ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

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Lista de Autores

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Sumário

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1. Identificação

Deliverable	E3.3.1. Relatório de procedimentos de incorporação de ingredientes corantes, na forma livre ou estabilizada, nos produtos de panificação e pastelaria
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A lista de publicações que compõem este entregável são as que se apresentam de seguida, anexas ao entregável:

- Anthocyanin-rich extract of jabuticaba epicarp as a natural colorant: optimization of heat- and ultrasound-assisted extractions and application in a bakery product. *Food Chemistry*, 316, 126364.

- Extracts from *Vaccinium myrtillus* L. fruits as a source of natural colorants: chemical characterization and incorporation in yogurts. *Food & Function*, 11, 3227.

- Characterization and application of pomegranate epicarp extracts as functional ingredients in a typical Brazilian pastry product. *Molecules*, 25, 1481.

- Betacyanins from *Gomphrena globosa* L. flowers: incorporation in cookies as natural colouring agentes. *Food Chemistry*, 328, 127178.

- *Ficus carica* L. and *Prunus spinosa* L. extracts as new anthocyanin-based food colorants: a thorough study in confectionery products. *Food Chemistry*, 333, 127457.

- Study on the potential application of *Impatiens balsamina* L. flowers extract as a natural colouring ingredient in a pastry product. *International Journal of Environmental Research and Public H*ealth, 18, 9062.

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Anthocyanin-rich extract of jabuticaba epicarp as a natural colorant: Optimization of heat- and ultrasound-assisted extractions and application in a bakery product

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ABSTRACT

Heat- and ultrasound-assisted extractions of anthocyanins from jabuticaba epicarp were optimized and the colouring potential of the developed extract was tested on macarons. The independent variables time (*t*), solvent concentration (*S*), and temperature (*T*) or power (*P*) were combined in a five-level central composite design coupled with response surface methodology. The delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside levels monitored by HPLC-DAD-ESI/MS were used as response criteria. The developed models were successfully fitted to the experimental data and used to determine optimal extraction conditions. HAE was the most efficient method yielding 81 ± 2 mg/g extract under optimal conditions (t = 21.8 min, T = 47.1 °C and S = 9.1% ethanol, *v/v*). Macarons were then produced using the optimized anthocyanin-rich colouring extract and their colour parameters and nutritional profile were monitored during shelf-life. The obtained results provided useful information for the development of anthocyanin-rich extracts from a bio-waste with potential use as natural food colorants.

1. Introduction

Colour is the main sensory aspect in foodstuff and an important requisite to consumer choice. The loss of natural colour in food during processing and storage and the search for more attractive aspect products are factors responsible for the use of colour additives by the food industry. For this finality, artificial colorants are preferred because they present higher stability, colouring power and are cheaper than natural colorants (Martins, Roriz, Morales, Barros, & Ferreira, 2016).

In recent decades there has been a growing concern about the potential risks of artificial colours to human health, which has lead the modern food industry replace these kinds of additive by alternative natural colorants, which, in addition to colour power, can also provide bioactive proprieties to formulated products (Basu & Kumar, 2015b, 2015a; Martins & Ferreira, 2017; Masone & Chanforan, 2015). For this reason, obtaining natural pigments for used as natural colorants has become an emerging research area. In addition, the valorisation of bio-residues has been of great interest for the production of value-added colorant compounds for use by various industries, such as nutraceutical and food (Martins & Ferreira, 2017), being a sustainable approach that allows meeting current needs of the industry and the demands of society without causing a defect in food demand.

Jabuticaba (*Myrciaria jaboticaba* (Vell.) Berg.) is a native Brazilian berry that is not usually consumed and generates high amount of residue during the manufacture of jabuticaba pulp-based products. The fruit has a purple-dark thick epicarp with a high amount of anthocyanins (Albuquerque et al., 2020). Anthocyanins are flavonoids that showed attractive colours, ranging from red to purple, and are allowed for use as food colorants by EFSA with the E163 code (EFSA, 2013). In addition to colour, anthocyanins have also gained attention due to their potential health benefits, such as diabetes control and prevention of cardiovascular diseases and neurological disorders (Leong, Show, Lim, Ooi, & Ling, 2018; Li, Wang, Luo, Zhao, & Chen, 2017; You et al., 2018).

The recovery of anthocyanins from natural sources, such as fruits and flowers, is not always easy and feasible for application on an

Abbreviations: C3G, cyanidin-3-*O*-glucoside; CCD, central composite design; D3G, delphinidin-3-*O*-glucoside; *P*, power; *S*, solvent proportion; *T*, temperature; *t*, time; TAC, total anthocyanin content; RSM, response surface methodology

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Table 1
Natural and coded values of the independent variables used in the 5-level central composite designs (CCD) implemented for optimization of heat- and ultrasound-assisted extraction methods using the response surface methodology (RSM).

Coded values	Natural values					
	Heat-assisted extraction (HAE)			Ultrasound-assisted extraction (UAE)		
	Time X_A (min)	Temperature X_B (°C)	Solvent proportion X_C (%, *v/v*)	Time X_A (min)	Power X_B (W)	Solvent proportion X_C (%, *v/v*)
−1.68	5	20	0	5	100	0
−1	21.2	34.2	20.3	9.1	181.1	20.3
0	55	45	50	15	300	50
1	68.8	75.8	79.7	20.9	418.9	79.7
1.68	85	90	100	25	500	100

industrial scale, since the extraction of these compounds is a delicate process due to their poor stability. High temperature, pH change, and presence of light are some factors that can lead to their degradation. Among them, temperature and pH are among the most relevant factors during processing (Ngamwonglumlert, Devahastin, & Chiewchan, 2017; Pinela et al., 2019). Nevertheless, applying heat to an extraction process is important to promote mass transfer phenomena, which increases process efficiency (Albuquerque et al., 2017). Therefore, the recovery of these compounds is very specific and requires a study to control the variables that can influence the process efficiency. A way around the extraction limitations of these compounds is by combining the variables or factors that provide the maximum yield of the tangent compound under viable experimental conditions. Also, the choice of an appropriate extraction method is crucial to the success of the process (Backes et al., 2018; Pinela et al., 2019). The response surface methodology (RSM) has been proposed to provide information regarding the optimal combination of extraction factors to obtain natural colorants from plant origin (Backes et al., 2018; López et al., 2018; Ordóñez-Santos, Pinzón-Zarate, & González-Salcedo, 2015; Pinela et al., 2019), and several methods have been described for anthocyanins recovery, such as maceration (Ćujić et al., 2016), heat-assisted extraction (HAE) (Backes et al., 2018; Khazaei, Jafari, Ghorbani, & Kakhki, 2016; López et al., 2018), ultrasound-assisted-extraction (UAE) (Backes et al., 2018; Espada-Bellido et al., 2017; He et al., 2016; López et al., 2018; Pinela et al., 2019), microwave-assisted extraction (Backes et al., 2018; Zou et al., 2012), accelerant-solvent extraction (Cai et al., 2016), and supercritical fluid extraction (Maran, Priya, & Manikandan, 2014). However, the specificities of each matrix have limited the extrapolation of condition conditions to the different anthocyanin sources. For example, UAE was more effective than HAE in the extraction of anthocyanins from *Hibiscus sabdariffa* calyces and *Ficus carica* L. peels (Backes et al., 2018; Pinela et al., 2019), but for *Arbutus unedo* L. fruit, HAE was the best method when compared to UAE (López et al., 2018).

This study was carried out to develop an anthocyanin-rich extract from jabuticaba epicarp, namely: i) by optimizing the extraction by the HAE and UAE methods using RSM; and ii) by validating the colouring capacity of the extract obtained under optimized conditions by incorporating it into a food matrix, namely macarons. A schematic representation of the different steps carried out in this work is illustrated in Fig. S1.

2. Material and methods

2.1. Plant material

Jabuticaba (*Myrciaria jaboticaba* (Vell.) O. Berg.) fruits were acquired from CEAGESP (*Companhia de Entrepostos e Armazéns Gerais de São Paulo*), Brazil, through the Unidos distributor. The fruits were washed and the epicarp was manually separated from the pulp. The epicarps were then frozen at −20 °C and lyophilized at 10 μHg and −55 °C (Enterprise I, Terroni, São Carlos, SP, Brazil) in the Instituto Mauá de Tecnologia in São Caetano do Sul, Brazil, and sent under the best condition to the Centro de Investigação de Montanha in Bragança, Portugal, where they were reduced to a fine and homogeneous powder (~20 mesh) and stored at −20 °C until analysis.

2.2. Standards and reagents

HPLC-grade formic acid and acetonitrile were supplied from Fisher Scientific (Lisbon, Portugal). The standards cyanidin-3-*O*-glucoside, fructose, glucose, sucrose, and melezitose were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade and purchased from common suppliers. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Experimental design for extraction optimization

A five-level central composite design (CCD) coupled with RSM was implemented to optimize the extraction of anthocyanins from jabuticaba epicarps. The coded and natural values of the independent variables X_A (time: *t*, min), X_B (temperature: *T*, °C, or power: *P*, W), and X_C (solvent proportion: *S*, % ethanol, *v/v*) are presented in Table 1. The 20 experimental points of the CCD design shown in Table 2 were generated using Design-Expert software, Version 11 (Stat-Ease, Inc., Minneapolis, USA) by entering factor ranges in terms of alphas ($\alpha = 1.68$). This rotatable design included 6 replicated centre points and a group of axial points chosen to allow rotatability, which ensures that the variance of the model prediction is constant at all points equidistant from the centre point of the design. The experimental runs were randomized to minimize the effects of unexpected variability in the observed responses.

2.4. Extraction methods

2.4.1. Heat-assisted extraction

The heat-assisted extraction (HAE) was performed in a thermostated water bath using sealed vessels to avoid solvent evaporation. Ethanol was used in the extractions due its low toxicity and efficiency in the recovery of phenolic compounds, including anthocyanins (Boulekbache-Makhlouf, Medouni, Medouni-Adrar, Arkoub, & Madani, 2013). Powdered jabuticaba epicarps (1 g) were mixed with 20 mL of solvent (ethanol: water mixtures acidified with citric acid at pH 3) and processed under continuous magnetic stirring at 500 rpm, according to the experimental design matrix in Table 2, where different levels of *t* (5–85 min), *T* (20–90 °C) and *S* (0–100%) are combined. The solid/liquid ratio (*S/L*) was kept at 50 g/L. After processing, the mixture was centrifuged at 480*g* for 10 min and the supernatant was carefully collected and stored at −80 °C until analysis.

2.4.2. Ultrasound-assisted extraction

The ultrasound-assisted extraction (UAE) was performed using an

Table 2
Experimental results obtained under the extraction conditions defined in the central composite design (CCD) matrix for extraction yield and contents of delphinidin-3-*O*-glucoside (D3G), cyanidin-3-*O*-glucoside (C3G), and both anthocyanins (TAC) as a function of the extraction method. The natural values of the independent variables X_A (time), X_B (temperature or power) and X_C (solvent proportion) are presented in Table 1.

Run	CCD design			Heat-assisted extraction (HAE)				Ultrasound-assisted extraction (UAE)			
	Coded values			Yield	D3G	C3G	TAC	Yield	D3G	C3G	TAC
	X_A	X_B	X_C	%	mg/g E	mg/g E	mg/g E	%	mg/g E	mg/g E	mg/g E
1	−1	−1	−1	52.4	6.11	54.61	60.73	26.7	2.24	24.10	26.34
2	−1	−1	1	51.4	3.72	13.92	17.64	32.5	2.61	9.33	11.94
3	−1	1	−1	54.5	12.25	55.82	68.08	48.0	3.05	30.96	34.01
4	−1	1	1	48.4	3.24	15.69	18.93	55.4	2.67	14.90	17.57
5	1	−1	−1	57.2	4.25	41.67	45.92	50.2	1.96	27.61	29.57
6	1	−1	1	50.9	3.64	15.55	19.19	44.7	2.52	11.04	13.56
7	1	1	−1	54.7	10.54	47.71	58.25	54.4	2.66	19.75	22.41
8	1	1	1	53.0	3.24	15.69	18.93	59.3	2.89	15.10	17.99
9	−1.68	0	0	54.8	3.68	21.62	25.30	58.7	3.23	17.62	20.85
10	1.68	0	0	58.1	3.36	20.19	23.55	54.0	3.69	23.52	27.21
11	0	−1.68	0	57.9	2.62	17.51	20.13	26.1	3.78	15.98	19.76
12	0	1.68	0	56.9	3.78	21.04	24.82	57.4	4.92	23.41	28.33
13	0	0	−1.68	44.5	10.47	49.23	59.70	48.8	1.90	17.66	19.55
14	0	0	1.68	32.8	3.78	21.04	24.82	24.9	2.51	7.30	9.81
15	0	0	0	54.7	3.41	20.24	23.65	56.8	4.03	23.04	27.07
16	0	0	0	57.0	3.32	20.83	24.15	52.8	4.34	25.90	30.25
17	0	0	0	57.2	3.33	20.47	23.80	53.9	4.15	24.36	28.51
18	0	0	0	55.1	3.80	22.08	25.82	56.0	4.05	24.17	28.23
19	0	0	0	56.3	3.49	20.70	24.19	56.3	4.34	24.86	29.20
20	0	0	0	54.8	3.32	19.99	23.31	54.9	3.47	22.05	25.52

E: dried extract.

ultrasonic system (Ultrasonic homogenizer, model CY-500, Optic Ivymen Systen, Barcelona, Spain) equipped with a titanium probe and a connector for the sample temperature control. Powdered jabuticaba epicarps (2.5 g) were placed in a beaker with 50 mL of solvent and processed according to the experimental design matrix in Table 2, where different levels of t (5–25 min), P (100–500 W; at a frequency of 20 kHz) and S (0–100%) are combined. The S/L was kept constant (50 g/L), as well as T (30–35 °C; a cold water bath was used to avoid heating the samples). The mixtures were then centrifuged and the supernatant collected as described for HAE.

2.5. Response variable analysis for extraction process optimization

2.5.1. Evaluation of the extraction yield

The extract weight resulting from each extraction was determined gravimetrically in crucibles by evaporation of 5 mL of supernatant (extract solution) at 105 °C for 24 h. The results were expressed as percentage of crude extract per plant material (%, *w/w*).

2.5.2. Chromatographic analysis of anthocyanin contents

A portion (1.5 mL) of each extract solution was filtered through 0.22 μm disposable syringe filters and analysed in a Dionex Ultimate 3000 HPLC system (Thermo Scientific, San Jose, CA, USA), operating under the conditions described in detail by Gonçalves et al. (2017). Compound detection was carried out with a diode array detector (DAD) at a wavelength of 520 nm. Chromatographic data were acquired and processed using Xcalibur software (Thermo Finnigan, San Jose, CA, USA). Anthocyanins were characterized according to their UV–Vis and mass spectra, and quantification was performed using a calibration curve constructed with cyanidin-3-*O*-glucoside (y = 134578x – 3000000; R^2 = 0.999). The total anthocyanin content was determined by sum of the quantified anthocyanins. The results were expressed as mg per g of dry extract (E).

2.6. Extraction process modelling and optimization

2.6.1. Response criteria and mathematical modelling

The dependent variables Y_1 (extraction yield), Y_2 (delphinidin-3-*O*-glucoside content, D3G), Y_3 (cyanidin-3-*O*-glucoside content, C3G), and Y_4 (total anthocyanin content, TAC) were used to optimize the recovery of anthocyanins from jabuticaba epicarp. The response surface models were fitted by means of last-squares calculation using the following second-order quadratic polynomial equation:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=!\\ J>1}}^{n-1} \sum_{j=2}^{n} b_{ij} X\ X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \quad (1)$$

where Y represents the dependent (response) variable to be modelled; b_0 is the constant coefficient and b_i, b_{ii} and b_{ij} indicate the regression coefficient for the linear, quadratic and interaction effects, respectively; X_i and X_j represent the independent variables; and n shows the number of variables involved (therefore, n = 3).

2.6.2. Fitting procedures and statistical analysis

Fitting procedures, coefficient estimates and statistical calculations were performed using Design-Expert software, Version 11. The analyses of variance (ANOVA) was used to determine the regression coefficients and to assess the significance of the data. The coefficient of determination (R^2) and the adjusted coefficient of determination (R^2_{adj}), interpreted as the proportion of variability of the dependent variable explained by the model (Albuquerque et al., 2018), were used to estimate the fitness of the polynomial equation to the response. Only the statistically significant terms (p-value < 0.05) were used to fit the mathematical models. The significance of all the terms of the polynomial equations was analysed statistically by computing the F-value at p < 0.05. The statistic lack of fit was used to evaluate the adequacy of the models. This test shows whether the model adequately describes the functional relationship between the independent variables and the obtained response. Thus, the lack of fit should be non-significant (p > 0.05). The software was also used to generate the response surface graphs.

2.7. Validation of the colorant potential of the anthocyanin-rich extract in a bakery product

2.7.1. Formulation of macarons

A traditional recipe of French macarons containing 28% almond flour, 28% sugar powder, 23% egg whites, and 23% sugar was followed. Due the amount of egg whites (pH ~7) added to the macaron formulation, lemon juice (pH ~2.2) was added (1:4, *v/w*) to decrease the pH and thereby increase the anthocyanin stability, preventing a change colour. The dough was then coloured with the anthocyanin-rich extract (AE) obtained under the optimized extraction conditions and with the commercial colorant E163 (used as control). The macarons were baked in a conventional oven for 13 min at 130 °C. The two batches of macarons were then divided into three groups: one was immediately analysed (T1) and the other two were analysed after three (T2) and six (T3) days of storage at ± 5 °C, protected from sunlight and in plastic bags. Then, after measuring the colour, the macarons were lyophilized, crushed, and analysed in triplicate for their nutritional value.

2.7.2. Colour analysis

The colour of macarons was measured with a colorimeter (model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan) previously calibrated against a standard white tile (López et al., 2019). Using illuminant C and an 8 mm diaphragm aperture, the CIE L^* (lightness), a^* (greenness-redness), and b^* (blueness-yellowness) colour space values were recorded using Spectra Magic Nx software (version CM-S100W 2.03.0006).

2.7.3. Nutritional composition analysis

The moisture content of macarons was determined using a moisture analyser (model PBM 163, Adam Equipment Inc., Oxford, EUA). The protein, fat, and ash contents were determined following the AOAC procedures (AOAC International, 2016). Briefly, the crude protein content (N × 5.18) was estimated by the macro-Kjeldahl method (AOAC 978.04), using an automatic distillation and titration unit (Pro-Nitro-A, JP Selecta, Barcelona); the crude fat content was determined by Soxhlet extraction with petroleum ether (AOAC 920.85); and the ash content was determined by incineration in a muffle furnace at 600 ± 15 °C for 5 h (AOAC 923.03). The total carbohydrate content was estimated by difference. The results were expressed as g per 100 g of dry weight (dw).

The energy value was calculated according to Regulation (EU) No 1169/2011 of the European Parliament and of the Council (2011) as follows: 4 × (g protein + g carbohydrates) + 9 × (g fat), and given as kcal per 100 g of dry weight (dw).

Free sugars were analysed in a high performance liquid chromatography system (HPLC, Knauer, Smartline system 1000, Berlin, Germany) coupled to a refractive index (RI) detector (Knauer Smartline 2300), following analytical procedures previously described by Barros et al. (2013). Chromatographic data were acquired and analysed using Clarity 2.4 software (DataApex). Quantification was performed by comparing the area of the sample peaks with calibration curves obtained from commercial standards, using the internal standard method (IS, melezitose). The results were expressed as g per 100 g of dry weight (dw).

2.7.4. Statistical analysis

Colour and nutritional analyses were performed in triplicate and the results were expressed as mean ± standard deviation. The statistical analysis was performed at a 5% significance level using SPSS Statistics software (IBM SPSS Statistics for Windows, Version 23.0, IBM Corp., Armonk, NY, USA). A two-tailed paired Student's *t*-test was applied for assessing statistical differences ($p < 0.05$) between AE and control samples, while a one-way ANOVA was applied for assessing the effect of storage time on the evaluated parameters. The fulfilment of the ANOVA requirements, specifically the normal distribution of the residuals and the homogeneity of variance, was tested by means of the Shapiro Wilk's and Levene's tests, respectively. Data were compared using Tukey's honestly significant difference (HSD) test.

3. Results and discussion

3.1. Optimization of the extraction of anthocyanins

Although the extraction of anthocyanins from jabuticaba epicarp has been described in previous studies (Paludo et al., 2019; Rodrigues, Fernandes, de Brito, Sousa, & Narain, 2015; Santos & Meireles, 2011; Santos, Veggi, & Meireles, 2012), this is the first work to optimize the HAE process. In addition, an easy-to-apply, low-investment, conventional method (HAE) is compared with a non-conventional method (UAE) whose acoustic cavitation effect can improve mass transfer and thus reduce processing time and increase the extraction yield (Pinela et al., 2019). It is also describes the individual behaviour of two anthocyanin previously identified by Albuquerque et al. (2019) in jabuticaba epicarp against the studied extraction variables (t, *T*, *P*, and *S*).

3.1.1. Experimental data obtained with the CCD design

The experimental results obtained with the 20 runs of the 5-level CCD design matrix implemented to optimize the HAE and UAE processes used to extract anthocyanins from jabuticaba epicarp are shown in Table 2. The extraction yield ranged from 32.8 to 58.1% with HAE and from 24.9 to 59.3% with UAE. In both cases, the lowest extraction yields were obtained with run 14, which combined medium *t* and *T* or *P* conditions (55 min and 45 °C for HAE and 15 min and 300 W for UAE; $\alpha = 0$) with a high solvent concentration (100% ethanol, *v/v*; $\alpha = 1.68$). Regarding anthocyanins, cyanidin-3-*O*-glucoside (C3G) was detected in greater quantity than delphinidin-3-*O*-glucoside (D3G) in all extracts, with concentrations ranging from 13.92 to 55.82 mg/g E and 7.30 to 30.96 mg/g E with the HAE and UAE processes, respectively, while the D3G levels ranged from 2.62 to 12.25 mg/g E with HAE and from 1.90 to 4.34 mg/g E with UAE. For both methods, the highest levels of total anthocyanins (TAC; 68.08 mg/g for HAE and 34.01 mg/g for UAE) were achieved with run 3, which employed a short-moderate *t* (21.2 min for HAE and 9.1 min for UAE; $\alpha = -1$), a medium–high *T* or *P* (75, 8 °C or 418.9 W; $\alpha = 1$) and a low-moderate *S* concentration (20.3% ethanol, *v/v*; $\alpha = -1$). As verified for yield responses, higher ethanol concentrations also had a negative effect on anthocyanin extraction by the UAE method, as only 9.81 mg TAC/g E were obtained with run 14 (Table 2). For HAE, the lowest TAC value (17.81 mg/g E) was reached with run 2 (21.2 min, 34.2 °C and 79.7% ethanol, *v/v*), while run 3 (which differed only in temperature, 75.8 °C) gave the highest content (68.08 mg/g E). In general, the HAE method resulted in higher anthocyanin extraction rates than UAE.

3.1.2. Analysis of the theoretical response surface models

RMS is a mathematical and statistical analysis tool suitable for modelling and analysing processes involving one or more response variables, which allows to minimize the number of laboratorial experiments and maximized responses (Leichtweis et al., 2019). When trying to develop theoretical models to predict and comprehend the effects of independent variables on certain response variables, it is necessary to evaluate their precision by fitting these models to experimental values. In this study, the response values of Table 2 were fitted to the second-order polynomial model of Eq. (1) to develop the mathematical models (Eqs. (2–5) for each response criteria. However, not all parameters of Eq. (1) were used in the development of the models since some coefficients were non-significant (Table 3); the significant ones were assessed at a 95% confidence level ($\alpha = 0.05$).

For the HAE process:

$$Y_{(Yield)} = 56.25 - 2.55S - 5.29S^2 \quad (2)$$

Table 3
Parametric values of the second-order polynomial equation (Eq. 1) for each extraction method and response criteria, and statistical information of the model fitting procedure. Parametric superscripted 1, 2 and 3 stand for the variables time (X_A), temperature or power (X_B), and solvent proportion (X_C), respectively.

		Heat-assisted extraction (HAE)				Ultrasound-assisted extraction (UAE)			
Effect		Yield	D3G	C3G	TAC	Yield	D3G	C3G	TAC
Intercept	b_0	56.25 ± 0.71	3.75 ± 0.68	20.89 ± 0.92	24.02 ± 1.08	54.50 ± 2.32	4.07 ± 0.35	22.57 ± 2.00	26.56 ± 2.03
Linear effect	b_1	ns	ns	−0.54 ± 0.85	−0.87 ± 0.99	ns	0.04 ± 0.27	ns	ns
	b_2	ns	0.96 ± 0.64	−0.41 ± 0.85	0.44 ± 0.99	8.64 ± 1.38	0.31 ± 0.27	1.55 ± 1.83	1.69 ± 1.86
	b_3	−2.55 ± 0.67	−2.32 ± 0.64	−9.69 ± 1.21	−1.63 ± 1.68	−2.85 ± 1.48	0.13 ± 0.27	−5.96 ± 2.23	−5.86 ± 2.27
Quadratic effect	b_{11}	ns	ns	ns	ns	ns	−0.35 ± 0.26	ns	ns
	b_{22}	ns	ns	ns	ns	−3.92 ± 1.70	ns	ns	ns
	b_{33}	−5.29 ± 0.65	1.42 ± 0.62	9.54 ± 1.03	9.82 ± 1.47	−2.53 ± 1.77	0.82 ± 0.24	−4.45 ± 2.32	−5.58 ± 2.37
Interactive effect	b_{12}	ns	ns	1.52 ± 1.43	4.58 ± 2.57	ns	ns	ns	ns
	b_{13}	ns	ns	3.98 ± 1.43	7.74 ± 2.52	ns	ns	ns	ns
	b_{23}	ns	−1.64 ± 0.84	−2.04 ± 1.43	−7.15 ± 2.53	2.53 ± 0.98	ns	ns	ns
Statistical analysis	SM	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0002	0.0002	< 0.0001
	LF	0.1454	0.0884	0.1693	0.1971	0.0769	0.1368	0.0531	0.0620
	R^2	0.8269	0.8807	0.9882	0.9909	0.9521	0.8075	0.7290	0.7484
	R^2_{adj}	0.8065	0.8488	0.9826	0.9867	0.9388	0.7388	0.6748	0.6981

Yield: extraction yield; D3G: delphinidin-3-*O*-glucoside; C3G: cyanidin-3-*O*-glucoside; TAC: total anthocyanin content; ns: not significant; SM: significance of the model; LF: lack of fit; R^2: coefficient of determination; R^2_{adj}: adjusted coefficient of determination.

$$Y_{(D3G)} = 3.75 + 0.96T - 2.32S + 1.42S^2 - 1.64TS \quad (3)$$

$$Y_{(C3G)} = 20.89 - 0.54t - 0.41T - 9.69S + 9.54S^2 + 1.52tT + 3.98tS - 2.04TS \quad (4)$$

$$Y_{(TAC)} = 24.02 - 0.87t + 0.44T - 1.63S + 9.82S^2 + 4.58tT + 7.74tS - 7.15TS \quad (5)$$

For the UAE process:

$$Y_{(Yield)} = 54.50 + 8.64T - 2.85S - 3.92T^2 - 2.53S^2 + 2.53TS \quad (6)$$

$$Y_{(D3G)} = 4.07 + 0.04t + 0.31T + 0.13S - 0.35t^2 + 0.82S^2 \quad (7)$$

$$Y_{(C3G)} = 22.57 + 1.55T - 5.96S - 4.45S^2 \quad (8)$$

$$Y_{(TAC)} = 26.56 + 1.69T - 5.86S - 5.58S^2 \quad (9)$$

The presented mathematical models reflect the complexity of extraction trends. The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs of the RSM design matrix (Table 2).

The parametric values of the second-order polynomial equation used to construct the mathematical models presented above and the statistical information of the model fitting procedure are presented in Table 3. All models presented a non-significant lack of fit, indicating that the equations adequately describe the effects of the variables on the evaluated responses with good predictability (Iberahim, Sethupathi, Goh, Bashir, & Ahmad, 2019). The obtained coefficients R^2 and R^2_{adj} were ≥0.83 and 0.81 for HAE and ≥0.73 and 0.67 for UAE (Table 3), which indicates that the variability of each response can be explained by the independent variables involved in the process. Additionally, these statistical data also indicate that the experimental values of HAE are closer to the ones predicted by the models, as can be seen in Part B of Figs. 1 and 2. The models proved to be statistically adequate and, therefore, were used to navigate the design space in the subsequent optimization steps. Although the model coefficients are empirical and do not reflect physical or chemical significance, they are useful to predict the outcome of untested experimental conditions (Pinela et al., 2018). In addition, the parametric value sign determines part of the response. Thus, for variables with a positive coefficient value, the response is higher at the high level, while for a negative coefficient value, the response is lower at the high level. The higher the parametric value, the more significant is the weight of the variable (Pinela et al., 2019).

Certain features regarding the overall effects of the independent variables can be inferred from the complexity of the parametric values (Table 3), *i.e.*, the variables can be ordered in a decreasing form as a function of its significance in the extraction processes as follows: $S > T$ or $P > t$. It is also possible to observe that some of the evaluated responses were significantly affected by linear and quadratic effects, mainly caused to the variable S (for HEA, t and T had no significant quadratic effects). The parametric values also show the existence of interactions between the three variables involved in the anthocyanin extraction by HAE, while only the yield was affected by $T \times S$ in the UAE process. These results justify the use of RSM as an optimization tool, since one-variable-at-a-time approaches do not evaluate the existence of interactions, which makes the determination of optimum values difficult.

3.1.3. Effect of the independent variables on the target responses

To visually describe the extraction trends, the results were presented as 3D response surface graphs, which illustrate the effect of the three studied independent variables on the extraction yield and anthocyanins content for both HAE (Fig. 1) and UAE (Fig. 2) methods. The net surfaces of each graph were predicted with the second-order polynomial Eq. (1), whose model equations are presented above. For representation of binary actions, the excluded variable in each graph was fixed constant (*cte*) as indicated. Additionally, the goodness of fit of the models was illustrated by the ability to simulate response changes between the observed and predicted data (Figs. 1 and 2, Part B).

For the extraction yield, it is possible to observe that, in the HAE process (Fig. 1), the response was significantly affected only by the ethanol concentration (Table 3), whose effect is perfectly illustrated by the curvature of the graphs surface where this variable is represented. The increase of this variable up to about 50% increased extraction yield, but thereafter it decreased through a quadratic effect. For UAE (Fig. 2), the ultrasound power was the most relevant variable, followed by the ethanol concentration (Table 3). The higher the ultrasound power, the higher the extraction yield. A positive interaction between the two variables ($P \times S$) was also verified (Table 3), which once again justifies the use of RSM for optimization. In both processes, the variable time had no significant effect on this response.

Regarding anthocyanins, the response surfaces obtained for HAE were more complex than those of UAE (Figs. 1 and 2), as predicted by the developed theoretical models. In general, the HAE extraction of both anthocyanins was favoured by higher temperatures and lower ethanol concentrations. The extraction time affected negatively the

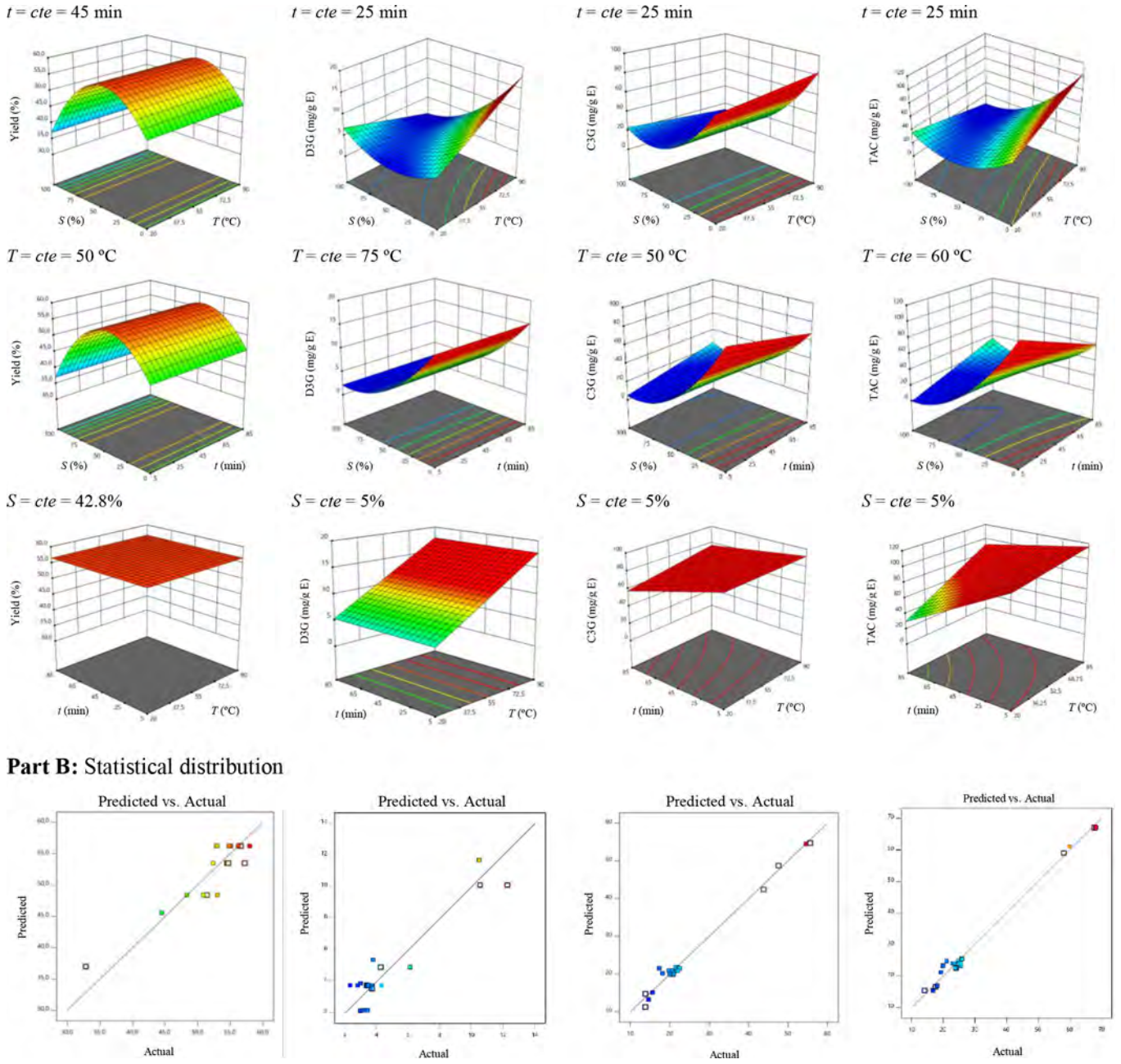

Fig. 1. Response surface graphs for extraction yield (%) and delphinidin-3-*O*-glucoside (D3G), cyanidin-3-*O*-glucoside (C3G) and total anthocyanin (TAC) contents (mg/g extract) obtained for the HAE method (Part A). To represent each graph, the excluded variable was fixed constant (*cte*) at the indicated value. Part B illustrates the goodness of fit through the ability to simulate response changes between actual and predicted values.

recovery of C3G (the anthocyanin found in higher amount) and, consequently, the total anthocyanin content (TAC); thus, the longer the extraction time, the lower the anthocyanin recovery. This is probably due to the breakdown of the structure of these sensitive compounds when subjected to longer processing times (López et al., 2019). Also noteworthy are the quadratic effects of the solvent and the positive interactions between $t \times S$ and $t \times T$, and the negative ones between $T \times S$ (Table 3). Meanwhile, in the UAE extraction, the highest amounts of anthocyanins were obtained when the jabuticaba epicarp samples were sonicated with a high power in a medium–low ethanol:water mixture. This last variable was the one that most affected the extraction, with quadratic effects marked on the surface graphs (Fig. 2). In the particular case of C3G, its extraction was also affected by the variable time; increasing the processing time up to about 15 min was beneficial for extraction but decreased thereafter.

3.1.4. Optimal extraction conditions and comparison of the HAE and UAE methods

From the response surface graphs shown in Figs. 1 and 2 it is possible to infer that an optimal extraction value can be found as a single point in almost all combinations. Therefore, the extraction conditions that lead to an absolute maximum were computed (Table S1). The conditions that maximized the HAE extraction were as follows:

- For extraction yield, the conditions were: t = 9.31 min, T = 34.47 °C, and S = 42.79% ethanol (*v/v*), and originated 56.6 ± 0.7% (*w/w*) of extract.
- For D3G, the conditions were: t = 5.68 min, T = 68.21 °C, and S = 0.09% ethanol (*v/v*), and yielded 14 ± 1 mg/g extract.
- For C3G, the conditions were: t = 7.89 min, T = 25.18 °C, and S = 11.78% ethanol (*v/v*), and yielded 71 ± 2 mg/g extract.

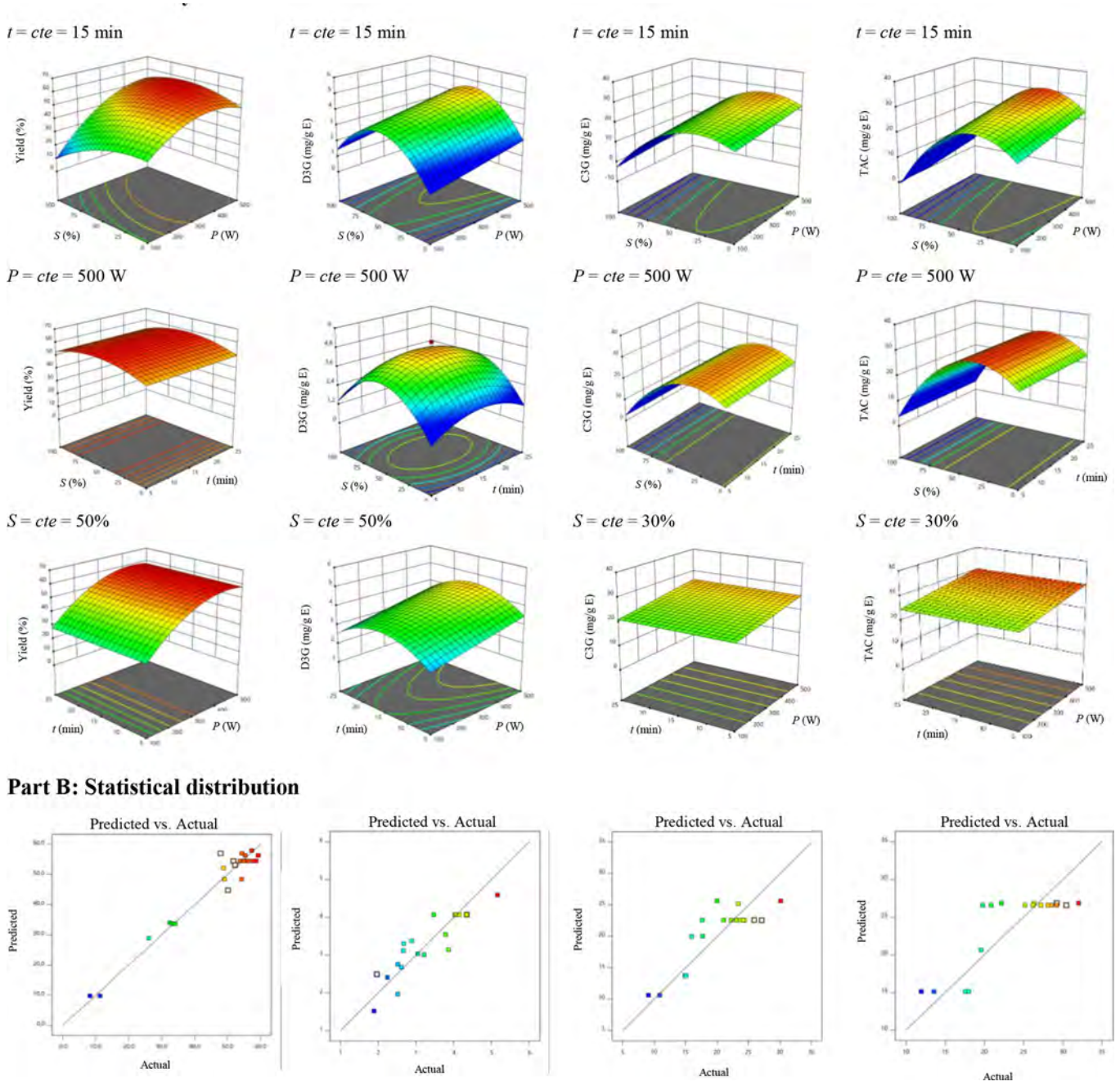

Fig. 2. Response surface graphs for extraction yield (%) and delphinidin-3-*O*-glucoside (D3G), cyanidin-3-*O*-glucoside (C3G) and total anthocyanin (TAC) contents (mg/g extract) obtained for the UAE method (Part A). To represent each graph, the excluded variable was fixed constant (*cte*) at the indicated value. Part B illustrates the goodness of fit through the ability to simulate response changes between actual and predicted values.

- For TAC, the conditions were: t = 21.8 min, T = 47.1 °C, and S = 9.1% ethanol (v/v), and yielded 81 ± 2 mg/g extract.

In general, the optimal HAE conditions were characterized by short extraction times and mean temperatures. Regarding the solvent, although 42.8% ethanol produced a higher amount of extract, the recovery of anthocyanins from jabuticaba epicarp was favoured by lower solvent percentages (Table S1), indicating that this variable contributes to the selectivity in anthocyanin recovery. This extraction trend can also be observed in the response surface graphs of Fig. 1.

In turn, the conditions that maximized the UAE extraction were as follows:

- For extraction yield, the conditions were: t = 7.49 min, P = 421.82 W, and S = 48.30% ethanol (v/v), and originated 59 ± 1% (w/w) of extract.
- For D3G, the conditions were: t = 15.37 min, P = 500 W, and S = 52.52% ethanol (v/v), and yielded 4.4 ± 0.2 mg/g extract.
- For C3G, the conditions were: t = 6.41 min, P = 500 W, and S = 30.26% ethanol (v/v), and yielded 27 ± 2 mg/g extract.
- For TAC, the conditions were: t = 24.44 min, P = 500 W, and S = 34.47% ethanol (v/v), and yielded 31 ± 2 mg/g extract.

The UAE process was characterized by short extraction times, high ultrasound power, and medium-low solvent percentages. No selectivity in extraction was verified.

The two extraction methods tested in this study were compared in order to conclude which one is the most suitable to produce an extract

with a higher amount of anthocyanins from the plant matrix to be recycled within the agri-food chain as a natural colorant. For extraction of intracellular solutes, UAE has shown some advantages over HAE, such as shorter extraction times, lower solvent consumption, and increased mass transfer. These UAE specifications are due to its ability to cause plant tissue/cell walls rapture by ultrasound waves, which facilitates solvent penetration and consequent solute recovery (Albuquerque et al., 2017; López et al., 2019). In fact, ultrasound has been a promising technology for anthocyanin extraction intensification (Backes et al., 2018; Leichtweis et al., 2019; Pinela et al., 2019). However, our results show the opposite, since the anthocyanin content obtained by HAE (81 ± 2 mg/g extract) was more than double obtained by UAE (31 ± 2 mg/g extract) (Table S1). This result may be due, in part, to the stirring system used in each method; in HAE a continuous magnetic stirring system was used, while in UAE the sample was "stirred" only by the power of the ultrasonic probe (therefore, lower powers caused less mixing movement). This result was different from those reported for optimal anthocyanin extraction conditions from other plant matrices, namely extraction of cyanidin-3-rutinoside from *Ficus carica* L. peels (Backes et al., 2018), delphinidin-3-*O*-sambubioside and cyanidin-3-*O*-sambubioside from *Hibiscus sabdariffa* calyces (Pinela et al., 2019), and cyanidin-3-rutinoside and peonidin-3-rutinoside from *Prunus spinosa* L. fruit epicarp (Leichtweis et al., 2019). However, López et al. (2018) showed that the extraction of cyanidin-3-*O*-glucoside (the major anthocyanin found in jabuticaba epicarp) from *Arbutus unedo* L. fruit is also more effective using the HAE technique than UAE. Thus, the nature of anthocyanin, such as its anthocyanidin and sugar molecule, may also be a factor that influences the extraction.

Rodrigues et al. (2015) also accomplished the optimization of cyanidin-3-*O*-glucoside extraction from jabuticaba epicarp by the UAE technique, but using an open ultrasonic bath (25 kHz, 150 W) instead of a probe. The best condition found by the authors were similar to those achieved in this study for the extraction time (~10 min) and yielded 4.9 mg C3G/g dry peel (against 14.85 mg/g dw obtained in this study). This difference may be due to the type of ultrasound system used for extractions. In fact, a probe system delivers the ultrasonic intensity on a small surface compared to an ultrasonic bath, thus being more powerful and widely used for bench-scale experiments (Chemat et al., 2017).

3.1.5. Experimental validation of the optimum extraction conditions

The optimal conditions that maximize the extraction of total anthocyanins (TAC) from jabuticaba epicarp (Table S1) were experimentally tested to confirm the accuracy of the results and to obtain the anthocyanin-rich extract for incorporation in macarons as a natural colorant. The HAE yielded 76 ± 1 mg TAC/g E and the UAE yielded 32 ± 1 mg TAC/g E, values that differed in about 6 and 3% from the predicted values, respectively.

3.2. Incorporation of anthocyanin-rich extract into macarons

An anthocyanin-rich extract (AE) of jabuticaba epicarp was produced employing the optimized extraction conditions that lead to the highest anthocyanin content (Table S1). The extract was then incorporated into a bakery product to assess its colouring capacity in a thermal processed foodstuff. For this purpose, the AE was added as natural colorant in macarons (Fig. S1) and the colour stability of these products was evaluated during a 6-day shelf-life at ~5 °C. In addition, the colour and nutritional value data of macarons formulated with AE were compared with those of control macarons produced and stored under the same conditions but containing the commercial colorant E163.

3.2.1. Effects on colour parameters

The results of the colour parameters L^* (lightness), a^* (redness) and b^* (yellowness) measured on macarons formulated with AE and E163 (control) during a 6-day shelf-life are present in Table 4. As illustrated in Fig. S1, macarons formulated with AE showed a distinct coloration from the control (p-values < 0.05). This difference in colour tone or intensity may be due to a distinct anthocyanin composition and/or content of the two tested colouring agents. It should be noted that the samples with the natural AE presented a more stable coloration during storage, since a decrease in the parameter b^* value of the stored control samples was verified (*i.e.*, for the T2 and T3 periods). This result highlights the interest in using the developed anthocyanin-rich ingredient as a food additive.

3.2.2. Effects on nutritional value

The control of moisture change during the shelf-life of bakery products is very important to preserve their safety and sensory quality (Smith, Daifas, El-Khoury, Koukoutsis, & El-Khoury, 2004). As shown in Table 4, the macarons formulated with AE had a moisture content similar to the control (p-values > 0.05) and there was no significant absorption or loss of water by the samples during the evaluated shelf-live.

Table 4 also shows the nutritional composition for the lyophilised macaron samples. Carbohydrates were the predominant macronutrients (~71 g/100 g dw), followed by crude fat (~17 g/100 g dw) and protein (~10 g/100 g dw). In general, all samples had a similar nutrition composition and no statistical differences were found between macronutrient levels or energy values (p-values > 0.05). The performed HPLC analysis revealed the presence of the monosaccharides fructose and glucose and the disaccharide sucrose, which corresponded to the estimated carbohydrate levels. The sucrose content (~70 g/100 g dw) showed no significant differences regardless of the used colouring agent or the evaluated shelf-life. However, the macarons formulated with AE presented higher fructose and glucose contents than those coloured with the commercial E163 (p-values < 0.05), which can be explained by the amount of reducing sugars that can be found in the jabuticaba epicarp (~33%) (Batista et al., 2017). A similar result was described by López et al. (2019) when a anthocyanin-rich *Arbutus unedo* L. fruit extract was used in the formulation of wafers, which also increased the levels of the same sugars in the obtained samples.

4. Conclusions

The extraction of anthocyanins from jabuticaba epicarp by the HAE and UAE methods was tested and optimized using RSM. HAE was more effective and selective than UAE, yielding 81 ± 2 mg of anthocyanins per g of extract. The solvent concentration was the most relevant variable in both extraction processes, followed by temperature or ultrasound power. After statistical validation of the theoretical models and prediction of the extraction conditions that maximize the anthocyanins recovery, an anthocyanin-rich extract was obtained under the best conditions and incorporated in macarons to validate its colouring capacity. This natural extract gave the macarons a more stable colour than the commercial colorant E163 during a 6-day shelf-life. Overall, this study provides important information for obtaining an anthocyanin-based colorant from jabuticaba epicarp and validates the colouring ability of this natural ingredient in a thermal processed pastry product, which highlights is potential to be used as a natural food colorant.

CRediT authorship contribution statement

Bianca R. Albuquerque: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **José Pinela:** Conceptualization, Methodology, Investigation, Formal analysis, Writing - review & editing. **Lillian Barros:** Methodology, Project administration, Writing - review & editing. **M. Beatriz P.P. Oliveira:** Conceptualization, Project administration, Writing - review & editing. **Isabel C.F.R. Ferreira:** Conceptualization, Methodology, Funding acquisition, Project administration, Writing - review & editing.

Table 4
Colour and nutritional value of macarons formulated with anthocyanin-rich extract and colorant E163 (control) during shelf-life.

	T1 (day 0)			T2 (3 days of storage)			T3 (6 days of storage)		
	AE	Control	*p*-value	AE	Control	*p*-value	AE	Control	*p*-value
Colour parameters									
L^* (lightness)	80.9 ± 0.5^a	76.3 ± 0.3^b	< 0.001	80.8 ± 0.8^a	75.9 ± 0.7^b	< 0.001	79.6 ± 0.7^a	75.3 ± 0.5^b	< 0.001
a^* (redness)	6.3 ± 0.3^b	10.4 ± 0.3^a	< 0.001	6.1 ± 0.3^b	10.7 ± 0.7^a	< 0.001	6.1 ± 0.4^b	10.6 ± 0.2^a	< 0.001
b^* (yellowness)	7.6 ± 0.5^a	1.7 ± 0.3^b	< 0.001	7.6 ± 0.2^a	0.9 ± 0.1^c	< 0.001	6.8 ± 0.3^a	0.73 ± 0.05^c	< 0.001
Nutritional value									
Moisture (g/100 g)	5.4 ± 0.8	5.6 ± 0.3	0.794	5.3 ± 0.1	5.6 ± 0.2	0.121	5.3 ± 0.4	5.2 ± 0.2	0.584
Ash (g/100 g dw)	0.94 ± 0.01	0.95 ± 0.01	0.292	0.94 ± 0.04	0.96 ± 0.01	0.236	0.94 ± 0.01	0.94 ± 0.03	0.679
Fat (g/100 g dw)	17.45 ± 0.03	17.4 ± 0.1	0.129	17.4 ± 0.1	17.4 ± 0.2	0.215	17.6 ± 0.5	17.3 ± 0.4	0.912
Protein (g/100 g dw)	10.55 ± 0.04	10.51 ± 0.04	0.115	10.1 ± 0.4	10.4 ± 0.3	0.595	10.3 ± 0.3	10.4 ± 0.4	0.665
Carbohydrates (g/100 g dw)	71.1 ± 0.1	71.1 ± 0.1	0.597	71.2 ± 0.2	71.4 ± 0.01	0.182	71.1 ± 0.2	71.3 ± 0.5	0.581
Energy (kcal/100 g dw)	483.5 ± 0.1	483.4 ± 0.5	0.671	483.4 ± 0.6	483.1 ± 0.5	0.588	484 ± 2	483 ± 2	0.415
Free sugars									
Fructose (g/100 g dw)	0.576 ± 0.001	0.17 ± 0.02	< 0.001	0.56 ± 0.04	0.184 ± 0.004	< 0.001	0.56 ± 0.03	0.18 ± 0.02	< 0.001
Glucose (g/100 g dw)	0.32 ± 0.02	0.16 ± 0.05	< 0.001	0.33 ± 0.02	0.13 ± 0.04	< 0.001	0.35 ± 0.06	0.15 ± 0.01	0.030
Sucrose (g/100 g dw)	70.1 ± 0.1	70.4 ± 0.5	0.180	70.3 ± 0.3	70.7 ± 0.2	0.108	70.23 ± 0.04	70.8 ± 0.6	0.096
Total sugars (g/100 g dw)	71.1 ± 0.1	70.8 ± 0.5	0.272	71.3 ± 0.4	71.1 ± 0.2	0.329	71.1 ± 0.1	71.1 ± 0.6	0.964

In each line and for each time period, statistical differences (p-value < 0.05) between AE and control samples were assessed by a Student's t-test, while the effect of storage time was assessed by a one-way ANOVA (different letters indicate significant statistical differences; there are no differences when no letters are displayed). AE: macarons with anthocyanin-rich extract; Control: macarons with the commercial colorant E163; dw: dry weight.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126364.

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Extracts from *Vaccinium myrtillus* L. fruits as a source of natural colorants: chemical characterization and incorporation in yogurts

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The food industry is always seeking innovative approaches to maintain consumers' interest and increase their awareness towards the healthiness of diets. Therefore, much interest has been given to natural food additives, namely colourants. In this work, a bilberry extract was primarily characterized in terms of anthocyanin compounds by UPLC-DAD-ESI/MSn, and its colouring capacity was further compared with a synthetic anthocyanin colourant (E163) in different yogurt formulations. The prepared samples were evaluated in different periods (0 and 7 days) for nutritional profile, individual fatty acids, soluble sugars and external colour to determine the effects of each additive. Overall, the major anthocyanin compounds in bilberry were malvidin glycoside and delphinidin glycoside derivatives. With regard to the prepared yogurts, all samples maintained the nutritional profile, individual fatty acids and soluble sugars, independent of storage time. Nonetheless, bilberry extracts showed lower colouring capacity when compared to that of E163, despite the higher stability of bilberry extract throughout the storage time, when compared to that of E163. Furthermore, another advantage of this natural extract is that it has potential bioactive properties that can be conferred to foods, due to their high content of bioactive compounds, such as anthocyanins.

Introduction

Colour is an important sensory attribute of foods that often plays an important role in the success of a product, namely as a quality indicator of flavour, safety, nutritional value, and other parameters.[1] Beyond conferring colours, food colourants are also used to overcome colour variation in foods and other related imperfections. The popularity of artificial colourants is associated with their low production cost, high resistance and chemical stability[1] but natural colourants are generally considered healthier than their synthetic counterparts, since most of them are derived from edible plants or fruits.[2] There are reports that link hyperactivity and allergies in children with exposure to artificial colourants, a fact that has stimulated the scientific community to search for new and improved natural extracts, which can be used as natural pigments and colourants.[2]

Flavonoids are a group of secondary plant metabolites characterized by a $C_6C_3C_6$ carbon backbone. Among these phytochemical compounds, anthocyanins are an important subclass of water-soluble pigments responsible for the colouration from blue to orange in plants.[3] *Vaccinium myrtillus* L., also known as bilberry, is one of the natural sources of this group of compounds. Besides being responsible for the bluish-red colour of the bilberry fruit, anthocyanins are also related to many health benefits, including cardioprotective, anti-inflammatory, antimicrobial, antioxidant, and hypoglycaemic effects, thus being of great interest to the food industry.[4] In bilberry fruits, the major anthocyanins are represented by C-3-*O*-glucosides, *O*-galactosides, and *O*-arabinosides of cyanidin, delphinidin, petunidin, peonidin, and malvidin.[5]

The market of functional foods and ingredients has increased paired with consumer awareness about the importance of healthy eating and lifestyle. The concern about the substitution of artificial colourants with natural counterparts is growing. There are several studies that prove the effectiveness of incorporating anthocyanins as natural pigments into various food matrices, with satisfactory results in terms of stability.[6] The European Food Safety Authority (EFSA) allows the use of anthocyanins extracted from fruits in various types of foods, namely dairy products, cereals and others, under the

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number E163.[7] The fruit sources used to produce E-163 are not detailed in the commission regulation 231/2012, the common sources being grape skins, blackcurrant, black carrot, or purple sweet potato. Bilberry fruits are not a common source for E163, but, given their high abundance (77000 ha worldwide in 2010), they could constitute an alternative source.[8]

Yogurts are one of the most consumed dairy products throughout the world due to their organoleptic and nutritional value. These lactic foods could benefit from colourings derived from natural compounds by appealing to the growing health-related awareness among consumers.[9] However, one of the drawbacks of using anthocyanins and other natural colourants is their instability, having a tendency to change hue and lose colour intensity after a few hours or days, highlighting the importance of stability studies. Herein, bilberry's anthocyanins have been used to colour yogurts, which were further compared in terms of stability (7 days) with a commercial anthocyanin (E163) extract from CHR Hansen.

2. Materials and methods

2.1. Sample preparation

Dried samples of fruits of *Vaccinium myrtillus* L. (bilberry) were provided by RBRfoods (Castro D'aire, Portugal). After reception, samples were reduced to a fine dried powder, using a domestic grinder (~20 mesh; model A327R1, Moulinex, Spain) and mixed to obtain homogenous samples.

2.2. Bilberry extract preparation and compound identification by LC-DAD-ESI/MS

To prepare the extracts, 1 g of each sample was extracted by maceration with 30 mL of distilled water (25 °C, 150 rpm, 1 h), followed by filtration through a Whatman filter paper no. 4. Afterwards, the residue was extracted with one additional portion of water (30 mL) and the pooled extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland), frozen and lyophilized.[10]

The lyophilized extract was then re-dissolved in ethanol/water (80 : 20 v/v) at 5 mg mL^{-1}, for phenolic profile evaluation, using a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA), linked to a diode array detector (preferential wavelength of 520 nm) and to a mass spectrometer Linear Ion Trap LTQ XL (Thermo-Finnigan, San Jose, CA, USA) working in positive mode.[11] Anthocyanins were identified based on their retention, absorption spectra and mass characteristics in comparison with commercial standards when available and our library data. Calibration curves of available phenolic standards (Polyphenols, Sandnes, Norway) were constructed from the areas of the peaks recorded at 520 nm to perform quantitative analysis: cyanidin-3-*O*-glucoside ($y = 630276x - 153.83$, $R^2 = 0.999$); delphinidin-3-*O*-glucoside ($y = 557274x + 126.24$, $R^2 = 0.999$); malvidin-3-*O*-glucoside ($y = 477\,014.9x + 38.376$, $R^2 = 0.999$); and peonidin 3-*O*-glucoside ($y = 537017x - 71.469$, $R^2 = 0.999$). The compounds were quantified *via* the calibration curve of the most similar available standard. The results were expressed as mg g^{-1} of dry extract.

2.3. Fortification of yogurts with the natural and commercial colorant additive

2.3.1 Incorporation process. Natural yogurts (no colour) were acquired at a local market (Bio Natural brand) and had a labelled nutritional composition (per 100 g) of 3.8 g of fat, 5 g of protein and 4.7 g of carbohydrates, which corresponded to 73 kcal. Yogurts were then divided into three groups with three samples (70 g each) per group. Group A consisted of the plain yogurt group, without any incorporation, while group B was incorporated with 0.02% of E163 (0.014 g; CHR Hansen, Denmark, prepared from grapes from the Mediterranean region), and group C was incorporated with 0.42% of bilberry extract (0.294 g). All samples were stored for 7 days (5 °C) and the analysis was conducted on day 0 and day 7 to evaluate their stability.

2.4 Nutritional and chemical composition

2.4.1 Nutritional composition. The proximate composition was analysed according to AOAC procedures,[12] including protein (991.02), crude fat (989.05) and ash (935.42) contents. The Kjeldahl method was used for crude protein ($N \times 6.25$); incineration at 600 ± 15 °C for 5 hours was used for ash content, while crude fat was determined using a Soxhlet apparatus with petroleum ether as the recycling solvent; total carbohydrates were calculated by difference. The total energy was calculated using the following equation: energy (kcal) = 4 × (g protein + g carbohydrates) + 9 × (g fat).

2.4.2. Chemical composition. Soluble sugars and individual fatty acids were analysed using HPLC and GC, respectively. Free sugars were determined by HPLC coupled to a refraction index detector (HPLC-RI; Knauer, Smartline system 1000, Berlin, Germany), using melezitose as the internal standard.[13,14]

The fatty acids were determined by gas chromatography coupled with a flame ionization detector (GC-FID/capillary column, DANI model GC 1000, Contone, Switzerland), using a split/splitless injector and a Macherey-Nagel column. The identification of fatty acids was performed by comparing the relative retention times of the fatty acid methyl esters (FAME) peaks from the samples with commercial standards.[13,14]

2.5. External colour

External colour was analyzed using a portable colorimeter CR400 (Konica Minolta, Chiyoda, Tokyo, Japan) using the C65 illuminant, which represents the midday light in Europe, and a standard from the International Commission of Illumination (CIE). The CIE L^*, a^*, and b^* colour space coordinates were used, where L^* represents lightness, a^* represents redness (red-green), and b^* represents yellowness (yellow-blue), with a 10° observer angle and 8 mm aperture. The variation in total color difference (ΔE^*) between yogurt samples was calculated using the following equation:

$$\Delta E^* = \sqrt{(L_2^* - L_1^*)^2 + (a_2^* - a_1^*)^2 + (b_2^* - b_1^*)^2}$$

2.6. Statistical analysis

Throughout the manuscript, all data are expressed as mean ± standard deviation. Samples were analyzed by a two-way

ANOVA with type III sums of squares using the SPSS Software, version 25. This multivariate general linear model evaluates the effect of each individual factor, storage time (ST) and incorporation type (IT), independent of each other. If a significant interaction (<0.05) among the two factors (ST × IT) occurred, these were evaluated simultaneously, and any possible general conclusions or tendency had to be inferred from the estimated marginal means (EMM). If there was no significant interaction (>0.05), each factor was evaluated independently using a simple Student's *T* test (for ST) or a Tukey's multiple comparison test (IT) when the means were homoscedastic, and a Tamhane's T2 for heteroscedastic samples. Homoscedasticity was evaluated using Levene's test.

Additionally, principal components analysis (PCA) was applied to evaluate the affinity (correlation) of each studied variable (nutritional parameters, individual compounds, color parameters and pH) with different mathematical functions (principal components). The number of dimensions kept for data analysis was assessed by the respective eigenvalues (which should be greater than one), by the Cronbach's alpha value (which must be positive) and also by the total percentage of variance (which should be the highest possible) explained by the selected components. Principal components were plotted considering different incorporation types (IT).

All analyses were carried out using a significance level of 0.05.

3. Results and discussion

The phenolic profile of bilberry extract was characterized to identify compounds associated with its bioactivity and colouring potential. Then, the colouring capacity of the extract was tested by incorporation in yogurts and compared with a plain yogurt formulation (without any colourant) and with a positive control, namely E163 which is an industrially produced colourant.

3.1. Identification and quantification of phenolic compounds in bilberry extract

Due to the prevailing colouring capacity of anthocyanins, these compounds were thoroughly characterized in the extract obtained from bilberry fruit. Eleven anthocyanin glycosides were detected derived from delphinidin (Dp; peaks 1, 2 and 4), cyanidin (Cy; peaks 3 and 6), petunidin (Pt; peaks 5 and 8), peonidin (Pn; peaks 7 and 10), and malvidin (Mv; peaks 9 and 11). Compounds were identified based on their chromatographic behaviour and absorption and mass spectra (Table 1). Peaks presented MS^2 fragments corresponding to distinct losses of hexosyl (−162 u) and pentosyl (−132 u) moieties, with elution orders coherent with the expected polarity of the sugar substituents. The position and nature of the sugar moieties were assigned taking into account the previously described literature regarding bilberry extracts,[5–17] as well as by comparison with available commercial standards and data from our compound library.

The Mv glycosides, especially malvidin-3-*O*-galactoside and malvidin-3-*O*-arabinoside, were the main compounds present, comprising 48% of the total anthocyanin content, whereas Dp derivatives (Dp-galactoside, Dp-glucoside and Dp-arabinoside) represented 22%, and Pt 20% (Pt-galactoside and Pt-arabinoside).

3.2. Nutritional profile

The main objective of analysing the nutritional profile was to verify if any change took place along the seven days of storage at 5 °C in the different yogurt formulations. Since two different factors contributed to the result variability, a 2-way ANOVA was used, allowing us to evaluate the effect of each factor, independent of the others. Table 2 shows the nutritional profile of each yogurt formulation (plain, E163, and bilberry extract) in the lower section, and the two analysed times (0 and 7 days) in the upper section. The nutrient values indicated in each part of the table correspond respectively to the means of each storage time (ST) including the three incorporation types (IT),

Table 1 Retention time (R_t), wavelengths of the maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (mg g^{-1} of extract) of the anthocyanins present in the hydromethanolic extracts of bilberry fruits

Peak	R_t (min)	λ_{max} (nm)	$[M]^+$ (m/z)	MS^2 (m/z)	Tentative identification	Quantification (mg g^{-1} of extract)
1	14.4	524	465	303 (100)	Delphinidin-3-*O*-galactoside[a]	2.65 ± 0.01
2	15.9	526	465	303 (100)	Delphinidin-3-*O*-glucoside[a]	0.162 ± 0.001
3	16.9	518	449	287 (100)	Cyanidin-3-*O*-galactoside[b]	0.62 ± 0.02
4	17.7	524	435	303 (100)	Delphinidin-3-*O*-arabinoside[a]	1.91 ± 0.02
5	19.2	528	479	317 (100)	Petunidin-3-*O*-galactoside[c]	2.50 ± 0.02
6	20.2	514	419	287 (100)	Cyanidin-3-*O*-arabinoside[b]	0.464 ± 0.004
7	21.7	518	463	301 (100)	Peonidin-3-*O*-galactoside[d]	0.45 ± 0.01
8	22.6	516	449	317 (100)	Petunidin-3-*O*-arabinoside[c]	1.67 ± 0.03
9	22.8	528	493	331 (100)	Malvidin-3-*O*-galactoside[c]	5.64 ± 0.05
10	26.1	522	433	301 (100)	Peonidin-3-*O*-arabinoside[d]	0.550 ± 0.004
11	28.9	528	463	331 (100)	Malvidin-3-*O*-arabinoside[c]	4.45 ± 0.02
					Total anthocyanins	**21.1 ± 0.2**

[a] Standard calibration curves used for quantification: delphinidin-3-*O*-glucoside ($y = 557274x + 126.24$, $R^2 = 0.999$). [b] Cyanidin-3-*O*-glucoside ($y = 630276x - 153.83$, $R^2 = 0.999$). [c] Malvidin-3-*O*-glucoside ($y = 477\,014.9x + 38.376$, $R^2 = 0.999$). [d] Peonidin 3-*O*-glucoside ($y = 537017x - 71.469$, $R^2 = 0.999$).

Table 2 Nutritional profile of the yogurt samples as a function of the added colorant and the storage time expressed in g per 100 g of fresh weight, and energy in kcal per 100 g of fresh weight

		Moisture	Fat	Proteins	Ash	Carbohydrates	Energy
Storage time (ST)	0 days	85.8 ± 0.3	3.2 ± 0.2	5.2 ± 0.2	0.8 ± 0.1	5.0 ± 0.3	70 ± 2
	7 days	85.3 ± 0.1	3.4 ± 0.2	5.3 ± 0.2	0.7 ± 0.4	5.3 ± 0.4	73 ± 2
p-Value ($n = 27$)	Student's *t*-test	<0.001	<0.001	0.139	0.569	0.033	<0.001
Incorporation Type (IT)	Plain yogurt	85.7 ± 0.5	3.3 ± 0.1[b]	5.3 ± 0.3	0.5 ± 0.2	5.2 ± 0.5	72 ± 4
	E163	85.3 ± 0.1	3.5 ± 0.1[a]	5.3 ± 0.3	1.0 ± 0.2	4.9 ± 0.1	72 ± 1
	Bilberry	85.5 ± 0.3	3.1 ± 0.2[c]	5.1 ± 0.1	0.9 ± 0.2	5.4 ± 0.3	70 ± 1
p-Value ($n = 18$)	Tukey's HSD test	0.010	<0.001	0.003	<0.001	<0.001	0.069
ST × IT ($n = 54$)	*p*-Value	<0.001	0.166	<0.001	<0.001	<0.001	<0.001

In each row and within each storage period, different letters mean significant statistical differences between plain yogurts, yogurts incorporated with E163 and yogurts incorporated with bilberry extract ($p < 0.05$).

and *vice versa*, therefore the standard deviation values should not be regarded as accuracy measure. Besides the effect of each individual factor, the significance of their interaction (ST × IT) was also evaluated. If a significant interaction was found, the classification obtained for multiple comparisons could not be observed, since the effect of each factor was not equal for all levels of the other. In those cases, the presented general tendencies were obtained from the Estimated Marginal Means (EMM) plots. Inversely, if no interaction was found ($p > 0.050$), the factor was classified individually using either Tukey's or Tamhane T2 tests (depending on the homoscedasticity of the distribution), for IT, and a Student's *t*-test for ST.

Yogurts presented a moisture content above 85 g per 100 g and approximately the same levels (~5 g per 100 g) of proteins and carbohydrates, with slightly lower (~3.3 g per 100 g) fat content, corresponding to energy values around 70 kcal per 100 g, validating the labelled information, and showing to be consistent with the typical nutritional composition of plain yogurt[18] ST and IT did not show to exert a cooperative effect over fat content, which was significantly higher in yogurts containing E163 and in stored samples. In turn, the interaction among factors (ST × IT) was significant ($p < 0.050$) for all other parameters in Table 2. Nonetheless, some conclusions could be obtained from the estimated marginal means corresponding to the combined results of these parameters, particularly the higher energy values in samples at 7 days (73 ± 2 kcalper 100 g fw), as it seems logical owing to the moisture loss during storage. Curiously, the ash content is significantly higher in yogurts containing E163 (1.0 ± 0.2 g per 100 g fw) and the bilberry extract, indicating that minerals were incorporated as a part of the composition of those additives. Despite the mathematical significance of these differences, all yogurt samples showed very slight nutritional differences. This fact should be expected as food additives are added to food for a technological goal and should not change in any way the nutritional and chemical aspects of food, except for the cases it is intended for.

3.3. Individual compound analysis

In terms of individual compounds (Table 3) the fatty acids present in the highest percentages were palmitic acid: C16:0 (~35%), oleic acid: C18:1n9 (~21%), myristic acid: C14:0 (~12%), and stearic acid: C18:0 (~11%), which is agreement with the results reported in a similar work.[18] As expected, lactose constitutes the main sugar. In line with the results obtained in the nutritional analysis, the differences obtained for each of the studied individual compounds reflected the significant interaction of both factors (ST and IT), except for the case of lactose, which showed statistically higher contents (despite low magnitude) in yogurts added with bilberry. Accordingly, the few overall tendencies had to be obtained from the EMM plots, which indicated lower C18:0 percentages and galactose contents in plain yogurts.

Table 3 Individual fatty acids found in the yogurt samples as a function of the added colorant and the storage time, expressed as relative percentages of themselves

		C14:0	C16:0	C18:0	C18:1n9	SFA	MUFA	PUFA	Galactose	Lactose
Storage time (ST)	0 days	12.0 ± 0.5	35.0 ± 0.5	11.3 ± 0.4	21 ± 1	71 ± 1	24 ± 1	4.8 ± 0.5	0.6 ± 0.1	4.1 ± 0.2
	7 days	12.2 ± 0.2	35.8 ± 0.2	11.3 ± 0.1	20 ± 1	73 ± 1	23 ± 1	4.8 ± 0.2	0.6 ± 0.1	4.1 ± 0.3
p-Value ($n = 27$)	Student's *t*-test	0.190	<0.001	0.451	0.001	<0.001	<0.001	0.916	0.465	0.985
Incorporation Type (IT)	Plain yogurt	11.9 ± 0.4	35.2 ± 0.5	11.0 ± 0.2	21 ± 1	72 ± 1	23 ± 1	5.0 ± 0.1	0.5 ± 0.1	3.9 ± 0.1[b]
	E163	12.0 ± 0.3	35.2 ± 0.5	11.3 ± 0.1	21 ± 1	72 ± 1	23 ± 1	5.2 ± 0.5	0.7 ± 0.1	4.0 ± 0.1[b]
	Bilberry	12.5 ± 0.5	35.8 ± 0.4	11.6 ± 0.3	20 ± 1	73 ± 1	23 ± 1	4.1 ± 0.4	0.6 ± 0.1	4.4 ± 0.3[a]
p-Value ($n = 18$)	Tukey's HSD test	<0.001	0.004	<0.001	0.240	0.119	0.244	<0.001	<0.001	<0.001
ST × IT ($n = 54$)	*p*-Value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.748

In each row and within each storage period, different letters mean significant statistical differences between plain yogurts, yogurts incorporated with E163 and yogurts incorporated with bilberry extract ($p < 0.05$).

3.4. External colour and pH

Owing to the incorporation of the colouring materials, the main differences were expected to be observed for colour parameters. Besides the Cartesian coordinates (L^*, a^* and b^*), the cylindrical coordinates (C^* and h) were also obtained; C^*, which stands for chroma (relative to saturation), was calculated as $\sqrt{a^{*2} + b^{*2}}$, while the formula used to calculate the hue angle (h) depended on the values of a^* and b^*, *e.g.*, if a^* and b^* were negative, the formula was $180 + \left(\arctan\frac{\frac{b^*}{a^*}}{2\pi}\right) \times 360$.

As depicted in Table 4, all parameters showed significant differences among different yogurt formulations, while the storage time had a very low effect, but for L^* values, which were slightly lower in samples at day 0. Despite the significant effect of IT, the statistical classification results could not be presented, since the interaction among factors was statistically significant in all cases. Nonetheless, the EMM plots allowed concluding that L^*, b^*, and C^* were higher in plain yogurt samples, which in turn showed lower h^* and, in particular, a^* values, as it is logical taking into account due to the very low quantity of red colour existing in the samples without the colorants. A visual representation of the yogurt colour is shown in Fig. 1, where it is clear that both colorants provided colour to the yogurts, although there was a lower colour intensity for the E163 yogurts, particularly at day 7 and mostly due to a decrease in the values of the a^* component (Fig. 2). This is corroborated by the calculated ΔE (total colour difference), which after 7 days reached a value of 3.86 for the E163 yogurts compared to day 0, while the difference for bilberry coloured yogurts was 0.91, very similar to the plain yogurt sample variation of 0.73. This proves the higher stability of the bilberry extract and its viability as a food colourant for yogurt. Similar results of enhanced stability of colorants from natural sources were reported by Nontasan *et al.*[19] who found stable L^* and C^* values in yogurts added with black rice bran for 21 days under 4 °C storage, and by Mohammadi-Gouraji *et al.*[20] following the addition of phycocyanin extracted from *Spirulina platensis* under similar storage conditions. This trend of incorporation of natural colourants in yogurts, not only with pigments from fruits but also flowers and other plant parts, seems to have gained traction, having good consumer acceptance due to a higher correlation in the colour-flavor perception.[20–22]

The pH values for each IT were statistically different, with yogurts containing E163 presenting less acidic values. On the other hand, ST had no effect at all on pH values, independent of IT.

Table 4 External colour profile of the yogurts as a function of the added colorant and along the storage time expressed as L^*, a^*, b^*, C^* and h, as well as pH

		L^*	a^*	b^*	C^*	h	pH
Storage time (ST)	0 days	89 ± 3	1 ± 3	7 ± 2	7 ± 2	199 ± 65	4.6 ± 0.2
	7 days	91 ± 2	0 ± 2	8 ± 1	8 ± 1	208 ± 70	4.6 ± 0.2
p-Value ($n = 27$)	Student's t-test	0.030	0.325	0.179	0.602	0.636	0.365
Incorporation Type (IT)	Plain yogurt	93 ± 1	−3.5 ± 0.1	9.9 ± 0.4	10.5 ± 0.4	110 ± 1	4.4 ± 0.1^{c}
	E163	89 ± 1	3.1 ± 0.5	6.4 ± 0.5	7.3 ± 0.3	244 ± 12	4.9 ± 0.1^{a}
	Bilberry	88 ± 1	1.5 ± 0.3	6.2 ± 0.5	6.4 ± 0.5	256 ± 2	4.6 ± 0.1^{b}
p-Value ($n = 18$)	Tukey's HSD test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ST × IT ($n = 54$)	p-Value	<0.001	<0.001	<0.001	<0.001	<0.001	0.006

In each row and within each storage period, different letters mean significant statistical differences between plain yogurts, yogurts incorporated with E163 and yogurts incorporated with bilberry extract ($p < 0.05$).

Fig. 1 Variation of the yogurt colours during the storage time.

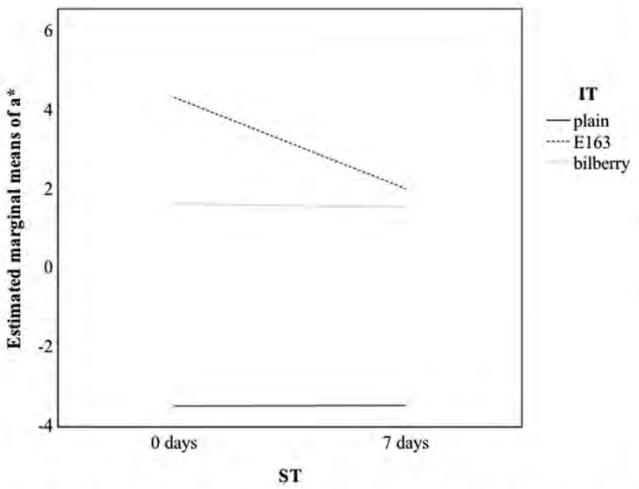

Fig. 2 Estimated marginal mean plots of a^* in yogurt formulations along the storage time.

3.5. Principal component analysis

This analysis was performed to verify the variables with the highest differences among each assayed IT. The first two defined dimensions (first: Cronbach's α = 0.920, eigenvalue = 8.232, explained variance = 47.4%; second: Cronbach's α = 0.871, eigenvalue = 5.940, explained variance = 37.0%) are plotted in Fig. 3. As can be observed, three main groups were formed, two distinct groups corresponding to plain yogurt markers and a big third group containing markers corresponding to yogurts added with E163 or added with bilberry extracts. In an initial analytical approach, it is easy to observe that markers corresponding to plain yogurts and those added with E163 or bilberry extracts were basically separated by dimension 1; considering the variable placement, it is also straightforward that color parameters were the ones with the highest contribution for this separation. Specifically, plain yogurts were mainly characterized by their high L^*, b^* and C^* values, while yogurts containing coloring agents present high a^* and h^* as the most distinguishable features.

In turn, the second dimension separated two distinct groups of markers, both belonging to plain yogurts. According to the SPSS output these two groups correspond exactly to the two assayed periods: 0 days and seven days, which indicates that these types of yogurts did not maintain their characteristics throughout the storage time. The bottom left group includes the 0 days markers (characteristically containing high moisture, C18:1n9 and MUFA contents), while the upper left group contains the markers corresponding to

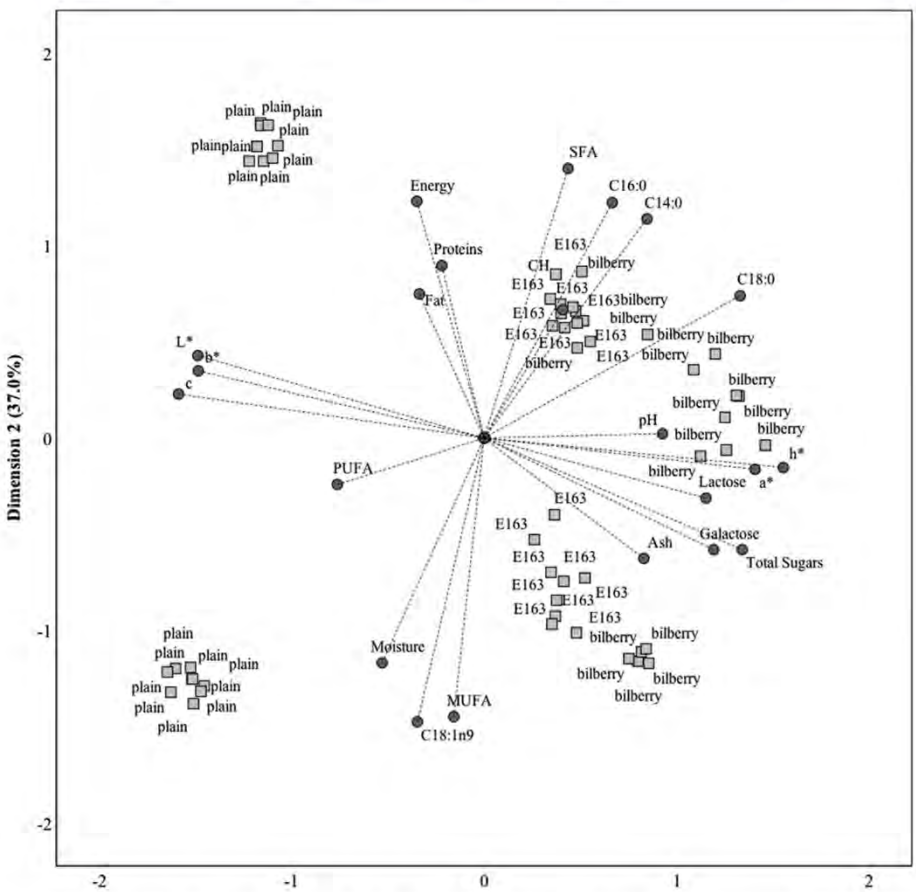

Fig. 3 Canonical discriminant function coefficients defined from the evaluated parameters and plotted to highlight differences among incorporation types.

plain yogurts stored for 7 days (high fat, protein and energy contents).

Nonetheless, with regard to the yogurts with E163 or bilberry extracts, there was no separation among non-stored and stored samples, which is solid evidence of the stability provided by both additives. Despite the resemblance among yogurts with E163 or bilberry extract, the markers corresponding to this last IT were generally placed on the right of those with E163, which is an overall indicator of a higher coloring effectiveness in the case of bilberry extracts (closest location to the a^* variable).

4. Conclusions

Bilberry, when used as a natural colorant for yogurts, showed very interesting potential not only as a stable colourant, but also as a functionalizing agent for this lactic product; moreover, it shows higher stability than its commercial counterpart E163 (counting grape anthocyanins). Likewise, the anthocyanin level of this berry might present additional health benefits to the consumers of products functionalized with its extracts.

Conflicts of interest

The authors declare that they have no conflicts of interest regarding this manuscript.

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Characterization and Application of Pomegranate Epicarp Extracts as Functional Ingredients in a Typical Brazilian Pastry Product

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Abstract: Currently, there is a clear tendency to incorporate natural ingredients into food and pharmaceutical formulations. Besides being well-accepted by consumers, these ingredients have less adverse side effects than their artificial counterparts. The pomegranate processing industry produces large quantities of by-products that are discarded as bio-residues, despite containing bioactive compounds. Accordingly, the epicarp of two pomegranate varieties (*Mollar de Elche* and *Purple Queen*) was tested as a potential source of bioactive compounds with food application. The phenolic profile was identified by HPLC–DAD–ESI/MS, revealing fourteen phenolic compounds in both varieties (*Purple Queen* showed also three anthocyanins), with punicalagin isomers as the major compounds. Nonetheless, *Mollar de Elche* presented greater antioxidant and antibacterial activities. Despite this result, *Purple Queen* was selected to be tested as a new natural colouring and functionalizing ingredient in a Brazilian pastry product. The incorporation of the selected extract maintained the nutritional profile and provided a higher antioxidant activity compared to the traditional product. In this way, this work confirmed the possible use of pomegranate epicarp as a natural ingredient in the food industry, conferring dyeing and functionalizing effects, and anticipating a possible valorisation of this bio-residue.

Keywords: *Punica granatum* L.; phenolic compounds; pectin; bioactivity; food application

1. Introduction

The utilization of products remaining from industrial processing of vegetal products represents an approach with high probability of success, besides holding ecological advantages, considering that the intense growth of the global horticultural sector has produced alarming amounts of discards [1]. However, these bio-residues often contain high-value compounds such as polyphenols, vitamins and fibres [2].

Punica granatum L. (pomegranate), is currently placed in the family Lythraceae, although it was previously included in the Punicaceae [3]. It is ranked among the twenty fruits with highest production levels, and its phenolic compounds have been extensively studied [4]. More than 150 phenolic compounds have been identified in pomegranates, including ellagitannins, anthocyanins, flavonols (e.g., quercetin), phenolic acid (e.g., caffeic acid, chlorogenic acid, gallic acid and quinic acid) and ellagic acid [5,6]. Actually, punicalin and punicalagin were named after the pomegranate

genus, due to the high concentrations of these compounds in this fruit [7,8]. Some of the phenolic compounds present in the pomegranate composition are described as being responsible for its significant biological activity, namely anti-inflammatory action, prevention against certain types of cancer [9], hypoglycaemic and hypocholesterolaemic action [10,11], or preventive action against neurodegenerative diseases [12].

Considering that more than 50% of the whole fruit is discarded as bio-residue, pomegranate has high potential as a source of bioactive compounds for different uses [13]. In addition, the pomegranate epicarp has a concentration of phenolic compounds (essentially tannins, catechins, gallocatechins and prodelfinidines) as much as three times higher than the pulp [14]. Likewise, the percentage of anthocyanins found in the epicarp is very relevant, namely, cyanidin-3,5-*O*-diglucoside, pelargonidin-3,5-*O*-diglucoside, pelargonidin-3-*O*-glucoside, cyanidin-3-*O*- glucoside and cyanidin-3-*O*-rutinoside [5]. In this sense, the food industry has been exploring this type of matrix as a source of natural ingredients with possible application in new products. Accordingly, pomegranate was tested as a new natural colourant and bioactive ingredient, which may represent an effective way to valorise this this bio-residue.

2. Results and Discussion

2.1. Phenolic Characterization of Extracts of Pomegranate Epicarp

From the point of view of bioactive compounds content, pomegranate is particularly interesting for its phenolic compound profile, already described as exerting excellent physiological activities (antioxidant, antimicrobial and anti-inflammatory) and disease-preventive actions [9,15]. Phenolic compounds are essentially present in the epicarp, a non-edible component of the fruit which may represent more than 50% of its total weight [13]. In this study, two varieties (sold as *Mollar de Elche* and *Purple Queen*) were selected for their different phenotypic characteristics and commercial diffusion. Besides the potential differences induced by each variety, the influence of maceration solvent (water, ethanol or an equal w/w mixture of the former) used in the maceration was also verified.

The phenolic profile of *Mollar de Elche* included fourteen compounds, while that of *Purple Queen* presented seventeen (including three anthocyanins). Table 1 describes the chromatographic parameters and spectral data that were considered in the identification of the compounds present in extracts of pomegranate epicarps. The phenolic profile and quantification of the ethanolic, aqueous and hydroalcoholic extracts of both varieties are presented in Table 2. The compounds were identified considering the chromatographic and spectral data previously described in the literature for pomegranate [5,6,8,15–21]. The identified compounds included five phenolic acids, seven hydrolysable tannins and five flavonoids (two flavonols and three glycosylated anthocyanins).

Among phenolic acids (or derivatives), peak 1 was identified as galloylglucose, having a molecular ion $[M - H]^-$ at *m*/*z* 331, which produced an MS^2 fragment at *m*/*z* 169 ($[M - 162]^-$ corresponding to the loss of one hexose relative to gallic acid. Peak 12 was identified as ellagic acid according to the mass and UV spectra and retention times of a commercial standard. On the other hand, peaks 7 ($[M - H]^-$, *m*/*z* 463), 10 ($[M - H]^-$, *m*/*z* 433) and 11 ($[M - H]^-$, *m*/*z* 447) presented UV–Vis and mass spectra characteristic of ellagic acid derivatives, producing a major MS^2 fragment at *m*/*z* 301 (ellagic acid) after the release of hexosyl units (−162 mu, peak 7), pentosyl (−132 mu, peak 10), or rhamnosyl (−146 mu, peak 11). These phenolic acid derivatives were previously described in pomegranate extracts [8,16,17].

The compounds identified as ellagitannins corresponded to peaks 2–6, 8 and 9. Peaks 3 and 4, in particular, were identified as isomers I and II of punicalagin, based on the detected pseudomolecular ion ($[M - H]^-$ at *m*/*z* 1083) and the fragmentation standard previously described by Ambigaipalan et al. [16], Qu et al. [20] and Mena et al. [6]. These molecules were also indicated as being the majority ellagitannins described in pomegranate juice, which was also observed in the present study (see next Section). Peak 2 ($[M - H]^-$ at *m*/*z* 783) presented fragments at *m*/*z* 481 and 301, allowing its identification as pedunculagin (bis-HHDP-glucose) [6]. The mass spectral characteristics of peak 3

($[M - H]^-$ a *m*/*z* 633, fragments *m*/*z* 463 and 301) coincide with galloyl-HHDP-glucose, also described in pomegranate [5,6]. Similarly, peak 6 was identified as digalloyl-HHDP-glucose, another common pomegranate compound [6,16,17,21]. Peaks 8 and 9 presented the same molecular ion ($[M - H]^-$ at *m*/*z* 633), which in this case was attributed to granatin B (digalloyl-HHDP-DHHDP-hexose) and identified as two distinct derivatives of this compound [6,17,21].

In what concerns flavonoids, peaks 13 and 14 were identified as kaempferol-3-*O*-rutinoside and kaempferol-3-*O*-glucoside, respectively, considering their maximum absorbance (λ_{max}) at 348 nm, the characteristic fragmentation standard of glycosylated kaempferol derivatives, UV spectrum and retention time; both compounds were previously described in pomegranate [6,18].

According to the quantification results, the major compounds in *Mollar de Elche* were the two isomers of punicalagin and galloyl-HHDP-glucose, and this tendency was verified in the three extracts. Generally, the extracts obtained with ethanol presented significantly higher contents. The higher efficacy of ethanol for extracting phenolic compounds from pomegranate epicarp was also verified in the case of *Purple Queen*. Likewise, the major compounds in *Purple Queen* variety were the two isomers of punicalagin, although in lower quantities than those obtained in *Mollar de Elche*.

On the other hand, anthocyanins, specifically cyanidin-3,5-*O*-diglucoside, cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside, were only detected in *Purple Queen*. In the case of this subgroup, the aqueous extract allowed the greatest yield, which is in agreement with the greater polarity of this type of compounds. Cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside were also identified after comparison of the chromatographic and spectral data with commercial standards. A third anthocyanin ($[M-H]^-$ at *m*/*z* 611) was identified as cyanidin-3,5-*O*-diglucoside, based on its fragmentation standard. The three anthocyanins identified are also characteristic of pomegranate [5,6,8,17].

2.2. Characterization of Bioactivity of Extracts of Pomegranate Epicarp

As could somehow be anticipated, considering the contents of phenolic compounds described in the previous section, all the extracts presented a significant antioxidant activity. In the case of *Mollar de Elche* pomegranate, the highest activity was measured in the aqueous extract (10 μg/mL), while in *Purple Queen* the extract with the greatest lipid peroxidation inhibition capacity was the hydroalcoholic (10 μg/mL). Thus, there was no direct correlation with the levels of phenolic compounds which, as described in the previous section, were higher in the ethanolic extract, indicating that other compounds with antioxidant activity may be present in the extracts. Even so, the lowest EC_{50} values were obtained in *Mollar de Elche*, which correlates with the highest concentrations of phenolic compounds quantified in this variety.

In addition to antioxidant activity, the cytotoxicity evaluated in *Mollar de Elche* epicarp extracts was also superior to that observed in *Purple Queen* extracts. However, in both cases, a greater effectiveness of the ethanolic extracts was verified. The cell line with the highest growth inhibition (Table 3) was HeLa (91 μg/mL for *Mollar de Elche* ethanolic extract, 153 μg/mL for *Purple Queen* ethanolic extract), whereas the one that showed to be less affected was NCI-H460 (194 μg/mL for *Mollar de Elche* ethanolic extract, 268 μg/mL for *Purple Queen* ethanolic extract). This differentiation in inhibitory capacity was verified independently of the extract type. In the case of hepatotoxicity, GI_{50} values were higher in all cases, which can be considered as a good result, despite the lower proliferation rate that characterizes this cell line, when compared with the evaluated tumour cell lines.

Table 1. Retention time (Rt), wavelengths of maximum absorption (λ_{max}), mass spectral data and attempt to identify the phenolic compounds.

Peak	R_t (min)	$\lambda_{máx}$ (nm)	Molecular Ion $[M - H]^-$ (*m*/*z*)	MS^2 (*m*/*z*)	Identification Attempt
1	4.4	267	331	169(100), 125(33)	Galloylglucose
2	4.6	258, 368	783	481(44), 301(100)	Pedunculagin (bis-HHDP glucose)
3	6.1	256, 378	1083	781(26), 601(13), 301(100)	Punicalagin isomer I
4	7.4	254, 378	1083	781(33), 601(21), 301(100)	Punicalagin isomer II
5	8.9	278	633	463(31), 301(100)	Galloyl-HHDP-glucose
6	13.5	278	785	633(17), 615(5), 483(100), 419(8), 301(50)	Digalloyl-HHDP-glucose
7	15.3	253, 358	463	301(100)	Ellagic acid-hexoside
8	16.6	276	951	933(100), 631(12), 613(9), 463(17), 301(48)	Granatin B (Digalloyl-HHDP-DHHDP-hexose) isomer I
9	17.3	276	951	933(100), 631(15), 613(5), 463(12), 301(49)	Granatin B (Digalloyl-HHDP-DHHDP-hexose) isomer II
10	20.6	256, 364	433	301(100)	Ellagic acid-pentoside
11	21.0	256, 364	447	301(100)	Ellagic acid-rhamnoside
12	22.0	256, 364	301	284(10), 245(5), 185(11), 173(4), 157(6), 145(5)	Ellagic acid
13	23.4	346	593	285(100)	Kaempferol-3-*O*-rutinoside
14	25.0	348	447	285(100)	Kaempferol-3-*O*-glucoside
			Anthocyanins		
15	8.9	515	611	449(23), 287(100)	Cyanidin-3,5-*O*-diglucoside
16	16.9	514	449	287(100)	Cyanidin-3-*O*-glucoside
17	20.0	505	433	271(100)	Pelargonidin-3-*O*-glucoside

Table 2. Quantification of phenolic compounds (mg/100 g dry weight) in different extracts of pomegranate peel of the *Mollar de Elche* and *Purple Queen* varieties.

Phenolic Compound	Variety of Pomegranate	Type of Extract			Homoscedasticity [1] (*p*-Value) (*n* = 27)	ANOVA[2] (*p*-Value) (*n* = 27)
		Aqueous	Ethanolic	Hydroalcoholic		
Galloylglucose [A]	*Mollar de Elche*	5.9 ± 0.1 b	8.4 ± 0.4 a	5.7 ± 0.3 b	0.003	<0.001
	Purple Queen	1.0 ± 0.1 c	2.1 ± 0.2 a	1.3 ± 0.1 b	<0.001	<0.001
Pedunculagin (bis-HHDP glucose) [B]	*Mollar de Elche*	3.5 ± 0.4 c	11.8 ± 0.4 a	7.0 ± 0.4 b	0.896	<0.001
	Purple Queen	1.2 ± 0.1 c	2.4 ± 0.2 a	1.7 ± 0.1 b	0.217	<0.001
Punicalagin isomer I [B]	*Mollar de Elche*	23 ± 2 c	61 ± 2 a	40 ± 2 b	0.975	<0.001
	Purple Queen	12 ± 1 c	30 ± 1 a	16 ± 1 b	0.348	<0.001
Punicalagin isomer II [B]	*Mollar de Elche*	18 ± 2 c	73 ± 3 a	28 ± 2 b	0.403	<0.001
	Purple Queen	15 ± 1 c	41 ± 3 a	20 ± 1 b	<0.001	<0.001
Galloyl-HHDP-glucose [B]	*Mollar de Elche*	12 ± 1 c	21 ± 1 a	15 ± 1 b	0.477	<0.001
	Purple Queen	2.4 ± 0.2 c	6.6 ± 0.5 a	3.2 ± 0.2 b	<0.001	<0.001
Digalloyl-HHDP-glucose [B]	*Mollar de Elche*	8 ± 1 c	11 ± 1 a	10 ± 1 b	0.029	<0.001
	Purple Queen	1.1 ± 0.1 b	1.7 ± 0.2 a	0.9 ± 0.1 c	0.004	<0.001
Ellagic acid-hexoside [B]	*Mollar de Elche*	5.2 ± 0.3 c	7.7 ± 0.3 a	4.7 ± 0.3 b	0.936	<0.001
	Purple Queen	1.2 ± 0.1 c	3.2 ± 0.2 a	1.7 ± 0.1 b	0.188	<0.001
Granatin B (digalloyl-HHDP-DHHDP-hexose) isomer I[B]	*Mollar de Elche*	8.0 ± 0.5 c	10.6 ± 0.4 a	8.7 ± 0.4 b	0.190	<0.001
	Purple Queen	2.8 ± 0.1 c	5.4 ± 0.2 a	3.3 ± 0.2 b	0.403	<0.001
Granatin B (digalloyl-HHDP-DHHDP-hexose) isomer II [B]	*Mollar de Elche*	2.4 ± 0.3 c	4.3 ± 0.3 a	2.1 ± 0.2 b	0.225	<0.001
	Purple Queen	1.1 ± 0.1 b	1.6 ± 0.1 a	0.6 ± 0.1 c	0.337	<0.001
Ellagic acid-pentoside [B]	*Mollar de Elche*	1.9 ± 0.2 a	2.0 ± 0.2 a	1.5 ± 0.2 b	0.290	<0.001
	Purple Queen	0.6 ± 0.1 a	0.7 ± 0.1 a	0.8 ± 0.1 a	<0.001	<0.001
Ellagic acid-rhamnoside [B]	*Mollar de Elche*	1.3 ± 0.1 c	2.1 ± 0.3 a	0.9 ± 0.2 b	0.043	<0.001
	Purple Queen	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.190	0.207
Ellagic acid [B]	*Mollar de Elche*	4.3 ± 0.2 c	6.2 ± 0.5 a	3.1 ± 0.5 b	0.143	<0.001
	Purple Queen	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.009	0.944
Kaempferol-3-*O*-rutinoside [C]	*Mollar de Elche*	1.3 ± 0.1 a	1.0 ± 0.1 b	0.8 ± 0.1 c	0.652	<0.001
	Purple Queen	0.4 ± 0.1 ab	0.3 ± 0.1 c	0.5 ± 0.1 a	0.835	0.013
Kaempferol-3-*O*-glucoside [D]	*Mollar de Elche*	0.5 ± 0.1 a	0.5 ± 0.1 ab	0.4 ± 0.1 b	0.029	0.013
	Purple Queen	0.18 ± 0.02 b	0.19 ± 0.04 b	0.27 ± 0.04 a	0.058	0.013

Table 2. *Cont.*

Anthocyanins						
Cyanidin-3,5-*O*-diglucoside [E]	*Purple Queen*	7.9 ± 0.4 a	4.5 ± 0.2 c	5.7 ± 0.3 b	0.039	<0.001
Cyanidin-3-*O*-glucoside [E]	*Purple Queen*	7.3 ± 0.2 a	4.0 ± 0.1 c	5.1 ± 0.3 b	0.097	<0.001
Pelargonidin-3-*O*-glucoside [F]	*Purple Queen*	4.3 ± 0.2 a	1.8 ± 0.1 c	2.5 ± 0.2 b	0.062	<0.001

Calibration curves: A: ellagic acid (y = 365.2x − 38.923; R^2 = 0.999); B: gallic acid (γ = 38.466x + 35.44; R^2 = 0.999); C: kaempferol -3-*O*-rutinoside (y = 182.94x + 96.644; R^2 = 0.997); D: kaempferol-3-*O*-glucoside (y = 236.33x + 70.006; R^2 = 0.999); E: cyanidin-3-*O*-glucoside (y = 134578x − 3E6; R^2 = 0.999); F: pelargonidin-3-*O*-glucoside (y = 61493x − 628875; R^2 = 0.996). [1] *p*-Values less than 0.05 indicate heteroscedastic distributions, and the multiple comparison was made by the Tamhane's T2 test; *p*-values greater than 0.05 indicate homoscedastic distributions, and the multiple comparison was made by Tukey HSD test. [2] If *p*-value is less than 0.05, the corresponding parameter shows significant differences in at least one of the extract types (identified with different letters within the same raw).

Table 3. Results of inhibition of the formation of thiobarbituric acid reactive substances (TBARS) and cytotoxicity in different extracts of the epicarp of the *Mollar de Elche* and *Purple Queen* varieties.

Antioxidant and Cytotoxicity	**Extract Type**			**Homoscedasticit [1] (*p*-Value) (*n* = 27)**	**ANOVA [2] (*p*-Value) (*n* = 27)**
	Aqueous	**Ethanol**	**Hydroalcoholic**		
Mollar de Elche					
Inhibition of TBARS formation (EC_{50}, μg/mL)	10 ± 1 c	18 ± 1 a	16 ± 1 b	0.002	<0.001
PLP2 (GI_{50}, μg/mL)	333 ± 9 a	276 ± 12 b	295 ± 34 b	<0.001	<0.001
HeLa (GI_{50}, μg/mL)	141 ± 2 b	92 ± 3 c	178 ± 5 a	0.042	<0.001
HepG2 (GI_{50}, μg/mL)	196 ± 6 b	111 ± 4 c	228 ± 4 a	0.153	<0.001
MCF7 (GI_{50}, μg/mL)	178 ± 4 b	122 ± 6 c	216 ± 6 a	0.191	<0.001
NCI-H460 (GI_{50}, μg/mL)	211 ± 16 b	194 ± 3 c	259 ± 5 a	<0.001	<0.001
Purple Queen					
Inhibition of TBARS formation (EC_{50}, μg/mL)	29 ± 1 c	37 ± 1 a	19 ± 3 b	0.001	<0.001
PLP2 (GI_{50}, μg/mL)	354 ± 24 a	299 ± 12 b	357 ± 7 b	0.012	<0.001
HeLa (GI_{50}, μg/mL)	223 ± 6 b	153 ± 6 c	321 ± 13 a	0.010	<0.001
HepG2 (GI_{50}, μg/mL)	205 ± 5 b	216 ± 2 c	318 ± 6 a	0.026	<0.001
MCF7 (GI_{50}, μg/mL)	246 ± 13 b	228 ± 8 c	269 ± 6 a	0.030	<0.001
NCI-H460 (GI_{50}, μg/mL)	260 ± 11 b	268 ± 36 c	250 ± 14 a	0.001	0.293

[1] Values of *p* less than 0.05 indicate heteroscedastic distributions, the multiple comparison being made by the Tamhane's T2 test; *p*-values greater than 0.05 indicate homoscedastic distributions, and multiple comparisons were made by the Tukey HSD test. [2] If the *p*-value is lower than 0.05, the corresponding parameter presents significant differences in at least one of the types of extract (identified with different letters within the same raw).

According to Table 4, all samples showed inhibitory activity against the analysed microorganisms. However, they did not show bactericidal capacity up to the maximum tested concentration of 20 mg/mL.

In general, Gram-positive bacteria were the most sensitive, as lower concentrations (MIC = 0.625–5 mg/mL) of extracts were required to inhibit their growth. Within these bacteria, MRSA showed higher sensitivity (MIC values of 0.625–1.25 mg/mL), followed by *E. faecalis* (MIC = 0.625–2.5 mg/mL) and *L. monocytogenes* (MIC = 2.5–5 mg/mL); the measured activity was higher than that reported by Alexandre et al. (MRSA: MIC = 3.91 mg/mL; *L. monocytogenes*: MIC = 15.63 mg/mL) [22].

For Gram-negative bacteria, *M. morganii*, *P. mirabilis* and *E. coli* were the most sensitive (MIC = 1.25–2.5 mg/mL), followed by *K. pneumoniae* (MIC = 1.25–5 mg/mL), and *P. aeruginosa*, which proved to be the most resistant (MIC in the range of 10–20 mg/mL). Fawole et al. [4] reported lower MIC values with methanolic extracts (*E. coli*: 0.78 mg/mL; *K. pneumoniae*: 0.39 mg/mL), but higher MIC values for aqueous extracts (12.50 mg/mL for both bacteria).

Comparing the pomegranate varieties, *Mollar de Elche* presented higher antibacterial activity (for all extracts), as evidenced by lower MIC values. The best extract analysed was obtained with ethanol (MIC of 0.625 and 1.25 mg/mL in most cases), which agrees with the higher concentration of phenolic compounds in *Mollar de Elche*. In contrast, the aqueous extract from *Purple Queen* epicarp showed the weakest antibacterial activity (MIC = 1.25–20 mg/mL).

It is important to note that the tested samples showed lower MIC than ampicillin against some bacteria. In the study of the inhibitory effect against *K. pneumoniae*, for example, 10 mg/mL of ampicillin was required, while the extracts tested showed the same capacity at lower concentrations (1.25 to 5 mg/mL). Similarly, a MIC of 20 mg/mL was required for *M. morganii*, while the extracts exerted the same effect at concentrations among 1.25 and 2.5 mg/mL. For *P. aeruginosa*, ampicillin had no inhibitory activity up to 20 mg/mL, whereas the extracts inhibited this species with concentrations of 10 to 20 mg/mL. These results interesting, given the increasing resistance of these microorganisms to the antibiotics used in clinical practice. Thus, the search for natural alternatives with similar or better effectiveness is a global priority in the fight against these resistant bacteria.

Some studies have highlighted the high number of compounds present in the pomegranate peel, which has aroused great curiosity, mainly by the food industry, aiming using a bio-waste with high application potential [23]. The presence of a high concentration of phenolic compounds has been justifying the different bioactivities revealed by the skin of different varieties of pomegranate. A study by Gullon et al. [24] revealed the antimicrobial ability of pomegranate peel (Mollar de Elche) for all of the microorganisms studied both Gram positive (*S. aureus*, *L. monocytogenes* and *L. innocua*) and Gram negative (*E. coli*, *P. aeruginosa* and *Salmonella* sp.). Similarly, the antioxidant activity of the peels has also been highlighted. In particular Derakhshan et al. [23]., highlighted greater antioxidant activity in the peels when compared to seed and juice of their extracts.

2.3. Characterization of New Formulations of "Casadinhos"

In general, the different extracts (mainly ethanolic extracts) obtained from the epicarp of both pomegranate varieties represent a source of phenolic compounds that allow it to exhibit excellent bioactive properties with potential application in the food industry. However, when developing novel foods, the end product may not exhibit certain types of bioactivity, e.g., antimicrobial, for the well-known reasons associated with the appearance of multi-resistant microbial strains, or cytotoxic effects, since this particularity could cause undesirable effects on normal cell proliferation in the body (for example along the gastrointestinal epithelium). For this reason, associated with the greater colouring ability of the extracts obtained from *Purple Queen* epicarp, the ethanolic extracts of this variety were used in the preparation of a traditional Brazilian pastry product, called *casadinho*s.

Table 4. Results of the antibacterial activity of different extracts of the pomegranate epicarp of the varieties *Mollar de Elche* and *Purple Queen*.

Bacteria Strain	Variety of Pomegranate	Extract type			Ampicillin (20 mg/mL)	Imipenem (1 mg/mL)
		Aqueous (MIC)[A]	Ethanol (MIC) [A]	Hydroalcoholic (MIC) [A]		
Gram-negative bacteria						
Escherichia coli	*Mollar de Elche*	2.5	1.25	2.5	MIC < 0.15	MIC < 0.0078
	Purple Queen	2.5	1.25	1.25	MBC < 0.15	MBC < 0.0078
Klebsiella pneumoniae	*Mollar de Elche*	2.5	2.5	1.25	MIC = 10	MIC < 0.0078
	Purple Queen	5	2.5	2.5	MBC = 20	MBC < 0.0078
Morganella morganii	*Mollar de Elche*	1.25	1.25	2.5	MIC = 20	MIC < 0.0078
	Purple Queen	2.5	1.25	1.25	MBC > 20	MBC < 0.0078
Proteus mirabilis	*Mollar de Elche*	1.25	1.25	2.5	MIC < 0.15	MIC < 0.0078
	Purple Queen	2.5	1.25	1.25	MBC < 0.15	MBC < 0.0078
Pseudomonas aeruginosa	*Mollar de Elche*	10	10	10	MIC > 20	MIC = 0.5
	Purple Queen	20	10	10	MBC > 20	MBC = 1
Gram-positive bacteria						
Enterococcus faecalis	*Mollar de Elche*	1.25	0.625	0.625	MIC < 0.15	nt
	Purple Queen	2.5	2.5	2.5	MBC < 0.15	
Listeria monocytogenes	*Mollar de Elche*	5	2.5	2.5	MIC < 0.15	MIC < 0.0078
	Purple Queen	5	5	5	MBC < 0.15	MBC < 0.0078
MRSA	*Mollar de Elche*	0.625	0.625	0.625	MIC < 0.15	nt
	Purple Queen	1.25	0.625	0.625	MBC < 0.15	

A: The minimum bactericidal concentration (MBC) values for the extracts were not presented because this activity was not verified until the maximum concentration tested (20 mg/mL). MIC: minimum inhibitory concentration. MBC: minimum bactericidal concentration. MRSA: Multi-resistant *Staphylococcus aureus*.

Using *Mollar de Elche* epicarp would not provide such an obvious change in the appearance of the products, besides increasing the possibility of obtaining a final product with undesired bioactive features (cytotoxic and antimicrobial). In order to increase the usefulness of pomegranate, a third formulation of this product was prepared in which the traditionally used filling, guava jelly, was substituted for a jelly prepared from pomegranate juice and pectin extracted from the epicarp.

In summary, three formulations were prepared: (i) traditional (CT), (ii) *casadinhos* with incorporation of pomegranate epicarp extract and guava filling (CEC), and (iii) *casadinhos* incorporating pomegranate epicarp extract and pomegranate jelly filling (CECP).

The different formulations were compared for their nutritional composition, free sugars and fatty acids profile and the colour parameters: L^*, a^* and b^*. In order to verify the functionalization of the product, its ability to inhibit lipid peroxidation was also evaluated.

It should be noted that, in any case, two factors of statistical variability were considered, namely the formulation (F) and the storage time (ST). Thus, in addition to assessing the significance of the changes induced by each individual factor, it is also necessary to verify if the two factors have significant (p-value < 0.05) interaction (F × ST). Presenting the results in this way implies that the value for each storage period is obtained from the average of the values of the three formulations for that period, and also, obviously, that the result obtained for each formulation is the result of the average of the three storage times for that formulation (this justifies some relatively high standard deviations values).

2.3.1. Nutritional Composition and Sugar Profile of "Casadinhos"

Table 5 shows the average values, in g/100 g of fresh product, obtained for the nutritional composition, free sugars and energy value of the analysed formulations. In general, carbohydrates were the most abundant component (≈74%), followed by fat (≈20%) and water (≈3–7%). In relation to the effects of each individual factor, while there were significant differences between the formulations for all parameters, the same was verified for ST only in the case of water, carbohydrates, sucrose, ash and energy content. The interaction was significant for all parameters, except for protein content, justifying why the classification obtained from the multiple comparison tests was performed only in the case of this parameter, and in relation to the effect of the formulation, which presented higher levels in the non-stored samples and in CECP. In relation to the other parameters, it was possible to obtain some conclusions from the estimated marginal means. For instance, fructose and glucose presented lower values in CT samples, while sucrose was detected in lower concentrations in CEPC. On the other hand, CEC had the lowest values of carbohydrates (73 g/100 g), fat (19 g/100 g) and energy (469 kcal/100 g) being, on the other hand, those that registered higher levels of water (7 g/100 g). Regarding ST effect, the induced differences were not so evident, except for the lower carbohydrate content (72 g/100 g) observed in non-stored samples. The incorporation of natural ingredients in different food matrices has been tested in order to verify its potential application as substitutes for artificial additives. Some studies on different pastry products have proven that this same application does not cause significant changes in the food matrix [25,26].

2.3.2. Fatty Acids Profile of "Casadinhos"

As a previous note, it should be highlighted that only fatty acids with percentages greater than 1% for at least one of the formulations or storage times were tabulated.

Once again, there was a significant interaction between the two factors (F × ST) for almost all cases, except for the percentage of C15:0 (p = 0.355), which did not show differences for each single factor. However, in the case of fatty acids, ST effect was more significant than that of the formulation (Table 5), as can be verified by the number of fatty acids with significant differences for each factor.

Despite these differences, all formulations presented similar profiles, characterized by high percentages of palmitic acid (≈30%) and oleic acid (≈21%), the major fatty acids.

Similarly, and despite the observed differences, it was not possible to identify unequivocal trends resulting from the individual effects of each factor, regardless of the statistically significant differences that were detected in some cases.

Thus, it can be concluded that the addition of the extracts in the dough and using pomegranate jelly as filling did not cause significant changes in the fatty acid profile of the traditional formulation of *casadinhos*.

2.3.3. Colorimetric Parameters and Textural Characteristics of "Casadinhos"

In addition to the nutritional aspects and the individual components discussed in the previous sections, the analysis of the colour parameters (Table 5), namely *L**, *a** and *b**, has an increased relevance, considering the objective of achieving a more appealing appearance for this food product.

The changes observed in physical properties, namely in colorimetric parameters and structural characteristics, resulted, again, from the cooperative action of both factors, since their interaction was always significant.

Regarding the individual effect of each factor, a higher influence of the formulation was observed, since ST only caused significant changes in fracturability ($p = 0.034$) and chewiness ($p = 0.004$). Therefore, it was once again necessary to observe the graphs of the estimated marginal means to infer some trends.

In relation to lightness, the addition of ethanolic extract of *Purple Queen* caused a slight lowering, having, on the other hand, and as expected, increased the intensity of the red hue (*a**). As for parameter *b**, the CEC formulation presented statistically lower values, although the magnitude of the difference is negligible. It should be noted that the small variations in the colour hue detected may also be due to the Maillard reactions. The result of these reactions is the golden aspect of the food when toasted or caramelized. This reaction occurs between amino acids or proteins (amine groups) and sugars (carbonyl groups) when the food is made, leading to the production of melanoidins [27].

The *casadinhos* prepared with the incorporation of pomegranate extract also presented lower hardness and fracturability. Thus, it is consistent with chewiness (the energy needed to chew food), which is superior in the case of the traditional formulation, raising the possibility of increasing the range of consumers.

2.3.4. Bioactivity of the "Casadinhos"

In addition to the appearance and texture of samples added with pomegranate extract, it was also important to verify if these new products maintained the antioxidant activity determined in the extracts, which would be an advantageous feature.

In the inhibition of thiobarbituric acid reactive substances, the traditional formulation showed $EC_{50} = 2268 \pm 714$ μg/mL, while CEC and CECP presented incomparably lower values: $EC_{50} = 79 \pm 11$ μg/mL and $EC_{50} = 67 \pm 21$ μg/mL, respectively. In addition to this important improvement in the antioxidant activity, it was also possible to verify that, while there was a loss of the already weak activity of the formulation over the storage time, in the cases of CEC and CECP, the antioxidant activity remained unchanged over the 14 days of storage. The incorporation of antioxidant activity has been proven by other authors, who have even proved the effectiveness of natural ingredients compared to artificial additives [28,29].

Table 5. Nutritional composition, free sugars (g/100 g), energy value (kcal/100 g), profile in fatty acids, colorimetric parameters and textural characteristics of the different formulations of *casadinhos*.

Factors Analyzed		Water	Fat	Proteins	Carbohydrates	Fructose	Glucose	Sucrose	Energy	Ashes
Formulation(F)	CT	3 ± 1	21 ± 1	0.7 ± 0.1 c	75 ± 1	4.1 ± 0.2	4 ± 1	39 ± 1	494 ± 5	0.5 ± 0.1
	CEC	7 ± 1	19 ± 1	0.8 ± 0.1 b	73 ± 1	5.8 ± 0.5	7 ± 1	38 ± 1	469 ± 8	0.5 ± 0.1
	CECP	4 ± 1	21 ± 1	0.9 ± 0.1 a	73 ± 1	5.8 ± 0.5	6 ± 1	32 ± 3	491 ± 4	0.5 ± 0.1
p-Value (*n* = 27) [A]		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Storage time (ST)	0 days	6 ± 2	21 ± 1	0.9 ± 0.1	72 ± 1	5 ± 1	6 ± 1	35 ± 5	478 ± 14	0.4 ± 0.1
	7 days	4 ± 2	21 ± 1	0.8 ± 0.1	74 ± 1	5 ± 1	6 ± 1	36 ± 4	485 ± 12	0.6 ± 0.1
	14 days	3 ± 1	21 ± 1	0.8 ± 0.1	74 ± 1	5 ± 1	5 ± 1	38 ± 2	490 ± 9	0.5 ± 0.1
p-Value (*n* = 27) [B]		<0.001	0.134	0.166	<0.001	0.262	0.212	0.006	<0.001	<0.001
F × ST *p*-value (*n* = 81) [A]		<0.001	0.016	0.103	0.013	0.002	<0.001	<0.001	<0.001	<0.001

		C6:0	C8:0	C10:0	C12:0	C14:0	C14:1	C15:0	C16:0	C16:1	C18:0	C18:1n9	C18:3n6	C18:3n3	SFA	MUFA	PUFA
Formulation (F)	CT	3 ± 1	2.0 ± 0.4	3.7 ± 0.4	4.4 ± 0.3	12 ± 1	1.0 ± 0.1	1.0 ± 0.1	30 ± 1	1.4 ± 0.1	10.9 ± 0.3	21 ± 1	6.1 ± 0.5	0.9 ± 0.1	69 ± 2	24 ± 2	7.2 ± 0.5
	CEC	4 ± 1	1.9 ± 0.2	3.5 ± 0.2	4.3 ± 0.3	12 ± 1	1.0 ± 0.1	1.0 ± 0.1	31 ± 1	1.4 ± 0.1	10.9 ± 0.3	21 ± 1	6.0 ± 0.5	0.9 ± 0.1	69 ± 1	23 ± 1	7.1 ± 0.5
	CECP	3 ± 1	1.8 ± 0.3	3.4 ± 0.4	4.2 ± 0.3	12 ± 1	0.8 ± 0.1	1.0 ± 0.1	31 ± 1	1.4 ± 0.1	11.2 ± 0.5	21 ± 1	6.6 ± 0.5	1.0 ± 0.1	69 ± 2	24 ± 1	7.8 ± 0.5
p-Value (*n* = 27) [A]		0.166	0.084	0.015	0.057	0.128	<0.001	0.676	0.135	0.120	0.007	0.219	<0.001	0.202	0.155	0.817	<0.001
Storage time (ST)	0 days	3 ± 1	1.8 ± 0.2	3.4 ± 0.3	4.4 ± 0.2	12.2 ± 0.5	0.9 ± 0.1	1.0 ± 0.1	31 ± 1	1.4 ± 0.1	10.9±0.3	21 ± 1	6.3 ± 0.3	1.0 ± 0.1	69 ± 2	24 ± 2	7.3 ± 0.3
	7 days	3 ± 1	1.9 ± 0.4	3.5 ± 0.5	4.4 ± 0.3	12.2 ± 0.4	1.0 ± 0.1	1.0 ± 0.1	31 ± 1	1.5 ± 0.1	11.1±0.4	21 ± 1	5.7 ± 0.5	0.9 ± 0.1	69 ± 2	24 ± 1	6.8 ± 0.5
	14 days	4 ± 1	2.1 ± 0.1	3.6 ± 0.2	4.1 ± 0.2	11.4 ± 0.5	0.9 ± 0.1	1.0 ± 0.1	30 ± 1	1.4 ± 0.1	11.0±0.4	20 ± 1	6.7 ± 0.5	1.0 ± 0.1	69 ± 1	23 ± 1	7.9 ± 0.5
p-Value (*n* = 27) [B]		<0.001	0.001	0.048	<0.001	<0.001	0.006	0.061	<0.001	<0.001	0.361	0.012	<0.001	<0.001	0.155	0.511	<0.001
F × ST *p*-value (*n* = 81) [A]		<0.001	<0.001	<0.001	<0.001	<0.001	0.002	0.355	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

		*L**	*a**	*b**	Hardness	Fracturability	Chewiness	Resilience
Formulation (F)	CT	78 ± 1	−1 ± 1	30 ± 1	4614 ± 685	4571 ± 828	172 ± 110	0.004 ± 0.002
	CEC	65 ± 2	8 ± 1	28 ± 1	1456 ± 252	1394 ± 311	29 ± 5	0.019 ± 0.003
	CECP	64 ± 1	8 ± 1	30 ± 1	2883 ± 187	2932 ± 152	46 ± 13	0.023 ± 0.002
p-Value (n = 27)[A]		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Storage time (ST)	0 days	68 ± 7	6 ± 5	30 ± 2	2553 ± 1121	2439 ± 1043	46 ± 16	0.02 ± 0.01
	7 days	70 ± 6	5 ± 4	29 ± 2	2972 ± 1288	3046 ± 1474	77 ± 51	0.02 ± 0.01
	14 days	70 ± 7	5 ± 4	29 ± 2	3428 ± 1564	3413 ± 1511	124 ± 138	0.01 ± 0.01
p-Value (*n* = 27) [B]		0.421	0.580	0.670	0.061	0.034	0.004	0.241
F × ST *p*-Value (*n* = 81) [A]		0.010	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[A] *p*-Values lower than 0.05 indicate that at least one of the formulations is significantly different from the others. [B] *p*-Values less than 0.05 indicate that at least one of the storage times is significantly different from the remaining ones. [C] *p*-Values less than 0.05 indicate a significant interaction between the factors, so it is not possible to classify the differences induced by each of the individual factors.

3. Materials and Methods

3.1. Preparation of Samples

Two different types of pomegranates sold as *Purple Queen* (three samples of red pomegranate) and *Mollar of Elche* (three samples of yellow pomegranate) were purchased from a local market in Bragança, Portugal. The epicarp was finely separated from the pulp and both were frozen and then lyophilized and stored in a desiccator at room temperature (average 25 °C), protected from light, until further analysis.

3.2. Extracts Preparation

The lyophilized samples (1 g) of both varieties were submitted to maceration at room temperature with 30 mL of different solvents (water, ethanol or ethanol:water 50:50, *v*/*v*), during 1 h with agitation (150 rpm). Subsequently, extracts were filtered through a filter paper (Whatman No. 4) and the retained residue was re-extracted under the same conditions. Finally, the alcoholic fraction of the extracts obtained was evaporated under reduced pressure (rotary evaporator, Büchi R-210, Flawil, Switzerland) and lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA).

3.2.1. Identification and Quantification of Phenolic Compounds

The phenolic compounds (non-anthocyanin and anthocyanin compounds) were separated, identified, and quantified following a previously optimized methodology [30,31] and using a Dionex Ultimate 3000 UPLC system (Thermo Scientific, San Jose, CA, USA). The detection was performed with a DAD and a mass spectrometer (LTQ XL mass spectrometer, Thermo Finnigan, San Jose, CA, USA) working in negative mode for non-anthocyanin compounds and positive mode for anthocyanin compounds.

Analytical curves (200–5 μg/mL) of the available phenolic standards were constructed based on the UV-Vis signal: gallic acid ($y = 365.2x - 38.923$, $R^2 = 0.999$); ellagic acid ($y = 38466x + 35.44$, $R^2 = 0.9994$); kaempferol-3-*O*-rutinoside ($y = 182.94x + 96.644$, $R^2 = 0.997$); kaempferol-3-*O*-glucoside ($y = 236.33x + 70.006$, $R^2 = 0.999$); cyanidin-3-*O*-glucoside ($y = 134578x - 3 \times 10^6$; $R^2 = 0.9986$) and pelargonidin-3-*O*-glucoside ($y = 61493x - 628875$; $R^2 = 0.9957$). Results were expressed in mg/g of dry extract.

3.2.2. Evaluation of Extracts' Antioxidant Activity

The lyophilized samples were re-dissolved in ethanol and successively diluted (5–500 μg/mL) to allow determining the corresponding EC_{50} values. The lipid peroxidation inhibition in porcine (*Sus domesticus*) brain homogenates was evaluated by the decrease in thiobarbituric acid reactive substances (TBARS) following the protocol described by Barros et al. [32]. The results were expressed in EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay) and Trolox was used as the standard.

3.2.3. Evaluation of Extracts' Toxicity

All the extracts were tested in four human tumour cell lines: MCF- 7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma), using the sulforhodamine B assay to measure the cell growth inhibition [33]. The hepatotoxicity was measured by using a freshly harvested porcine liver cell culture, PLP2 (acquired from certified slaughterhouses) [32].

In both assays, a phase contrast microscope was used to monitor the growth of cell cultures, which were sub-cultured and plated in 96-well plates (density of 1.0×10^4 cells/well). Dulbecco's modified Eagle's medium (DMEM) supplemented with FBS (10%), penicillin (100 U/mL) and streptomycin (100 μg/mL) were used. As a positive control, ellipticin was used. The results were expressed as GI_{50} values (μg/mL).

3.2.4. Evaluation of Extracts' Antibacterial Activity

The bacterial strains were clinical isolates obtained from patients hospitalized in various departments at the Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Five Gram-negative bacteria (*Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Morganella morganii*) and three Gram-positive bacteria (*Enterococcus faecalis*, *Listeria monocytogenes* and methicillin-resistant *Staphylococcus aureus* (MRSA)) were tested. All these microorganisms were incubated at 37 °C in appropriate fresh medium for 24 h before analysis to maintain the exponential growth phase.

The MIC determinations on all bacteria were conducted using colorimetric assay according to described by Pires et al. [34]. Ampicillin and Imipenem were used for all Gram-negative bacteria tested and *Listeria monocytogenes*. Ampicillin and vancomycin were selected for *E. faecalis* and MRSA. For the determination of MBC, 10 µL of liquid from each well that showed no change in colour was plated on solid medium, Blood agar (7% sheep blood) and incubated at 37 °C for 24 h.

3.3. Incorporation of Natural Ingredient in a Traditional Brazilian Bakery Product

Purple Queen was selected to be tested as a new natural colouring and functionalizing ingredient and three groups of samples were prepared: (i) control samples (*casadinhos* with traditional cake dough and guava jam); (ii) samples with functional dough (*casadinhos* with cake dough functionalized with red pomegranate epicarp ethanolic extract and guava jam); (iii) samples with functional dough and jam (*casadinhos* with cake dough functionalized with red pomegranate epicarp ethanolic extract and pomegranate jelly).

Casadinhos dough was prepared by mixing wheat flour, sugar and butter. After that in both functionalized groups the red pomegranate epicarp extract was added to the mixture. The dough was then divided into small spherical portions and baked at 180 °C for 15 min. The filling was placed directly between two cooked portions. Guava jam was purchased at a local store. The pomegranate jam was prepared with water, lemon, sugar and juice of a red pomegranate. The mixture was warmed and stirred slightly for about two hours.

All samples were lyophilized, finely crushed and analysed, in triplicate, immediately after preparation and after seven and fourteen days of storage (at room temperature and packed in a sealed plastic bags covered with aluminium paper).

3.3.1. Evaluation of Colour Parameters and Texture of "Casadinhos" during Storage Time

The colour of the samples was measured using a colorimeter (model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan). The illuminate C was used and a diaphragm aperture of 8 mm and previously calibrated against a standard white tile. The CIE L^* (lightness), a^* (greenness/redness), b^* (blueness/yellowness) colour space values were registered using a data software ''Spectra Magic Nx" (version CM-S100W 2.03.0006) [35]. The texture was determined using a TA-XT plus Texture Analyser implemented with the Exponent software version 6.1.11.0 (Stable Micro System, London, UK) with an acrylic disk (40 mm) in order to measure different parameters: hardness, adhesiveness, resilience, cohesion, elasticity, gum and chewability.

3.3.2. Nutritional Composition

The contents of protein, fat, carbohydrates and ash, were determined following the AOAC methods [36] and following a procedure previously reported by Barros et al. [32]. Total energy was calculated following the equation:

$$\text{Energy (kcal)} = 4 \times (\text{g protein} + \text{g carbohydrates}) + 9 \times (\text{g fat}) \quad (1)$$

3.3.3. Chemical Composition

Free Sugars Present in Epicarps of Both Varieties

One gram of dried sample was mixed with melezitose (internal standard (IS) 5 mg/mL), and extracted with 40 mL of ethanol:water (80:20) 80 °C for 30 min. After that, the suspension was centrifuged (Centurion K24OR refrigerated centrifuge, West Sussex, UK) at 15,000 *g* for 10 min and the supernatant was concentrated at 60 °C under reduced pressure and defatted three times with 10 mL of ethyl ether, successively. The solid residues were dissolved in water to a final volume of 5 mL and filtered through 0.2 μm Whatman nylon filters. HPLC coupled to a refraction index (RI) detector was used to determine the free sugars following a previously described procedure [35]. The free sugars were identified by comparison with standards and further quantified considering the internal standard.

Fatty Acids in Pulp and Epicarp of Both Varieties

The fatty acids were determined by gas chromatography coupled with a flame ionization detector (GC–FID, DANI model GC 1000, Contone, Switzerland), following a procedure previously described [32]. The results were expressed as relative percentage of each fatty acid.

3.3.4. Evaluation of Antioxidant Activity

The lyophilized samples (3 g) were extracted with ethanol/water (80:20) at room temperature during 1 h under stirring. The extract was filtered with Whatman paper filter Nº 4 (Sigma-Aldrich, St Louis, MO, USA) and the remaining solid residue subjected to an additional extraction at the same conditions. The extracts were evaporated under reduced pressure in a rotatory evaporator until complete removal of methanol. Finally, the evaporated extract was dissolved in methanol at a concentration of 50 mg/mL and submitted to TBARS previously described in Section 3.2.2.

3.4. Statistical Analysis

For each extract (aqueous, ethanolic or hydroalcoholic), three independent samples were analysed in triplicate. Likewise, from each *casadinho* formulation, three independent samples were also selected, and each sample was analysed in triplicate. Data were expressed as mean±standard deviation. The statistical tests were applied considering a value of $\alpha = 0.05$ (95% confidence) using the IBM SPSS Statistics for Windows software version 25.0 (IBM Corp., New York, NY, USA).

An analysis of variance (ANOVA) was performed, based on the Tukey test (when homoscedasticity of the distributions was verified) or the Tamhane's T2 test (heteroscedastic distributions) to classify the statistical differences between the different parameters evaluated in each of the extracts. Compliance with the ANOVA requirements, specifically the normality of the distribution of results and the homogeneity of variances, was verified through the Shapiro-Wilk and Levene tests, respectively.

In the case of the *casadinhos*, an analysis of variance (ANOVA) was performed with type III sum of squares, using the generalized linear model (GLM) procedure. All dependent variables were analysed using a 2-factor ANOVA, specifically the formulation (F) and storage time (ST). In cases where there was a significant interaction between the two factors, the results were compared through the estimated marginal means, in all cases where the effect of each individual factor was statistically significant.

4. Conclusions

This work focused on the extraction of phenolic compounds from bio-residues (epicarps) from *Punica granatum* L. to test its potential as a natural functionalising ingredient to be incorporated in a pastry product. The characterization of the phenolic profile of the extracts obtained from the three solvents expressed the existence of fourteen phenolic compounds in ME, the most abundant being the two isomers of punicalagin and galloyl-HHPD-glucose, and seventeen compounds in *Purple*

Queen, of which the most abundant were the two isomers of punicalagin, in smaller amounts than those obtained in *Mollar de Elche*. In turn, the presence of anthocyanins, namely cyanidin-3,5-*O*-diglucose, cyanidin-3-*O*-glucose and pelargonidin-3-*O*-glucose, was verified only in *Purple Queen* (being maximum in water extracts). The phenolic composition and the bioactive characteristics presented by the extracts favour the development of applications in the food industry. Thus, given the moderate bioactivity (characteristic required for food application) of the extract obtained from *Purple Queen*, it was incorporated in the formulation of a typical Brazilian pastry product, *casadinhos*. This incorporation has a double application as functional food and as a colouring agent. The incorporation of the natural ingredient did not cause significant changes in the nutritional profile or chemical composition but added antioxidant capacity. The rose colour and soft texture added to the biscuit presented by the new formulations will be pleasant points to present to consumers. This work demonstrates that with the use of eco-friendly solvents and with raw material that until now is considered waste, it is plausible the development of economically interesting products of public interest. Sensory studies that assess the acceptance of this type of food by consumers should also be carried out.

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Betacyanins from *Gomphrena globosa* L. flowers: Incorporation in cookies as natural colouring agents

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ABSTRACT

A betacyanin rich extract was obtained from the flowers of *Gomphrena globosa* L. by ultrasound-assisted extraction and dried either by lyophilization or spray-drying, was tested as a natural colourant in cookies and compared to a commercial colourant. The extracts were characterized in terms of betacyanin content and antioxidant potential. The effects of the colourants incorporation in the cookies were assessed through proximate composition, soluble sugars, fatty acids, color, texture and microbial load, over a shelf life of 30 days. Considering all the assays and analyzing the results through a 2-way analysis of variance, the cookies incorporated with spray-dried colourant showed the most intense pink coloration while cookies incorporated with lyophilized extract lost less color intensity over time. Thus, betacyanin extracts have potential as pink natural alternatives to synthetic colourants in the food industry.

1. Introduction

Natural food additives have been used in the food industry for a long time (Tomaska & Brooke-Taylor, 2014). Nevertheless, these natural additives present some stability problems as is the case of natural colourants (Wu et al., 2020), which are sensitive to temperature, pH, light and storage conditions, leading to the assumption that these additives are not viable for industrial applications (Leong et al., 2018). The synthetic counterparts present higher stability under the same conditions as well as higher colouring capacity and maintenance (Martins, Roriz, Morales, Barros, & Ferreira, 2016). Recent scientific studies have shown that artificial additives have several side effects and are often associated with toxicity problems (Zhang et al., 2020), such as hyperactivity and allergies (Kumar, Singh, Sharma, & Kishore, 2019). Aware of these evidences, the regulatory agencies, namely the European Food Safety Authority (EFSA) and the Food and Drug Administration of the United States of America (FDA) established several restrictions on the use of these additives (Carocho, Morales, & Ferreira, 2015). Together with these scientific facts, consumers also started to show extra care about their health and started to seek for safer and natural additives, causing an enormous pressure to the industry and scientific community (Roriz, Barreira, Morales, Barros, & Ferreira, 2018). This fact, associated with current food trends, where visual traits are an important attribute (Stich, 2016), together with health benefits (Martins, Sentanin, & De Souza, 2019), a new era of scientific challenges focused on the exploitation of natural resources for industrial applications, with safer and health-promoting agents (Maqsood, Adiamo, Ahmad, & Mudgil, 2020). These functional products should provide consumers with new experiences and, at the same time, be able to meet nutritional needs, showing preventive disease effects (Roriz et al., 2018). All of these different functions can be obtained from natural sources due to the richness in bioactive compounds, able to provide health benefits and at the same time act as food preservatives, colouring agents among other properties (Majerska, Michalska, & Figiel, 2019).

One of the most important agents in the food industry are colourants, which are especially responsible for the attractiveness of foods. In this particular case, there are already some natural colourants approved by EFSA, namely carotenoids, chlorophylls, anthocyanins and betalains (Martins et al., 2016). The latter, for instance, are chromoalkaloids derived from tyrosine, divided into two subgroups, the betaxanthins that present a yellow/orange colour and the betacyanins, with colours between pink and violet (Martins, Roriz, Morales, Barros, & Ferreira, 2017). Betacyanins are very similar to anthocyanins, regarding their

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colour, but showing a stronger colouring power (Roriz, Barros, Prieto, Morales, & Ferreira, 2017). The preferable natural source to extract this class of compounds from is beetroot (*Beta vulgaris* L.) although there are also flowers and fruits that can be considered as good sources of this natural colourant (Calogero et al., 2012; Melgar et al., 2017; Roriz, Barros, Prieto, Barreiro et al., 2017). Since natural colourants became a global hot topic, new extraction, purification and stabilization procedures needed to be developed (Corrêa et al., 2019) to guarantee the viability of these colourants as candidates for effective applications at the industrial level (Roriz et al., 2018; Roriz, Barros, Prieto, Barreiro et al., 2017).

Gomphrena globosa L. flowers have purple colour and are a rich source of betalains, namely betacyanins with strong colouring capacity as described in a recent work, where the incorporation of *G. globosa* extracts in ice cream showed promising results regarding their colouring potential (Roriz et al., 2018).

Thus, the objective of the present work was to evaluate the potential of *G. globosa* flowers as a source of betalains and their viability as colouring agents in the food industry, namely in pastry products. The extracts were obtained by ultrasound-assisted extraction (UAE) and further dried through spray-drying and lyophilization techniques. The stability of these extracts after incorporation into cookies, was studied, namely the centesimal composition, chemical and physical parameters, over a shelf-life of 30 days. All the performed assays with cookie formulations were compared with samples incorporated with a widespread commercial colourant (E162).

2. Material and methods

2.1. Plant samples

Gomphrena globosa L. plants were acquired from Ervital, a company established in a mountain region of great biodiversity, Castro D'Aire, Portugal. Ervital has a wide certified collection of vegetable materials from different origins and applies sustainable harvesting of spontaneous local species, and organic farming of exogenous species. The botanical identification was made by the botanical expert responsible for the collection of medicinal plants in the herbarium of Escola Superior Agrária (BRESA), Polytechnic Institute of Bragança (Trás-os-Montes, Portugal). Subsequently, the plants were subjected to a mechanical treatment to separate the coloured parts of the flower (intense purple-pink colour), obtaining the pigmented floral parts (bracts and bracteoles) from the inflorescences, as described by Roriz, Barros, Prieto, Barreiro et al. (2017).

2.2. Preparation of the betacyanin's rich extracts

G. globosa extracts rich in betacyanins were obtained by UAE (QSonica sonicators, model CL-334, Newtown, CT, USA), working at 500 W, for 22 min, using water (treated in a Milli-Q water purification system, TGI Pure Water Systems, Greenville, SC, USA) as the preferred extraction solvent and a liquid-to-solid ratio of 5 g/L, as described previously by Roriz et al. (2018). The extracts were further dried using two different methodologies: i) lyophilization (FreeZone 4.5, Labconco, Kansas City, MO, USA) and ii) spray-drying, to evaluate the impact of these technologies towards the stability of the extracts. In the lyophilization process, the extracts were placed in appropriate containers and frozen at −80 °C through a fast-freezing process, in order to avoid compromising the stability of the colouring compounds. After freezing, the extracts were placed in the lyophilizer at a constant temperature of −45 °C and 0.04 mBar of pressure until the samples were completely dried (4 days). For spray-drying, the samples were dried using 20% of maltodextrin (w/w), a drying adjuvant (Molina et al., 2019). The percentage of maltodextrin (20%, w/w) was relative to the total solids content of the extract sample to be spray-dried, being established in previous studies (Molina et al., 2019). The solutions containing the betacyanins and maltodextrin were prepared immediately before atomization. Briefly, the extracts were mixed with maltodextrin and further homogenized by stirring for 10 min at room temperature, using a stirring plate and a magnetic bar. The used spray-drying equipment was a Mini Spray Dryer B-290 Büchi (Flawil, Switzerland) programmed in normal operation mode (nozzle diameter: 0.7 mm; atomized volume: 200 mL, solids content < 33%). The used operation conditions were established according to previous works using this drying co-adjuvant (inlet temperature 140 °C, outlet temperature 72 °C, aspiration 90% and pump 20% (6 mL/min)) (Molina et al., 2019). The collected dry samples were kept in sterile flasks protected from light (4 °C) until further analysis. The overall yield was estimated as the ratio between the weight of recovered powder (dry basis) and the weight of the initial solids in the atomized solution (dry basis). In the lyophilization process, a non-powdered and heterogeneous sample was obtained displaying a dark purple colour, while the spray-drying technique rendered a homogeneous and shiny powder displaying a deep purple-pink hue.

2.3. Betalains quantification and identification

Betacyanin were profiled using a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) couple to a DAD (using 530 nm as the preferred wavelength) and to a mass spectrometer working in positive mode (Linear Ion Trap LTQ XL, Thermo Finnigan, San Jose, CA, USA) and equipped with an ESI source, following a procedure previously reported (Melgar et al., 2017). The separation was carried out in an AQUA® (Phenomenex) reverse phase C_{18} column (5 μm, 150 × 4.6 mm i.d) operating at 35 °C. The betacyanin's identification was performed by comparing the obtained information with available data reported in the literature giving a tentative identification. For quantitative analysis, a calibration curve using an isolated compound gomphrenin III (isolated from *Gomphrena globosa* L.) was constructed based on the UV signal ($y = 14670x - 19725$, $R^2 = 0.9997$) (Roriz et al., 2017). The results of betacyanins were expressed as mg per g of extract.

2.4. Evaluation of the anti-haemolytic activity

The antioxidant capacity of the extracts was evaluated by the oxidative haemolysis inhibition assay (OxHLIA). An erythrocyte solution (2.8%, v/v; 200 μL) was mixed with 400 μL of either extracts dissolved in phosphate-buffered saline (PBS), having one control with only PBS solution, or water (for complete haemolysis). After a pre-incubation period of 10 min with shaking at 37 °C, 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) (200 μL, 160 mM in PBS, from Sigma-Aldrich) was added, and the optical density (690 nm) measured every 10 min using a microplate reader (Bio-Tek Instruments, ELX800) until complete haemolysis (Lockowandt et al., 2019). Trolox was the positive control. The results were expressed as IC_{50} values (μg/mL) at a Δt of 60 min, representing the concentration required to keep 50% of the erythrocyte population intact for 60 min.

2.5. Preparation of the cookies with the colouring agents

To prepare the cookies, 450 g of butter (pasteurized butter Continente®; composition per 100 g: 3016 KJ, or 733 Kcal energetic value; 81 g of lipids of which 58 g were saturated; 0.6 g of carbohydrates, being the majority sugars; 0.5 g of protein; and 1.8 g of salt) was mixed with 540 g of sugar (granulated white sugar Continente®; composition per 100 g: 1700 KJ, or 400 Kcal energetic value; 0 g of lipids; 100 g of carbohydrates of which 100 g were sugars; 0 g fibres; 0 g of protein; and 0 g of salt). Then, 840 g of flour (self-raising wheat flour Continente®; composition per 100 g: 1421 KJ, or 333 Kcal energetic value; 1 g of lipids of which 0.3 g were saturated; 71 g of carbohydrates of which 2 g were sugars; 3 g of fibres; 9 g of protein; and 2 g of salt), 20 g of yeast powder (yeast powder Continente®; composition per

100 g: 589 KJ, or 139 Kcal energetic value; 0.8 g of lipids of which < 0.1 g are saturated; 32.5 g of carbohydrates of which < 0.1 g are sugars; 0.1 g fibres; 0.5 g of protein; and 31.1 g of salt), 50 g of water and 100 g of vegetable oil (cooking oil Continente®; composition per 100 mL: 3397 KJ, or 826 Kcal energetic value; 92 g of lipids of which 10 g are saturated; 0 g of carbohydrates; 0 g of protein; and 0 g of salt) mixed with a stand mixer (Food Processor SKM 550 A1, SilverCrest, Hamburg, Germany). The cookie dough was divided into four equivalent parts, and identified as: i) control (cookie dough without colouring agents); ii) cookie dough coloured with E162 (commercial natural food colourant; 2 g, i.e. ≈4 mg/100 g cookie dough); iii) cookie dough coloured with lyophilized *G. globosa* extract (1 g, i.e. ≈2 mg/100 g cookie dough); iv) cookie dough coloured with spray-dried *G. globosa* extract 1 g, i.e. ≈2 mg/100 g cookie dough). For the batches where the colourant was added, the dough was continuously kneaded until the dough color presented was homogeneous. For each batch, the cookie dough was divided into approximately 12 g balls, on a tray lined with parchment paper and baked for 25 min at ≈ 140 °C. After baking, the cookies were separated to be analysed through at four different storage times, using 10 cookies per time. Prior to analysis, all cookie samples were lyophilized, finely crushed and analyzed (in triplicate), immediately after preparation, and at three more sampling times (7; 15; and 30 days of storage). Cookies were stored at room temperature and packed in a sealed plastic bag (zip lock bag, ≅ 0,1mm of thickness) covered with aluminium paper.

2.6. Chemical composition of the prepared cookies

2.6.1. Nutritional value

The pulverized samples were analysed according to the AOAC procedures in terms of macronutrients (moisture, proteins, fat, carbohydrates and ash) (AOAC, 2010). The macro-Kjeldahl method was used to estimate the crude protein content (Nx5.70). To determine the crude fat content, a Soxhlet extraction with petroleum ether was performed. Incineration at 600 ± 15 °C was used to measure ash content. Total carbohydrates were calculated by difference, and the energetic value calculated as: Energy (kcal) = 4× (g protein + g carbohydrate) + 9× (g fat).

2.6.2. Soluble sugars

A HPLC coupled to a refraction index (RI) detector (Knauer, Smartline system 1000, Berlin, Germany) was used to determine soluble sugars, using the internal standard method (IS, melezitose, Sigma-Aldrich, St. Louis, MO, USA), as previously described by Barros, Pereira, Calhelha et al. (2013). The mobile phase was a mixture of acetonitrile:water (70:30 v/v, acetonitrile HPLC-grade, Lab-Scan, Lisbon, Portugal), and separation was achieved using a Eurospher 100–5 NH_2 column (4.6 × 250 mm, 5 µm, Knauer). The results were recorded and processed using Clarity 2.4 software (DataApex, Prague, Czech Republic).

2.6.3. Tocopherols

Tocopherols determination followed the procedure previously described by Barros, Pereira, Calhelha et al. (2013). Briefly, the method comprises a HPLC system (Knauer, Smartline system 1000, Berlin, Germany) coupled to a fluorescence detector (FP-2020; Jasco, Easton, USA) programmed for excitation at 290 nm and emission at 330 nm, using the IS method (tocol, Matreya, Pleasant Gap, PA, USA) for quantification. The mobile phase was a mixture of hexane:ethyl acetate (70:30, v/v, hexane and ethyl acetate HPLC-grade, Lab-Scan, Lisbon, Portugal), and chromatographic separation was performed using a Polyamide II column (250 × 4.6 mm, 5 µm; YMC, Kyoto, Japan). The results were recorded and processed using Clarity 2.4 software (DataApex, Prague, Czech Republic).

2.6.4. Fatty acids

A gas chromatographer (DANI1000, Contone, Switzerland) equipped with a split/splitless injector, and a flame ionization detector (GC-FID at 260 °C) operating in the conditions described by Barros, Pereira, and Ferreira (2013) was chosen. The identification and quantification of fatty acids was achieved by comparing the relative retention times of fatty acid methyl esters (FAME) reference standard mixture, Sigma-Aldrich, St. Louis, MO, USA) standards. The results were recorded and processed using CSW 1.7 software (Data Apex 1.7, Prague, Czech Republic).

2.7. Stability of the prepared cookie formulations

2.7.1. Physical parameters

2.7.1.1. Texture. A Stable Micro Systems (Vienna Court, Godalming UK) TA.XT) Plus Texture Analyser with a 30 Kg load cell, using the P/45 45 mm aluminium cylinder probe was used to carry out the texture analysis. A Texture Profile Analysis (TPA) was performed on the samples using a 5 mm/s as the pre- and post-test speed, and 3 mm/s as the test speed. The target mode was set to "strain" at 25% strain level for 5 consecutive seconds, while the trigger was set to "force" with measurement starting at 50 g of force. After the analysis, a macro was performed to measure various dimensions of texture, namely hardness, adhesiveness, springiness, cohesiveness, chewiness and resilience. The texture results were achieved through the Exponent program, proprietary of Stable Micro Systems.

2.7.1.2. Moisture. The cookie samples (2 g) were put in the metal plate and placed in a moisture analyzer (Adam Equipment, PMB 163). This equipment increases the temperature gradually to 105 °C to force moisture to evaporate from the food sample. When the weight achieves a constant value, i.e. no evaporation is detected, the sample is weighed a second time. The results were obtained using the following equation: % Moisture = (mi-mf)/mi × 100. Where mi is the initial weight and mf is the weight after reaching a constant weight.

2.7.1.3. Colour analysis. The external colour was measured in three different points of the cookies for the various storage times. Colour was on the exterior and interior of the cookie, as well as on the powder of ground cookies. This assay was performed with a portable CR400 colourimeter from Konica Minolta (Chiyoda, Tokyo, Japan) with the D65 illuminant, a standard illuminant defined by the International Commission on Illumination (CIE) which represents the midday light in Europe (daylight illuminant). The CIE L* a* b* colour space of 1976 was used, with L* representing lightness, a* representing redness (red-green), and b* representing yellowness (yellow-blue), with a 10° observation angle and 8 mm aperture.

2.7.2. Microbiological analysis

The final cookies were analysed for their stability regarding microbial growth control over the shelf life. Briefly, 1 g of the cookie powder was mixed with 9 mL of peptone water (PW, Liofilchem, Italy) and, from this suspension, serial decimal dilutions were prepared until achieving 10^{-3}. Afterwards, different counts were performed:

Aerobic plate count (total viable count; ISO 4833-2:2013: 1 mL of the prepared suspensions were inoculated in 15 mL of melted PCA (plate count agar, Liofilchem, Italy) (kept at 50 °C in a water bath or incubator), using the pour plate technique, in duplicate (LOQ = 1 log UFC/g). The plates were homogenized and left to solidify. The plates were then incubated at 30 °C for 72 h, in a reversed position. The counting was performed only in the plates presenting between 15 and 300 colonies.

Coliforms (and E. coli; ISO 4832:2006): 1 mL of the prepared suspensions were inoculated in 15 mL of melted VRBLA (violet red bile lactose agar, Liofilchem, Italy) (kept at 50 °C in a water bath or incubator), using the pour plate technique, in duplicate (LOQ = 1 log

UFC/g). The plates were homogenized and left to solidify. On the top of the medium, a top layer of 4 mL of VRBLA was poured, and it was left to solidify. Afterwards the plates were incubated at 30 °C for 48 h, in a reversed position. The counting was performed only in the plates presenting between 10 and 150 colonies.

Yeasts and Moulds (ISO 21527-1/2:2008): 0.2 mL of the prepared suspensions were pipetted onto a plate containing 15 mL of DRBC (dichloran rose bengal chloramphenicol, Liofilchem, Italy) by the spread plate technique, in duplicate (LOQ = 1.7 log UFC/g). The plates were further incubated at 25 °C for 5 days, in the upright position. The counting was performed in the plates having less than 150 colonies; the count of yeast and mould colonies was performed separately after 2 and 5 days of incubation, respectively.

Bacillus cereus (ISO 7932:2004). 0.2 mL of the prepared suspensions were pipetted onto a plate containing 15 mL of MYP (mannitol yolk polymyxin, Liofilchem, Italy) using the spread plate technique, in duplicate (LOQ = 1.7 log UFC/g). The plates were incubated at 30 °C for 24–48 h, in reversed position. The counting was performed in the plates showing between 10 and 150 colonies.

The microbial load of the different cookies was assessed after their preparation (t0) and after 30 days of storage.

2.8. Statistical analysis

Throughout the manuscript, all data is expressed as mean ± standard deviation. For the betacyanin's characterization of the two different natural extracts, an ANOVA was used followed by a Tukey's test to classify the differences among the three samples. If only two samples showed specific betacyanin compounds, the differences were sought by means of a simple Student's T-Test, using a significance level of 0.05 for both statistical analysis. For the cookie characterization, a two-way ANOVA with type III sums of squares using the SPSS Software, version 25. This multivariate general linear model treats the two factors, storage time (ST) and colourant type (CT) as independent, thus allowing the effect of each one to be analyzed independently, providing more insight to their contribution towards the outcome. If a significant interaction ($p < 0.05$) was recorded among the two factors (ST × CT), these were evaluated simultaneously, and some general conclusions and tendencies were extracted from the estimated marginal means (EMM) plots. If a significant interaction was not detected ($p > 0.05$), each factor was classified independently using a Tukey's multiple comparison test when the means were homoscedastic, and a Tamhane's T2 for non-homoscedastic samples. Homoscedasticity was evaluated using a Levene's test. All analyses were carried out using a significance level of 0.05.

3. Results and discussion

Cookies are bakery products of great commercial interest considering their production, commercialization and consumption characteristics, high demand, relatively long shelf life and good acceptability, particularly by children. Knowing the optimal proportions, and the impact of each ingredient in shape, appearance and product acceptance is very important, as demonstrated by Chin (1988). Therefore, several studies focusing on the improvement of this type of product, either by changing some of its ingredients, by using healthier alternatives, such as flour (Bassinello et al., 2011; Cheng & Bhat, 2016; Kaur, Singh, & Kaur, 2017), sugars (Aggarwal, Sabikhi, & Sathish Kumar, 2016), fibre supplementing (Baumgartner, Özkaya, Saka, & Özkaya, 2018; Galla, Pamidighantam, Karakala, Gurusiddaiah, & Akula, 2016; Mudgil, Barak, & Khatkar, 2017), or bioactive compounds (Infante et al., 2017) have been conducted. This trend can be justified by the growing appetence for natural additives, in response to the increasing need of industry, consumers, and to access the possibility to include these new product formulations in the functional product line.

The main objective of this work was to understand the influence of different colourants, namely a commercial one, E162, and another extracted from natural sources and dried in two different ways (lyophilization and spray-drying) all compared to a control sample, without any colouring. The influence of the colourants was analysed in different parameters, namely microbial load, nutritional profile, tocopherols presence, texture and exterior and interior colour.

3.1. Drying processes

Two different techniques were used to dry the betacyanin extracts, lyophilization and spray-drying. The spray-drying process had a yield of 20% due to inherent equipment losses. In the lyophilization process, a non-powdered and heterogeneous sample was obtained due to the presence of interfering molecules. With the spray-drying technique, a homogeneous, and shiny powder without agglomeration was produced due to the presence of maltodextrin. Even having a lower yield, the spray-drying technique provided an extract with better appearance and homogeneity. The main objective of using these two techniques was to evaluate which of them allows higher stability of the betacyanins and enables the obtaining of a homogeneous extract.

3.2. Betacyanins quantification and identification

Table 1 presents the peak characteristics (retention time, wavelength of maximum absorption and mass spectral data), tentative identification and quantification (mg/g of extract) of the betacyanin compounds present in the commercial colourant, lyophilized or spray dried extract of *G. globosa* flowers. Eleven different compounds were tentatively identified in the three samples. The tentative identification of the betacyanin extracts profile was performed following several descriptions already published by other authors. For *G. globosa* samples the tentative identification of peaks 3 ($[M+H]^+$ at *m/z* 551, gomphrenin I), 4 ($[M+H]^+$ at *m/z* 551, isogomphrenin I), 7 ($[M+H]^+$ at *m/z* 727, *cis*-Isomer of gomphrenin III), 8 ($[M+H]^+$ at *m/z* 727, *cis*-Isomer of isogomphrenin III), 9 ($[M+H]^+$ at *m/z* 697, gomphrenin II), 10 ($[M+H]^+$ at *m/z* 727, gomphrenin III), and 11 ($[M+H]^+$ at *m/z* 727, isogomphrenin III) was performed using the previously description made by Roriz, Barros, Carvalho, Santos-Buelga, and Ferreira (2014) of the same *G. globosa* sample, but also following the description made by Kugler, Stintzing, and Carle (2007) in crude extracts, Ferreres, Gil-Izquierdo, Valentão, and Andrade (2011) in aqueous extracts, and Cai, Xing, Sun, and Corke (2006) in hydroethanolic extracts of *G. globosa*.

The profile of the commercial extract obtained from *B. vulgaris*, as can be seen in Table 1, is completely different from *G. globosa*. Peaks 1 ($[M+H]^+$ at *m/z* 551, betanin), 2 ($[M+H]^+$ at *m/z* 551, isobetanin), 5 ($[M+H]^+$ at *m/z* 549, unknown compound) and 6 ($[M+H]^+$ at *m/z* 549, neobetanin), have been previously identified in beetroot juice (except for peak 5), being tentatively identified following the descriptions made by Nemzer et al. (2011). The presence of orange neobetanin pigments in *B. vulgaris* sample can also be a determining factor for the colorimetric difference between this commercial extract and the ones analyzed in this manuscript. The amount of betacyanins present in the commercial sample of beetroot is four-fold lower (39.36 ± 0.01 mg/g of extract) than in globe amaranth samples (161.6 ± 3.4 and 134 ± 2 mg/g of extract in the lyophilized and spray-dried sample, respectively), thus revealing that these extracts are excellent sources of these type of compounds. The large quantity obtained in the globe amaranth samples is mainly due to the presence of gomphrenin II (61 ± 2 and 45.25 ± 0.04 mg/g of extract) both in lyophilized and spray-dried samples, respectively. Between the two extracts (lyophilization and spray-drying), significant differences were detected, with lyophilization revealing the highest amount of total betacyanins, although the spraydrying extract showed a more intense coloration (Fig. 2). A more in-depth study should be carried out to uncover if the observed differences in the total amount of betacyanins justifies the expenses that the spray-drying technique implies.

Table 1
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification (mg/g of extract) of the betacyanins compounds present in the three samples studied (mean ± SD).

Peak	Rt	λmax (nm)	$[M-H]^+$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	C	L	Sd	*t*-Studentstest *p*-value
1	17.26	533	551	389(1 0 0),345(5),150(5)	betanidin-5-*O*-*β*-glucoside (Betanin)	19.3 ± 0.2[c]	nd	nd	–
2	18.64	530	551	389(1 0 0),345(6),150(5)	Isobetanidin-5-*O*-*β*-glucoside (Isobetanin)	20.1 ± 0.2	nd	nd	–
3	19.63	533	551	389(1 0 0),345(6),150(7)	Gomphrenin I	nd	6.6 ± 0.1	6.9 ± 0.1	0.010
4	20.78	531	551	389(1 0 0),345(6),150(7)	Isogomphrenin I	nd	3.2 ± 0.1	3.3 ± 0.1	0.009
5	21.96	544	549	387(1 0 0)	Unknown compound	nq	nd	nd	–
6	22.65	544	465	387(1 0 0)	14,15-Dehydrobetanin (Neobetanin)	5.1 ± 0.1	nd	nd	–
7	30.11	544	727	551(52),389(1 0 0)	*cis*-Isomer of gomphrenin III	nd	31 ± 1	42 ± 1	< 0.001
8	31.19	542	727	551(49),389(1 0 0)	*cis*-Isomer of isogomphrenin III	nd	23.2 ± 0.1	16 ± 1	< 0.001
9	30.47	544	697	551(38),389(1 0 0)	Betanidin-6-*O*-(6′-*O*-*trans*-4-coumaroyl)-*β*-glucoside (gomphrenin II)	nd	61 ± 2	45.25 ± 0.04	< 0.001
10	31.67	545	727	551(60),389(1 0 0)	Betanidin-6-*O*-(6′-*O*-*trans*-feruloyl)-*β*-glucoside (gomphrenin III)	nd	19 ± 1	19.8 ± 0.2	0.030
11	32.11	543	727	551(54),389(1 0 0)	Isobetanidin-6-*O*-(6′-*O*-*trans*-feruloyl)-*β*-glucoside (isogomphrenin III)	nd	17.2 ± 0.2	nd	–
					Total	39.36 ± 0.01[c]	161.6 ± 3.4[a]	134 ± 2[b]	–

In each row, different letters mean significant differences. Classification of samples was carried out with a Tukey's post-hoc test for polyphenols present in all three samples, and a Student's T-test for polyphenols detected in only two samples. Nd – not detected. Nq – not quantifiable. C – commercial colourant; L – lyophilized extract; Sd – spray dried extract. Standard calibration curve used: Gomphrenin III ($y = 14670x - 19725$, $R^2 = 0.9997$).

3.3. Antioxidant activity

The best result was sought for the lyophilised extract of *G. globosa*, with an IC_{50} of 80 ± 2 μg/mL, followed by the spray-dried extract, with an IC_{50} of 176 ± 11 μg/mL, which was significantly different. These values translate the extract concentration required to protect 50% of the erythrocyte population from the haemolytic action caused by the oxidative agent, AAPH, for 60 min. Thus, the lower the IC_{50} value, the higher the antihaemolytic activity of the tested extracts. In turn, a higher concentration (699 ± 12 μg/mL) of the commercial colourant was required to protect the erythrocytes population during the same time period. Trolox, the used positive control, was more efficient in protecting the erythrocyte membranes than the tested extracts, with an IC_{50} value of 19 ± 1 μg/mL. However, trolox is a pure compound while the extracts are complex mixtures of different compounds with or without antioxidant activity. This *ex vivo* erythrocyte system offers test conditions close to *in vivo* since the oxidant AAPH generates peroxyl radicals in the *in vitro* system, which are also found in the human body (Takebayashi, Iwahashi, Ishimi, & Tai, 2012). These radicals are formed from the thermal decomposition of AAPH and attack the erythrocytes membrane, eventually causing its lysis. As a consequence, lipophilic radicals are generated through peroxidation phenomena, which also attack the polyunsaturated fatty acid-rich membranes. Overall, these results highlight the higher antioxidant capacity of the *G. globosa* extracts compared to the tested commercial colourant.

3.4. Chemical composition of the prepared cookie formulations

The two factors varying in this analysis were, the colourant type (CT) and storage time (ST), that, through a simple analysis of variance would not be correctly analysed due to the possibility of the combined effect of them to the outcome. Thus, a 2-way analysis of variance was used, allowing for and individual assessment of each parameter individually. In this way, Tables 2–4 are divided into two sections, the upper one with the different ST, ranging from 0 to 30 days, and the lower one representing the TC (control, commercial, lyophilized and spray dried). For each tested day in the upper section, all tested colourant types are included, and for each tested colourant in the lower section, all storage times are included. This type of representation and results interpretation allows for the aforementioned individual assessment of each parameter. Thus, the standard deviations should not be regarded as the accuracy of one individual analysis, but rather of a range of variation for the non-fixed parameter (CT or ST). If a significant interaction between these parameters is detected, by having the *p*-value of CT × ST lower than 0.05, no multiple comparisons can be extracted, meaning that both parameters (CT and ST) had significant contributions for the changes, which only allows tendencies to be extracted from the Estimated Marginal Means (EMM) plots. If inversely, the *p*-value of CT × ST is higher than 0.05, each parameter is analyzed individually.

3.4.1. Nutritional profile

The centesimal profile, expressed in g/100 of dry weight, is listed in the left section of Table 2. The profile included fat, protein, ash and carbohydrate content (calculated by difference) and energy. The nutrients detected in the highest quantity were the carbohydrates (although they are comprised of fibres present in the flour, and also the added sucrose), followed by fat and protein. A significant interaction among the two analysed parameters (storage time and colourant type) was detected (ST × CT < 0.05), which hindered any individual assessment of the individual contribution of each parameter. Still, given the low amount of water in the cookies, significant variations in the nutritional profile were not expected.

Table 2
Nutritional profile of the different biscuit types along the 30-day storage time, represented in g/100 g of dry weight (dw) (left section), as well as the different dimensions of texture (right section).

		Fat	Proteins	Ash	Carbohydrates	EnergyKcal	Hardness (g)	Adhesiveness(g.sec)	Springiness(%)	Cohesiveness (%)	Chewiness	Resilience(%)
Storage time (ST)	0 days	23 ± 1	5.2 ± 0.2	1.8 ± 0.1	70 ± 1	509 ± 5	15688 ± 4953	−29 ± 28	0.49 ± 0.09	0.30 ± 0.05	2727 ± 1664	0.16 ± 0.04
	7 days	25 ± 1	5.5 ± 0.3	1.79 ± 0.09	67.71 ± 0.09	519 ± 8	8442 ± 3640	−4 ± 4	0.8 ± 0.1	0.54 ± 0.07	1881 ± 740	0.23 ± 0.04
	15 days	23.4 ± 0.7	5.3 ± 0.3	1.7 ± 0.2	69.6 ± 0.7	510 ± 3	6656 ± 977	−3 ± 1	0.69 ± 0.05	0.39 ± 0.07	1860 ± 579	0.220 ± 0.01
	30 days	24.7 ± 0.7	5.3 ± 0.3	1.7 ± 0.1	68.3 ± 0.7	517 ± 3	3394 ± 1310	−22 ± 22	0.6 ± 0.1	0.3 ± 0.1	1394 ± 813	0.2 ± 0.1
p-value (n = 5)	Tukey's HSD test	< 0.001	< 0.001	0.022	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Colourant Type (CT)	Control	25 ± 1	5.2 ± 0.2	1.8 ± 0.1	68 ± 1	517 ± 8	7836 ± 4286	−16 ± 24	0.5 ± 0.1	0.4 ± 0.1	1337 ± 648	0.3 ± 0.1
	Commercial	24.6 ± 0.7	5.3 ± 0.3	1.81 ± 0.08	68.2 ± 0.9	516 ± 4	9531 ± 6545	−8 ± 5	0.67 ± 0.09	0.44 ± 0.08	2702 ± 1304	0.2 ± 0.1
	Lyophilized	24 ± 1	5.5 ± 0.1	1.7 ± 0.2	69 ± 1	512 ± 7	9092 ± 6796	−8 ± 8	0.8 ± 0.2	0.32 ± 0.09	2079 ± 1292	0.22 ± 0.05
	Spray Dried	23.2 ± 0.8	5.3 ± 0.3	1.7 ± 0.1	69.4 ± 0.7	509 ± 4	7721 ± 3986	−27 ± 30	0.6 ± 0.1	0.3 ± 0.1	1743 ± 667	0.23 ± 0.09
p-value (n = 20)	Tukey's HSD test	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
ST × CT (n = 80)	*p*-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

The presented standard deviations were calculated from results obtained under different operational conditions. Thus, they should not be regarded as a measure of precision, rather as a range of values.

3.4.2. Soluble sugars and tocopherols

Sucrose was the only detected sugar by HPLC-RI, and is represented in g/100 g of dry weight on Table 3 Furthermore, tocopherols, isoforms of vitamin E were detected through HPLC coupled to a fluorescence detector, and are also presented in Table 3, expressed in mg/100 g of dry weight. Sucrose was detected since it is the sugar added to the recipe and thus it is expected to be found at a high amount, ranging from 47 to 51 g/100 g. Also, it was expected that after 30 days of storage, sucrose would breakdown to fructose and glucose, and despite this having occurred, the detected amounts were residual, probably due to the very low content of water found in the cookies that halted the breakdown. A significant interaction was detected for sucrose, and thus, both parameters promoted the verified changes, which due to the low moisture in the cookies was not significant. Regarding tocopherols, only three of the four isoforms were detected, namely α-, β-, and δ-tocopherol. The most abundant isoform detected was α-tocopherol, followed by δ-tocopherol, and finally γ-tocopherol. Vitamin E could be present in any of the three main ingredients, namely butter, sunflower oil and flour, although none of the labels stated the amounts of vitamin E. Still, and appreciable quantity of tocopherols was found, amounting to 39 mg/100 g. Moreover, and once again, a significant interaction was verified, and little to no variations were detected for tocopherols, once again due to residual amounts of water and the fact that the cookies did not undergo lipidic oxidation, which would reduce the content of tocopherols over the storage time.

3.4.3. Individual fatty acids

Table 3 also represents the individual fatty acids composition, namely monounsaturated (MUFA), polyunsaturated (PUFA) and saturated fatty acids (SFA), as detected through GC-FID along the cookie's storage time of 30 days. Values are expressed as relative percentages. Although a higher number of individual fatty acids were detected, only the ones representing at least, 1% of the total amount are shown in Table 3. The most abundant individual fatty acid was palmitic acid (C16:0), followed by oleic (C18:1) and linoleic acid (C18:2). This profile is consistent with the used ingredients, being mainly composed by butter, sunflower oil and flour. In terms of the fatty acid groups, the SFA prevailed over the unsaturated ones, with roughly 60% of the total amount, while the MUFA reached an average of 24%, and PUFA only 12–13%. As can be inferred by the *p*-value of the interaction, *p*-value < 0.05, there was a significant interaction between the two parameters, CT and ST, in terms of the variation of the fatty acids. Furthermore, the EMM did not show any general tendencies, but, observing the variations of the fatty acids, it is clear that there was no major variation along the storage time and the influence of the colourant was not enough to drastically change the fatty acid profile. These results were expected mainly because the cookies had very low water quantities and 30 days was not enough time to promote an oxidative cascade in the lipid fraction.

3.5. Cookie stability over storage time

3.5.1. Physical parameters

The right section of Table 2 displays the different dimensions of the texture analysis, including hardness, adhesiveness, springiness, cohesiveness, chewiness and resilience. Once again, the overall changes in texture were a result of a significative interaction between the colourant type and storage time. Still, over time, the cookie hardness decreased, probably due to retrogradation, in which moisture is captured from the surrounding environment, often softening the biscuit. Furthermore, these variations could also be explained by the low values of glutein which, in high amounts result in a more stable dough with lower hardness changes over time (Barak, Mudgil, & Khatkar, 2013). Adhesiveness, springiness, cohesiveness and resilience did not undergo drastic changes, and the ones registered were a product of the interaction of the storage time and colourant type, showing a consistency

Table 3
Sucrose (g/100 g dw), tocopherol isoforms (mg/100 g dw) (left section) and individual fatty acids, MUFA, PUFA and SFA of the different cookies along the 30 days of storage time, represented as a relative percentage of themselves (right section).

		Sucrose	α-tocopherol	β-tocopherol	δ-tocopherol	Total tocopherols	C6:0	C8:0	C10:0
Storage time (ST)	0 days	48 ± 3	29.5 ± 0.7	2.0 ± 0.2	7.9 ± 0.7	39.5 ± 0.1	2.9 ± 0.2	1.42 ± 0.08	2.9 ± 0.1
	7 days	50 ± 1	28.2 ± 0.8	2.3 ± 0.3	8.8 ± 0.9	39.4 ± 0.1	3.4 ± 0.6	1.4 ± 0.1	2.7 ± 0.2
	15 days	48 ± 2	29.2 ± 0.8	2.0 ± 0.1	8.4 ± 0.6	39.6 ± 0.2	3.3 ± 0.3	1.5 ± 0.1	2.8 ± 0.1
	30 days	48 ± 2	30 ± 1	1.9 ± 0.2	7 ± 1	39.6 ± 0.2	3.1 ± 0.2	1.4 ± 0.1	2.6 ± 0.1
p-value (n = 5)	Tukey's HSD test	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Colourant Type (CT)	Control	51 ± 2	29.8 ± 0.9	1.9 ± 0.2	7.9 ± 0.7	39.6 ± 02	3.0 ± 0.2	1.4 ± 0.1	2.7 ± 0.2
	Commercial	49 ± 2	29.2 ± 0.4	1.97 ± 0.07	8.4 ± 0.3	39.5 ± 0.1	3.2 ± 0.3	1.44 ± 0.08	2.79 ± 0.07
	Lyophilized	47 ± 2	28.1 ± 08	2.20 ± 0.09	9.1 ± 0.6	39.4 ± 0.1	3.2 ± 0.2	1.4 ± 0.1	28 ± 0.3
	Spray Dried	48 ± 2	30 ± 1	2.2 ± 0.4	7 ± 1	39.6 ± 0.2	3.3 ± 0.7	1.4 ± 0.1	2.7 ± 0.1
p-value (n = 20)	Tukey's HSD test	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
ST × CT (n = 80)	*p*-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1n9c	C18:2n6c	SFA	MUFA	PUFA
Storage time (ST)	3.9 ± 0.1	10.7 ± 0.3	30.2 ± 0.8	1.30 ± 0.02	10.3 ± 0.3	22.0 ± 0.4	12 ± 1	64 ± 1	24.1 ± 0.4	12.0 ± 0.9
	3.6 ± 0.1	10.1 ± 0.3	29.2 ± 0.4	1.24 ± 0.03	10.1 ± 0.4	23 ± 1	12.8 ± 0.7	61 ± 2	25 ± 1	13.2 ± 0.7
	3.55 ± 0.07	10.10 ± 0.1	29.7 ± 0.4	1.24 ± 0.04	10.6 ± 0.2	22.3 ± 0.2	12.4 ± 0.8	62.8 ± 0.9	24.4 ± 0.2	12.8 ± 0.8
	3.6 ± 0.2	10.2 ± 0.3	29.5 ± 0.4	1.29 ± 0.03	10.6 ± 0.1	22.6 ± 0.2	12.3 ± 0.9	62 ± 1	24.8 ± 0.2	12.7 ± 0.9
p-value (n = 5)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Colourant Type (CT)	3.5 ± 0.1	10.0 ± 0.1	29.1 ± 0.2	1.250 ± 0.02	10.3 ± 0.2	22.7 ± 0.2	13.4 ± 0.2	61.4 ± 0.4	24.7 ± 0.2	13.8 ± 0.2
	3.7 ± 0.2	10.2 ± 0.3	29.8 ± 0.3	1.28 ± 0.03	10.7 ± 0.1	22.5 ± 0.4	11.9 ± 0.2	63.1 ± 0.4	24.6 ± 0.4	12.3 ± 0.1
	3.7 ± 0.2	10.4 ± 0.4	29.9 ± 0.6	1.26 ± 0.06	10.4 ± 0.5	23 ± 1	11 ± 1	63 ± 2	25 ± 1	12 ± 1
	3.7 ± 0.2	10.4 ± 0.3	29.9 ± 0.9	1.28 ± 0.02	10.3 ± 0.3	22.0 ± 0.5	12.3 ± 0.7	63 ± 1	24.1 ± 0.6	12.7 ± 0.7
p-value (n = 20)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
ST × CT (n = 80)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

C6:0 – Caproic acid; C8:0 – Caprylic acid; C10:0 – Capric acid; C12:0 – Lauric acid; C14:0 – Myristic acid; C16:0 – Palmitic acid; C16:1 – Palmitoleic acid; C18:0 – Stearic acid; C18:1n9 – Oleic acid; C18:2n6 – Linoleic acid. SFA: Saturated Fatty Acids; MUFA: Monounsaturated Fatty Acids; PUFA: Polyunsaturated Fatty Acids. The presented standard deviations were calculated from results obtained under different operational conditions. Thus, they should not be regarded as a measure of precision, rather as a range of values.

Table 4
Representation of the external (Cookies), internal (Interior) and the biscuit powder (Milled Sample) colours, according to the L*, a*, b* colour space.

		Cookies			Interior			Milled Sample		
		L*	a*	b*	L*	a*	b*	L*	a*	b*
Storage time (ST)	0 days	62 ± 7	18 ± 11	15 ± 9	58 ± 9	14 ± 8	12 ± 8	67 ± 8	16 ± 10	16 ± 9
	7 days	62 ± 9	17 ± 11	14 ± 10	60 ± 7	14 ± 8	12 ± 8	66 ± 9	16 ± 10	16 ± 10
	15 days	62 ± 9	17 ± 11	15 ± 10	61 ± 7	14 ± 8	13 ± 8	67 ± 8	17 ± 10	17 ± 9
	30 days	62 ± 8	17 ± 11	16 ± 9	64 ± 7	14 ± 9	12 ± 7	68 ± 8	17 ± 11	17 ± 9
p-value (n = 5)	Tukey's HSD test	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Colourant Type (CT)	Control	75 ± 1	−1.0 ± 0.2	28.7 ± 0.6	73 ± 2	−0.2 ± 0.5	22.9 ± 0.5	82.3 ± 0.8	−0.8 ± 0.2	29.1 ± 0.5
	Commercial	60.7 ± 0.6	23.2 ± 0.7	18.1 ± 0.5	59 ± 2	18.7 ± 0.8	15.6 ± 0.7	64.8 ± 0.5	22.1 ± 0.9	21 ± 1
	Lyophilized	56.3 ± 0.6	22.1 ± 0.2	8 ± 1	56 ± 2	17.5 ± 0.4	7.4 ± 0.4	61 ± 1	21.5 ± 0.8	11.0 ± 0.7
	Spray Dried	56 ± 1	25.5 ± 0.4	4.3 ± 0.4	55 ± 4	20.5 ± 0.3	2.9 ± 0.4	61.2 ± 0.9	24.8 ± 0.6	5.9 ± 0.6
p-value (n = 20)	Tukey's HSD test	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
ST × CT (n = 80)	*p*-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

The presented standard deviations were calculated from results obtained under different operational conditions. Thus, they should not be regarded as a measure of precision, rather as a range of values.

along the storage time. Chewiness, which is defined as the energy required to masticate the food, also decreased over time, in line with the decrease of hardness, providing that softer foods require less energy to masticate. In this way, and although both parameters (CT and ST) contributed, the cookies became softer overtime, and their chewiness decreased in line with this trend.

Cookies are snacks that, beyond being appealing to the palate, also have to be appealing to the eye of the consumer, hence the need to colour them or to intensify their natural colours. Thus, the colour analysis is of crucial importance, and the resistance of colourants to oven cooking and storage time is of utmost importance for their success in the industry. To obtain a better understanding of the colouring capacity of these colourants, the colour, measured with a portable colourimeter, was analysed on the outer part of the cookies, but also in the internal one, and finally in their powder after milling. This procedure allows some insights into the effects of cooking and storage time, both on the outer part of the cookies, but also the inner section. Colour was analysed using the CIELab colour space, that measures coordinates of L* (lightness, variation between −100 and +100, black to white), a* (greenness and redness, variation between −100 and +100, red to green), and b* (yellowness/blueness, variation between −100 and +100, yellow to blue). There was a significative interaction between storage time and colourant type in Table 4, although some tendencies could be extracted from the EMM plots. This table shows the L*, a* and b* coordinates of the external and internal colour of cookies, as well as their powder.

The main objective of this work was to achieve a pink colouration on the cookies, the ideal coordinates are an average of 0 for L*, +100

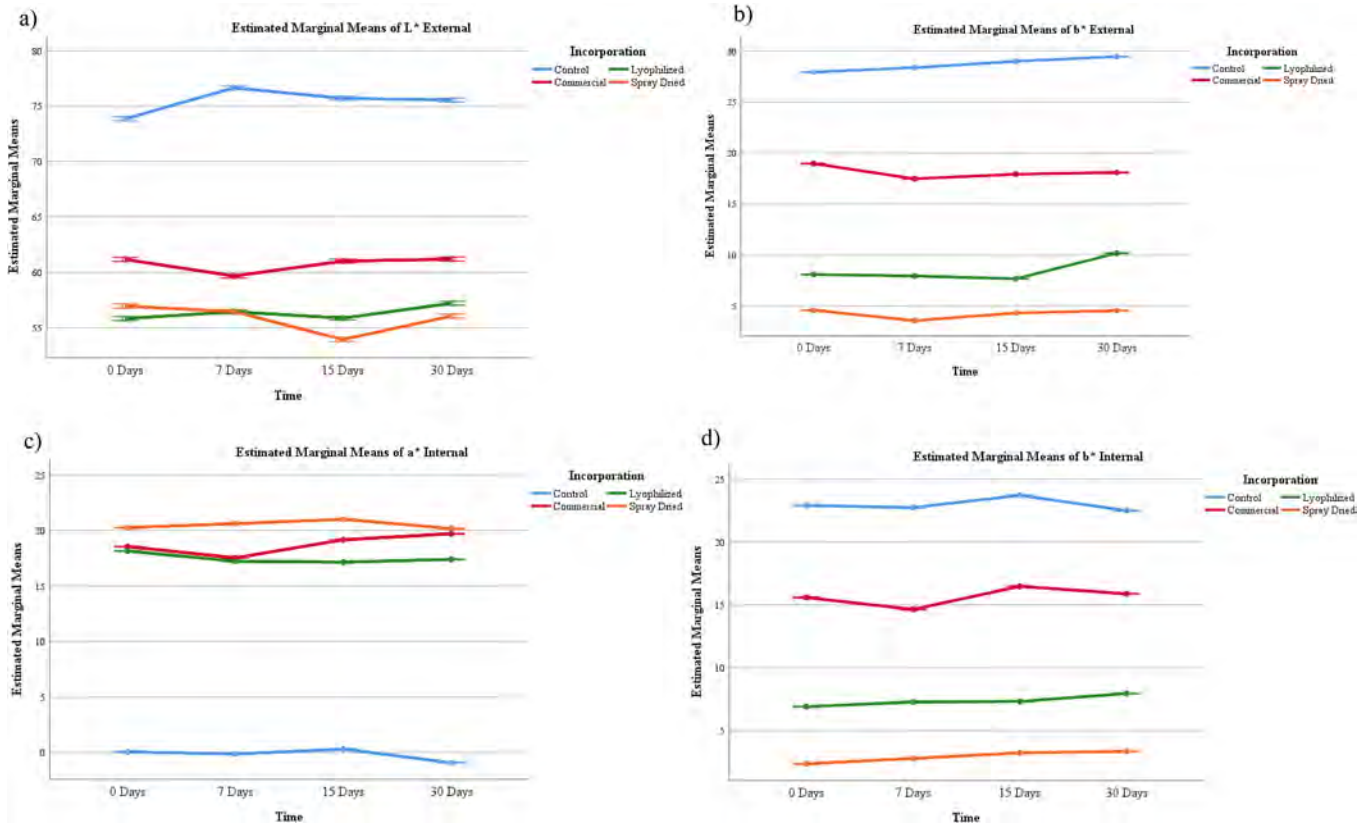

Fig. 1. EMM plots of the external and internal colour of the cookies during the 30-day storage time.

for a* and 0 for b*. Fig. 1 shows the different coordinates of the external and internal colour for the different incorporated cookies along the 30-days of storage time. It is clear that, in line with other parameters, the variation in colour is quite low due to the low water content and the absence of oxidation during the storage time; still, some changes did occur. As stated above, to achieve the pink colouration, the lightness (L*) should be near 0, and thus, both the lyophilized and spray dried cookies showed the lowest values throughout the storage time (Fig. 1a) for the external colour. In terms of b*, where the desired amount is 0, both cookies with spray-dried extract and lyophilized of *G. globosa* showed similar values, thus proving a more homogenous pink colour (Fig. 1b). The same trend was noticed for the colours measured in the interior samples, as can be seen in Fig. 1c and d, that displays the EMM plots of a* and b*. Fig. 2a shows the colour of the three different colourants used by converting the L*, a* and b* coordinates to RGB colours. A darker colour is shown for the commercial sample and lyophilized extract, showing a high similarity, while the lyophilized extract is lighter and shows a color resembling violet. Fig. 2b shows the overall colour of the cookies themselves by averaging the colours during the 30 days of storage. The external colour of the natural coloured cookies was more consistent with a pink, while the commercial samples showed a shift to orange. The deeper pink colour can be observed for all cookies added with the spray-dried extract of *G. globosa*, followed by the lyophilized form, especially in the internal section of the cookie. The orange colour, detected in commercial sample does not fit the pink colouration intended for the enhancement of the biscuit's appearance, thus representing an opportunity for these natural colourants to fill a void in the food industry in terms of food colouring.

3.5.2. Microbiological analysis

To guarantee the safety of the developed colourants, the prepared cookies were analysed for their microbial counting over their shelf life (30 days), due to the risk that microorganisms could hinder the cookie's stability due to degradation. No microbial contamination was detected along the 30 days of analysis, meaning that the cooking process was able to eliminate possible microorganisms present in the flour or extracts.

4. Conclusions

The fact that the spray-drying samples showed a deeper pink coloration can be related to the protective effect of the spray-drying process, which seems to be effective for the betacyanins presented in the extracts. The enveloping of the extract with maltodextrin, contributed to this protection, and played an important role during the baking process. Inversely, the lyophilized extract and the commercial colourant did not achieve this protective effect conferred by maltodextrin, and thus, the color did not maintain the intended deep pink. However, the lyophilized extract seemed to lose at a lower extent the colour intensity when compared to the commercial sample (which is expected to resist high temperatures). This lyophilized extract can be considered a natural alternative, with a somewhat better performance than the commercial sample, probably associated with the extraction procedure that allowed a higher yield of coloring molecules, and also a comparably reduced extraction time. Furthermore, both drying techniques provided a higher stability over time, beyond presenting no degradation and no microbial count. Moreover, the incorporation did not significantly alter the chemical composition of the cookies, which is paramount for all food additives to be considered for these purposes. In terms of marketing viability, when comparing the lyophilized and spray-dried extracts, the lyophilized seem to show better results, provided that the pink intensity can be achieved by adding a higher amount of extract. Because the production costs of the lyophilized extract are considerably lower, the corresponding extract can be an interesting alternative to commercial colourants.

Fig. 2. Colours of the cookies, obtained from the L*, a*, b* coordinates, considering an average of the colours during all storage times.

CRediT authorship contribution statement

Custódio Lobo Roriz: Methodology, Investigation, Writing - original draft. **Sandrina A. Heleno:** Methodology, Writing - review & editing. **Márcio Carocho:** Methodology. **Paula Rodrigues:** Methodology. **José Pinela:** Methodology, Writing - original draft. **Maria Inês Dias:** Methodology. **Isabel P. Fernandes:** Methodology. **Maria Filomena Barreiro:** Methodology. **Patricia Morales:** Methodology, Writing - review & editing. **Lillian Barros:** Conceptualization, Methodology, Writing - review & editing. **Isabel C.F.R. Ferreira:** Conceptualization, Methodology, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Ficus carica L. and *Prunus spinosa* L. extracts as new anthocyanin-based food colorants: A thorough study in confectionery products

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ABSTRACT

The present work describes the evaluation of fig peels and blackthorn fruit extracts as natural purple colorants in doughnuts (icing) and in a typical Brazilian pastry called "beijinho". The extracts were screened for their antioxidant activity as well as their antibacterial capacity. Nutritionally, the employed extracts did not induce significant changes, contrarily to the observed for the rheological features, mainly the darker purple tone observed when blackthorn extract was used in the icing solution. After 24 h, both prepared formulations showed a decrease in color intensity, with no significant differences between fig and blackthorn extracts. In turn, the firmness and consistency of the doughnuts benefited from using natural colorants in the icing solution, while "beijinhos" became softer and chewier, which are valued attributes. A significant increase in the antioxidant and antimicrobial activities was also observed for both natural extracts. Accordingly, the evaluated extracts are promising candidates as natural food colorants.

1. Introduction

Food colorants are substances of natural or synthetic origin used to convey, restore or standardize the color and appearance of food, making it more attractive to consumers (Gordillo et al., 2018). Due to consumer preference for natural food colorants, the quest for pigments from natural sources has become widespread, quickly turning into a trend in the current market (Agcam, Akyildíz, & Balasubramaniam, 2017; Gordillo et al., 2018).

Anthocyanins represent the largest group of phenolic pigments and the most important group of water-soluble pigments in plants. These compounds are responsible for colors in fruits, vegetables, cereal grains and flowers (Mojica, Berhow, & Gonzalez de Mejia, 2017), and, beyond this, they also display health benefits, namely in reducing the risk of coronary heart disease, stroke, and other diseases, while also showing cytotoxic and anti-inflammatory effects (Agcam et al., 2017). As water-soluble and innocuous pigments, anthocyanins have high potential as food colorants, besides adding value due to their bioactivities. Color formulations based on anthocyanins exhibit some limitations due to their lack of chemical stability. Factors such as pH variation, exposure to heat, light, oxygen, temperature, metals, bleaching agents, among others, can affect their behavior and stability, compromising the desired color. In light of these limitations, the incorporation of these compounds constitutes a challenge for the food industry (Gordillo et al., 2018).

The fig tree (*Ficus carica* L., family Moraceae) is native to southwestern Asia and the eastern part of the Mediterranean. Its fruit, is a rich source of sugar, vitamins and phenolic compounds (Buenrostro-Figueroa et al., 2017; Duenas et al., 2008), being consumed fresh or dehydrated. Figs are also consumed as processed foods, mainly as ingredients for wine, liquor, juice and jelly, although these foods use the pulp and discard the peel, which is the largest reservoir of phenolic compounds (Buenrostro-Figueroa et al., 2017). In fact, Vallejo, Marín, and Tomás-Berberán (2012), showed that high concentrations of phenolic compounds (mainly anthocyanins) were found in the peel of mature figs (Barolo, Ruiz Mostacero, & López, 2014).

Prunus spinosa L. has also proved its worth as a fruit with high amounts of antioxidant molecules and natural pigments. The fruits of this plant, also known as blackthorn, presents high amounts of cyanidin 3-*O*-rutinoside and peonidin 3-*O*-rutinoside, as well as an overall high

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amount of antioxidant compounds (Guimarães et al., 2013, 2014; Barros, Carvalho, Morais, & Ferreira, 2010). The fruits of *P. spinosa* are commercially unexplored due to their astringent taste, being basically used for the production of liqueurs and jams and also for some medicinal purposes (Morales et al., 2013; Pinacho et al., 2015).

The present study aims to evaluate the bioactive properties and applicability of natural food colorants based on anthocyanins from the residues (peels and epicarps) of *Ficus carica* L. and *Prunus spinosa* L. These extracts were tested on two highly appreciated deserts, namely doughnuts (as an icing solution) and a Brazilian soft dairy pastry called "beijinho", being their application monitored in terms of chemical and physical properties immediately after manufacture and after 24 h, according to their typical shelf-life.

2. Material and methods

2.1. Standards and reagents

The reference standard mixture of fatty acid methyl esters (FAME) (47885-U standard), 2,2′-azobis (2-amidinopropane) dichlorohydrate (AAPH), acetic acid, formic acid, trichloroacetic acid) and Tris were purchased from Sigma-Aldrich, as well as the sugar standards used. Hydrogen chloride, formic acid, HPLC grade acetonitrile and P.A. grade ethanol were obtained from Fisher Scientific. Toluene, sulfuric acid, and the remainder of the chemical reagents used were purchased from Sigma Chemical Co. Water was treated in a Milli-Q purification system (TGI Pure Water Systems). Anthocyanin standards (cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-glucoside) were purchased from Extrasynthèse.

2.2. Preparation of the extracts

The figs and blackthorns were obtained from a local producer in Bragança, Portugal (N 41° 47.422, W 6° 45.606). The samples were peeled, and the peels of *F. carica* and the epicarp of *P. spinosa* were lyophilized (FreeZone 4.5, Labconco), crushed to 20 mesh and stored in a freezer at −20 °C for subsequent analysis. The lyophilized extracts were extracted under the best conditions according to a previous study (Backes et al., 2018; Leichtweis et al., 2019), relying on an ultrasonic probe apparatus (QSonica sonicators, model CL-334, Newtown, CT, USA), where each plant was extracted with 100 mL of acidified solvent (pH 3, using citric acid). For the fig peel extraction, the solvent used was 100% ethanol (180 g/L, 21 min, 310 W), whilst for blackthorn, the solvent used was a mixture (50:50 *v*/*v*) of ethanol:water (75 g/L, 5 min, 400 W). The samples were centrifuged (6000 rpm for 20 min at 10 °C) and filtered through filter paper Whatman n° 4 to remove suspended solids. The supernatants were lyophilized (FreeZone 4.5, Labconco) and stored in a refrigerator (−20 °C) for subsequent analyzes.

2.3. Bioactivities

2.3.1. Evaluation of antioxidant activity: Inhibition of lipid peroxidation (TBARS)

The antioxidant activity of the extracts was evaluated by the lipid peroxidation inhibition using thiobarbituric acid reactive substances (TBARS) in pig brain homogenates. Successive dilutions of the extract were prepared and mixed with ascorbic acid, brain tissue, and thiobarbituric acid. The potential formation of malodialdehyde (MDA) from brain tissue, which occurs in the absence of antioxidant agents, was verified by the formation of MDA-TBA complex (intense pink color) at 532 nm (Barros et al., 2010). A percentage inhibition *versus* concentration curve of the extract was created using the following equation:

$$\text{Inhibition percent}(\%) = \left[\frac{\text{CA-EA}}{\text{CA}}\right] \text{x } 100 \quad (1)$$

CA represents the control absorbance and EA the extract solution absorbance. The concentration capable of causing 50% inhibition of lipid peroxidation (IC_{50}) was determined for each of the extracts.

2.3.2. Evaluation of antioxidant activity: Inhibition of oxidative hemolysis (OxHLIA)

A known mass of extract was dissolved in a phosphate buffered saline solution, obtaining concentrations ranging from 312.5 to 5000 μg/mL. To determine the inhibition capacity of the oxidative hemolysis (OxHLIA) associated with each of the extracts, blood from healthy sheep was harvested and used as a substrate. After filtering the blood, the leucocytes were discarded, and the red cell suspension was used after washing with a NaCl solution. The erythrocytes were resuspended and incubated at 37 °C for 10 min, prior to an optical reading at 690 nm every 10 min for 2 h. The percentage of the intact erythrocyte population (EP) was calculated using the following equation:

$$EP\ (\%) = (S_t - CH_0 / S_0 - CH_0) \times 100 \quad (2)$$

S_t and S_0 correspond to the optical density of the sample at time t and 0 min, respectively, and CH_0 is the optical density of complete hemolysis at 0 min. The results were expressed as hemolysis delay time (Δt). Subsequently, linear correlations were established between the Δt values and the different sample concentrations (Takebayashi et al., 2012). The extract concentration capable of delaying 80% of the hemolysis (IC_{80}) in 60 min ($IC_{80(60\ min)}$, mg/mL) and 120 min ($IC_{80(120\ min)}$, mg/mL) were calculated.

2.3.3. Evaluation of antibacterial activity

The assay used microorganisms obtained from clinical isolates (Hospital Centre from Trás-os-Montes and Alto Douro, Vila Real, Portugal). Four Gram-positive bacteria (*Enterococcus faecalis*, isolated from urine; *Listeria monocytogenes*, isolated from cerebrospinal fluid; methicillin-resistant *Staphylococcus aureus* - MRSA, isolated from expectoration and methicillin-sensitive *Staphylococcus aureus* - MSSA, isolated from wound exudate) and five Gram-negative bacteria (*Pseudomonas aeruginosa* and *Proteus mirabilis*, isolated from expectoration; *Escherichia coli*, *Klebsiella pneumoniae* and *Morganella morganii*, isolated from human urine).

Microorganism identification and susceptibility tests were performed on the MicroScan panels (MicroScan®; Siemens Medical Solutions Diagnostics, West Sacramento, CA, USA) using the microdilution method. The interpretation criteria was based on Interpretive Breakpoints as indicated in the Clinical and Laboratory Standards Institute (CLSI, 2008) and in the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2013).

The colorimetric assay was performed using *p*-iodonitrotetrazolic chloride (INT) according to Kuete et al. (2011) with some modifications. Successive extract dilutions were made in microplate wells (20 to 0.156 mg/mL) prior to inoculation with a suitable turbidity suspension. Three negative controls (MHB/TSB; MHB/TSB with bacterial inoculum and antibiotic; extract) and a positive control (MHB/TSB with bacterial inoculum) were also prepared. For the Gram-positive bacteria, Vancomycin was used as a positive control, while Ampicillin and Imipenem were used for the Gram-negative ones. The minimum inhibition concentration (MIC) was defined as the lowest extract concentration to inhibit bacterial growth (color change from yellow to pink), while the minimum bactericidal concentration (MBC) was the minimum concentration that killed all bacteria present in the well.

2.3.4. Evaluation of cytotoxic potential in a non-tumor cell line

Pig liver obtained from a local slaughterhouse was used to obtain a non-tumor cell culture, henceforth named PLP2 (primary culture of pig liver cells). The liver tissue was washed with Hank's solution and divided into explants of approximately 1 × 1 mm^2, obtaining a cell culture with adequate density (190 μL, 1.0 × 10^4 cell/well), which was pipetted in 96-well microplates with 10 μL of different dilutions of the extracts (400 to 1.5 μg/mL) and incubated at 37 °C for 48 h. Analysis of

the cytotoxicity of the extract was evaluated according to the Sulforhodamine B (SRB) colorimetric assay. Ellipticine was used as a positive control and the results were expressed as GI_{50}.

2.4. Incorporation of extracts into foodstuffs

After confirming the antioxidant and antibacterial activity of fig peel and blackthorn extracts, as well as their lack of toxicity, the extracts were incorporated in the doughnut icing and "beijinhos"

2.4.1. Preparation of doughnut icing

White chocolate was melted in a heated bath and homogenized with milk cream at a 3:1 *m/m* (840 g of white chocolate to 280 g of milk cream) ratio. Then, citric acid was added at 1:200 *m/m* (4.2 g) for chocolate (below the maximum value of 5 g/kg stipulated by legislation (European Parliament, 2011). The mixture was then divided into three portions, in one portion, acting as a dye, the extract of fig peel was added, in the second portion, the extract of blackthorn was added (both at 0.7% of the total volume of the icing), and finally, in portion three, no colorant was added. Each of these portions was further divided into two three replicates for each analysis, at 0 h and 24 h measuring both physical, chemical and nutritional parameters.

2.4.2. Preparation of "beijinho"

In the preparation of the "*beijinho*" 800 g of condensed milk, 200 g of milk cream and 24 g of butter were used. These ingredients were heated and stirred until the mixture was easily detached from the bottom of the pan. Then, 4.8 g of citric acid was added, and the mixture separated in three equal parts. One of them was used as control, and the other two were incorporated with 2.4 g one of the two natural extracts. After cooling, the dough was divided into 10 g portions and rolled into small spheres (typical presentation of this pastry).

2.5. Evaluation of the nutritional profile

The nutritional composition of the doughnut icing and "beijinhos" samples was performed by analyzing the moisture content, ashes, proteins, fat and carbohydrates according to the procedures described by AOAC (2016). Moisture was determined by lyophilization (FreeZone 4.5, Labconco) following AOAC 925.09 method. The ashes were determined by muffle incineration (Optic ivymen system) at 550 ± 5 °C following AOAC 923.05. The total proteins were estimated by the macro-Kjeldahl technique in a specific equipment (Pro-Nitro A, Selecta) by determining the total nitrogen present in the sample (N × 6.25 for the icing and N × 6.38 for "beijinho") following method AOAC 920.87. Total fat was determined by extraction through a Soxhlet apparatus using method AOAC 989.05. The carbohydrate content (g/100 g fw), in fresh product (fw), was calculated by difference and the total energy (kcal/100 g fw) was determined according to the following equation.

$$\text{Energy} = 4 \times (CH + PC) + 9 \times CF \quad (3)$$

CH represents carbohydrates (g/100 g fw), PC the protein content (g/100 g fw), CF the crude fat (g/100 g fw).

2.6. Individual molecules

Free sugars and individual fatty acids were also determined, namely through gas chromatography coupled to flame ionization detector (GC-FID) and high-performance liquid chromatography coupled to refraction index detector (HPLC). The free sugars followed the methodology published by Barros et al. (2013), where they were analysed by HPLC coupled to a refraction index detector (Knauer, Smartline system 1000). The compounds were identified by chromatographic comparisons with authentic standards (D(-)-fructose, D(+)-sucrose, D(+)-glucose, and D(+)-lactose, as also melezitose which was applied as the internal standard (IS) and used in the quantification method. Data was analyzed using Clarity 2.4 software (DataApex, Podohradska, Czech Republic), and the results were expressed in g/100 g fw.

Fatty acids were determined by GC-FID (DANI model GC 1000, Contone, Switzerland). The separation was achieved with a Macherey-Nagel (Düren, Germany) column (50% cyanopropyl-methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 μm d_f). Fatty acids were identified by comparing their retention times to the ones of FAME peaks of commercial standards (FAME reference standard mixture, standard 47885-U, Sigma-Aldrich, St. Louis, MO, USA). The results were recorded and processed using the CSW 1.7 software (DataApex 1.7, Prague, Czech Republic) and were expressed in relative percentage (%).

2.7. Evaluation of pH, color and texture parameters

The pH was measured directly in three different points of the samples using a portable pH-meter (Hanna Instruments, Woonsocket, Rhode Island, USA). The external color was measured in three different points of the three replicates using a portable colorimeter (model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan) relying on the illuminant C with a diaphragm aperture of 8 mm, as well as the CIElab L^* (luminosity), a^* (green/red) and b^* (blue/yellow) color space values. The total color difference (ΔE^*) between the colored samples and the control sample was also calculated using the following equation:

$$\Delta E^* = \sqrt{(L_2^* + L_1^*)^2 + (a_2^* + a_1^*)^2 + (b_2^* + b_1^*)^2} \quad (4)$$

Texture was determined using a TA XT. Plus Texture Analyzer (Stable Micro System) to measure the following parameters: firmness, consistency, cohesiveness and the viscosity index of the icing, using an inverted extrusion probe, due to be a semiliquid. The "beijinhos", being a solid, underwent a normal TPA (texture profile analysis) analysis, describing the hardness, adhesiveness, resilience, cohesiveness, springiness, gumminess and chewiness, using an acrylic disc (40 mm), a 30 kg load cell, speeds of 3 mm/s and 25% strain with a trigger force of 50 g.

2.8. Statistical analysis

All samples are expressed as mean ± SD, using 3 replicates for all assays. In relation to the incorporation of the extracts, samples were analyzed through a two-way analysis of variance (ANOVA) with type III sums of squares, after verification of the homoscedasticity through a Levene's test. The Tukey (homoscedastic) or Tamhane's T2 (heteroscedastic) *post-hoc* tests were used to classify differences induced by the colorant type (CT), while a student-T test was used with the same purpose for the analysis time (AT). Using a two-way ANOVA, the contribution of each CT or AT can be assessed individually, and their influence in the variation of each parameter can be better understood. When a significant interaction ($p < 0.05$) among the two factors (CT × AT) was found, they are evaluated simultaneously and, in some cases, general tendencies are extracted from the Estimated Marginal Means (EMM) plots. If not, they were classified using the *post-hoc* tests described above. All statistical analysis was carried out using the IBM SPSS, version 25.0. (IBM Corp.).

3. Results and discussion

3.1. Antioxidant activity

The antioxidant potential of the fig peel extract, rich in cyanidin 3-rutinoside (Backes et al., 2018), and blackthorn extract, rich in cyanidin 3-rutinoside and peonidin 3-rutinoside (Leichtweis et al., 2019), were evaluated by two distinct methods, which involve the use of biological material, namely the TBARS and OxHLIA assays. In what concerns the OxHLIA method, for the fig extract, the results obtained did not allow the establishment of EC_{50} values, because the different extract

Table 1
Nutritional profile, energy and free sugars detected in the icings and "beijinhos" over the 24 h. Nutritional profile and free sugars are expressed as g/100 g fresh weight and energy as kcal/100 g.

Icing												
		Moisture	Ash	Proteins	Fat	Carbohydrates	Energy	Fructose	Glucose	Sucrose	Lactose	Total Sugars
Colorant type (CT)	Control	13.4 ± 0.1[b]	1.17 ± 0.04	6.0 ± 0.1	33.2 ± 0.4	55 ± 1	581 ± 5	n.d.	n.d.	18.7 ± 0.4[a]	7.0 ± 0.1[b]	25.6 ± 0.6[a]
	Fig	12.6 ± 0.3[a]	1.22 ± 0.04	6.0 ± 0.1	32.0 ± 0.5	61 ± 1	555 ± 2	0.52 ± 0.01	0.71 ± 0.01	23.8 ± 0.5[c]	6.2 ± 0.1[a]	31.3 ± 0.6[c]
	Blackthorn	12.7 ± 0.1[a]	1.20 ± 0.03	5.9 ± 0.2	32.8 ± 0.9	60 ± 1	559 ± 5	0.19 ± 0.01	0.34 ± 0.03	21.6 ± 0.5[b]	6.4 ± 0.2[a]	28.4 ± 0.6[b]
p-value (n = 6)	Tukey's HSD Test	< 0.001	0.054	0.699	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Analysis time (AT)	T 0	13.0 ± 0.3	1.22 ± 0.04	6.0 ± 0.1	32 ± 1	59 ± 2	563 ± 10	0.2 ± 0.2	0.3 ± 0.3	21 ± 2	6.5 ± 0.4	28 ± 2
	T 24	12.8 ± 0.4	1.18 ± 0.04	6.0 ± 0.1	33 ± 1	58 ± 3	568 ± 15	0.2 ± 0.2	0.4 ± 0.3	21 ± 2	6.5 ± 0.3	29 ± 3
p-value (n = 9)	Student's T Test	0.014	0.032	0.800	0.081	0.005	0.002	0.885	< 0.001	0.170	0.748	0.315
CT × AT (n = 18)	*p*-value	0.074	0.438	0.056	0.006	< 0.001	0.001	< 0.001	< 0.001	0.834	0.438	0.892
"beijinho"												
Colorant type (CT)	Control	10.1 ± 0.2	1.1 ± 0.1	9.4 ± 0.3	16.5 ± 0.3	73 ± 1	477 ± 1[b]	0.3 ± 0.1	0.31 ± 0.04	34 ± 1	5.1 ± 0.2	40 ± 1
	Fig	11.8 ± 0.1	1.2 ± 0.1	9.2 ± 0.1	16.0 ± 0.3	74 ± 1	475 ± 1[b]	0.5 ± 0.1	0.59 ± 0.05	33 ± 1	4.5 ± 0.2	39 ± 1
	Blackthorn	12.0 ± 0.2	1.3 ± 0.2	9.3 ± 0.2	15.0 ± 0.5	74 ± 1	470 ± 2[a]	0.7 ± 0.1	0.67 ± 0.05	32 ± 1	4.7 ± 0.3	38 ± 1
p-value (n = 6)	Tukey's HSD Test	< 0.001	< 0.001	0.083	< 0.001	< 0.001	< 0.001	0.002	< 0.001	< 0.001	< 0.001	< 0.001
Analysis time (AT)	T 0	11 ± 1	1.3 ± 0.2	9.2 ± 0.1	15.8 ± 0.5	74 ± 1	473 ± 3	0.6 ± 0.2	0.5 ± 0.1	33 ± 2	4.9 ± 0.4	39 ± 1
	T 24	11 ± 1	1.3 ± 0.2	9.4 ± 0.2	15.9 ± 0.5	73 ± 1	474 ± 4	0.4 ± 0.1	0.5 ± 0.2	33 ± 1	4.7 ± 0.2	39 ± 1
p-value (n = 9)	Student's T Test	0.012	< 0.001	0.002	0.712	0.464	0.296	< 0.001	0.948	0.121	0.002	0.026
CT × AT (n = 18)	*p*-value	< 0.001	< 0.001	< 0.001	0.007	< 0.001	0.188	< 0.001	< 0.001	0.002	< 0.001	0.021

In each column for CT, different letters mean significant differences among the samples; in the case of AT, with an overall significance value of 0.05 for both cases. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values. n.d. – amount under the limit of detection.

concentrations presented different antioxidant behaviors. These effects are shown in Fig. S1 (supplementary material), where it is possible to observe the overlap and/or crossing of the lines corresponding to the different hemolytic times (Ht) of each concentration. Similar results were found in previous studies, leading to the same type of conclusions (Takebayashi et al., 2012). The IC_{80} value, corresponding to the concentration capable of delaying hemolysis was determined at 60 ($IC_{80\ (60\ min)}$) and in 120 min ($IC_{80\ (120\ min)}$), which are also shown in Table S1 (supplementary material). For the blackthorn extract the IC_{50} was calculated, although for comparison sake between the two extracts, only the IC_{80} are reported. None of the extracts showed IC_{50} and IC_{80} values as low as Trolox (used as positive control), although it should be noted that this compound is analyzed in its pure form. Whole extracts, despite potential synergies among different compounds, are not expected to be as antioxidant as pure compounds (owing the purity degree). Overall, blackthorn presented greater cell protection against oxidation when compared to the fig peel extract. These results are in agreement with those found in other studies, which had already reported high antioxidant activity for both extracts (Amessis-Ouchemoukh et al., 2017; Barros et al., 2010; Pereira et al., 2017; Pinacho et al., 2015; Solomon et al., 2006). A considerable number of studies have shown the antioxidant activity of *F. carica* peel, namely Harzallah et al. (2016) which determined the IC_{50} values of 9.71 and 4.52 mg/mL for the purple and black variety peel, respectively, using the (2,2-diphenyl-1-picrylhydrazil) DPPH free radical method. Oliveira et al. (2009) obtained IC_{25} values of 2.95 mg/mL for the "Pingo de Mel" variety of figs, while the pulp of the same did not present a decent DPPH capacity, even at a concentration of 5.56 mg/mL.

Concerning blackthorn, Barros et al. (2010) reported an IC_{50} value of 0.154 ± 0.002 mg/mL for methanolic extracts of whole fruits using the TBARS assay. When compared, these results corroborate the antioxidant potential of the epicarp extract of *P. spinosa*. The small difference between them is justified by the different amount and/or phenolic profile present in the analyzed extracts, due to factors like solvent type, extraction conditions, part of the fruit used and conditions of soil and climate of the year of harvest, along with variety and individual variation. The IC_{50} values determined in this study for the TBARS method regarding fig and blackthorn are higher than those determined in other studies in natural matrices already commercially reported as rich sources of antioxidant compounds such as blackberry (*Aristotelia chilensis*) with an IC_{50} of 0.024 mg/mL (Céspedes et al., 2008) and olive oil (*Olea europaea*) with an IC_{50} of 44.71 μg/mL (Khaliq et al., 2015). However, compared to other alternative sources used to recover phenolic compounds, both extracts investigated in the present study had superior antioxidant activity.

3.2. Antibacterial activity

The samples were tested against a set of 9 Gram-positive and Gram-negative bacteria strains with importance in the health area, some of them with known resistance to commercial antibiotics. All bacteria were somehow inhibited, making it possible to obtain minimum inhibition concentrations (MIC) values. For both extracts, there were no specific groups of bacteria that presented greater sensitivity or resistance for both extracts, since the results were heterogeneous. This suggests that the samples have compounds with an ample range of activity, being able to act against Gram-positive and Gram-negative bacteria. For the fig extract, the best MIC result (the lowest concentration required to inhibit the growth of the microorganism), was detected in one Gram negative (*Escherichia coli*) and in two Gram positive (MRSA and MSSA) species, all with a MIC of 2.5 mg/mL. Regarding blackthorn, the lowest result was also 2.5 mg/mL, observed in MSSA. In general, MIC values obtained for fig extract were all lower or equal to that obtained for blackthorn, which demonstrates a slightly higher antibacterial activity than the one obtained for fig peel. Pertuzatti et al. (2016) evaluated the antimicrobial activity of ten

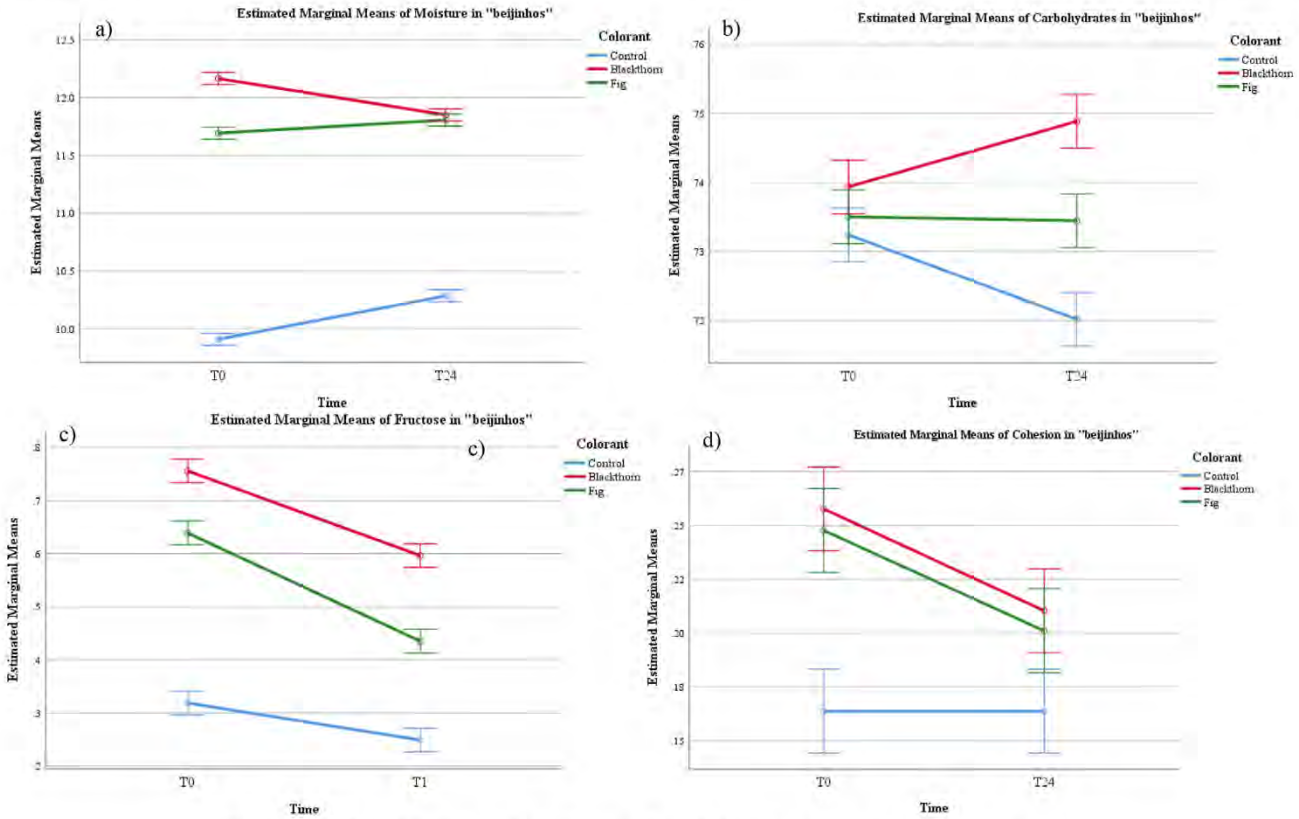

Fig. 1. EMM plots of "beijinhos" for a) Moisture, b) carbohydrates, c) fructose and d) cohesiveness.

blueberry cultivars and verified higher MIC's (20 mg/mL) than those obtained in the present study for the fruit epicarp extract of *P. spinosa*. In a study by Liu et al. (2017) with grapefruit peels, the MIC obtained for *E. coli* was 4.5 mg/mL, presenting an antimicrobial activity slightly higher to the one of blackthorn, but inferior to fig extract. These two extracts presented better antimicrobial activity than that reported in the study by Diao et al. (2013), who determined a MIC value of 10 mg/mL for *E. coli* and 20 mg/mL for *P. aeruginosa* using the essential oil extracted from *Zanthoxylum schinifolium*, a common edible plant from Asia. Still, and comparatively to *E. coli*, Yao et al. (2011) determined a MIC of 8.28 mg/mL of the Bayberry fruit (*Myryca gale*), widely consumed in China, which also had MIC higher than that of both extracts studied herein.

3.3. Cytotoxic potential

From the performed analysis, both extracts showed no activity against the non-tumor porcine liver cell line PLP2, revealing no toxicity to these cells, even at a maximum concentration of 400 μg/mL. This is important to make a first validation of the use and incorporation of these extracts in food matrices. The same conclusion was reached by (Uz et al., 2015), who described fig extract (whole infructescence) as a natural matrix that did not present a cytotoxic effect on the investigated normal cell line (rat epithelial cell).

3.4. Application of the colorant extracts in a food product

The extracts were used as natural food colorants in two dairy confectionery products, doughnut icings and "beijinho" pastry, being analyzed immediately after production and after 24 h of shelf-life. This narrow window for analysis is due to the high perishability of these food products, which are made without any preservatives (used to keep the external color and avoid oxidation and microbial contamination).

3.5. Nutritional profile and individual sugars

The nutritional profile encompassed the analysis of moisture, ash, proteins, fat, carbohydrates and energy of all samples (colored with fig extract, colored with blackthorn and control sample: no colorant). As stated in section 2.8., a two-way ANOVA was used to obtain conclusions regarding the contribution of each individual factor: colorant type (CT) and analysis time (AT). In all cases showing independent effects for each parameter (p-value CT × AT $>$ 0.05), each sample was classified individually; on the other hand, when CT × AT had a p-value $<$ 0.05, the overall conclusions were extracted from the plots of the EMM. Considering this, Table 1 is divided into 4 horizontal sections. The two upper ones pertain to the nutritional profile of the doughnut icing, and the two bottom ones to "beijinhos". The results for each confectionery product are further divided in two sections: CT (top) and AT (bottom). For each colorant type, both analysis times are included, and for each analysis time, all the colorants are included, justifying the apparently high standard deviation values (results obtained in different conditions). Still, this allows to understand the contribution of each factor independently. Considering the nutritional profile of the icings (Table 1), carbohydrates were the highest nutrient, followed by fat. For moisture the highest differences were probably due to the incorporation of the extracts. Beyond this, the incorporation of the extracts and the 24 h of storage had a very slight effect. For "beijinhos", a significant interaction was found for all nutrients except energy value, in which blackthorn showed lower values. Fig. 1 shows the EMM plots of "beijinhos", where it became clear that moisture (Fig. 1a) was higher for samples with natural colorants (when compared to the control), and these tended to increase their moisture after 24 h, probably due to its absorption from the environment. Fig. 1b shows a carbohydrates reduction in the control samples of "beijinhos" after 24 h, while the same parameter increased in samples with blackthorn extract and showed no variation in samples with fig extract. Once again, these are general tendencies extracted from the EMM plots, and do not show a significant

Table 2
Fatty acid profile of the icings and "beijinhos" detected over the 24 h, expressed as relative percentage.

Icing											
		C4:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	MUFA	PUFA	SFA
Colorant type (CT)	Control	1.6 ± 0.1[a]	1.6 ± 0.1	2.5 ± 0.1	5.9 ± 0.1	33.4 ± 0.3	26.5 ± 0.2	14.1 ± 0.1	5.26 ± 0.02[b]	16.8 ± 0.1	8.5 ± 0.2	75.3 ± 0.2
	Fig	1.7 ± 0.1[b]	1.7 ± 0.1	2.6 ± 0.1	5.9 ± 0.1	33.3 ± 0.2	26.3 ± 0.2	14.3 ± 0.1	5.21 ± 0.05[a,b]	16.8 ± 0.1	8.2 ± 0.2	75.5 ± 0.2
	Blackthorn	1.8 ± 0.1[c]	1.6 ± 0.1	2.4 ± 0.1	5.8 ± 0.1	33.1 ± 0.2	26.5 ± 0.3	14.2 ± 0.1	5.20 ± 0.05[a]	16.8 ± 0.1	8.4 ± 0.1	75.4 ± 0.2
p-value (n = 6)	Tukey's HSD Test	< 0.001	< 0.001	0.006	0.082	0.001	0.090	0.107	0.032	0.335	0.001	0.094
Analysis time (AT)	T 0	1.6 ± 0.1[a]	1.6 ± 0.1	2.5 ± 0.5	5.9 ± 0.1	33.5 ± 0.2	26.5 ± 0.2	14.2 ± 0.1	5.20 ± 0.04	16.8 ± 0.1	8.3 ± 0.1	75.6 ± 0.1
	T 24	1.9 ± 0.1	1.6 ± 0.1	2.5 ± 0.1	5.8 ± 0.1	33.1 ± 0.1	26.5 ± 0.3	14.2 ± 0.1	5.24 ± 0.01	16.8 ± 0.1	8.5 ± 0.1	75.2 ± 0.2
p-value (n = 9)	Student's T Test	< 0.001	0.764	0.208	0.006	< 0.001	0.734	0.458	0.051	0.107	< 0.001	0.303
CT × AT (n = 18)	*p*-value	0.054	0.001	0.033	0.036	0.048	0.003	0.038	0.126	0.073	0.009	0.357
"beijinho"												
Colorant type (CT)	Control	4 ± 1	3.6 ± 0.1[b]	5.3 ± 0.1	13.1 ± 0.2	37 ± 1	9.4 ± 0.4	10.1 ± 0.3	5.1 ± 0.1[a]	14.4 ± 0.3	8.2 ± 0.1	78.2 ± 0.2
	Fig	5 ± 1	3.3 ± 0.1[a]	4.6 ± 0.4	11.8 ± 0.5	35 ± 1	9.5 ± 0.2	10.3 ± 0.1	5.5 ± 0.1[b]	14.7 ± 0.1	9.8 ± 0.5	76.7 ± 0.4
	Blackthorn	3 ± 1	3.3 ± 0.2[a]	4.8 ± 0.1	12.2 ± 0.5	37 ± 1	10.2 ± 0.2	10.7 ± 0.3	5.4 ± 0.1[b]	15.3 ± 0.5	8.6 ± 0.5	77.3 ± 0.4
p-value (n = 6)	Tukey's HSD Test	< 0.001	0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.001	< 0.001	< 0.001	< 0.001	< 0.001
Analysis time (AT)	T 0	4 ± 2	3.3 ± 0.2*	4.8 ± 0.4	12 ± 1	36 ± 1	9.7 ± 0.5	10.3 ± 0.5	5.3 ± 0.2	14.8 ± 0.5	9 ± 1	77.4 ± 0.5
	T 24	4 ± 1	3.6 ± 0.1	5.0 ± 0.2	13 ± 1	36 ± 1	9.7 ± 0.4	10.4 ± 0.2	5.4 ± 0.2	14.8 ± 0.1	9 ± 1	77.3 ± 0.5
p-value (n = 9)	Student's T Test	< 0.001	0.001	< 0.001	0.200	0.361	0.668	0.432	0.072	0.515	0.035	0.449
CT × AT (n = 18)	*p*-value	< 0.001	0.222	< 0.001	< 0.001	< 0.001	0.002	0.002	0.947	< 0.001	< 0.001	0.014

In each column, for CT, different letters mean significant differences among the samples; in the case of AT, an asterisk (*) means different statistical differences among the two analysis times, with an overall significance value of 0.05 for both cases. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values.

change.

The same four sugars were found in both confectionery products, namely fructose, glucose, sucrose, and lactose, probably due to having very similar ingredients. In both, sucrose was the highest sugar, followed by lactose and then, fructose and glucose in similar amounts. Interestingly, the control sample did not show any fructose or glucose. Sucrose, lactose and total sugars seemed to be more affected by CT than AT, considering the significant differences found among the colored samples. For sucrose, samples with fig extract showed the highest values, followed by those prepared with blackthorn extracts and control samples, all with significant differences. For lactose, CT did not induce differences, but a significantly lower amount was quantified, when compared with the control sample. Finally, for total sugars, significant differences were also found between all three samples, with products prepared with fig extract showing the highest amount, followed by those containing blackthorn extract and control samples. Regarding "beijinhos", the sugar profile showed the same prevalence of sugars as the icing used on doughnuts, with a significant interaction between CT and AT in all cases. Considering the EMM, it was possible to observe (Fig. 1c) that fructose reached the highest values in samples prepared with blackthorn extract, followed by those added with fig extract, and that all samples showed a decrease of this sugar after 24 h. Overall, the changes induced by CT in the nutritional profile were hardly noticeable for both foods, while for the individual sugars, slight changes were found in the icing solution. AT did not induce noticeable differences in these profiles.

3.6. Fatty acids profile

Table 2 shows the individual fatty acids (relative percentages) found in both confectionery products. Beyond the tabled fatty acids, 21 other were detected and identified, but in percentages under 1%. The most abundant fatty acid was palmitic acid (C16:0) in both foods, with an average of 30 to 35%, followed by stearic acid (C18:0), which was found in higher quantities in doughnut icing (about 26%), reaching only 10% in "beijinhos". The most abundant unsaturated fatty acid was oleic acid (C18:1) with 14% in the icing and 10% in "beijinhos". As expected, and due to the inclusion of dairy products, both foods showed higher amounts of saturated fatty acids (SFA), averaging 75%, followed by monounsaturated (MUFA), at 16% in the icing solution and 14% in "beijinhos", and polyunsaturated (PUFA), at roughly 8% for both cases. Individually, for the icing solution, C4:0 suffered a significant increase from T0 to T24, and all three samples showed significant differences among each other, although slight. The same was found for linoleic acid (C18:2), in which the control sample had a significant difference between blackthorn, but no difference towards the fig extract colored sample. For all other individual fatty acids, a significant interaction was found, as well as for the SFA, MUFA and PUFA, with a very low change in the profile of these molecules. Regarding "beijinhos", a significant interaction was also found in all but two fatty acids, namely C10:0 and C18:2:0. Regarding linoleic acid, it was also detected in higher amounts in samples with the natural colorants, although there was no significant difference from AT. Overall, as intended, the colorants did not significantly change the profile in fatty acids of either foods.

3.7. Physical analysis

Table 3 displays the physical analysis performed in the two food samples, which included a pH determination, external color and texture analysis. Regarding pH, measured with a portable probe, the values did not vary significantly in both foods, ranging from 4.6 to 5.1.

External color analysis was one of the most important analyses, provided that coloring was the main intended effect of the extracts. Thus, the comparison was performed with a portable colorimeter that read the values in three distinct spots of the surface of the foods immediately after production and 24 h after. The CIELab spherical

Table 3
pH, external color (L*, a* and b*) and texture profile of the icings and "beijinhos" over the course of 24 h.

Icing												
		pH	L*	a*	b*	Firmness (g)		Consistency (g.*sec*)		Cohesiveness (g)		Viscosity(g.*sec*)
Colorant Type (CT) *p*-value (n = 6)	Control	5.08 ± 0.05	82 ± 2	−6 ± 1	28.0 ± 0.5	2288 ± 1119		13739 ± 5741		−1483 ± 741		−4439 ± 2484
	Fig	4.98 ± 0.05	60 ± 2	17 ± 2	10.8 ± 0.5	1616 ± 600		9490 ± 1519		−3897 ± 3087		−4309 ± 811
	Blackthorn	4.55 ± 0.03	46 ± 1	24 ± 2	2.6 ± 0.4	1522 ± 360		10417 ± 755		−820 ± 107		−5079 ± 122
	Tukey's HSD Test	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001		< 0.001		< 0.001		< 0.001
Analysis Time (AT) *p*-value (n = 9)	T 0	4.8 ± 0.2	62 ± 17	13 ± 14	15 ± 12	1176 ± 88		8777 ± 735		−2748 ± 2975		−4136 ± 1474
CT × AT (n = 18)	T 24	4.9 ± 0.3	63 ± 14	11 ± 12	13 ± 10	2441 ± 666		13653 ± 3996		−1385 ± 585		−5083 ± 1362
	Student's T Test	< 0.001	0.062	< 0.001	0.001	< 0.001		< 0.001		< 0.001		< 0.001
	p-value	0.001	< 0.001	0.002	0.001	< 0.001		< 0.001		< 0.001		< 0.001
"beijinhos"		pH	L*	a*	b*	Hardness (g)	Adhesiveness (g/*sec*)	Resilience (%)	Cohesiveness (%)	Springiness (mm)	Gumminess	Chewiness
Colorant Type (CT)	Control	5.1 ± 0.1	81 ± 2	−4 ± 1	29 ± 2	1250 ± 174^{c}	−611 ± 149	2.4 ± 0.1^{a}	0.16 ± 0.01	28 ± 3^{a}	205 ± 30$^{a, b}$	61 ± 13^{a}
	Fig	4.8 ± 0.1	59 ± 5	17 ± 2	9 ± 4	983 ± 50^{b}	−680 ± 43	2.6 ± 0.1^{b}	0.22 ± 0.03	46 ± 4^{b}	217 ± 17^{b}	101 ± 10^{c}
	Blackthorn	4.8 ± 0.1	60 ± 6	17 ± 3	9 ± 5	772 ± 75^{a}	−598 ± 69	2.4 ± 0.1^{a}	0.23 ± 0.03	47 ± 3^{b}	177 ± 24^{a}	83 ± 10^{b}
p-value (n = 6)	Tukey's HSD Test	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.383	0.002	< 0.001	< 0.001	0.033	< 0.001
Analysis Time (AT)	T 0	4.9 ± 0.1	66 ± 12	10 ± 11	15 ± 11	907 ± 296	−617 ± 106	2.5 ± 0.1	0.22 ± 0.05	42 ± 11	192 ± 33	83 ± 21
	T 24	4.9 ± 0.2	67 ± 11	10 ± 11	15 ± 11	1097 ± 173	−642 ± 96	2.4 ± 0.1	0.19 ± 0.02	38 ± 8	208 ± 22	80 ± 20
p-value (n = 9)	Student's T Test	0.683	0.316	0.788	0.988	0.005	0.620	0.719	0.001	0.100	0.177	0.686
CT × AT (n = 18)	*p*-value	0.049	< 0.001	< 0.001	< 0.001	0.091	0.724	0.671	0.033	0.132	0.425	0.432

In each column, for CT, different letters mean significant differences among the samples; in the case of AT, with an overall significance value of 0.05 for both cases. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values. Chewiness and gumminess are dimensionless.

Fig. 2. Detail of variation of colors of the different foods along 24 h, obtained by converting the L*, a* and b* coordinates.

coordinates (L*, a* and b*) are shown in Table 3 for doughnut icing and "beijinhos". A significant interaction was found for all coordinates, which was expected for CT, but also for AT, which tended to reduce the intensity of the colors for both foods and colorants. As stated above, the statistical treatment implies that in the tables, the different colorant type encompass both analysis times, and each time includes all three samples (control, fig and blackthorn colored). For the specific color assay, the information from the tables can be misleading, and thus, the total difference between the colored samples and the control was calculated using Eq. (4), and the true color (obtained from the conversion of L*, a*, b* coordinates) was calculated and is shown in Fig. 2. This image shows the true colors of the foods, allowing for a better visual interpretation of CT effect. For the doughnut icing, blackthorn rendered a more intense purple color, which lost some of its intensity after 24 h, while the fig colorant did not have such an intense color, despite maintaining it more efficiently. It is also clear that the sample darkened over the 24-hour period. In the case of "beijinhos", both colorants showed very similar coloring capacity, and both lost intensity with AT, becoming lighter. In order to better understand the coloring capacity of these natural colorants, the total difference between the control samples and each colored samples was calculated, for both times, as stated above using Eq. (4). The difference among the fig colored icing and control sample at T0 was 39.3 and after 24 h reduced to 31.0, while the blackthorn samples started off with a difference from the control sample of 56.4 and reduced to 49.2, which is consistent with Fig. 2, where a lighter tone is seen for the fig colored sample (lower difference to control sample) and a darker one for blackthorn, and the reduction of color intensity over the 24 h. Regarding "beijinhos", the difference between samples and the control was very similar at T0, namely 42.8 for fig and 44.0 for blackthorn, which reduced considerably for both to 30.1 and 29.3, respectively. The colors, present in Fig. 2, show this very similar behavior between the samples colored with the two different natural extracts, which, compared to the icing colors, shows a harmonization in colors at T0 and T24. This could be due to the different ingredients that induced a loss of color to both samples over the 24 h. The icing color changes were also observed from the EMM plots (Fig. 3), in which it is clear that the darkest color was found in the blackthorn colored samples, which became lighter with time, as with the fig colored samples, while the control sample became darker, as detailed in Fig. 3a, that shows the L* values. The a* coordinates pertain to the green/red variation, and in Fig. 3b the higher redness is visible in the colored samples (higher values of a*), with blackthorn showing the highest, and a slight reduction over time. Finally, the b* values (Fig. 3c) correspond to the blue/yellow variation, and thus, the colored samples showed the lowest values, closer to the blue tones, while the control sample shifted towards the yellow, with a loss of this tone during the analysis which was not verified for the naturally colored samples.

A texture analysis was also carried out for both foods, although different dimensions were sought for each (the icing solution was a semi-solid, while "beijinhos" are a solid food). The texture analysis is also shown in Table 3. Regarding the icing samples, a significant interaction was found for all dimensions of texture revealing that both AT

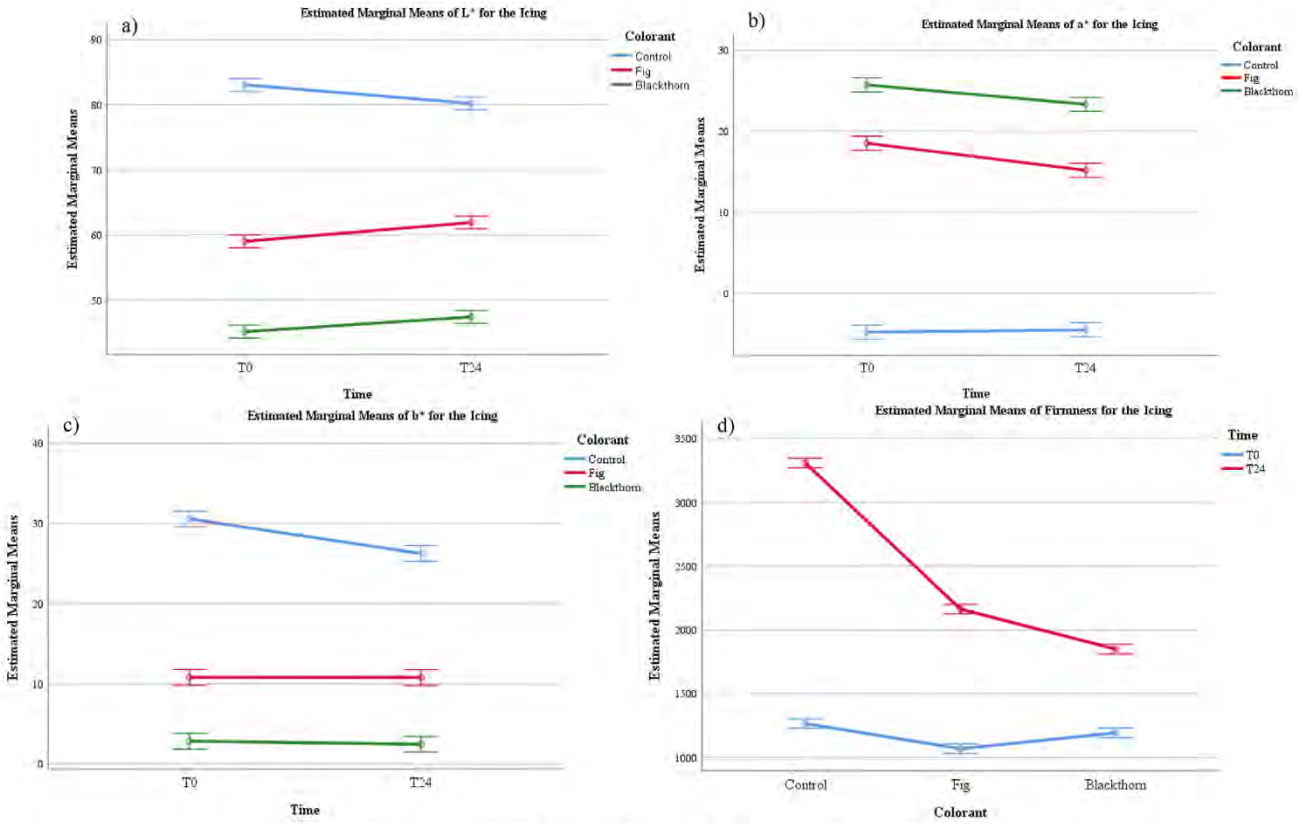

Fig. 3. EMM plots of the icing for a) L*, b) a*, c) b*, and d) Firmness.

and CT contributed to the differences observed in terms of texture. Still, some tendencies were extracted from the EMM plots, namely for firmness (Fig. 3d), in which the plots show that firmness was overall higher for T0; at T24 the control sample showed a much higher firmness value. Firmness is defined as the resistance that a semi-solid food excerpts against deformation of an applied force, and thus, it can be implied that the natural colorants make the icing less firm, making them creamier which is a valued asset in doughnut icing. Another dimension, consistency, can be described as how thick or smooth a substance can be, and is also important for the overall perception of icings. The EMM plot (Fig. S2, supplementary material) shows that at T24 the consistency was higher for all samples, although the control sample showed much higher values, making the icings seem thicker. Overall, in terms of texture profile, the natural colorants seemed to improve the consistency and firmness of the doughnut icing, without having statistically significative influence on the other dimensions, namely cohesiveness and viscosity. The bottom section of Table 3 shows the texture profile of "beijinhos", which, as a solid, underwent a traditional texture profile analysis, having been calculated their hardness, adhesiveness, resilience, cohesiveness, springiness, gumminess and chewiness. Contrarily to the icing texture profile, a significative interaction was only found for cohesiveness, where through the EMM plot, Fig. 1d, it is possible to see that the control sample showed a constant cohesiveness during the 24 h, while the colored samples stared off with a higher cohesiveness and decreased after the 24 h. Cohesiveness is the extent of destruction or deformation a product can endure when a load is applied to it, and thus, the colorants improve this resistance in "beijinhos", making them more resistant to deformation, and to not collapse or deform. Considering hardness (Table 3), the variation along the storage time had a lower influence than the colorants, in which these helped to soften the "beijinhos", with the softer one being the blackthorn colored "beijinho" followed by fig colored, with statistical differences among each other and towards the control sample. Adhesiveness did not show a significant interaction, but there were no significant differences among AT and CT. Resilience showed similar behavior to hardness, without significant changes induced by AT, contrarily to CT, namely the fig coloring, which induced higher resilience with significative differences to the control and blackthorn colored samples. Springiness, gumminess and chewiness did not have significative changes induced by the AT, but CT did induce some statistically significant changes. The colorants induced a significant increase in springiness and, therefore a higher chewiness, while gumminess increased more drastically in the fig colored sample. Overall, the colorants showed a higher impact over "beijinhos" than over the icings, probably due to the specific method of making them into a round shape. Overall, the colorants, beyond the coloring effect, increased the softness and made them chewier, which are favored attributes for these candies.

4. Conclusion

The main objective of this work was to uncover the effects that two natural colorants (fig peel and blackthorn fruit) could have in two different food uses, doughnut icings and "beijinhos", two appreciated dairy snacks. Overall, the main objective of maintaining an acceptable coloring effect for at least 24 h without changing nutritional properties (in line with food additive regulations) was achieved. For doughnut icing, blackthorn conferred a darker purple color, while fig only render a light pink color. In turn, while blackthorn lost a considerable tone after 24 h, the fig maintained its color reasonably unaltered. Inversely, both colorants showed a pleasing dark purple color at the beginning of the experiment for "beijinhos", and both tended to lose this tone and become closer to pink. This change in the first color could be due to some ingredient of the icing which quickly oxidized the fig compounds, thus promoting a loss in color intensity. In terms of texture, the icings profited in terms of lower firmness and consistency offered by the colorants, although these were just general tendencies. The "beijinhos", on the other hand, showed significant changes induced by CT, namely on hardness reduction and increase in springiness and chewiness. The

chemical profile of these foods only underwent very slight changes, with moisture reduction in the icings, and a slight increase in soluble sugars (fatty acids were kept nearly unaltered). Likewise, "beijinhos" were also very slightly altered, with a small increase in moisture and carbohydrates and no considerable change in fatty acids. Overall the colorants improved the texture properties, while conferring color with no apparent change in the chemical profile. Still, during the 24 h, no statistical changes occurred in the studied parameters, and both foods could profit from an extension of the shelf-life (although no legal shelf-life is advised from competent authorities). More studies in terms of oxidation and bacterial growth are needed to further corroborate this claim. Fig peel and blackthorn are interesting colorants that could be considered as food colorants due to their natural origin which might be consumed without posing toxicological risks towards consumers.

CRediT authorship contribution statement

Emanueli Backes: Investigation. **Maria G. Leichtweis:** Investigation. **Carla Pereira:** Investigation, Conceptualization. **Marcio Carocho:** Data curation, Writing - original draft. **João C.M. Barreira:** Data curation, Writing - review & editing. **Aziza Kamal Genena:** Writing - review & editing. **Ilton José Baraldi:** Writing - review & editing, Conceptualization. **Maria Filomena Barreiro:** Writing - review & editing. **Lillian Barros:** Investigation, Conceptualization, Writing - review & editing. **Isabel C.F.R. Ferreira:** Project administration, Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.127457.

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Article

Study on the Potential Application of *Impatiens balsamina* L. Flowers Extract as a Natural Colouring Ingredient in a Pastry Product

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Abstract: Flowers of the genus *Impatiens* are classified as edible; however, their inclusion in the human diet is not yet a common practice. Its attractive colours have stirred great interest by the food industry. In this sense, rose (BP) and orange (BO) *I. balsamina* flowers were nutritionally studied, followed by an in-depth chemical study profile. The non-anthocyanin and anthocyanin profiles of extracts of both flower varieties were also determined by high-performance liquid chromatography coupled to a diode array and mass spectrometry detector (HPLC-DAD-ESI/MS). The results demonstrated that both varieties presented significant amounts of phenolic compounds, having identified nine non-anthocyanin compounds and 14 anthocyanin compounds. BP extract stood out in its bioactive properties (antioxidant and antimicrobial potential) and was selected for incorporation in "bombocas" filling. Its performance as a colouring ingredient was compared with the control formulations (white filling) and with E163 (anthocyanins) colorant. The incorporation of the natural ingredient did not cause changes in the chemical and nutritional composition of the product; and although the colour conferred was lighter than presented by the formulation with E163 (suggesting a more natural aspect), the higher antioxidant activity could meet the expectations of the current high-demand consumer.

Keywords: edible flowers; nutritional composition; phenolic compounds; bioactivities; natural colorants; food industry

1. Introduction

Food colouring is defined as any substance of artificial or natural origin that has the ability to confer, improve or even intensify the colouring of a food [1,2]. The popularity of the colorant class in the food industry is due to its power to win over consumers by enhancing the visual aspects, from the processing and storage stages to the purchase of the final product [3,4]. However, there has been a noticeable resistance to consumers about adopting artificial colorants in food formulations due to the relationship of these substances with some human symptoms and their possible side effects [1,5–7]. In this sense, the industry has been exploring cost-effective, safe and stable natural colour matrices to meet consumer needs [8,9].

Colorants from plant matrices such as leaves, stems, fruits and flowers have proven to be promising alternatives for the industrial sector due to their numerous positive health effects, and due to their increasing consumption worldwide [1,10,11]. However, the search for more stable, safe and habitable natural matrices is still a challenge to be overcome [12,13].

Due to their different attractive colours, edible flowers can be considered innovative and engaging alternatives for application in food formulations, in particular by the discovery of new compounds related to their natural pigments, such as carotenoids, chlorophylls and anthocyanins [14,15].

Many of these compounds present in flowers are known to have the ability to act against numerous human symptoms, including free radical inactivation, bactericidal and bacteriostatic activity against some strains of microorganisms and even in the remediation of inflammation and anti-aging [16].

Plants of the genus *Impatiens* are classified as edible but are mainly known for their intense cultivation in landscape interventions, and their flowers present themselves as promising matrices for the extraction of bioactive colorants due to their intense colouration [17,18].

In this sense, the present work investigated the colouring potential of the hydroethanolic extract of the pink (BP) and orange (BO) petals of the species *Impatiens balsamina* L. through the characterisation and quantification of its phenolic compounds (anthocyanin and non-anthocyanin), the study of its bioactivities, and the chemical and nutritional characterisation present in its petals. After characterisation, the extract with the greatest colorimetric and bioactive potential was applied to a Portuguese pastry product "bombocas", in order to evaluate its behaviour as an alternative colorant compared to a control (without colouring additive) and a formulation based on strawberry gelatin (with E163 colouring additive).

2. Materials and Methods

2.1. Preparation of the Samples

The specimens of flowers of the species *I. balsamina* were collected in July and August 2019, from an urban park in Medianeira city (25°17′24.8″ S 54°05′19.2″ W) in the state of Paraná (Brazil). A portion of the plant material was identified botanically and preserved as a witness in the FLOR herbarium at the Federal University of Santa Catarina, Florianópolis, SC, Brazil. The remaining collected samples were washed in running water and deposited on absorbent paper. After drying at room temperature, the flowers were separated by colour (orange and pink). Soon after separation, they were frozen in plastic containers, lyophilised (Freezone 4.5, Labconco, Kandas City, MO, USA), crushed and finally stored in airtight bottles protected from light until further analysis.

2.2. Evaluation of Colour Parameters

The colouring of the fresh petals was measured using a portable colourimeter (model CR-400, Konica Minolta Sensing, Inc., Osaka, Japan), as described by Pereira et al. [19]. The values found for the CIE colour space L^* a^* b^* were recorded using illuminant C with an 8 mm diaphragm opening, which was subsequently processed according to the software "Spectra Magic Nx" (version CM-S100W 2.03. 0006), by Konica Minolta.

2.3. Nutritional Composition

The nutritional profile of *I. balsamina* petals was determined according to official food analysis methodologies [20]. Macronutrients were therefore quantified by analysing the content of proteins, fat and carbohydrates. Subsequently, the amounts of ash, moisture and total energy value of the samples were also determined.

2.4. Chemical Composition

2.4.1. Sugars

The free sugar content was evaluated following the methodology previously described by Barros, Pereira and Ferreira [21]. The extraction was performed with 40 mL of ethanol 80% during 30 min at 80 °C. Then the suspension was centrifuged at 15,000× *g* for 10 min (Centurion K24OR refrigerated centrifuge, West Sussex, UK) and then concentrated under reduced pressure and defatted three times with 10 mL of ethyl ether, successively. After concentration at 40 °C, the solid residues were dissolved in water to a final volume of 5 mL and filtered through 0.2 μm Whatman nylon filters using an HPLC system coupled to a refractive index detector (Knauer, Smartline 1000 and Smartline 2300 systems, respectively). The identification of free sugars was made by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI; Knauer, Smartline system 1000, Berlin, Germany) using melezitose as an internal standard. The results presented in g/100 g of fresh weight (fw).

2.4.2. Fatty Acids

After extraction by Soxhlet with petroleum ether, the obtained fat extract was subjected to a methylation process with 5 mL of 2:1:1 (v/v/v) methanol/sulphuric acid/toluene in a water bath (50 °C, at 160 rpm, during 12 h); then, 3 mL of deionised water were added to obtain the phase separation. The FAME was recovered by adding 3 mL of diethyl ether, stirring on a Vortex stirrer. The sample was collected and filtered with 0.2 μm Whatman nylon filter. The fatty acids were identified by gas chromatography with flame ionisation detection (GC-FID), as previously described by Pereira, Barros, Martins and Ferreira [22]. The identification of fatty acids was made according to their relative retention times of FAME peaks of samples with known standards. CSW 1.7 software (DataApex 1.7, Prague, Czech Republic) was used to process the results, and these were expressed as a relative percentage (%) for each fatty acid detected.

2.4.3. Organic Acids

Dry sample (2 g) was extracted with 25 mL of metaphosphoric acid (25 °C, 150 rpm, 45 min) and subsequently centrifuged (10,000× *g* for 5 min) and then filtered through 0.2 μm Whatman nylon filters. The organic acids were determined by high-performance liquid chromatography coupled to a photodiode detector (UFLC-PDA) according to a procedure previously described by Barros et al. [23]. The detection of organic acids was achieved using a DAD system, applying a wavelength of 215 nm (and 245 nm for ascorbic acid). The quantification of the compounds was carried out by comparing the area of their recorded peaks at the wavelengths mentioned above with the calibration curves obtained from the standards of the respective compound. The results were expressed in g/100 g (fw).

2.5. Phenolic Composition and Bioactive Potential of Impatiens Flower Extracts

2.5.1. Extract Preparation

For the extract preparation, 0.5 g of freeze-dried petals of *I. balsamina* of both colours (pink and orange) were used. Initially, the samples were macerated at room temperature with the addition of a solution (30 mL) of ethanol/water (80:20, v/v), for 1 h (150 rpm). Subsequently, the hydroethanolic extract was filtered using a filter (Whatman No. 4), and the retained content was extracted again using the same procedure. The filtered content was deposited in a rotary evaporator (Büchi R-210, Flawil, Switzerland), and the ethanol removed under reduced pressure. Finally, the aqueous phase of both extracts was frozen and lyophilised. It should be noted that to obtain an extract rich in anthocyanins, the same methodology was used, adding 0.5% of trifluoroacetic acid (TFA) to the extraction solvent.

2.5.2. Identification and Quantification of Phenolic Compounds

The identification and quantification of phenolic compounds (non-anthocyanin and anthocyanin compounds) was performed following the previously optimised methodology [24,25] and using a Dionex Ultimate 3000 UPLC system (Thermo Scientific, San Jose, CA, USA). DAD and mass spectrometer (LTQ XL mass spectrometer, Thermo Finnigan, San Jose, CA, USA) were used, working in negative mode for the detection of non-anthocyanin compounds and in positive mode for the detection of anthocyanin compounds.

Analytical curves (200–5 μg/mL) of the available phenolic standards were constructed based on the UV-Vis signal: *p*-coumaric acid (y = 30.1950*x* + 6966.7, R^2 = 1, LOD = 0.68 μg/mL and LOQ = 1.61 μg/mL, peaks 1 and 2); apigenin-7-*O*-glucoside (y = 10,683*x* − 45,794, R^2 = 0.996, LOD = 0.10 μg/mL; LOQ = 0.53 μg/mL, peak 3); quercetin-3-*O*-glucoside (y = 34,843*x* − 160,173, R^2 = 0.9998, LOD = 0.21 μg/mL; LOQ = 0.71 μg/mL, peaks 4, 5, 6, 7, 8, and 9); pelargonidin-3-*O*-glucoside (y = 268,748*x* − 71,423; R^2 = 0.9986, LOD = 0.24 μg/mL and LOQ = 0.76 μg/mL, peaks 10 to 23). The results were presented in mg/g of dry extract.

2.5.3. Bioactivities Evaluation

Evaluation of Antioxidant Activity: To observe the antioxidant activity, the dry extract was redissolved (2.5 mg/mL) in an ethanol/water solution (80:20, v/v) and sequentially diluted to determine its EC_{50} value. The oxidative haemolysis inhibition test (OxHLIA) was performed on sheep blood samples as previously described by Lockowandt et al. [26]. In this assay, the results were expressed by their inhibitory concentrations (EC_{50} value, μg/mL) capable of producing a haemolysis delay Δt of 60 and 120 min. Trolox was used as a positive control in both tests.

Evaluation of Anti-Inflammatory Activity: The anti-inflammatory activity was performed according to the previous methodology described by Jabeur et al. [27]. The dry extracts were dissolved in water at a concentration of 8 mg/mL and evaluated in contact with the RAW 264.7 mouse macrophage cell line. The positive control adopted was dexamethasone 50 μM and the results were expressed in terms of EC_{50} (μg/mL).

Evaluation of the Hepatotoxic Activity: For the cytotoxicity test, the dried extracts were diluted in water at a concentration of 8 mg/mL [23]. The cytotoxic potential was observed by using human tumour cell lines: MCF- 7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma) and using the sulforhodamine B assay to measure the cell growth inhibition. The hepatotoxic potential was determined using a freshly harvested porcine liver cell culture (acquired from certified slaughterhouses), designated as PLP2. Ellipticine was used as a positive control, and the results were expressed as GI_{50} values (μg/mL) as a positive control.

Evaluation of the Antimicrobial Activity: The dried extracts were dissolved in water (10 mg/mL), and the antibacterial potential was evaluated applying a methodology previously described by Soković, Glamoćlija, Marin, Brkić and Griensven [28]. In this assay, two Gram-negative bacteria strain *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella typhimurium* (ATCC 13311), and two Gram-positive bacteria strains: *Bacillus cereus* (human isolate), *Staphylococcus aureus* (ATCC 11632) and *Listeria monocytogenes* (NCTC 7973), were used. The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined using streptomycin and ampicillin as positive controls.

For the antifungal activity, the methodology described by Soković and Griensven [29] was applied and used for four fungal strains: *Aspergillus fumigatus* (ATCC 1022), *Aspergillus niger* (ATCC 6275), *Aspergillus versicolor* (ATCC 11730), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrachloron* (ATCC 9112) and *Penicillium verrucosum var. cyclopium* (food isolate). The MIC and minimum fungicidal concentration (MFC) were evaluated using ketoconazole as the positive control.

The microorganisms are deposited at Mycological Laboratory, Department of Plant Physiology, Institute for biological research "Sinisa Stanković", University of Belgrade, Serbia.

2.6. Incorporation of Natural Colorant in "Bombocas"

2.6.1. Formulation of the "Bombocas"

Three groups of "bombocas" samples were prepared: (i) "bombocas" Control (BC) prepared with neutral powder gelatin; (ii) Strawberry "bombocas" (BS) prepared with strawberry powder gelatin; and (iii) "bombocas" containing *Impatiens* extract (BI).

The "bombocas" were made according to a traditional recipe based on the preparation of a base syrup, obtained from the dilution of 400 g of sugar and 80 mL of glucose syrup in 120 mL of water at room temperature. After dilution, the mixture was heated to 100 °C until it reached boiling point, after which the temperature was raised to 180 °C, and the mixture remained on heating for 5 min. Simultaneously, three sheets of animal gelatine (4.2 g) and 80 g of gelatine powder were diluted in 120 mL of water for 2 min in a microwave oven (Eletric Co, MW70017SG, Guangdong, China) at the power of 1200 W. The hot diluted gelatine was beaten with an electric mixer (BRAUN, HM3135WH, Walldürn, Germany) until a thick foam developed in order to promote the incorporation of air into the mixture. The cooled base syrup was placed in contact with the gelatine foam, and 10 mL of lemon juice and the mixture was whipped at maximum speed with a power of 500 W until the marshmallow consistency was obtained. For the BI formulation, in this last step, 600 mg of *I. balsamina* extract was added. All groups of samples were analysed in triplicate, immediately after preparation and after three and seven days of storage kept at room temperature and protected from light, with each group consisting of 12 marshmallows. After freezing, all samples were lyophilised and then ground, and the mass was homogenised.

2.6.2. Evaluation of Colour Parameters, Nutritional Composition, Sugar and Fatty Acid Content and Antioxidant Activity of "Bombocas" during Storage Time

The colour of the samples was assessed in triplicate, at three different points of each sample, according to the procedure described in Section 2.3.

The nutritional compositions as well as the sugar and fatty acid content of all samples from all times were determined following the procedures described previously in Sections 2.3, 2.4.1 and 2.4.2, respectively.

The antioxidant activity of all "bombocas" samples, from all times, was evaluated following the methodology described previously in Section 2.5.3.

2.7. Statistical Analysis

All the assays were performed in triplicate, and the results expressed in the mean ± standard deviation (SD) format. An independent-samples t-test was used to classify the two extracts. The data from the "bombocas" was analysed using a two-way analysis of variance (ANOVA) followed by a Tukey's test for homoscedastic samples and a Tahmane T2 for non-homoscedastic sample. A significance of 0.05 was used for all analysis throughout the whole manuscript (SPSS v. 23.0; IBM Corp., Armonk, NY, USA).

3. Results

3.1. Evaluation of Colour Parameters

The flowers of the genus *Impatiens balsamina* present extremely attractive colours, ranging from white, yellow and orange to more reddish tones, which in turn are directly associated with the presence of numerous compounds, especially the anthocyanins class [17]. Thus, the chromatic analysis in the CIE colour space L^* (brightness), a^* (green/red) and b^* (blue/yellow) was performed on the petals of specimens of the species *I. balsamina*, in which the results are expressed as shown in Table 1. Regarding the orange petals (BO),

it was possible to verify that only the values obtained for *L** do not present statistically significant differences. In turn, the differences in the *a** and *b** parameters resulted in higher values for *I. balsamina*. Regarding the pink flowers (BP), these showed statistically significant differences for *L** and b* values justified by the slightly darker and brighter shade of BP that was observed and recorded in Table 1. Reports on the colourimetry of *Impatiens* flowers are scarce. However [30], when observing petals of the species *Impatiens walleriana* L., the orange petals presented *L** and *b** values higher than those presented by the pink variety, translating to light and yellowish, while the pink petals presented higher values in the *a** parameter, suggesting a more intense tone in the red range, a fact that corresponded to the information also obtained by this work.

Table 1. Colour parameters (CIE *L** *a** *b**), nutritional and chemical composition, fatty acids percentage (%) and quantity of organic acids (g/100 g fw) of the petals of *Impatiens balsamina* L. (orange_BO and pink_BP) (mean ± SD).

	BO	**BP**	***p*-value**
Colour Parameters			
*L**	46 ± 2	27 ± 1	<0.001
*a**	46 ± 2	45 ± 3	0.314
*b**	53 ± 3	14 ± 1	<0.001
Nutritional Composition			
Ash (g/100 g fw)	0.26 ± 0.02	0.26 ± 0.01	0.376
Protein (g/100 g fw)	0.33 ± 0.01	0.315 ± 0.001	0.001
Fat (g/100 g fw)	0.13 ± 0.01	0.10 ± 0.01	<0.001
Carbohydrates (g/100 g fw)	4.2 ± 0.1	4.76 ± 0.02	<0.001
Energy (kcal/100 g fw)	19.2 ± 0.4	21.145 ± 0.003	<0.001
Energy (kJ/100 g fw)	80 ± 2	88.53 ± 0.01	<0.001
Sugars			
Fructose (g/100 g fw)	0.866 ± 0.003	0.933 ± 0.001	<0.001
Glucose (g/100 g fw)	1.23 ± 0.02	1.34 ± 0.01	<0.001
Total sugars (g/100 g fw)	1.2 ± 0.02	1.34 ± 0.01	<0.001
Fatty Acids (%)			
Caprylic Acid (C8:0)	0.25 ± 0.01	0.11 ± 0.01	<0.001
Capric Acid (C10:0)	0.65 ± 0.02	0.26 ± 0.01	<0.001
Undecylic Acid (C11:0)	2.87 ± 0.04	0.90 ± 0.03	<0.001
Lauric acid (C12:0)	0.77 ± 0.03	0.38 ± 0.02	<0.001
Tridecyl acid (C13:0)	0.061 ± 0.002	0.048 ± 0.002	<0.001
Myristic acid (14:0)	2.29 ± 0.06	1.28 ± 0.06	<0.001
Myristoleic acid (C14:1)	0.97 ± 0.04	0.59 ± 0.03	<0.001
Pentadecenoic acid (C15:1)	9.8 ± 0.1	9.2 ± 0.3	<0.001
Palmitic acid (C16:0)	1.66 ± 0.07	1.04 ± 0.02	<0.001
Palmitoleic acid (C16:1)	0.51 ± 0.01	0.43 ± 0.02	<0.001
cis-10-Heptadecenoic acid (C17:1)	2.64 ± 0.06	2.4 ± 0.1	<0.001
Stearic acid (C18:0)	31.65 ± 0.09	24.2 ± 0.2	<0.001
Linoleic acid (C18:2n6)	20.8 ± 0.6	26.1 ± 0.3	<0.001
γ-linoleic acid (C18:3n6)	14.4 ± 0.1	21.4 ± 0.4	<0.001
Linolenic acid (C18:3n3)	0.56 ± 0.01	0.62 ± 0.01	<0.001
Arachidic acid (C20:0)	0.35 ± 0.02	0.37 ± 0.01	<0.001
Eicosenoic acid (20:1)	0.169 ± 0.002	0.15 ± 0.01	<0.001
Eicosadienoic acid (C20:2)	0.31 ± 0.01	0.217 ± 0.008	<0.001
Eicosatrienoic acid (C20:3n3)	3.9 ± 0.1	3.6 ± 0.2	<0.001
Dihomo-γ-linolenic acid (C20:3n6)	0.073 ± 0.001	0.202 ± 0.003	<0.001

Behenic acid (C22:0)	0.396 ± 0.004	2.18 ± 0.01	<0.001
Tricosanoic acid (C23:0)	3.5 ± 0.2	3.3 ± 0.1	<0.001
Tetracosanoic acid (C24:0)	0.491 ± 0.005	0.31 ± 0.01	<0.001
Tetracosenoic acid (C24:1)	0.16 ± 0.01	0.18 ± 0.01	<0.001
SFA	44.9 ± 0.5	34.37 ± 0.06	<0.001
MUFA	14.13 ± 0.08	12.9 ± 0.5	<0.001
PUFA	40.9 ± 0.6	52.7 ± 0.5	<0.001
Organic Acids (g/100 g fw)			
Oxalic acid	8.1 ± 0.7	6.13 ± 0.03	<0.001
Quinic acid	13.4 ± 0.2	11.6 ± 0.2	<0.001
Malic acid	16.7 ± 0.1	15.1 ± 0.3	0.336
Succinic acid	43.9 ± 0.9	59.8 ± 0.9	<0.001
Ascorbic acid	40.4 ± 0.3	41.3 ± 0.5	0.002
Total	123 ± 2	134.0 ± 0.4	<0.001

*L** luminosity; *a** chromatic axis from green (-) to red (+); *b** chromatic axis from blue (-) to yellow (+); fw—fresh weight, dw—dry weight; SFA—Saturated fatty acids; MUFA—Monounsaturated fatty acids; PUFA—Polyunsaturated fatty acids. Standard calibration curves: oxalic acid (y = 1E + 07*x* + 231.891, R^2 = 0.9999); quinic acid (y = 671.557*x* + 14.583, R^2 = 0.9998); malic acid (y = 950.041*x* + 6255.6, R^2 = 0.9999); succinic acid (y = 640.365*x* – 17.602, R^2 = 0.9995) and ascorbic acid (y = 4E + 07*x* + 1E + 06, R^2 = 0.9909).

3.2. Nutritional Composition

The nutritional composition of *Impatiens balsamina* L. petals in pink and orange varieties have been evaluated by the Official Food Analysis Methodologies (AOAC). Their respective amounts of protein, fat, carbohydrates and energy, as well as the values of ashes and sugars, were reported in Table 1. At first, it can be noted that the ash content was similar between the two colours of flowers studied. Meanwhile, orange-coloured petals (BO) had a higher amount of protein and fat, while pink petals (BP) stood out in terms of carbohydrates and had a higher energy value (21.1 kcal/100 g dw (BP) and 19.2 kcal/100 g dw (BO)). Regarding sugar content, fructose and glucose were found in both varieties, the latter being the majority sugar and the quantity being higher in the BP variety compared to BO.

Few studies report on the nutritional profile of the *Impatiens* genus. However, Fernandes, Casal, Pereira, Saraiva and Ramalhosa [31] state that the components commonly present in edible flowers, in general, do not differ much from the nutritional composition found in other plant organs. Among the few existing studies, Szewczyk et al. [32] investigated the presence of water-soluble polysaccharides of four distinct aquose extracts of species of *Impatiens* (*Impatiens glandulifera* Royle, *Impatiens parviflora* DC., *Impatiens balsamina* L. and *Impatiens noli-tangere*). From the practical aspects, it was demonstrated that the main sugars present are arabinose, ramnose, galactose, mannose, xylose and glucose, which varied their proportions according to the species. While for protein composition, quantities of 3.1 g/100 g dw [31] and 4.60 g/kg dw [33] were reported to be present in the species *I. walleriana*. In the bibliographical review by Fernandes et al. [31], the nutritional profiles of more than thirty species of edible flowers were grouped. Results were tabled for moisture (71.6 to 93.4%), total carbohydrates (10 to 90.20 g/100 g dw), proteins (2 to 52.3 g/100 g dw), fats (1.3 to 6.1 g/100 g dw), ash (2.6 to 15.9 g/100 g dw) and energy (75 to 465 kJ/100 g dw). In general, it can be noted that the values found in the nutritional composition of the petals studied in our research correspond to the values obtained for edible flowers in the literature.

3.3. Chemical Composition

The individual, saturated, monounsaturated and polyunsaturated fatty acids present in the BO and BP samples are detailed in Table 1. Twenty-four individual fatty acids were identified in the samples of BO and BP, in which the majority were: stearic (C18:0; 31.65 ± 0.09%), linoleic (C18:2n6; 20.8 ± 0.6%) and γ-linoleic (C18:3n6; 14.4 ± 0.1%) for BO, and linoleic (C18:2n6; 26.1 ± 0.3%), stearic (C18:0; 24.2 ± 0.2%) and γ-linoleic (C18:3n6; 21.4 ± 0.4%) for BP. However, some differences in the composition of fatty acids were observed in the varieties analysed, since saturated fatty acids were mainly in BO, whereas polyunsaturated acids stood out in BP, according to the following proportions: 44.9 ± 0.5% (BO) and 34.37 ± 0.06% (BP) for saturated fatty acids, 14.13 ± 0.08% (BO) and 12.9 ± 0.5% (BP) for monounsaturated fatty acids and 40.9 ± 0.6% (BO), and 52.7 ± 0.5% (BP) for polyunsaturated fatty acids. Szewczyk et al. [34] studied the lipophilic composition of the hexanoic extract of leaves, roots and seeds of the species *Impatiens glandulifera* Royle *and Impatiens noli-tangere* L. From the results obtained, ten different fatty acids were observed (Caprylic (C8:0); Capric (C10:0); Azelaic (C9:0); Palmitic (C16:0); Stearic (C18:0); Oleic (C18:1); Linoleic (C18:2) ω-6; α-linolenic (C18:3) ω-3; γ-linolenic (C18:3) and arachidonic (C20:4), as well as it was reported that polyunsaturated fatty acids had a higher percentage, with a predominance for α-linolenic, oleic and palmitic acids. Furthermore, the author also reinforced that leaves and seeds of the two studied species had higher amounts of saturated fatty acids than their own roots. The fatty acid profile of flowers of the species *I. balsamina* showed a particular discrepancy with the literature due to its greater diversity of individual fatty acids and its divergence in the percentages of saturated, monounsaturated and polyunsaturated fatty acids. However, these facts can be justified by the difference between the organs studied, the environmental conditions and also by the difference between the species.

The profile of organic acids found for orange (BO) and pink (BP) flowers of the species *I. balsamina* was represented by five distinct compounds (oxalic acid, quinic acid, malic acid, succinic acid, ascorbic acid), in which their proportions are described in Table 1. Among the organic acids identified, succinic acid was the majority in both samples, at 59.8 ± 0.9 g/100 g dw in the BP sample and 43.9 ± 0.9 g/100 g dw in the BO variety. However, oxalic, quinic and malic acids had a higher proportion for BO samples, while succinic and ascorbic acids were more evident in the BP sample. Still, the BP sample (134.0 ± 0.4 g/100 g dw) showed a total of organic acids higher than the BO sample (122.6 ± 2.1 g/100 g dw). Chua [35] researched small metabolites from the methanolic extract (50%, v/v) of stems of *I. balsamina* L. and identified thirteen different organic acids (hydroxybutyric acid, glyceric acid, fumaric acid, succinic acid, tartronic acid, malic acid, citramalic acid, dehydrossikimic acid, hydroxiterpenilic acid, quinic acid, gluconic acid, lauric acid, cafeic trihydroxyl acid). From these results, only three compounds corresponded to those quantified (quinic acid, malic acid, succinic acid) in our study. This justifies the need for further study on the quantification of organic acids of the genus *Impatiens*.

3.4. Identification and Quantification of Phenolic Compounds

Polyphenols are secondary metabolites that are present in various plant matrices, including plants, fruits, flowers, seeds, bark, leaves and roots [36]. There has been a growing interest from the investigative and industrial sector about this class of compounds due to their numerous bioactive properties presented [37]. With this perspective, the chromatographic data of each peak in terms of retention times (Tr), wavelength of maximum absorption in the UV-Vis region (max), pseudo molecular ion ($[M-H]^-/[H]^+$), and the fragmentation of the molecular ion (MSn) were used for the identification of the non-anthocyanin and anthocyanin compounds.

Regarding the non-anthocyanin group, nine compounds were tentatively identified, including two phenolic acids (*O*-*p*-coumaroyl) and seven flavonoids (*O*-glycosylated

derivatives of apigenin, quercetin and kaempferol). Peaks 1 and 2 ([M-H]$^-$ at *m*/*z* 325) presented a UV-Vis spectrum very characteristic of *p*-coumaric acid, with a maximum between 308 nm and 325 nm, and were tentatively identified as *O*-*p*-coumaroyl-α-hexoside and *O*-*p*-coumaroyl-β-hexoside, respectively, following the chromatographic characteristics previously described by Jaiswal and Kuhnert [38] in fruits of *Lagenaria siceraria* Stand.

Regarding the detected flavonoids, only one *O*-glycosylated apigenin was identified (peak 3, [M-H]$^-$ at *m*/*z* 517, apigenin-*O*-malonyl-hexoside), showing MS^2 fragments at *m*/*z* 311 and *m*/*z* 269, and its tentative identification was confirmed by comparison with the previously described in *Cynara cardunculus* var. Altilis [39]. Two *O*-glycosylated quercetin derivatives were also detected in *I. balsamina* samples, peaks 5 ([M-H]$^-$ at *m*/*z* 667) and 6 ([M-H]$^-$ at *m*/*z* 595) being tentatively identified as quercetin-acetyl-*O*-hexoside and quercetin-*O*-hexoside-pentoside, respectively [40]. The most representative aglycone of the flavonoid group is kaempferol, of which four *O*-glycosylated derivatives were found: peak 8 (kaempferol-3-*O*-glucoside) identification was carried out by comparing the retention time and UV-vis spectra together with the available standard; peak 4 ([M-H]$^-$ at *m*/*z* 609), 7 ([M-H]$^-$ at *m*/*z* 651) and 9 ([M-H]$^-$ at *m*/*z* 543), kaempferol-*O*-hexoside-*O*-hexoside, kaempferol-acetyl-*O*-hexoside-*O*-hexoside and kaempferol-*O*-hexoside-*O*-deoxyhexoside, respectively, were previously described by by Sut et al. [41] and Ning et al. [42] in *Paeonia* species and *Cyclocarya paliurus* tea leaves, respectively.

Table 2 shows a markedly *O*-glycosylated flavonoid-rich profile and only two phenolic acids derived from *p*-coumaric acid, with kaempferol-acetyl-*O*-hexoside-*O*-hexoside (peak 7) being the majority compound in this sample (2.23 ± 0.03 and 4.014 ± 0.004 mg/g extract, in BO and BP, respectively). The pink variety (BP) showed higher content in phenolic compounds, 17.19 mg/g extract, respectively, mainly due to the content in flavonoids and phenolic acids, respectively.

Table 2. Retention time (Rt), wavelengths of maximum absorption in the UV-Vis region (λmax), tentative identification and quantification of phenolic compounds in *Impatiens balsamina* L. (orange_BO and pink_BP) hydroethanolic extract (mean ± SD).

Non-Anthocyanin Phenolic Compounds							
Peak	**Rt (min)**	**λ_{max} (nm)**	**[M-H]$^-$**	**Main Fragment ESI- MS^2 [Intensity (Relative %)]**	**Tentative identification**	**Quantification (mg/g)**	
						BO	**BP**
1	7.99	309	325	307(5),265(76), 235(100),205(5),163(5)	*O*-*p*-Coumaroyl-α-hexoside	0.52 ± 0.02	1.32 ± 0.05 *
2	9.13	308	325	307(5),265(82), 235(100),205(5),163(5)	O-*p*-Coumaroyl-β-hexoside	0.43 ± 0.02	1.04 ± 0.01 *
3	10.15	324	517	311(15),269(100)	Apigenin-*O*-malonyl-hexoside	1.175 ± 0.005	0.98 ± 0.02 *
4	16.07	346	609	447(21),285(100)	Kaempherol-*O*-hexoside-*O*-hexoside	1.92 ± 0.04	3.48 ± 0.02 *
5	17.13	348	667	625(100),463(10),301(20)	Quercetin-acetyl-*O*-hexoside-*O*-hexoside	0.9997 ± 0.0002	1.105 ± 0.003 *
6	17.93	342	595	301(100)	Quercetin-*O*-hexosyl-pentoside	1.27 ± 0.01	2.02 ± 0.01*
7	20.3	346	651	609(100),447(8),285(53)	Kaempherol-*O*-acetylhexoside-*O*-hexoside	2.23 ± 0.03	4.014 ± 0.004 *
8	21.94	451	447	285(110)	Kaempherol-3-*O*-glucoside	1.325 ± 0.005	1.804 ± 0.002 *
9	23.82	342	543	431(28),285(100)	Kaempherol-*O*-hexoside-*O*-deoxyhexoside	1.484 ± 0.002	1.43 ± 0.02 *
					TPA	0.95 ± 0.04	2.36 ± 0.04 *
					Tflav	10.4 ± 0.07	14.843 ± 0.005 *

					TNAC	11.4 ± 0.1	17.19 ± 0.04 *
Anthocyanin Phenolic Compounds							
Peak	**Rt (min)**	**λ_{max} (nm)**	**$[H]^+$**	**Main Fragment ESI- MS^2 [Intensity (Relative %)]**	**Tentative Identification**	**Quantification (mg/g)**	
						BO	**BP**
10	14.62	500	595	271(100)	Pelargonidin-*O*-dihexoside	7.4 ± 0.5	1.2 ± 0.1 *
11	21.01	501	637	475(30),271(100)	Pelargonidin-*O*-hexoside-*O*-acetylhexoside	0.82 ± 0.01	0.29 ± 0.02 *
12	34.04	506	741	579(100),271(12)	Pelargonidin-*O*-hexoside-*O*-deoxyhexosyl-hexoside	0.38 ± 0.02	0.62 ± 0.03 *
13	36.62	510	801	639(25),331(100)	Malvidin-*O*-coumaroylhexoside-*O*-hexoside isomer I	0.25 ± 0.01	1 ± 0.1 *
14	37.56	511	801	639(25),331(100)	Malvidin-*O*-coumaroylhexoside-*O*-hexoside isomer II	0.26 ± 0.03	0.73 ± 0.09 *
15	38.48	504	741	579(100),271(15)	Pelargonidin-*O*-hexoside-*O*-deoxyhexosyl-hexoside	0.8 ± 0.1	0.0001 ± 0.00003 *
16	39.73	510	801	331(100)	Malvidin-*O*-coumaroylhexoside-*O*-hexoside isomer III	0.57 ± 0.02	2.7 ± 0.3 *
17	40.28	509	783	579(100),475(34),271(25)	Pelargonidin-*O*-*p*-coumaroylhexoside-*O*-acetyl-hesoxide	0.89 ± 0.09	1.3 ± 0.2 *
18	41.11	511	813	609(100),301(14)	Peonidin-*O*-acetylhexoside-*O*-*p*-coumaroylhexoside	0.43 ± 0.09[c]	0.9 ± 0.2 *
19	41.71	504	783	579(100),475(34),271(35)	Pelargonidin-*O*-*p*-coumaroylhexoside-*O*-acetyl-hexoside	2.5 ± 0.2	1.5 ± 0.1 *
20	42.7	511	843	639(100),331(34)	Malvidin-*O*-acetylhexoside-*O*-coumaroylhexoside	n.d.	2.3 ± 0.1 *
21	43.12	511	813	609(100),301(17)	Peonidin-*O*-acetylhexoside-O-coumaroylhexoside	0.7 ± 0.1	n.d. *
22	43.35	511	639	331(100)	Malvidin-*O*-coumaroylhexoside	0.8 ± 0.2	n.d. *
23	44.14	515	843	639(61),331(23)	Malvidin-*O*-acetylhexoside-*O*-coumaroylhexoside	n.d.	6.4 ± 0.5 *
					TAC	15.7 ± 0.7	19 ± 1 *

Rt—retention time; TPA—Total Phenolic Acids; Tflav—Total Flavonoids; TNAC—Total non-anthocyanin compounds; TAC—Total Anthocyanin Compounds. n.d. —not detected (below detection limit) TPC—total phenolic compounds. Standard calibration curves: *p*-coumaric acid ($y = 301.950x + 6966.7$, $R^2 = 1$, LOD = 0.68 μg/mL and LOQ = 1.61 μg/mL, peaks 1 and 2); apigenin-7-*O*-glucoside (y = 10.683x – 45.794, $R^2 = 0.996$, LOD = 0.10 μg/mL; LOQ = 0.53 μg/mL, peak 3); quercetin-3-*O*-glucoside (y = 34.843x – 160.173, $R^2 = 0.9998$, LOD = 0.21 μg/mL; LOQ = 0.71 μg/mL, peaks 4, 5, 6, 7, 8, and 9); pelargonidin-3-*O*-glucoside ($y = 268.748x – 71.423$; $R^2 = 0.9986$, LOD = 0.24 μg/mL and LOQ = 0.76 μg/mL, peaks 10 to 23). * *t*-student test *p*-value <0.001.

Fourteen anthocyanin compounds were tentatively identified, corresponding to six glycosylated pelargonidin derivatives, six malvidin derivatives and two peonidin derivatives. Anthocyanin glycosyl acylation decreases the polarity of the acylated anthocyanins 13 to 23; consequently, their retention time in a reversed-phase column was increased. This occurs due to the change of the molecular size and spatial structure of the anthocyanin aglycone [43]. The pelargonidin derivatives were the compounds found with the highest numerical expression in the samples. The attempt to identify peak 10 ($[H]^+$ at *m*/*z* 595), pelargonidin-*O*-dihexoside was performed accordingly as previously described by Pires et al. [44] in *Vacinnum mirtylus* L. The attempt to identify 12/15 ($[H]^+$ at *m*/*z* 741) as pelargonidin-*O*-hexoside-*O*-deoxyhexoside-hexoside was carried out as previously described by Li et al. [45] in hydrolysate of vegetable extracts. The attempted identification of peaks 17/19 ($[H]^+$ at *m*/*z* 783) as pelargonidin-*O*-*p*-coumaroyl-hexoside-*O*-acetyl-hesoxide was made following the previously described by Hosokawa et al. [46] in red

flowers of *Hyacinthus orientalis*. For peak 11, also a glycosylated derivative of pelargonidin, no bibliographic reference was found with its characterisation, so identification was carried out by the presence of a pseudo molecular ion at $[H]^+$ at *m*/*z* 637, with subsequent losses of MS^2 fragments at *m*/*z* 475 (162 u) and 271 (pelargonidin aglycone, 162 u + 42 u) corresponding to the loss of one hexose residue and acetyl and hexose residues, respectively, being therefore tentatively identified as pelargonidin-*O*-hexoside-*O*-acetylhexoside. Regarding malvidin derivatives, peaks 13, 14 and 16 ($[H]^+$ at *m*/*z* 801), tentatively identified as malvidin-3-*O*-*p*-coumaroylhexoside-*O*-hexoside isomer I, II and III, respectively, had already been characterised and identified by other authors [47] in red wine grape pomace. Peak 22, malvidin-*O*-coumaroylhexoside ($[M]^+$ at *m*/*z* 639), was also already described by the same authors [47]. For peaks 20 and 23, the identification attempt was performed only by the chromatographic data obtained, in which both presented a pseudomolecular ion at $[H]^+$ at *m*/*z* 843, with subsequent losses of MS^2 fragments at *m*/*z* 639 (loss of an acetyl residue and a hexose) and *m*/*z* 331 (loss of a *p*-coumaric acid residue and a hexose), being therefore tentatively identified as malvidin-*O*-acetylhexoside-*O*-coumaroylhexoside. Finally, for peonidin derivatives, no bibliographic references were found to support the attempted identification of these peaks either, so the chromatographic data obtained were used. Peaks 18 and 21 were tentatively identified as peonidin-*O*-acetylhexoside-*O*-*p*-coumaroylhexoside, showing a pseudomolecular ion at $[M]^+$ at *m*/*z* 813, with subsequent losses of MS^2 fragments at *m*/*z* 609 (162 u + 42 u, acetyl and hexose residues) and 301 (peonidin aglycone, and 146 u + 162 u, which corresponds to *p*-coumaric acid and hexose residues).

Contrary to what was observed for the non-anthocyanin phenolic compounds, it was observed that at the level of anthocyanins, the samples presented a very similar qualitative and quantitative profile. However, similarly to what was observed for the non-anthocyanin phenolic compounds, it is again in the pink varieties of the plants under study that the highest concentration of anthocyanin compounds was obtained (18.9 ± 1.3 mg/g extract), mainly due to the presence of malvidin-3-*O*-coumaroylhexoside-*O*-hexoside derivatives, respectively (peak 7a).

3.5. Bioactivities Evaluation

The Oxidative Hemolysis Inhibition (OxHLIA) analysis method was used to verify the existence of antioxidant activity in the BP and BO extracts. Thus, it was possible to notice that both extracts possessed excellent antioxidant activity (Table 3), with values of 29 ± 2 μg/mL for BP and 42 ± 2 μg/mL for BO expressed in terms of EC_{50}. Oldenburg, Henning and Soendergaard [48] studied *Impatiens chinensisa* plant parts (seeds, leaves, stems, roots and flowers) by means of antioxidant assays (DPPH and ABTS), using as solvent 1% of HCl, 90% of aqueous methanol. The extracts revealed the capacity of radical scavenging in the various tissues, with the flowers exhibiting the highest amount of antioxidant capacity.

Table 3. Cytotoxic, hepatotoxic, anti-inflammatory, antioxidant, antibacterial (MIC and MBC mg/mL) and antifungal (MIC and MFC mg/mL) activity of the hydroethanolic extracts of *Impatiens balsamina* L. (orange_BO and pink_BP) samples (mean ± SD).

	BO	BP	Positive Control
Antioxidant activity (Ec_{50} values; μg/mL)			Trolox
Oxidative hemolysis inhibition assay (OxHLIA)	42 ± 2	29 ± 2 *	85.2 ± 2
Anti-inflammatory (GI_{50} values; μg/mL)			Dexamethasone
RAW264.7	281 ± 12	164 ± 7 *	6.30 ± 0.4
Tumour cell lines (GI_{50} values; μg/mL)			Ellipticine

HeLa			121 ± 3		90 ± 6 *		1.03 ± 0.09
HepG2			201 ± 6		135 ± 9 *		1.10 ± 0.09
MCF-7			253 ± 9		155 ± 15 *		1.02 ± 0.02
NCI-H460			293 ± 12		167 ± 13 *		1.01 ± 0.01
Non-tumour cell lines (GI_{50} values; μg/mL)							Ellipticine
PLP2			>400		>400		1.40 ± 0.1
Antibacterial activity			*B.c.*	*S.a.*	*L.m.*	*E.c.*	*P.a.*	*S.t.*
BO	MIC		0.10	0.20	0.20	0.05	0.10	0.20
	MBC		0.20	0.40	0.40	0.10	0.20	0.40
BP	MIC		0.05	0.20	0.20	0.075	0.20	0.20
	MBC		0.10	0.40	0.40	0.10	0.40	0.40
Antifungal activity			*A.fun.*	*A.v.*	*A.n.*	*P.f.*	*P.o*	*P.v.c*
BO	MIC		0.012	0.025	0.012	0.012	0.006	0.025
	MFC		0.025	0.05	0.025	0.025	0.012	0.05
BP	MIC		0.025	0.025	0.025	0.025	0.012	0.025
	MFC		0.05	0.05	0.05	0.05	0.025	0.05

GI_{50} concentration that inhibited 50% of cell growth. *B.c.: Bacillus cereus; S.a.: Staphylococcus aureos; L.m.: Listeria monocytogenes; E.c.: Escherichia coli; P.a..: Pseudomonas aeruginosa; S.t.: Salmonella Typhimirium; A.fum.: Aspergillus fumigatus; A.v.: Aspergillus versicolor; A.n.: Aspergillus niger; P.f.: Penicillium funiculosum; P.o.: Penicillium ochrochloron; P.v.c.: Penicillium verrucosum* var. *cyclopium.* * t-student test *p*-value <0.001.

The anti-inflammatory potential of the extracts (BP and BO) was proven by in vitro measurements with macrophage cells (RAW264.7) (Table 3). Thus, it was evidenced that the pink coloured extract (163.5 ± 6.8 μg/mL, BP) obtained the most promising GI_{50} value compared to the orange-coloured extract (280.8 ± 12.4 μg/mL, BO). Paun et al. [49] evaluated the anti-inflammatory activity of hydroethanolic extract (50:50, v/v) of leaves and stems of *Impatiens noli-tangere* against the inflammatory enzymes LOX, COX-1 and COX-2. The most promising IC_{50} values were, respectively, 2.46 μg/mL (LOX), 18.4 μg/mL (COX-1) and 1.9 μg/mL (COX-2) for the nanofiltration fraction (NF), which can be used for inflammatory diseases. Pires Junior et al. [30] in turn observed the anti-inflammatory activity of hydroethanolic extract (80:20, v/v) of pink and orange flowers of the species *I. walleriana*, in which diagnosed values were 312.1 ± 5.5 μg/mL for the orange extract and 349.21 ± 12.8 μg/mL for the pink extract. It can be observed that the extracts BP and BO present more promising values when compared to the similar study made with the extract of flowers of the species *I. walleriana*.

For the evaluation of the antitumour activity of BP and BO extracts, a set of human tumour cell lines (MCF-7, NCI-H460, HeLa and HepG2) was used, and the results were described according to GI50 values. The toxicity of the extracts was tested for the non-tumour cell line (PLP2) up to the maximum concentration as shown in Table 3. In general, it can be affirmed that BP and BO extracts provided an antitumor activity for all tumour cell lines. However, BP was responsible for the best bioactive concentrations, showing values of 90.4 ± 5.5 μg/mL (HeLa), 134.9 ± 9.2 μg/mL (HepG2), 154.9 ± 14.5 μg/mL (MCF7) and 167.2 ± 12.5 μg/mL (NCI-H460) when compared with the values obtained by the extract BO. Furthermore, both extracts showed no toxicity against PLP2 cell lines (GI_{50} > 400 μ g/mL). Ding et al. [50] evaluated the antitumor activity of the chloroform and ethanolic extracts of *I. balsamina* flowers and subsequently tested on HePG2 tumour cells, in which the IC_{50} values corresponded to 6.08 ± 0.08 μg/mL, while Wang et al. [51] investigated the behaviour of naphthoquinone (MeONQ) isolated from the aerial parts of

I. balsamina against adenocarcinoma cells (MKN45), which proved that the cytotoxic activity on the cells was comparable to amoxicillin, with an IC_{50} value of 4.52 μg/mL.

The antibacterial activity of ethanolic extracts obtained from the flowers of *I. balsamina* was tested against a panel of Gram-positive (Bacillus cereus; *Staphylococcus aureus* and *Listeria monicytogenes*) and Gram-negative (*Escherichia coli*; *Pseudomonas aeruginosa* and *Salmonella typhimurium*) bacteria. Next, the antifungal activity of *Impatiens* extracts (BO) and (BP) was also tested for a panel of fungi (*Aspergillus fumigatus*; *Aspergillus versicolo*r; *Aspergillus niger*; *Penicillium funiculosum*; Penicillium ochrachloron; *Penicillium verrucosum var. cyclopium*), in which the results obtained for each extract in MIC, MBC and MFC were expressed as shown in Table 3. Previous studies reported that methanolic extracts of *Impatiens* (80:20, v/v) show significant antibacterial activity against Gram-positive strains without acting effectively against Gram-negative bacteria [52]. These results are inconsistent with those obtained in this study since the Gram-negative bacteria *Escherichia coli* is overall the most sensitive for the two extracts tested (BO) and (BP). In another study, Yang et al. [53], after isolating the bioactive compound, 2-methoxy-1,4-naphthoquinone (MNQ), from the hydroethanolic extract (95%) of the aerial parts of *I. balsamina* L., tested its antifungal capacity against a panel of 8 fungi and found that all the microorganisms were susceptible. This was confirmed in our study since the extracts BP and BO presented themselves as promising for fighting fungi since the CMF values were significantly lower than CMB.

The phenolic composition referred to in Table 2, evidenced a higher number of phenolic compounds for the BP extract, which will justify the better bioactive performance of the extract in relation to BO.

3.6. Incorporation of Natural Colorant in "Bombocas"

The pastry is a culinary segment essentially linked to the production of sweets with attractive products. In the pastry industry, marshmallows stand out as an airy and smooth product, formulated from the combination of gelatin, sugar, glucose syrup and flavouring ingredients, highly appreciated by consumers [54,55].

The potential colouring of the extract (BP) obtained from pink flowers of *I. balsamina* (the most promising extract in terms of bioactivity) was analysed in the marshmallow-based filling for the preparation of a Portuguese pastry sweet, popularly known as "bombocas". Thus, the "bombocas" made with the addition of *Impatiens* extract (BI) were analysed in comparison with two other formulations: a control formulation (BC) with a filling without colouring additive and another similar to the traditional recipes (BS) made with strawberry gelatine and additive E163, where the results obtained over a period of seven days are reported in Figure 1.

Figure 1. Formulations of "bombocas" made and final colouration of the filing obtained by the coordinates *L* a* b** (created by the author).

The influence of the incorporation of *I. balsamina* extract (BI) in the shelf life of the "bombocas" and in the centesimal profile of the marshmallow was evaluated in comparison with the other formulations (BC and BS). Regarding the nutritional value of the "bombocas" (Table 4), the most abundant nutrient are carbohydrates, followed by moisture and protein. Regarding the statistical treatment, the results were treated using a two-way ANOVA, allowing the individualised understanding of each of the factors, type of colorant (TC) and time interval (TI). After analysing the interaction between the two factors (TC × TI), if TC × TI < 0.05, they were analysed simultaneously, and general tendencies can be extracted from the Estimated Marginal Means (EMM) plots. If TC × TI > 0.05, each factor was evaluated independently and classified according to the post-hoc test. For the centesimal composition, a significant interaction was sought for all assays, meaning that both factors, namely the colorant and storage time, influenced the outcome. Still, some general tendencies were extracted from the estimated marginal means for proteins and carbohydrates (Figure 2a,b). The samples incorporated with *Impatiens* showed a higher quantity of proteins, which did not vary much over the seven days, while the control sample showed a lower value which decreased over time, revealing that the *Impatiens* extract, beyond providing proteins, also shows the potential of preserving them over storage time. In terms of the carbohydrates, the EMM plots reveal that once again, the control sample showed the least and generally reduced their quantity over the storage time, while the samples incorporated with *Impatiens* and strawberry showed a higher quantity. While the *Impatiens*-incorporated sample did reduce quantity over time, the strawberry incorporated sample maintained it. To date, there are few literature data reporting the nutritional profile of marshmallows. However, Periche, Heredia, Escriche, Andrés and Castelló [56] studied the substitution of isomaltulose in marshmallows and stated that the recommended moisture range for this class of products tends to vary between 15–22 g of water/100 g. In turn, Yudhistira, Affandi and Nusantari [57] observed the effects of adding spinach (*Amaranthus tricolor* L.) and tomato (*Solanum lycopersicum*) on the physical, chemical and sensory properties of marshmallows, reporting moisture values between 11.71% and 17.56% and ash values between 0.22% and 0.44%. Thus, in this work, the moisture of the marshmallows was higher than the values pointed out by the cited literature. However, a certain similarity regarding the ash content was confirmed.

Table 4. Nutritional, chemical, and antioxidant activity profile of the formulations Control (BC), Strawberry (BS) and *Impatiens* (BI) in relation to shelf life (0, 3, and 7 days).

		Humidity (g/100 g)	Ash (g/100 g)	Protein (g/100 g)	Fat (g/100 g)	Carbohydrates (g/100 g)	Energy (Kcal)	Energy (Kj)
Colourant Type (CT)	Control	27 ± 2	0.174 ± 0.004	2.9 ± 0.2	0.066 ± 0.004	69 ± 2	290 ± 7	1214 ± 30
	Strawberry	20 ± 1	0.191 ± 0.002	2.2 ± 0.2	0.067 ± 0.003	77 ± 2	319 ± 5	1337 ± 23
	Impatiens	24 ± 3	0.182 ± 0.006	4.4 ± 0.1	0.06 ± 0.04	74 ± 2	311 ± 5	1301 ± 19
p-value (n = 27)	Tukey Test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Time Interval (TI)	T0	25 ± 2	0.181 ± 0.006	3 ± 1	0.062 ± 0.003	74 ± 2	308 ± 8	1289 ± 33
	T3	22 ± 3	0.187 ± 0.008	3 ± 1	0.069 ± 0.003	74 ± 4	310 ± 14	1296 ± 59
	T7	25 ± 4	0.180 ± 0.001	3 ± 1	0.066 ± 0.003	73 ± 5	305 ± 18	1276 ± 75
p-value (n = 3)	Tukey Test	<0.001	<0.001	<0.001	<0.001	0.009	0.177	0.178
TC×IT (n = 81)	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

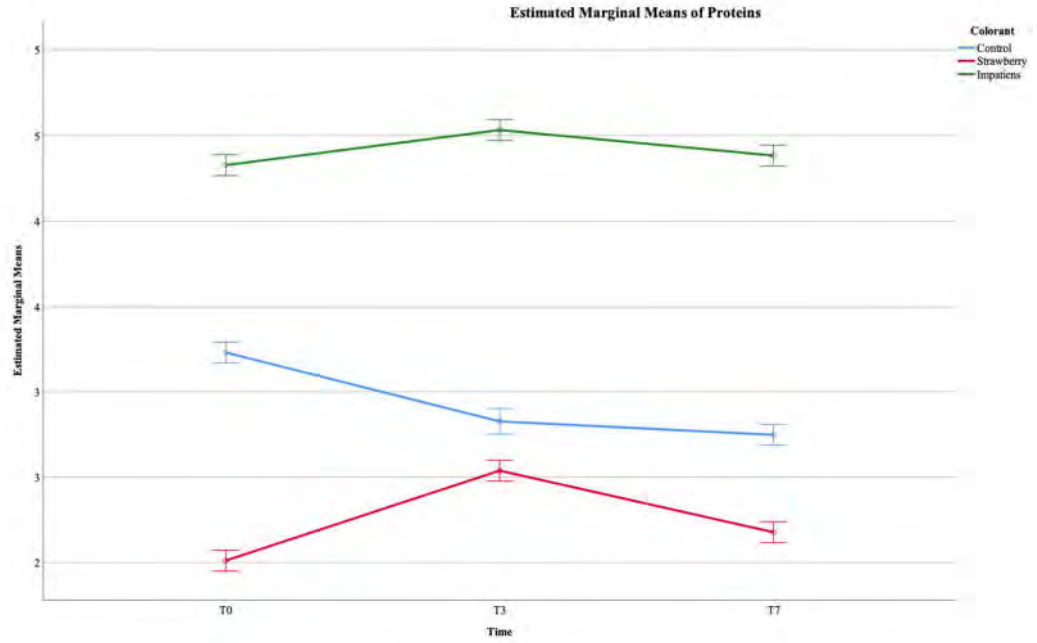
		C16:0 (%)	C18:0 (%)	C18:1 (%)	C18:2 (%)	SFA (%)	MUFA (%)	PUFA (%)	Fructose	Glucose	Sucrose
Colourant Type (CT)	Control	55 ± 4	16 ± 2	18 ± 2	10.7 ± 0.5	71 ± 2	18 ± 2	10.7 ± 0.5	12.8 ± 0.4	13.8 ± 0.3	33 ± 1
	Strawberry	49 ± 2	16.3 ± 0.7	25 ± 2	9 ± 1	65 ± 1	25 ± 2	10 ± 1	16.1 ± 0.6	16.8 ± 0.6	31 ± 1
	Impatiens	56 ± 5	16 ± 2	18 ± 2	10 ± 1	72 ± 3	18 ± 2	10 ± 1	13.9 ± 0.7	15 ± 1	35 ± 2
p-value (n = 27)	Teste Tukey	<0.001	0.008	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Time Interval (TI)	T0	56 ± 4	14.6 ± 0.9	19 ± 3	10.1 ± 0.9	71 ± 3	19 ± 3	10.1 ± 0.9	14 ± 1	14 ± 1	32 ± 2
	T3	55 ± 4	15.9 ± 0.4	20 ± 4	9.2 ± 0.7	65 ± 1	20 ± 4	9.2 ± 0.7	14 ± 2	15 ± 1	33 ± 3
	T7	48 ± 2	18.2 ± 0.9	23 ± 3	10 ± 1	72 ± 3	23 ± 3	10 ± 1	15 ± 2	16 ± 1	34 ± 1
p-value (n = 3)	Tukey Test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
TC × IT (n = 81)	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.005	<0.001	0.007

OxHLIA		Control (BC)	Strawberry (BS)	*Impatiens* (BI)	Trolox
(IC_{50}, μg/mL)	T0	w.a.	124 ± 8	212 ± 29	8.8 ± 0.5
	T3	w.a.	w.a.	267 ± 222	-
	T7	w.a.	w.a.	486 ± 57	-

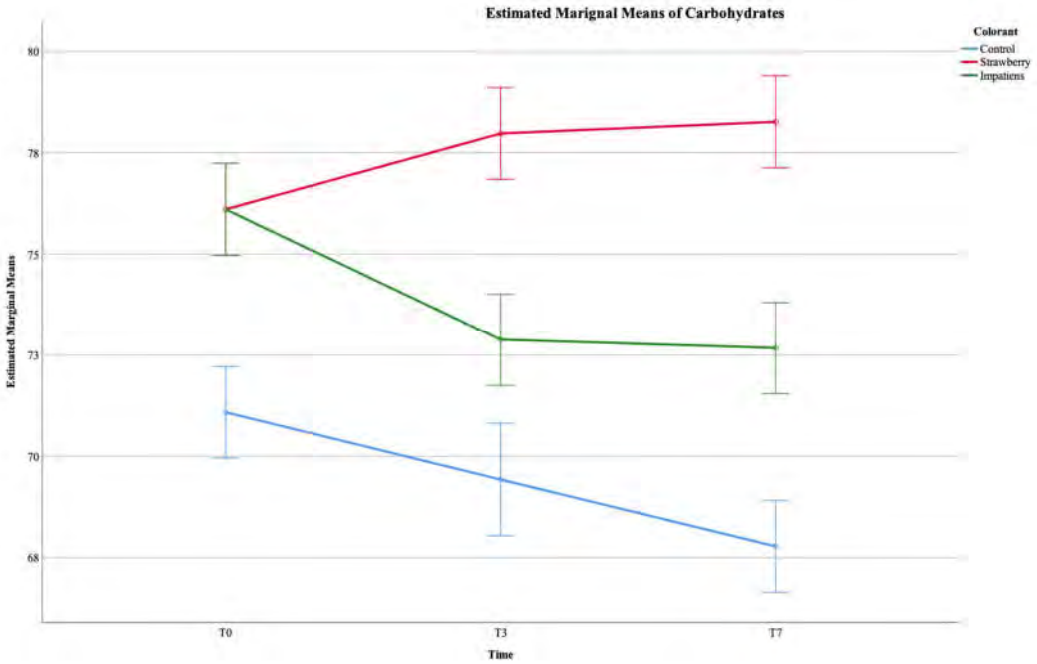
T0—0 days; T3—3 days; T7—7 days; Palmitic acid (C16:0); Stearic acid (C18:0); Oleic acid (C18:1); Linoleic acid (C18:2); SFA—Saturated fatty acids; MUFA—Monounsaturated fatty acids; PUFA—Polyunsaturated fatty acids; w.a. —without antioxidant activity.

A recent study conducted by de Oliveira Melo et al. [58] aimed to develop strawberry gelatine gummies enriched with *Hibiscus sabdariffa* L. extract (acidified aqueous solution, citric acid (1%)), verifying that the replacement of the strawberry pulp by the anthocyanin extract of Hibiscus did not influence in general the nutritional profile (protein, lipid, total fat and carbohydrate content) of the product. However, it was noted that hibiscus enrichment was able to promote changes in pH, acidity, total solids content, ash and moisture. A different phenomenon was observed in the present study, as it is possible to

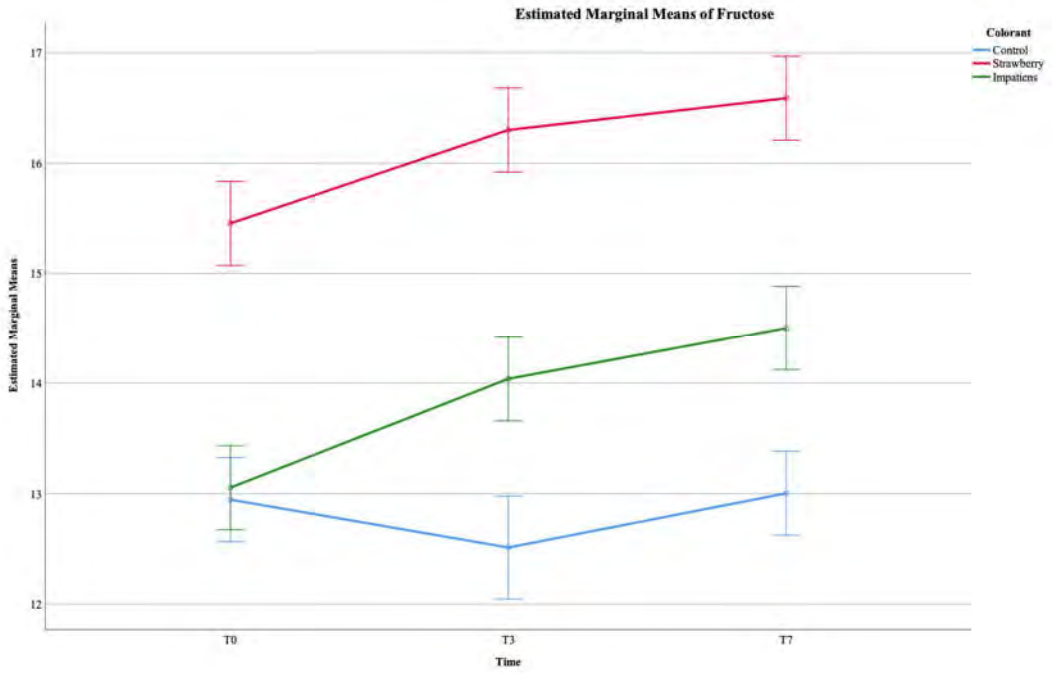
observe in Figure 2a,b; due to the addition of *Impatiens* flower extract, the corduroy filling showed some changes in the carbohydrate and protein, although relatively slight.



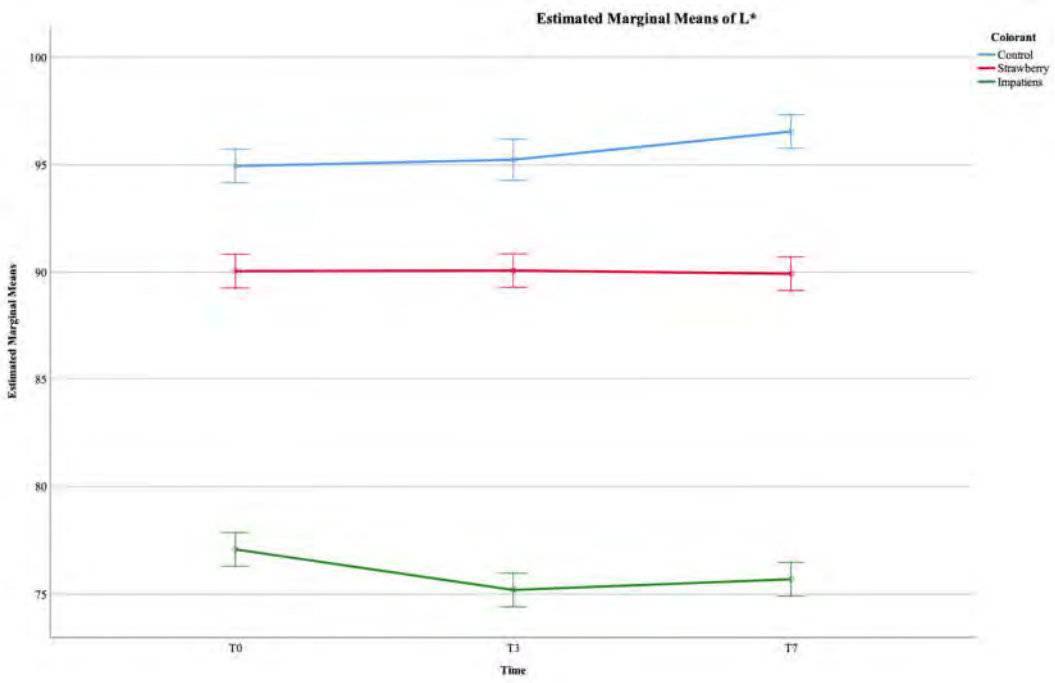
(a)



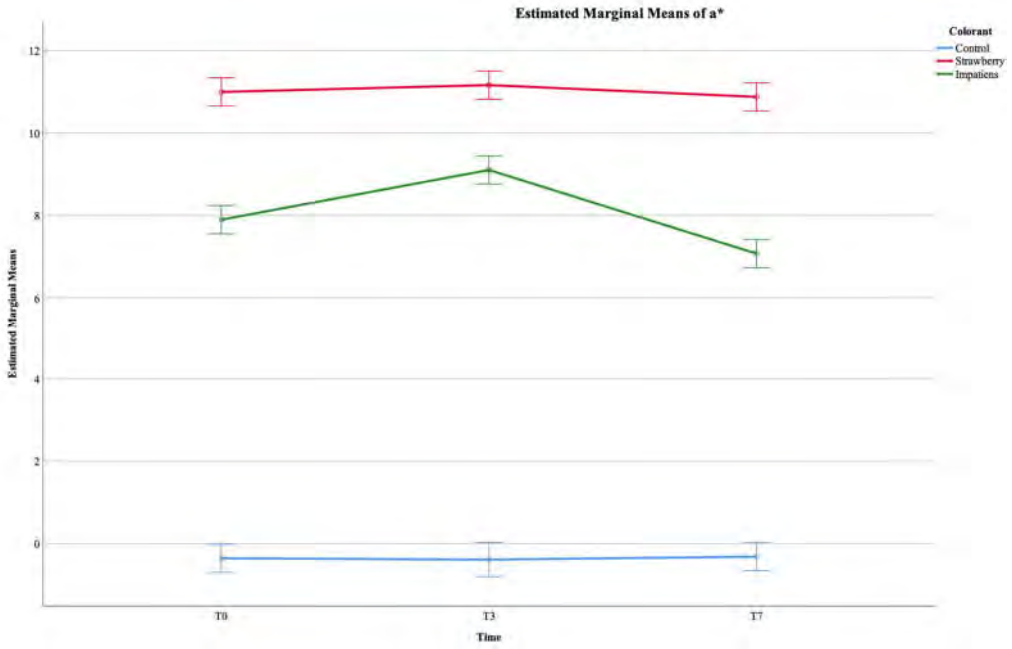
(b)


Estimated Marginal Means of Fructose
Colorant
Control
Strawberry
Impatiens
Estimated Marginal Means
17
16
15
14
13
12
T0
T3
T7
Time

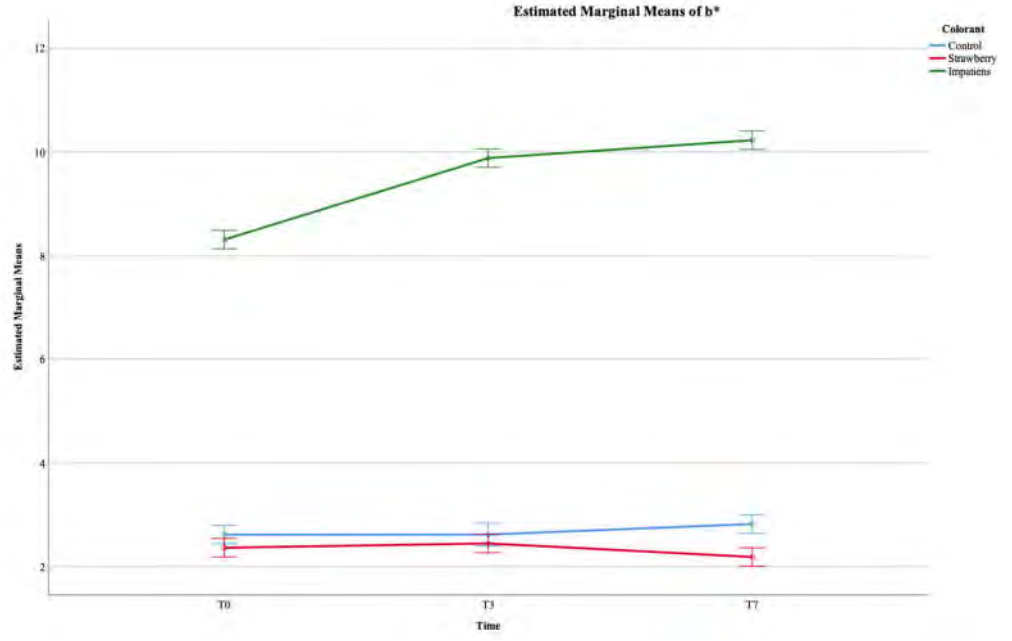
(c)


Estimated Marginal Means of L*
Colorant
Control
Strawberry
Impatiens
Estimated Marginal Means
100
95
90
85
80
75
T0
T3
T7
Time

(d)



(e)



(f)

Figure 2. Estimated Marginal Means plots for (**a**) proteins, (**b**) carbohydrates; (**c**) fructose; (**d**) *L**; (**e**) *a**, and (**f**) *b**.

Considering the fatty acids (Table 4), palmitic acid was the most abundant fatty acid, followed by oleic, stearic and linoleic acids. As for soluble sugars (Table 4), three were detected, namely fructose, glucose and sucrose, the latter being the most abundant (Table 4). Again, a significant interaction was detected between the TC and TI, thus not being possible to classify them independently. For fructose, it was possible to draw some generic conclusions from the EMM (Figure 2c). The artificial strawberry colorant provided a

higher amount of fructose for the filling of the "bombocas". The control sample (BC) and the sample with *Impatiens* (BI) showed similar values with a similar progression over time. Again, although these small changes were observed in the fatty acid and soluble sugar profile, the changes in these profiles were residual.

The colour coordinates of the filling of the different "bombocas" throughout the seven days of storage are expressed in the *L**, *a**, *b** colour space, shown in Figure 1. Again, there was a significant interaction between the type of colorant and the time interval of the trial. Thus, some conclusions were from the EMA represented in Figure 2d. With this, it was possible to verify that the filling containing *Impatiens* extract was the most intense sample, revealing lower *L** values (lower brightness), while the control sample was the one with the highest brightness, as it was very close to the maximum value of the colour space (100). In Figure 2e, for the a* coordinates, it was possible to verify that the red colour intensity level was higher for the strawberry filling, although the filling containing Impatiens extract revealed a slightly lower red tone, losing intensity from the 3rd to 7th day. Finally, for the *b** coordinates, Figure 2f, it is possible to verify that the Impatiens extract added a greater yellow intensity to the filling, a shade that increases over the seven days of storage. The colour coordinates (*L**, *a** and *b**), when combined, form the final colour of the filling, presented in Figure 1, where the strawberry gives a bright pink colour to the "bombocas" filling, while the *Impatiens* extract shows off a softer colour which integrates well with the chocolate covering the "bombocas" and may appeal to consumers who currently tend to reject food products with solid colours by association with less healthy products. A recent study testing the enrichment of gelatine gummies with *Hibiscus sabdariffa* L. flower extract revealed that the taste and texture were not altered when compared to the original formulation, unlike the colour, which became more pronounced, proving the aqueous extract's colouring potential [58].

The values obtained in the oxidative haemolysis inhibition assay (OxHLIA) are shown in Table 4, with the results were expressed as IC_{50} values (μg/mL) at a Δt of 30 min, which translates to the concentration of the extract required to maintain 50% of the red blood cell population intact for 30 min. The BI samples showed higher antioxidant activity when compared with the BC and BS samples, considering the storage time. Moreover, IC_{50} values were higher immediately after baking, and pink balsam extract (BP) showed antioxidant activity for the marshmallow filling, although it varied with shelf life. Al-Askalany and Ghandor [59] evaluated the colouring potential of golden mulberry (*Physalis peruviana*) and beetroot (*Beta vulgaris* rubra) juice for the preparation of marshmallows. Among the results found, it was observed that the use of anthocyanin colorants resulted in an increase in antioxidant activity by 32.76% and 44.87% compared to samples with added artificial colorants and samples without colours, respectively. The cited literature reinforces the antioxidant characteristics provided by the addition of natural extracts in the composition of marshmallows, a factor that is in line with the expectations of this research since the petal extract showed antioxidant capacity when introduced into the filling of marshmallows.

Artamonova et al. [60] investigated the organoleptic, physicochemical and antioxidant properties of six different marshmallow samples according to the type of structuring and colouring agent (water, hydroethanolic extract of Sudan rose or blackcurrant). The antioxidant capacity value found in the samples with natural colorant was 2 to 2.5 times higher than the results for the samples made without the colorants. Moreover, the marshmallows made with natural colouring resisted storage for two days without any packing material and remained stable for an extended period—thirty days in airtight polyethylene packaging and carton box—with high-quality indexes and colour stability. Note that the *Impatiens* extract presented a dual functionality in marshmallow filling because, in addition to providing colour, it was also responsible for ensuring less oxidation of the filling throughout the shelf life studied (seven days), indicating that this extract can be exploited as an alternative for formulations to obtain food additives such as colorants and preservatives.

4. Conclusions

The colorants obtained from natural matrices have gathered great interest in the food industry; however, there are still challenges to be overcome for their exploitation and application. Many of the flowers classified as edible have attractive colours with great potential; however, further characterisation studies are still needed. In this perspective, the petals of the two varieties (pink and orange) of *I. balsamina* were chemically, bioactively and nutritionally characterised. Both varieties had bioactive potential, highlighting the pink extract that went on for testing as a colouring ingredient in the filling of a pastry product, "bombocas". The natural ingredient gave a softer colouring to the filling when compared to the synthetic additive, providing a more natural appearance, and guaranteeing antioxidant activity throughout the shelf life without changing the chemical and nutritional composition of the food product. Thus, flowers of the *Impatiens* genus may represent auspicious colouring ingredients, with great interest in exploitation by the food industry as a new alternative to synthetic colorants.

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