Table 3. Creep-recovery analysis of the cookies doughs incorporated with different forms of *A. platensis.* Results are presented as mean ± standard deviation.

Dough	Creep						
	Compliance (1/Pa)		Retardation time (s) λ_{1C}	Coefficient of viscosity (Pa.s) η_0	Max. Strain (%)	Max. Compliance (1/Pa)	r^2
	J_{0C}	J_{1C}					
Control	0.0037^{b} ± 0.0002	0.0256^{b} ± 0.0009	29.46^{ab} ± 3.26	12169^{a} ± 11	3.09^{b} ± 0.06	0.056^{b} ± 0.001	0.99
Biomass	0.0017^{a} ± 0.0003	0.0111^{a} ± 0.0018	31.60^{b} ± 1.15	24343^{b} ± 4073	1.45^{a} ± 0.24	0.026^{a} ± 0.004	0.99
Free Extract	0.0039^{b} ± 0.0003	0.0265^{b} ± 0.0023	28.75^{ab} ± 1.56	11827^{a} ± 27	3.18^{b} ± 0.14	0.058^{b} ± 0.003	0.99
Encapsulated Ext.	0.0073^{c} ± 0.0007	0.0484^{c} ± 0.0037	22.29^{a} ± 1.48	7118^{a} ± 741	5.54^{c} ± 0.50	0.109^{c} ± 0.002	0.99
Dough	**Recovery**						
	Compliance (1/Pa)		Retardation time (s) λ_{1R}	Equilibrium strain (%)	Equilibrium compliance (1/Pa)	Relative elastic portion (%)	r^2
	J_{0C}	J_{1C}					
Control	0.0055^{b} ± 0.0002	0.0169^{b} ± 0.0011	87.00^{b} ± 6.40	1.82^{a} ± 0.01	0.033^{a} ± 0.0003	58.90^{a} ± 1.60	0.90
Biomass	0.0033^{a} ± 0.0006	0.0109^{a} ± 0.0018	102.70^{b} ± 5.20	0.65^{a} ± 0.11	0.012^{a} ± 0.002	44.60^{a} ± 0.20	0.93
Free Extract	0.0062^{b} ± 0.0002	0.0179^{b} ± 0.0000	85.50^{b} ± 2.70	1.80^{a} ± 0.15	0.033^{a} ± 0.003	56.70^{a} ± 2.20	0.95
Encapsulated Ext.	0.0115^{c} ± 0.0004	0.0294^{c} ± 0.0004	64.60^{a} ± 3.20	3.18^{b} ± 0.55	0.058^{b} ± 0.010	53.30^{a} ± 10.50	0.94

Concerning the texture analysis, all doughs incorporated with different forms of *A. platensis* presented significant differences ($p < 0.05$) when compared to the control. As it can be observed in Fig. 3, the biomass cookie dough showed higher hardness, which agrees with the creep-recovery results. Nevertheless, the dough incorporated with the free extract, which had similar rheological behaviour as the control, exhibited a low hardness value. This decrease of hardness could be attributed to the reduced flour mass and its substitution for components of the freeze-dried antioxidant extract. Those constituents may further bind with water molecules through hydrogen bonds, suppressing the water absorption and the gluten protein swelling. Consequently, the free extract dough displayed a lower network strength, comparable to the one with encapsulated extract (Liu, Liang, Saeed, Lan, & Qin, 2019).

[Insert Fig. 3 here]

The dough properties during the cooking process were evaluated through a temperature-sweep analysis and results revealed that the obtained profiles were consistent with that of the creep-recovery test. Figure 2b and 2c show the viscoelastic properties (complex modulus and tanδ) against temperature increase. G^* reflects both contribution of elastic (G') and viscous (G'') moduli; while tanδ, which character prevail, is defined as G''/G'. In the mechanical spectra, tanδ was lower than 1 ($G' > G''$) for all temperatures, indicating that all doughs behave as a gel-like material. The dough incorporated with *A. platensis* biomass exhibited higher G^* values, as the biomass composition promotes a more structured network. Oppositely, the encapsulated extract dough presented lower G^* values due to its reduced viscosity. Nonetheless, tanδ spectra did not show great differences, as the proportion between elastic and viscous moduli was similar for all formulations.

Moreover, throughout the temperature-sweep profile it is also possible to detect a continuous decrease in G^* up to 40 °C, which may be related to the melting of the butter that accounts for a significant part (25 g/100 g) of the dough, as equally observed by Kim et al. (2019). Afterwards, the modulus remains almost constant until 105-110 °C, when it starts to increase. Nevertheless, this phenomenon occurred at a lower temperature for cookies incorporated with encapsulated extract (~ 90 °C). When the temperature rose to above 120 °C, a further decrease of G' and G'' was detected. It is inferred that the pyrolytic

decomposition and leaching of the amylose in flour starch granule were induced, which may have caused the corresponding decrease (Kim et al., 2019).

3.3 3D printing and post-processing

3D food printing is a digital manufacturing technology, which is used to fabricate three-dimensional structures in a layer-by-layer manner, using liquid-, gel-, or powder-type food materials as a printing medium; it includes three steps: modelling, 3D printing and post-processing. A range of 3D printing methods have been utilized for food printing, such as selective laser sintering/hot air sintering, hot-melt extrusion/room temperature extrusion, binder jetting, and inkjet printing (Holland, Tuck, & Foster, 2018; Sun, Zhou, Huang, Fuh, & Hong, 2015). Among them, extrusion-based 3D food printing is the most widely adopted method, which consists of a material being extruded through a nozzle moving in *x*-, *y*- and *z*-direction, building up a structure layer-by-layer (Kim et al., 2019; Sun et al., 2018).

For a successful 3D printing step, it is required a material which can be smoothly extruded through the nozzle and, at the same time, can support the weight of the subsequent printed layers without deformation. In this context, the knowledge of the material's rheological and mechanical profiles is imperative to achieve proper extrudability and structure stability during the process (Liu, Zhang, Bhandari, & Yang, 2018b; Wang, Zhang, Bhandari, & Yang, 2018; Yang et al., 2018). Furthermore, 3D food printing is not only affected by the physicochemical properties of the ingredients used, but also by the process parameters, such as nozzle moving speed, extrusion rate, nozzle diameter, and layer and nozzle heights. This correlation between the food formula attributes and the operational conditions influences the printing precision and, thus, is a key factor in the end-product quality (Dankar et al., 2018; He, Zhang, & Fang, 2019; Pérez et al., 2019).

Lastly, the printed food pieces may require a further post-deposition cooking process (e.g. baking and boiling), which involves different levels of heat penetration in the food matrix; resulting in texture modifications and, possibly, misshapen structures (Dankar et al., 2018; Sun et al., 2018). Cookie dough is a material that unavoidably requires post-processing; however, it rapidly deforms after baking. Kim *et al.* (2019) have studied the addition of hydrocolloids as a structuring agent, aiming to suppress product deformation in the high-temperature environment of the post-processing step, whereas maintaining the ingredients and product characteristics of the desired cookie. Xanthan gum in the concentration of 0.5 %

was reported to promote the best shape accuracy of the selected 3D printed model after the baking process; therefore, it was chosen to develop the functional cookies of this work.

3D food printing is a tool which allows the creation of unique, innovative, products that other methods cannot emulate. Among numerous applications, this technology is becoming popular due to the possibility to design foods with appealing forms, new textures and personalized nutritional values, where several raw materials can be blended according to individual's physical and nutritional status. Additionally, this 3D printing flexibility enables the use of alternative ingredients in food processing, such as microalgae, insects and fungi; in the production of tasty, healthy and tailored foods (An, Guo, Zhang, & Zhong, 2019; Godoi, Prakash, & Bhandari, 2016; Lille, Nurmela, Nordlund, Mets€a-Kortelainen, & Sozer, 2018; Severini, Azzollini, Albenzio, & Derossi, 2018; Voon, An, Wong, Zhang, & Chua, 2019).

Fig. 4 shows the 3D printed cookies incorporated with different forms of the microalga *A. platensis* right after the 3D printing step and after the post-processing treatment.

[Insert Fig. 4 here]

All 3D printed cookies presented shape fidelity in the range of 100 ± 5.0 %, demonstrating dimensional consistency with the 3D model (Fig. 5a). The effect of the cooking process over the cookies is showed in Fig. 5b. Upon baking, the cookie thickness varied from 3.37 % for the biomass cookies, until 18.22 % for the encapsulated extract cookies. The increase in this dimension is related to the gas production from the water vaporization and a higher dough elasticity, which explains the fact that the cookies incorporated with fresh alginate microbeads have an enlargement superior to the other formulations, corroborating the results obtained in the rheology analyses (Chevallier, Della Valle, Colonna, Broyart, & Trystram, 2002).

Oppositely, the diameter of the cookies had a smaller variation after the post-processing treatment, as it can also be observed in Fig. 5b. Except for the encapsulated extract cookies, which suffered a shrinkage of around 7 % probably due to the high water loss, all the other formulations suffered a positive influence of the temperature during baking. The heat promotes the melting of fat, which confers plasticity and an ease flow, resulting in

an initial spreading followed by a width retraction at the end of the process (Walker, Seetharaman, & Goldstein, 2012).

[Insert Fig. 5 here]

3.4 Cookies physical-chemical characterization

A number of parameters can be scrutinized from baked cookies that are of crucial importance to determine their adequacy. Colour is an attribute which impacts food quality, contributing to consumer's attraction to a product. The incorporation of *A. platensis* in different forms into cookie doughs stimulated a decrease in luminosity when compared to the control, which was more prominent in the biomass and free extract cookies (Fig. 6). Biomass cookies were distinguished by the microalga green tonality, showing negative values of a^*; while the free extract cookies tended to the blue colour, represented by the C-phycocyanin distinctive pigmentation (Lucas, Morais, Santos, & Costa, 2018).

[Insert Fig. 6 here]

Table 4 presents the total colour differences (ΔE^*) between the raw dough and baked cookies, as well as the differences for each formulation over 30 days of storage time. *A. platensis* cookies showed significantly colour variation upon baking, with ΔE^* varying from 17.47 to 25.50. This outcome may be explained primarily by the browning of cookie surface (lightness decrease and colour parameter increase), possibly due to formation of Maillard reaction products (MRP) through the interaction of reducing sugars with proteins, but also possibly owing to starch dextrinization and sugar caramelization. Moreover, changes in tonality may be related to pigment loss upon exposure to high temperatures (Batista et al., 2017; Chevallier et al., 2002).

On the other hand, along the conservation time, all cookie formulations presented a low ΔE^*, principally the encapsulated extract cookie. According to Mokrzycki & Tatol (2011), ΔE^* values between 1 and 2 indicate that only experienced observers can notice a

588 colour difference, and values between 2 and 3.5 suggest that an inexperienced observer is
589 also able to see the difference. Hence, it is possible to conclude that the encapsulation of *A.*
590 *platensis* extract improved the cookie colour stability during the 30 days of storage time,
591 when compared to other formulations.
592
593 Table 4. Total colour variation (ΔE^*) between cooked and raw cookie samples and colour stability
594 along conservation time.

Total colour difference (ΔE^*)	**Raw *vs.* Baked**	**24 h *vs.* 30 days**
Control	9.71	2.85
Biomass	25.50	2.43
Free Extract	25.29	2.12
Encapsulated Extract	17.47	1.30

595
596 Another essential physical stability factor, which gives an identity to a food product,
597 is the texture (Carter, Galloway, Campbell, & Carter, 2015). In this work, the cookie's texture
598 was evaluated through a penetration test, and the results are represented in Fig. 7. As it has
599 been stated for the cookie dough texture analysis (see section 3.2), the addition of *A. platensis*
600 biomass promoted an increase in the cookie hardness. Inversely, the incorporation of
601 microalgae antioxidant extract in fresh alginate microbeads resulted in a softer texture, which
602 it was expected considering the water molecules present among the microparticles. Finally,
603 over the 30 days of storage time, there was no significant difference ($p > 0.05$) in the cookie
604 texture for all the developed formulations.
605 The incorporation of *A. platensis* into cookies by conventional methods has been
606 described in the literature by a few authors, promoting different effects on the final product
607 texture. Onacik-Gür and co-workers (2018) evaluated the addition of 1 %, 2 % and 3 % of
608 microalga powder and reported a decrease in the cookies hardness with the increase of
609 microalga concentration. On the other hand, Marcinkowska-Lesiak and co-workers (2017)
610 incorporated different powder amounts of the same species into shortbread biscuits, which
611 promoted an increase in the cookie hardness directly proportional to the addition of microalga
612 powder; therefore, corroborating with the results found for the 3D printed cookies of this
613 study.
614 Concomitantly with the food texture, water activity (a_w) is also a significant parameter
615 regarding the conservation of low moisture cookies, particularly for the maintenance of a

crispy texture. Furthermore, the food physical-chemical and microbiological stability depend greatly on the water content and its interaction with food ingredients. Water activity is a measure of the availability of water molecules to enter into microbial, enzymatic or chemical reactions. Therefore, this parameter has been used to assess the potential microbial growth and chemical stability of foods after manufacture. It is established that bacteria do not grow at a_w values of 0.80 or below, while the limit for mould and yeast growth is 0.6 (Hough. Buera, Chirife, & Moro, 2001; Khouryieh & Aramouni, 2012). As Fig. 7 shows, all cookie formulations presented a_w values below 0.3 throughout the 30 days of storage, indicating high microbiological stability.

[Insert Fig. 7 here]

3.4.1 Antioxidant activity

The microalga *A. platensis* is recognized to have notable free radical scavenging properties and antioxidant activity, due to the presence of natural pigments and other bioactive compounds in its composition. Its light-harvesting protein-pigment complexes called phycobilisomes are composed by phycobiliproteins, where C-phycocyanin and allophycocyanin are considered the most important ones. Moreover, this microalga contains phenolic compounds and a spectrum of natural mixed carotene and xanthophyll phytopigments that, together with phycocyanin, seem to be related to its distinguished antioxidant activity (Batista et al., 2017; Zaid, Hammad, & Sharaf, 2015).

The 3D printed cookies developed in this work had their antioxidant capacity evaluated through the ORAC and ABTS assays after the post-processing step and after 30 days of storage (Fig. 8). After the storage period, the encapsulated extract cookie exhibited a significantly higher ($p < 0.05$) ORAC value compared to all other formulations, showing its improvement against processing and environmental factors. On the other hand, no significant difference between the formulations was found for ABTS assay. One possible explanation for this discrepancy could be due to the differences in the mechanism of action of those antioxidant analyses. The ORAC assay measures the affinity of antioxidative compounds to neutralize the free radicals over a period of time, accounting for any potential lag phases in antioxidant activity rather than providing a measurement of only fast acting antioxidants;

whereas the ABTS assay neutralize free radicals at a particular point of time without accounting for slow-acting antioxidants (Nayak, Liu, & Tang, 2015).

Additionally, it is noticeable that antioxidant capacity was also found for the cookies with no incorporation of *A. platensis* (control). Cookies are usually prepared with reducing sugars and a protein source, which leads to the formation of MRPs. Those compounds are one of the main responsible for the browning process characteristic of many foods, and it has been associated to an increase of their antioxidant potential (Nooshkam, Varidi, & Bashash, 2019; Yalmaz & Toledo, 2005).

According to Manzocco et al. (2001), it can be stated that in the development of the Maillard reaction, there is a positive correlation between colour and antioxidant properties. This correlation was found in foods where Maillard reaction was the sole or the prevalent event related to the antioxidant activity. Such circumstance generally occurs in food products with no or low content of naturally occurring antioxidants; which means that eventual changes in the antioxidant capacity upon processing are only due to the formation of heat-induced antioxidants. Given that, the antioxidant capacity found for the control cookies could be explained by the formation of MRPs, as antioxidant compounds are absent in its composition.

[Insert Fig. 8 here]

4. Conclusions

The microalga *A. platensis* was used as a source of antioxidants in the development of 3D printed cookies, based on functional food-inks. The antioxidant extraction was optimized through a DoE approach. Optimal conditions were 1 h extraction, with 0 % ethanol and 2.0 % biomass. All cookie dough formulations were suitable for extrusion, forming a homogenous filament with a diameter close to the nozzle aperture, and presenting dimensional consistency with the 3D model after the post-deposition step. Furthermore, the fortification with *A. platensis* resulted in 3D printed cookies with an innovative appearance. The encapsulation of the antioxidant extract was capable of improving the antioxidant activity and colour stability along the storage time when compared to all other formulations. Therefore, cookies developed in a 3D printer could be considered a promising alternative for the incorporation of

new ingredients, such as microalgae, to obtain a novel functional food with antioxidant properties.

Conflict of interest

The authors declare no conflict of interest.

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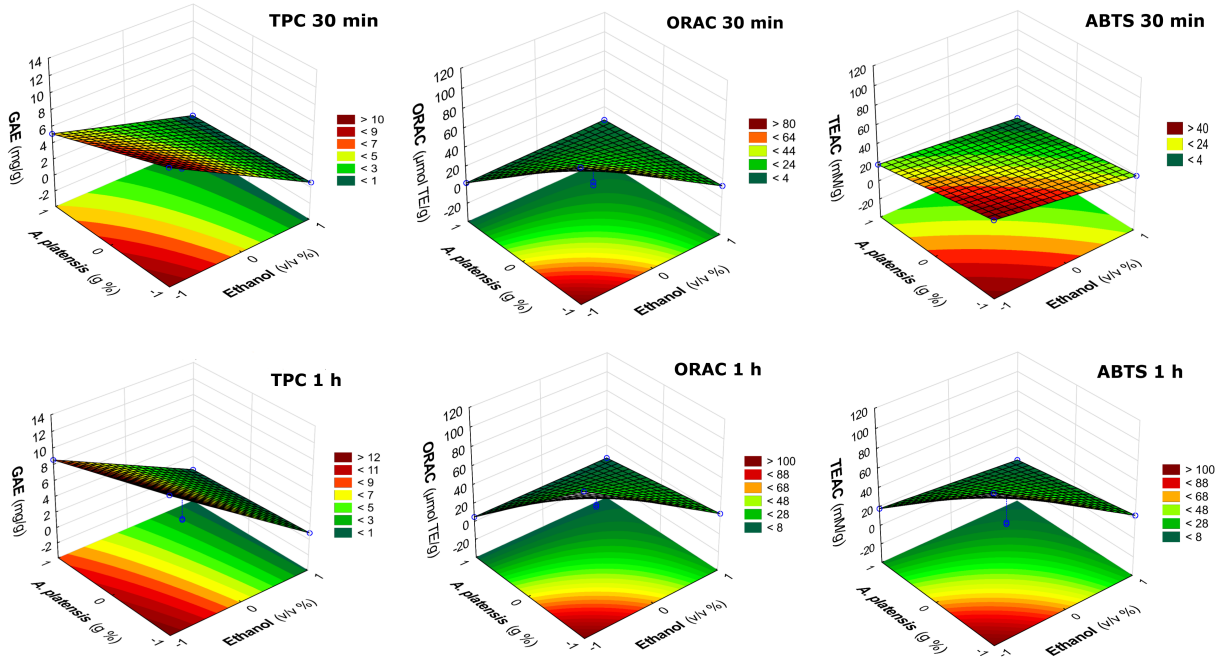
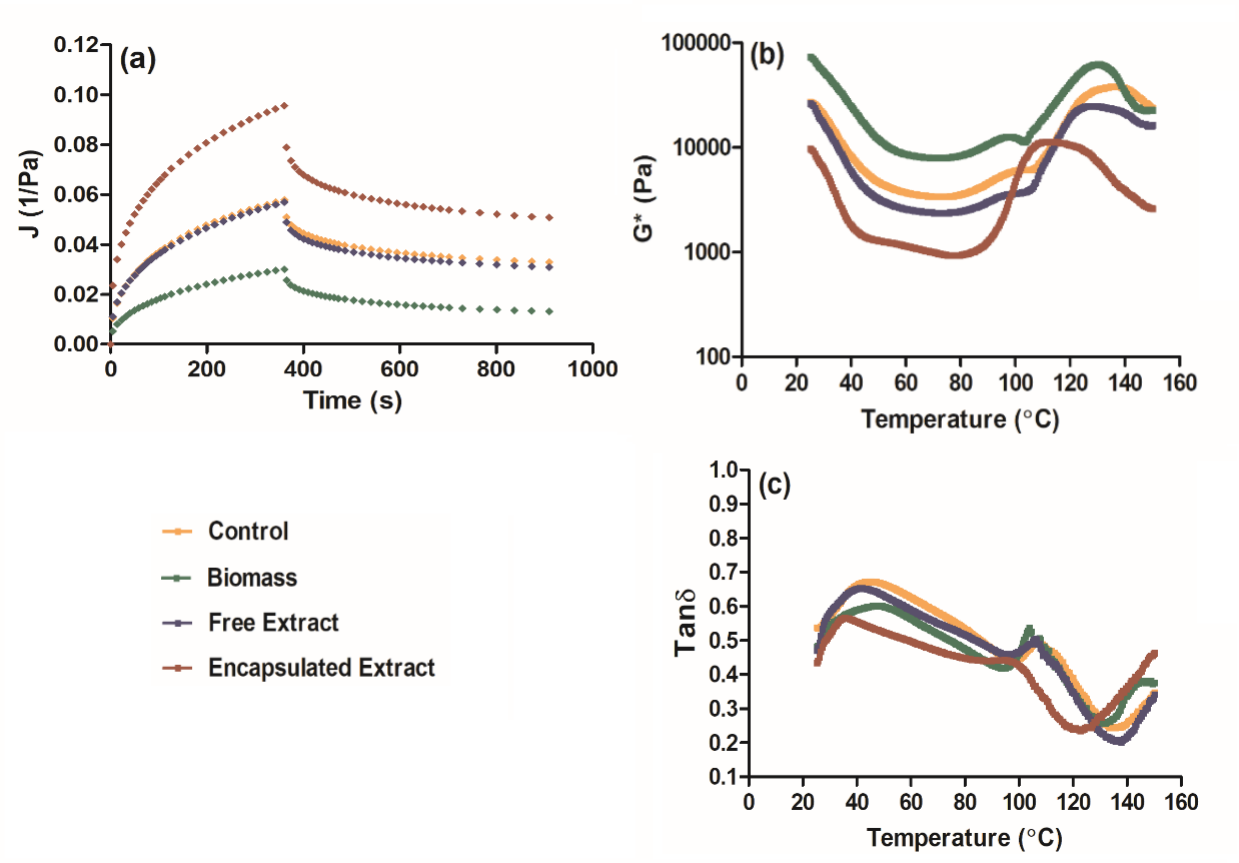
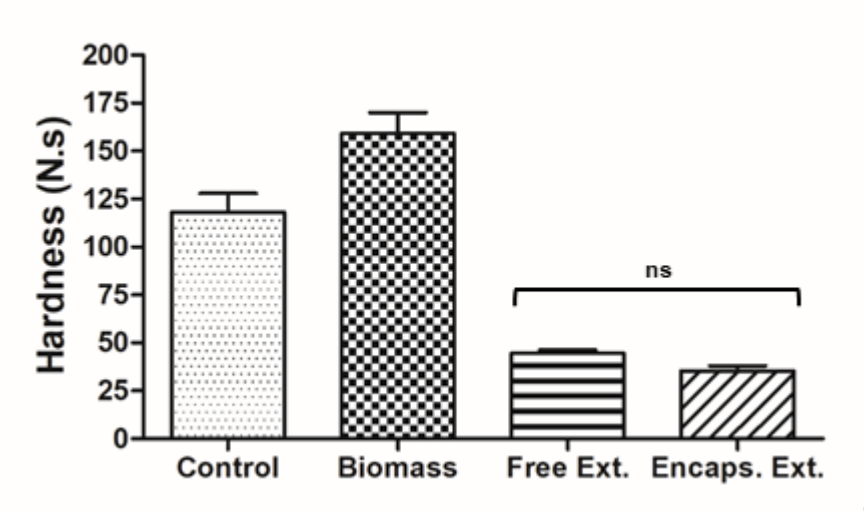
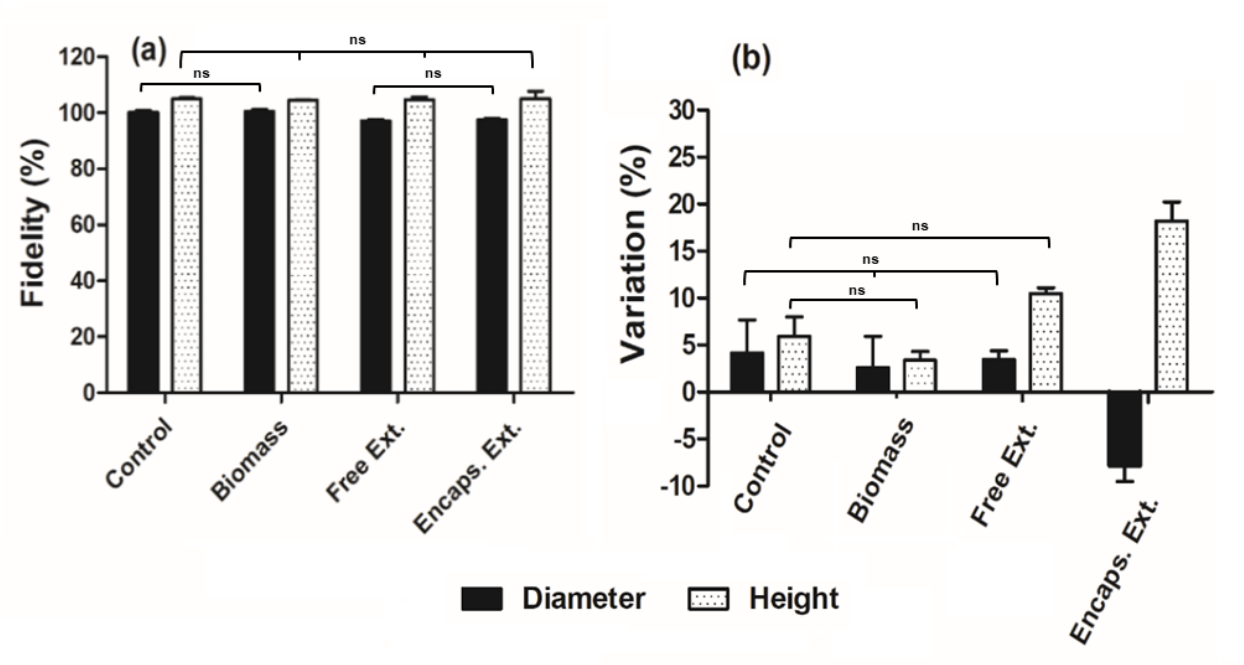
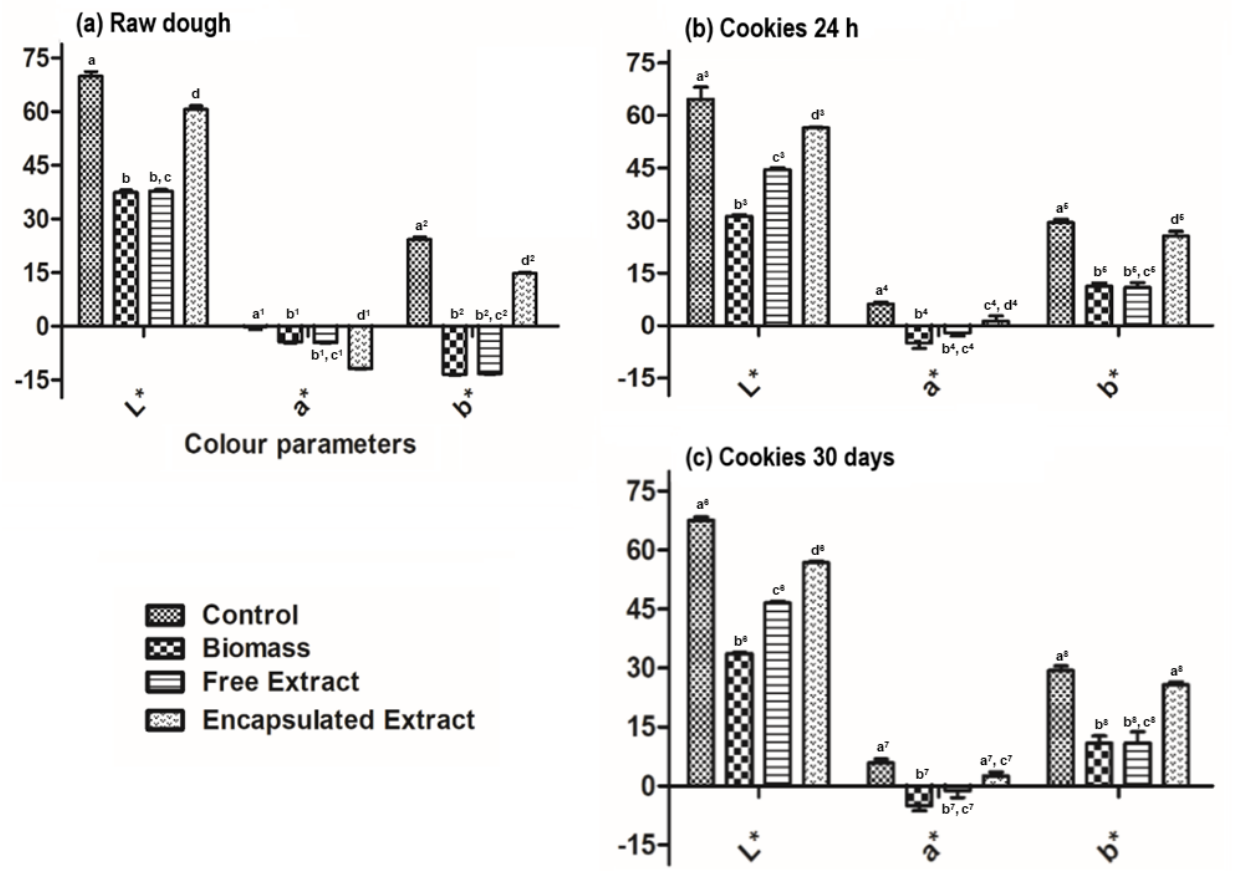
977 **Figure Captions**
978
979 Fig. 1. Response surfaces of the biomass and ethanol concentrations combined effect in *A.*
980 *platensis*' antioxidant activity and TPC.
981
982 Fig. 2. Dough rheology analyses. (c) Creep-recovery curves and temperature-sweep profiles
983 represented by G^* (b) and tanδ (c) against temperature.
984
985 Fig. 3. Texture analysis of the cookie doughs incorporated with different forms of *A.*
986 *platensis.* Results are presented as mean ± standard deviation. The term "ns" denotes a not
987 statistically significant difference.
988
989 Fig 4. 3D printed cookies incorporated with different forms of *A. platensis*. First row: after
990 the 3D printing step; second row: after the baking process.
991
992 Fig 5. (a) Shape fidelity of the raw 3D printed cookies and (b) Effect of the cooking process
993 on the 3D cookies measures. Results are presented as mean ± standard deviation. The term
994 "ns" denotes a not statistically significant difference.
995
996 Fig. 6. Colour parameters L^*, a^* and b^* for the raw cookie doughs and for the baked cookies
997 after 24 h and 30 days of storage. a) Raw dough; b) baked cookies after 24 h and c) baked
998 cookies after 30 days. Results are presented as mean ± standard deviation. For results with
999 the same letter, the difference between the means is not statistically significant.
1000
1001 Fig. 7. Texture (a) and water activity (b) analyses of the 3D printed cookies incorporated with
1002 different *A. platensis* forms over 30 days of storage time. Results are presented as mean ±
1003 standard deviation. The label "*" denotes a statistically significant difference.
1004
1005 Fig 8. Antioxidant activity of the 3D printed cookies incorporated with different *A. platensis*
1006 forms over 30 days of storage time. Results are presented as mean ± standard deviation. The
1007 label "*" denotes a difference statistically significant with respect to other formulations at the
1008 same sampling time (30 days).
1009

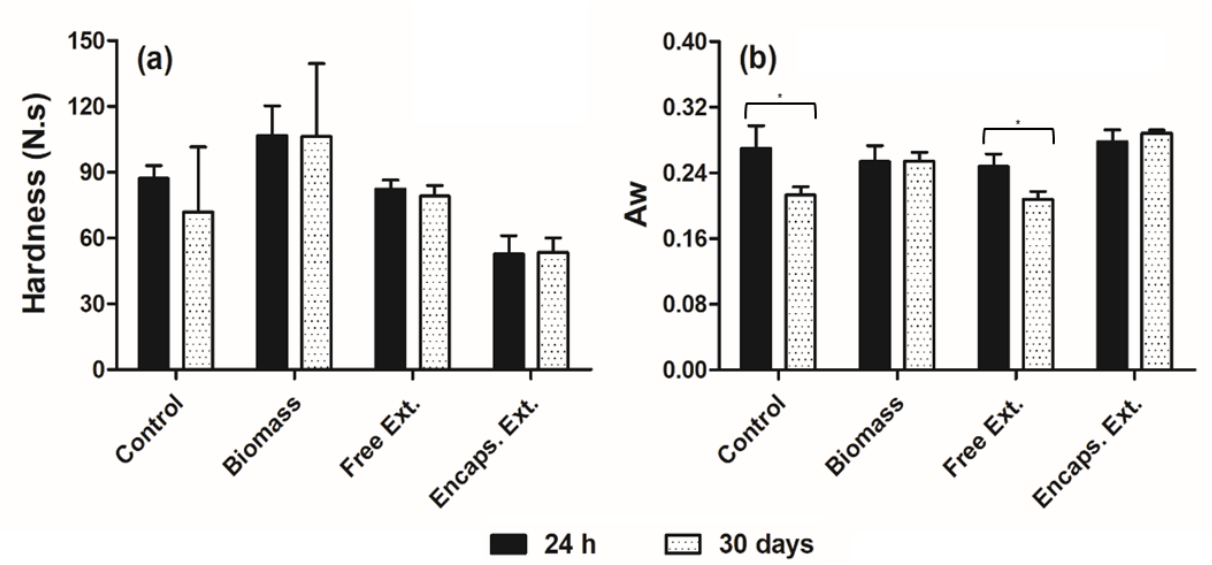


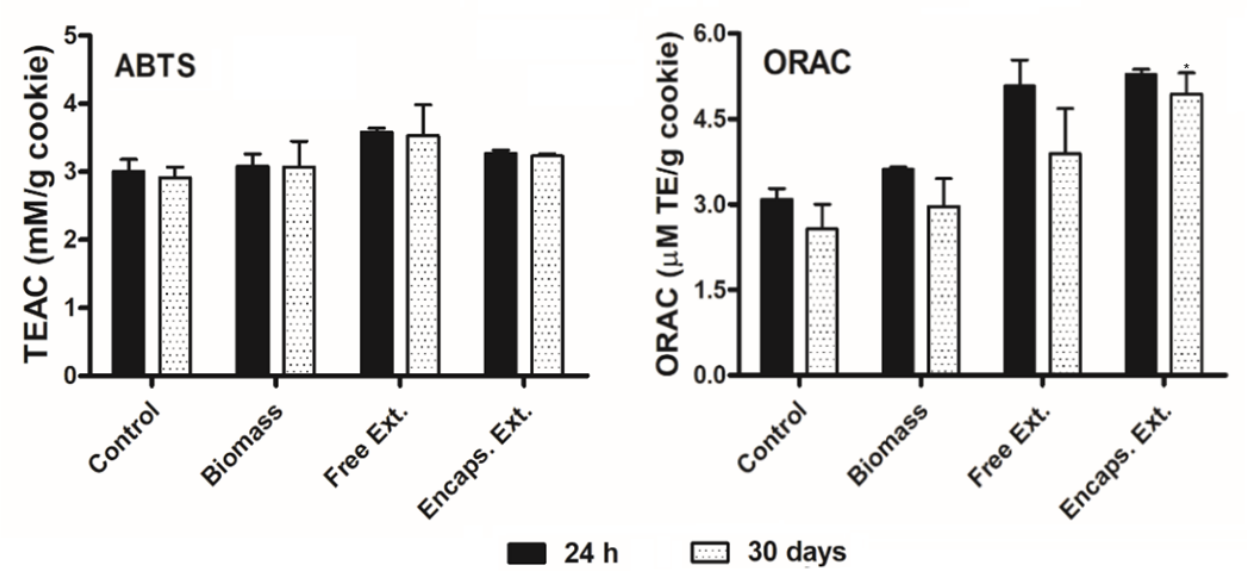

(a)
J (1/Pa)
Time (s)
(b)
G* (Pa)
Temperature (°C)
(c)
Tanδ
Temperature (°C)
Control
Biomass
Free Extract
Encapsulated Extract






(a) Raw dough
(b) Cookies 24 h
(c) Cookies 30 days
Colour parameters
L*
a*
b*
Control
Biomass
Free Extract
Encapsulated Extract





- Antioxidant extraction from the microalga A. platensis was optimised using DoE
- Edible inks were made using encapsulated microalgae extracts and food hydrocolloids
- All cookie dough formulations (edible inks) were suitable for 3D food printing
- 3D printed cookies exhibited colour, texture and microbiological stability over time
- Extract encapsulation improved cookies antioxidant potential and colour stability

INTERNATIONAL IBERIAN NANOTECHNOLOGY LABORATORY

Braga, September 29th 2019

Prof. P. A. Williams,
Editor-in-Chief of Food Hydrocolloids

Dear Prof. Williams,

I, hereby represent the authors of the manuscript "*3D printed functional cookies fortified with Arthrospira platensis: evaluation of its antioxidant potential and physical-chemical characterisation*" to be considered for publication in Food Hydrocolloids as an original article in the Special Edition of the 20th Gums and Stabilisers for the Food Industry Conference.

We declare that the content of our manuscript is original and that it has not been published or accepted for publication, either in whole or in part, in any form (other than an abstract or other preliminary publication). Moreover, all authors have approved the final version of this article, and none of them have any conflict of interest regarding this study.

Yours sincerely,
on behalf of the authors,

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