Enhancing the health benefits of yogurt with pinhão seed coat extract: optimization of extraction methods and in vitro bioaccessibility

DANIELLE SPECHT MALTA – GABRIEL GOETTEN DE LIMA – FERNANDO CASTRO LEAL – WÉDISLEY VOLPATO MAROLDI – ALVARO LUIZ MATHIAS – WASHINGTON LUIZ ESTEVES MAGALHÃES – CRISTIANE VIEIRA HELM – MARIA LUCIA MASSON

Summary

This study evaluated two extraction methods for pinhão (*Araucaria angustifolia*) seed coat (PSC), namely, infusion and shaker compared with Soxhlet, so as to evaluate the potential use of the resulting bioactive extract in dairy products. Also, to provide an accessible product to small and medium agribusinesses. The shaker method presented a higher extraction efficiency compared to infusion but lower than Soxhlet. In comparison to the other two extraction methods, the extract obtained by the former method had lower toxicity on *Artemia salina*. That extract was chosen to be incorporated in yogurt, which resulted in a significant enhancement of phenolics (100 %), flavonoids (19 %), antioxidant potential with 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay (17 %) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (116 %). Additionally, samples showed good stability during 30 days of shelf-life tests and low toxicity. In vitro gastrointestinal digestion increased the bioaccessibility of bioactive compounds such as phenolics, flavonoids and potential antioxidants. This bioactive extract has potential to be used as a functional ingredient in dairy products without affecting their taste, which is a significant contribution since the seed coat is considered a residue and can provide added value to food products.

Keywords

bioactive compound; antioxidant; phenolics; sterol

Pinhão is the edible seed of *Araucaria angustifolia*, a tree that grows within its own biome, the *Araucaria* forest, in the southern part of Brazil [1]. The edible portion, seed, is widely consumed due to its nutritional value [2]. However, pinhão seed coat (PSC) is considered a residue despite containing several bioactive compounds [3]. These compounds present several health benefits, including the inhibition of pancreatic lipase [4] and α-amylase [5], which reduce carbohydrate digestion and glucose absorption, and attenuating postprandial hyperglycemic excursions.

Several methods can be used to utilise PSC, including the extraction of its phenolic compounds. Various factors can influence the extraction of these compounds, such as particle size, solid-liquid ratio, time, solvent, method of extraction and temperature [6, 7]. However, the effect of particle size on PSC and the use of ethanol as solvent to extract its phytosterols have not been investigated.

In yogurt, a dairy food produced by fermenting milk with lactic acid bacteria, bioactive peptides are formed during fermentation. However, it is a weak source of bioactive ingredients [8] and since by-products from PSC are generated by the agri-food industry, it may be possible to combine both products. This could potentially increase the health benefits of yogurt and classify it as a functional food. To date, no products with PSC extract have been introduced in the dairy sector.

The inclusion of various bioactive extracts in milk and yogurt was found to enhance their gas-

Danielle Specht Malta, Fernando Castro Leal, Wédisley Volpato Maroldi, Alvaro Luiz Mathias, Maria Lucia Masson, Graduate Program in Food Engineering, Department of Chemical Engineering, Federal University of Paraná, Av. Cel. Francisco H. dos Santos 100, 81530-000 Curitiba, Brazil.

Gabriel Goetten de Lima, Graduate Program in Engineering and Materials Science - PIPE, Federal University of Paraná, Av. Cel. Francisco H. dos Santos 100, 81530-000 Curitiba, Brazil.

Washington Luiz Esteves Magalhães, Cristiane Vieira Helm, Embrapa Florestas, Br-476, Km 111, 83411-000 Colombo, Brazil.

Correspondence author:

Danielle Specht Malta, e-mail: daniellesmalta@gmail.com

trointestinal digestibility through the stimulation of peptic and pancreatic enzymes [9]. These enzymes releases polyphenols, bioactive peptides and amino acids linked to milk proteins [10]. Therefore, investigating the importance of PSC and its contribution when used in yogurt is instructive.

To test the usage of PSC extracts in food applications, toxicological studies are necessary to quantify the median lethal dose and maximum dosage. Various methods, such as the *Artemia salina* test, are proposed to assess general toxicity and are considered essential as a preliminary bioassay in the study of compounds with potential biological activity [11]. The choice of this toxicity test may be a low-cost alternative to evaluate the feasibility of the potential use of plant extracts. Artemias (brine shrimps) are sensitive species, which have demonstrated efficacy in toxicity testing of food ingredients [12]. To ensure the safety of incorporating Preserved Standard Culture extracts in yogurt, it is important to conduct toxicity analysis. This is important also due to the presence of live bacterial cultures in the yogurt.

In this work, a preliminary screening was used to optimize the extraction of phenolic compounds from PSC, including the particle size effect and lethality tests in which the best method was evaluated as a natural additive to yogurt. This was addressed by testing its bioaccessibility in vitro, the bioactive compounds content after gastrointestinal digestion in vitro and its shelf life for 30 days. To the best of our knowledge, there are no studies on incorporation of pinhão extract in dairy products, and this innovation may have an impact at both national and international levels.

MATERIALS AND METHODS

Pinhão samples

Pinhão seeds were collected in the forest area of Embrapa Florestas (S 25° 19.295', W 49° 09.577') from approximately 10 trees in various locations in Colombo (Paraná, Brazil). They were harvested when fell into the ground and at ripe condition. Selected seeds were oven-dried at 60 °C for 48 h to 7% moisture [13]. After seeds peeling, the seed coats were grounded in a Willye STAR FT-60 Macro knife mill (Fortinox, Piracicaba, Brazil). The procedure yielded 67 % (w/w) of seed, 31 % (w/w) of the seed coats and 2 % (w/w) of residues. Particle size distribution was determined with specific sieves and a granulometric agitator (Bertel, Caieiras, Brazil) for 10 min. High yields of particles smaller than 0.71 mm were obtained and, therefore, fractions between 0.71 mm and 0.43 mm (diameter 0.57 mm), 0.43 mm and 0.30 mm (diameter 0.36 mm) and 0.30 mm and 0.15 mm (diameter 0.23 mm) were chosen.

Pinhão seed coat extract

All extracts were obtained with the ethanol solvent, in order to achieve high content of phytosterols [3] and low toxicity for food use. The extractions were performed in an Erlenmeyer flask by pouring 10 ml of ethanol into PSC in the absence of light.

In infusion extraction (I), heated ethanol was poured in the flask with the seed coats until boiling (78.3 °C) [14]. In shaker extraction (S), ethanol was poured in the flask with the seed coats at room temperature (± 25 °C), closed with plastic film and shaken in a refrigerated orbital incubator-shaker TE-42I (Tecnal, Piracicaba, Brazil) at 15 Hz.

A screening was performed to obtain the best extraction conditions using a two-level complete factorial experimental design to optimize the concentration of phenolic compounds, with three replications at the central point. The extraction I was evaluated towards the effect of three factors (2^3): granulometric parameter (G), proportion of solids to solvent ratio (S/S) and time (t). The extraction S was evaluated with four factors (2^4), with the addition of temperature (T).

For I and S extraction conditions were (–1, 0 and 1): granulometric parameter 225 μm, 362 μm and 567 μm; S/S ratio 4 % (v/v), 8 % (v/v) and 12 % (v/v); time of I extraction 5 min, 17 min and 30 min; time of S extraction 15 min, 30 min and 45 min; temperature of S extraction 25 °C, 37.5 °C and 50 °C (Tab. 1).

Soxhlet extraction (So) was performed for comparison with the optimized extraction methods (I and S), using the optimum conditions (particle diameter of 225 μm, S/S of 4 %, v/v), with 8.34 g of PSC and 200 ml of ethanol for 6 h and approximately 78 °C, in duplicate. The extract yield was calculated and the product was frozen.

Recovery and yield of extracts

Extracts were recovered after centrifugation (relative centrifugal force 112 ×g, 5 min, 15 °C) employing a centrifuge Heraeus Fresco 21 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the yield was calculated and expressed in percent (v/v).

Powdered extracts

The phenolic extracts obtained by optimized I and S methods, as well as by So method, were ro-

tary evaporated (40 °C, 80 kPa) in a rotary vacuum evaporator MA120/THV (Marconi, Piracicaba, Brazil). Powder yield was calculated and expressed in percent (w/w) and the product was frozen.

Characterization of extracts

Total phenolics were evaluated in all extracts. Antioxidants, flavonoids and tannins were determined in the optimized I and S extracts and in the unoptimized Soxhlet extract. Total phenolics and antioxidant potentials by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and ferric reducing antioxidant power (FRAP) assay were determined following de Lima et al. [3], in triplicate. Total flavonoids were determined following Zhishen et al. [15], in triplicate. Condensed tannins were determined in triplicate [3]. The determination of total phytosterols was based on the Liebermann–Buchard (LB) method according to Porter et al. [16] and expressed as β-sitosterol equivalent per unit mass of PSC.

Measurement results were then expressed in terms of gallic acid equivalents (GAE) for phenolics, catechin equivalents (CE) for flavonoids and Trolox equivalent (TE) for antioxidant activity determined by ABTS, DPPH and FRAP assays. All results were expressed per mass unit of the tested material on a dry basis

Artemia salina toxicity test

The test with *A. salina* of the three extracts obtained was carried out according to Bellatto and Braguini [11], in order to verify the limits to add in food products. The concentrations evaluated were 1, 10, 100, 250, 500, 750 and 1000 $\mu g \cdot ml^{-1}$ (extract in synthetic seawater), in triplicate. The rate of death from nauplii specimens was evaluated after 24 h. The results were expressed as the concentration causing 50% lethality (LC_{50}). The toxicity tests were carried out in 2 batches and in each batch, all the concentrations used were applied in triplicate.

Application of the extracts in yogurt

Tirol pasteurized bovine milk (Tirol, Treze Tílias, Brazil) containing 47 $g \cdot kg^{-1}$ carbohydrates, 32 $g \cdot kg^{-1}$ proteins and 30 $g \cdot kg^{-1}$ fat, and Molico skimmed milk powder (Molico, Araçatuba, Brazil) containing 500 $g \cdot kg^{-1}$ carbohydrates, 320 $g \cdot kg^{-1}$ proteins and 0 $g \cdot kg^{-1}$ fat were purchased locally in Araçatuba, Brazil. A thermophilic starter culture for yogurt Yoflex YF-L812 (Chr. Hansen, Hørsholm, Denmark) was used, which is composed of *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*.

Tab. 1. Experimental conditions of pinhao seed coat extraction and content of total phenolics.

Run	Experimental conditions				Total phenolics [$g \cdot kg^{-1}$]
	G [μm]	*S/S* [%]	*t* [min]	*T* [°C]	
Infusion extraction					
I1	225	4	5		64.0 ± 0.9
I2	567	4	5		25.7 ± 0.3
I3	225	12	5		48.3 ± 1.0
I4	567	12	5		25.2 ± 0.1
I5	225	4	30		69.6 ± 1.1
I6	567	4	30		26.1 ± 0.4
I7	225	12	30		61.1 ± 2.0
I8	567	12	30		35.6 ± 0.5
I9	362	8	17.5		41.3 ± 0.4
I10	362	8	17.5		42.8 ± 1.2
I11	362	8	17.5		40.0 ± 0.8
Shaker extraction					
S1	225	4	15	25	75.2 ± 1.6
S2	567	4	15	25	45.0 ± 0.5
S3	225	12	15	25	76.0 ± 0.5
S4	567	12	15	25	35.8 ± 0.8
S5	225	4	45	25	80.6 ± 0.9
S6	567	4	45	25	45.5 ± 0.4
S7	225	12	45	25	76.2 ± 1.8
S8	567	12	45	25	45.1 ± 0.4
S9	225	4	15	50	83.9 ± 3.1
S10	567	4	15	50	40.8 ± 1.2
S11	225	12	15	50	78.9 ± 0.1
S12	567	12	15	50	45.1 ± 2.4
S13	225	4	45	50	98.5 ± 5.3
S14	567	4	45	50	48.1 ± 0.4
S15	225	12	45	50	80.5 ± 0.6
S16	567	12	45	50	49.6 ± 0.5
S17	362	8	30	37.5	62.1 ± 0.5
S18	362	8	30	37.5	64.1 ± 1.0
S19	362	8	30	37.5	65.0 ± 0.5

Runs I9, I10, I11, S17, S18, S19 represent repetitions at the central point.
Content of total phenolics is expressed as grams of gallic acid equivalents (values are on dry basis).
G – granulometric parameter (average granule size); *S/S* – solids to solvent ratio, *t* –extraction time, *T* – extraction temperature.

The preparation was performed according to the manufacturer's instructions. The packet containing the bacteria was homogenized and an aliquot of 0.1936 g of bacteria was used for each 10 l of yogurt. The content was diluted in 100 ml milk, previously boiled, and refrigerated, homogenized, and divided into equal parts (10 ml for each litre). The suspension was frozen until further use, for a maximum of 30 days.

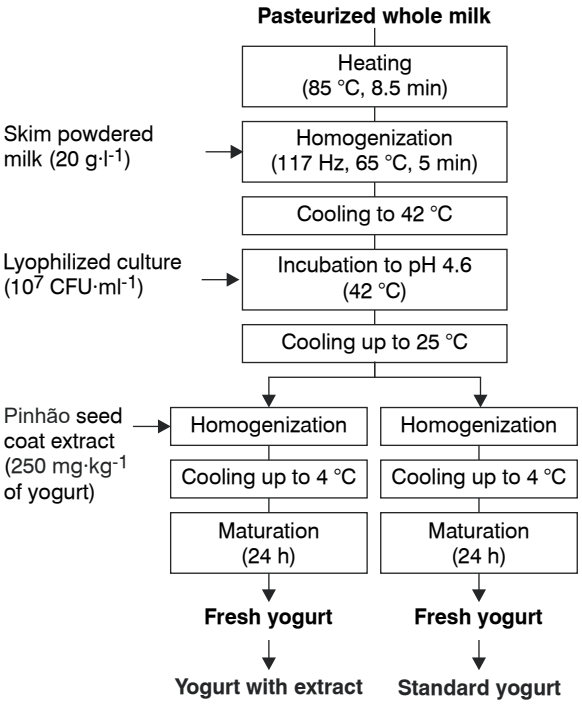

Fig. 1. Production of yogurt added with pinhão seed coat extract.

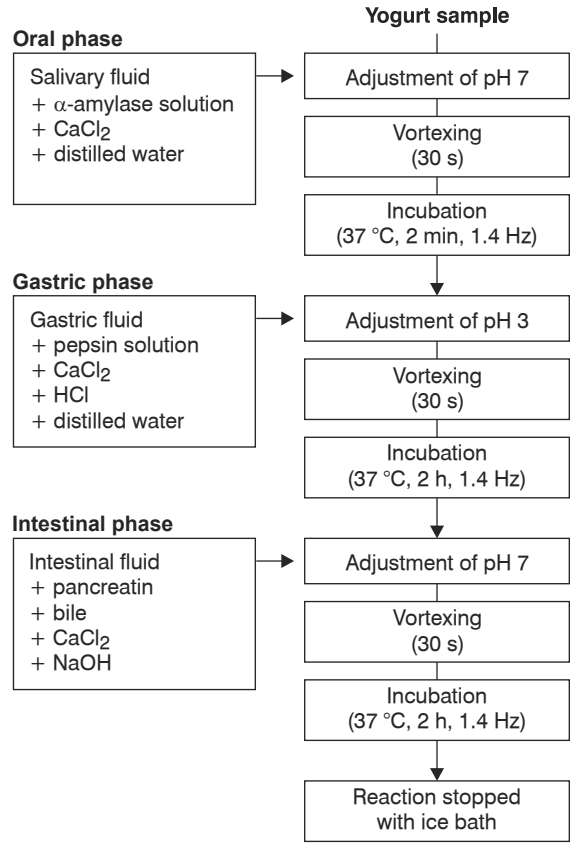

Fig. 2. In vitro gastrointestinal digestion of yogurts added with pinhão seed coat extract.

Yogurts were produced with 4 l of milk, which was heated at 85 °C for 8.5 min, according to the scheme in Fig. 1. Afterwards, milk was cooled to 65 °C and one quarter was mixed with 20 $g \cdot l^{-1}$ skim powdered milk during 5 min. Then, the mixture was homogenized with the rest of the milk (three quarters) and when the temperature reached 42 °C, the thermophilic culture was added to the milk, at a concentration of 10^7 $CFU \cdot ml^{-1}$ of culture. Subsequently, the mixture was fractionated into 4 sterilized jars (of approximately 900 g), 2 for the standard yogurt and 2 for the yogurt with addition of PSC extract after fermentation. Incubation was carried out at 42 ± 1 °C in a Q316 m bacteriological incubator (Quimis, Diadema, Brazil). The procedure was finished after reaching pH 4.6.

To ensure homogenous mixing of the extract (250 $mg \cdot kg^{-1}$ of yogurt), the yogurts were cooled to 25 °C after fermentation. They were then manually shaken in both clockwise and counterclockwise directions for 30 turns each. This process was performed after diluting the extract with the yogurt. The yogurts were packaged in 200 ml polypropylene pots (upper diameter of 55 mm, lower diameter of 50 mm, height of 60 mm), containing approximately 100 g of sample. The samples were refrigerated at 5 ± 1 °C for 12 h for maturation and then evaluated.

In vitro gastrointestinal digestion of yogurts

The in vitro bioaccessibility of the yogurt samples was evaluated through a three-phase gastrointestinal digestion process, including oral, gastric and intestinal phases. This process was performed in duplicate following the scheme shown in Fig. 2.

Gastrointestinal fluids were prepared using stock solutions of KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2$ (H_2O_6), $(NH_4)_2CO_3$, HCl, H_2O. NaCl was not added to salivary fluid, nor $(NH_4)_2CO_3$ was added to intestinal fluid. The fluids were prepared and kept under refrigeration until analysis. The enzymes were prepared on the day of the analysis with their respective fluids [17].

For oral phase, 2.10 ml of salivary fluid of pH 7 was used. To the salivary fluid 300 µl of α-amylase (EC 3.2.1.1) solution (1 500 $U \cdot ml^{-1}$ from stock solution of *Aspergillus niger* 12 143 $U \cdot ml^{-1}$, Sigma Aldrich, St. Louis, Missouri, USA), 15 µl $CaCl_2$ (0.3 $mol \cdot l^{-1}$) and 585 µl distilled water were added.

For gastric phase, 4.50 ml of gastric fluid of pH 3 was used. Pepsin (EC 3.4.23.1) solution (960 µl, 25 000 $U \cdot ml^{-1}$, from the gastric mucosa of pig of 727 $U \cdot mg^{-1}$, Sigma Aldrich), $CaCl_2$ (3 µl, 0.3 $mol \cdot l^{-1}$), HCl (120 µl, 1 $mol \cdot l^{-1}$) and 417 µl distilled water were added.

For intestinal phase, 6.60 ml of intestinal fluid of pH 7 was used. Pancreatin (3 ml, 800 U·ml^{-1}, from the porcine pancreas, 8× USB, Sigma Aldrich), bile (1.5 ml, 160 mmol·l^{-1}, Sigma Aldrich), $CaCl_2$ (24 µl, 0.3 mol·l^{-1}) and NaOH (90 µl, 1 mol·l^{-1}) were added.

After each digestion phase, the samples were subjected to centrifugation (1 800 ×g, 1 h). The resulting supernatant, which contained the absorbed material, was then lyophilized to facilitate further evaluation of the bioactive compounds.

To prepare the extracts for analysis, both undigested controls and samples from each digestion phase were utilized. For each extraction, 3 g of yogurt samples were resuspended in 4 ml of 80% (v/v) methanolic solution. The mixture was vortex-mixed for 1 min and incubated in an ultrasonic bath (SSBu 3.8 l; 7lab, Rio de Janeiro, Brazil) operating at 40 kHz for 10 min. Subsequently, centrifugation was performed at 1 800 ×g for 10 min using 80-2B centrifuge (Daiki, Araucária, Brazil). The supernatant was collected and the extraction process was repeated by adding 2 ml of the solvent.

The analyses of total phenolics, total flavonoids and antioxidant activities using ABTS, DPPH and FRAP assays were conducted in quadruplicate, following the procedures outlined in reference [17], using microplates.

Colour measurement

The colour analysis of the liquid extracts and yogurt samples was performed using the MiniScan XE Plus digital colorimeter (HunterLab, Reston, Virginia, USA), in the CIE $L^*a^*b^*$ system, D65 illuminan, and observation angle of 10°, in triplicate. The colour variation (ΔE) was calculated from the values of L^* (luminosity), a^* and b^* (chromaticity) (Eq. 1).

$$\Delta E = \sqrt{(L^* - L_0{}^*)^2 + (a^* - a_0{}^*)^2 + (b^* - b_0{}^*)^2} \quad (1)$$

where, L_0, a_0 and b_0 are parameters of the control sample (each extract) and L^*, a^* and b^* are parameters of the test sample.

Statistical analysis

Factorial designs were generated and evaluated using Statistica 7 software (StatSoft, Tulsa, Oklahoma, USA). The significance of the experimental model coefficients was evaluated by ANOVA with a confidence level of 95 % ($p < 0.05$). Subsequent analyses were performed using Jamovi Software, free version 2.2.5 (Jamovi, Sydney, Australia). All data were evaluated for normality by the Shapiro Wilk test ($p < 0.05$). subsequently submitted to ANOVA and treatments were analysed by Tukey's multiple comparison test ($p < 0.05$) for groups of greater than 3. For comparison between two groups, Student's T-test ($p < 0.05$) was performed in the analysis of yogurt formulations and paired T-test for shelf life analysis. Spearman's correlation analysis ($p < 0.05$) was employed for datasets that did not follow a normal distribution. Principal component analysis (PCA) was performed with data previously evaluated by Bartlett's test and Kaiser-Meyer-Olkin (KMO) test.

RESULTS AND DISCUSSION

Optimization of extraction

The extraction technique and the solvent used are important factors for extraction of bioactive compounds from plants. Specifically, the solvent must have maximum compatibility with the properties of the extracted materials. The yield of phenolics by I and S extraction (Tab. 1) could be predicted by Eq. 2 and Eq. 3. According to the model, regression coefficients for I and S extraction (Tab. 2) had significant ($p < 0.05$) negative effects regarding granulometry (G), 7-fold higher for both methods than other variables, together with S/S, while time showed a positive effect. Temperature had positive effect for S extraction. Interactions between G and S/S were significant, while interactions between G and T were significant for S extraction.

$$y = 43.3 - 16.7G - 2.3\,S/S + 3.3t + \\ + 3.8G \times S/S \quad (2)$$

$$y = 62.9 - 18.4G - 1.9\,S/S + 2.7t + \\ + 2.9T + 1.4G \times S/S - 1.3G \times T \quad (3)$$

Optimal results for I extraction were obtained at lower granulometry, lower S/S and longer time. For S extraction, the highest effect was observed for temperature. Such effect of granulometry and S/S was previously reported, as an increase in solids reduced the extraction of phenolics. The increase in solids content led to difficulties with mass transfer, which hampered the extraction. The temperature increase is associated with an increase in diffusion coefficient of the liquid solvent within the solid matrix, favouring the bioactive compounds extraction.

Repeatability of the best condition from each extraction I5 and S13 was determined. The repetitions showed no significant differences in relation to phenolics content ($p < 0.05$). I showing on average 66 g·kg^{-1} and S showing on average 94 g·kg^{-1} phenolics, with a 42 % increase. These

Tab. 2. Regression coefficients of predictive models for the response of total phenolic compounds and analysis of variance.

Source	Coefficients	Sum of squares	Degrees of freedom	Mean of squares	*F*-value	*P*-value
Infusion extraction						
*G**	–16.68	2 225	1	2 225.12	1 104	$< 10^{-4}$
S/*S**	–2.27	41.18	1	41.18	20.45	0.045
*t**	3.29	86	1	86.68	43.03	0.022
G × *S*/*S**	3.79	115	1	115.15	57.17	0.017
Lack of fit		54.91	4	13.73	6.815	0.132
Pure error		4.03	2	2.01	–	
Total		2 527	10		–	
Shaker extraction						
*G**	–18.42	5 430	1	5 430.52	2 438	0.004
S/*S**	–1.92	59	1	59.17	26.565	0.036
*t**	2.71	117	1	117.22	52.633	0.018
*T**	2.87	132	1	132.07	59.296	0.016
G × *S*/*S*	1.42	32	1	32.46	14.572	0.062
G × *T*	–1.35	29	1	29.24	13.127	0.068
Lack of fit		163	10	16.33	7.333	0.013
Pure error		4.45	2	2.22	–	
Total		5 968	18		–	

Coefficient of determination $R^2 = 0.9921$, pure error of the mean of squares is 2.0141.
G – mean granule size, *S*/*S* – proportion of solids in relation to solvent, *t* – extraction time, *T* – extraction temperature, *G* × *S*/*S* – interaction of variables *G* and *S*/*S*, and *G* × *T* – interaction of variables *G* and *T*, * – significant values.

results showed the application potential of fractions that would previously be discarded or reground due to particle size, which can reduce production costs.

Comparison of optimized and conventional methods

The extracts obtained by optimized I and S methods and by Soxhlet extraction were investigated regarding the bioactive compounds content (Tab. 3). All parameters evaluated exhibited a significant difference ($p < 0.05$) between the extraction methods. Extraction by Soxhlet presented the best condition with approximately 1.6-times higher yield than the optimized S and I extraction with equal yields ($p < 0.05$). Only the antioxidant potential values determined by FRAP method were higher for S extraction than for Soxhlet extraction

Tab. 3. Performance parameters of the extraction methods.

	Extraction method		
	Infusion	Shaker	Soxhlet
Yield [%]	83 ± 1^{b}	82 ± 1^{b}	89 ± 0^{a}
Phenolics [g·kg^{-1}]	670 ± 30^{c}	930 ± 50^{b}	$1\,370 \pm 20^{a}$
Flavonoids [g·kg^{-1}]	700 ± 20^{c}	770 ± 60^{b}	$1\,010 \pm 30^{a}$
AA_{ABTS} [μmol·kg^{-1}]	$6\,630 \pm 390^{c}$	$8\,580 \pm 1\,190^{b}$	$10\,910 \pm 1\,390^{a}$
AA_{DPPH} [μmol·kg^{-1}]	$5\,010 \pm 90^{c}$	$7\,320 \pm 50^{b}$	$10\,030 \pm 310^{a}$
AA_{FRAP} [μmol·kg^{-1}]	120 ± 10^{c}	210 ± 10^{a}	162.2 ± 12.8^{b}
Tannins [g·kg^{-1}]	232 ± 8^{c}	252 ± 8^{b}	331 ± 9^{a}
Phytosterols [g·kg^{-1}]	10.0 ± 0.10^{c}	13.5 ± 0.6^{b}	23.2 ± 0.4^{a}

Values of mean ± standard deviation are presented. Different lowercase letters in superscript in the same row indicate significant differences by Tukey's test ($p < 0.05$).
Values are expressed on a dry basis for pinhão seed coat. Content of phenolics is expressed as gallic acid equivalents. Content of flavonoids is expressed as catechin equivalents. Antioxidant activity is expressed as micromoles of Trolox equivalents. Tannins are expressed as condensed tannins. Phytosterols are expressed as β-sitosterol equivalent per unit mass of pinhão seed coat. AA_{ABTS} – antioxidant activity determined by ABTS assay, AA_{DPPH} – antioxidant activity determined by DPPH assay, AA_{FRAP} – antioxidant activity determined by FRAP assay.

($p < 0.05$). Significant findings were observed for S extraction indicating a higher content of bioactive compounds with iron ion-chelating capabilities. Previous studies reported yields of up to 80 % for this specific matrix [18].

The presence of condensable tannins in PSC extract is related to effective inhibitors of pancreatic lipase [4] and α-amylase. These findings are consistent with its anti-obesity potential, as suggested for other polyphenols or tannin-rich preparations [5].

The values of antioxidant potential determined by ABTS, DPPH and FRAP methods in the present study were 41-, 110- and 10-times higher, respectively, than those of the extracts obtained using extraction of the cooked PSC with a hydroalcoholic solution (80 %, v/v) [18]. Those values showed that the cooking process in PSC drastically reduced the antioxidant potential, due to migration of the solvent to the seed [19]. Thus, our results were superior to those obtained with cooked pinhão [3, 6, 18], being a promising new methodology to obtain bioactive compounds.

Phenolics exhibited high values and significant differences between samples ($p < 0.05$; Tab. 3). The enhancement of phenolics content agreed with their high antioxidant activity determined by ABTS and DPPH tests, as well as with levels of flavonoids and tannins. The total phytosterols content of the extracts ranged from 0.97 g·kg^{-1} to 2.32 g·kg^{-1}, which were lower than those obtained for raw PSC (7.11 g·kg^{-1}). The yield for powdered extracts was 8 % for extracts obtained by I, 11 % for S and 17 % for Soxhlet extraction.

The bioactive compounds presented high values in the extracts obtained by Soxhlet, related to the extraction characteristic, employing continuous reflux, facilitating the mass-transfer process. In addition, the extraction time selection reduces costs and avoid degradation of bioactive compounds. Longer times, at elevated temperatures, can cause thermal degradation due to oxidation, decomposition and polymerization reactions of phenolic compounds. Colorimetric tests were performed for the extracts presenting significant different values ($p < 0.05$) and perceptible difference to human eye ($\Delta E > 2$), extracts were negatively correlated with L^* and b^* colour, so with more phenolics extracted, darker ($-L^*$) and blue tendency ($-b^*$) occurs (Tab. 4).

Tab. 4. Colorimetric parameters of the extracts.

	Extraction method		
	Infusion	Shaker	Soxhlet
L^*	35.10 ± 0.02 ^{a}	29.61 ± 0.03 ^{b}	29.26 ± 0.03 ^{c}
a^*	28.42 ± 0.04 ^{c}	33.09 ± 0.01 ^{a}	29.08 ± 0.09 ^{b}
b^*	50.1 ± 0.4 ^{a}	48.7 ± 0.3 ^{b}	37.5 ± 0.4 ^{c}

Different lowercase letters in superscript in the same row indicate statistically significant differences by Tukey's test at $p < 0.05$.
L^* – degree of brightness (0 – black, 100 – white), a^* – degree on green to red colour gradient ($-a^*$ – green colour, $+a^*$ – red colour), b^* – degree on blue to yellow colour gradient ($-b^*$ – blue colour, $+b^*$ – yellow colour).

Toxicity by hatching test using *Artemia salina*

The *Artemia salina* toxicity test, also known as the lethality test, was performed to provide insights into the maximum allowable dosage of extract that can be added to yogurt without adversely affecting the live cultures present in the yogurt.

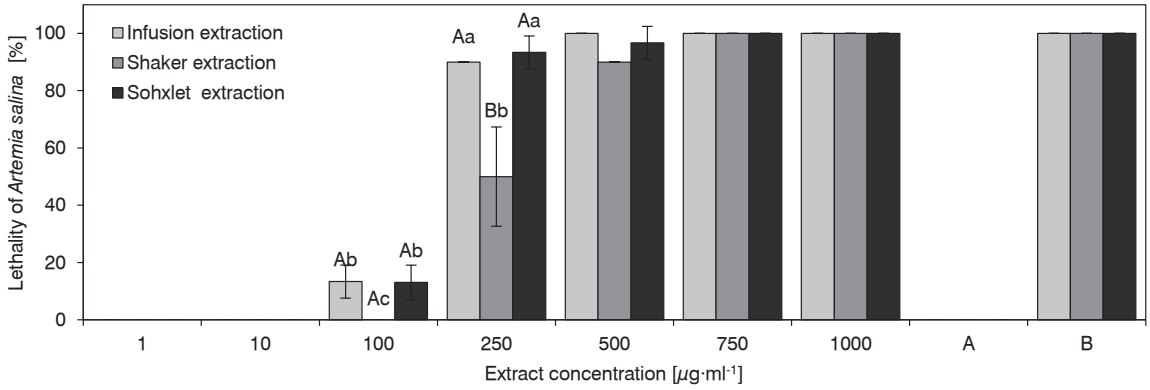

Fig. 3. Toxicity of extracts from pinhão seed coat in a test with *Artemia salina*.

Different capital letters indicate significant differences by Tukey's test ($p < 0.05$) between extracts obtained by various extraction methods. Different lowercase letters indicate significant differences between concentrations within the same method.
A – sea water, B – potassium dichromate (1 mg·ml^{-1}).

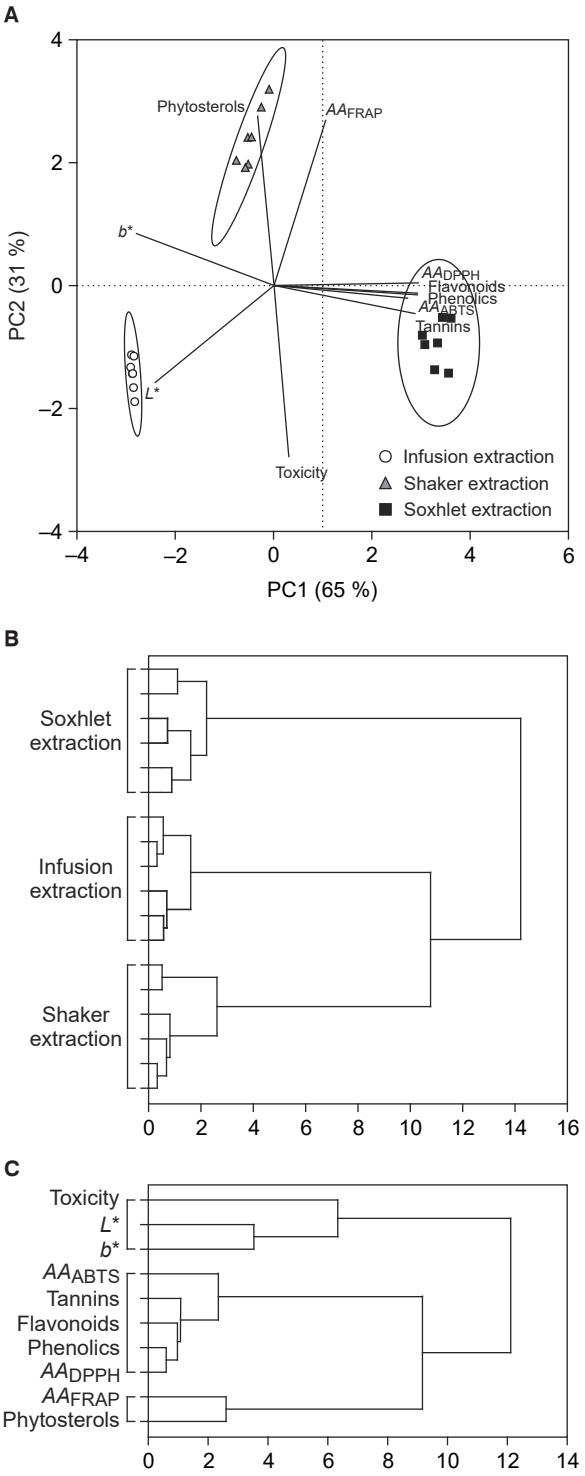

Fig. 4. Principal component analysis and clustering analysis of bioactive compounds extracted from pinhão seed coat.

A – principal component analysis, B – hierarchical clustering on principal components by extraction methods, C – hierarchical clustering on principal components analysis by parameters.
AA_{ABTS} – antioxidant activity determined by ABTS assay, AA_{DPPH} – antioxidant activity determined by DPPH assay, AA_{FRAP} – antioxidant activity determined by FRAP assay.

While *A. salina* is a sensitive species, its suitability as an indicator for the toxicity of plant extracts intended for human consumption has been previously reported [11]. It is worth noting that this test has been utilized in assessing natural products with potential pharmacological activity and establishing concentration limits. However, the interpretation and applicability of these results should be approached with caution and further investigation may be necessary.

The results of the lethality test (Fig. 3) showed that at concentrations up to 10 mg·l^{-1}, the extract caused no deaths of specimens and at 500 mg·l^{-1} the of extract caused 100% lethality. LC_{50} for the extracts ranged from 100 mg·l^{-1} to 250 mg·l^{-1}, indicating medium toxicity. At 250 μg·ml^{-1}, S extracts reached LC_{50}, while I and So were close to 90% lethality, with similar values and different from S ($p < 0.05$).

The best fit obtained by a polynomial equation [11] showed that the LC_{50} ranged from 165 μg·ml^{-1} to 250 μg·ml^{-1}, with coefficient of determination R^2 from 0.93 to 0.95. Based on this, the extracts presented medium toxicity (LC_{50} of 100–500 mg·l^{-1}) [20]. Therefore, in agreement with previous works, the PSC extract can be employed as food but should be used within the safe concentration limits [11].

The toxicity of the extracts significantly correlated ($p < 0.05$) with the phytosterols content and antioxidant activity determined by FRAP assay. Also, it correlated with colour parameter a^*. Spearman's correlation coefficient values ranged between –0.63 and –0.75, indicating that samples with lower phytosterol content, lower antioxidant activity determined by FRAP assay and more reddish ($+a^*$), were less toxic. Furthermore, approximately 56 % of the variation in extract toxicity could be attributed to the extraction temperature. This suggests that the studied extraction methods only partially extracted the relevant compounds.

The results obtained in the present study corroborate those obtained previously, using the same raw material and 25% (v/v) ethanolic extract [11]. Thus, in addition to suggestions for the food application with fish or shrimps, and to pharmaceutical application as adsorbent material for antibiotics and drugs [11], PSC can be used to prepare extracts that can be incorporated into food products for human consumption and provide a significant amount of phenolics and flavonoids, as well as antioxidant activity.

Core components and cluster analysis

PCA (Fig. 4) was used to describe the extraction of bioactive compounds by I, S and Soxhlet ex-

traction methods. The colour parameter a^* was removed from the analysis because it had $P = 0.432$; while the other parameters showed $p > 0.54$. PCA explained 95 % of the total variation in the extraction methods. When evaluating the PCA biplot, together with clustering of variables (Fig. 4A and Fig. 4C), it was verified that the extracts presented characteristics that were distant from each other. The most important parameters for the extract obtained by I extraction were toxicity and colour parameters L^* and b^*. For S extraction, the most important parameters were antioxidant activity determined by FRAP assay and concentration of phytosterols. For Soxhlet extraction, the most important parameters were antioxidant activity determined by ABTS assay, concentrations of tannins, flavonoids and phenolics, as well as antioxidant activity determined by DPPH assay. Fig. 4B shows the presence of two main clusters, while one of them is divided into two smaller ones. The extraction by Soxhlet was the most different and was in a unique cluster, while S and I were close to each other.

Although Soxhlet extraction yielded the highest amount of bioactive compounds [11], this technique requires longer extraction times. The employment of S extraction is advantageous for the industry in terms of energy, time and cost and it is commonly utilised [4]. I extraction requires less energy, since the process after boiling of the solvent takes place at room temperature and without agitation. Moreover, S extraction method could be selected for yogurts because it presented the lowest toxicity with cost-effectiveness.

Effects of extract addition to yogurt

In a preliminary study, addition of 25 mg and 50 mg of PSC extract to 100 g of commercial yogurt caused no change in acidity and pH ($p < 0.05$). In addition, increase in acidity was determined in the standard yogurt over time ($p < 0.05$). The colour of the samples added with the extract showed a perceptible variation ($\Delta E > 2$) as the concentration increased. This result was already expected considering the enhancement of the extract concentration. In addition, content of total phenolics increased with the addition of the extract. However, the addition of 25 mg or 50 mg presented no significant difference ($p < 0.05$).

The results of shelf-life investigation with standard yogurts and the samples added PSC extract obtained by S extraction (250 mg·kg^{-1} of yogurt) showed that the yogurt formulations at the initial time, 0 days, were statistically different by T-test ($p < 0.05$), except for flavonoids ($p > 0.05$; Tab. 5). After 30 days of cold storage, there was a 29% reduction in the flavonoids content of yogurt with the extract ($p < 0.05$ by the paired T-test) and thus content of these compounds also showed a significant difference between the two types of samples ($p < 0.05$ by the T-test). The addition of the extract to yogurt significantly improved the content of bioactive compounds measured by total phenolics, antioxidant activity determined by ABTS, DPPH and FRAP assays in two time periods, after 0 and 30 days ($p < 0.05$), except for the flavonoids content. The addition of the extract an increase by 20–120 % in bioac-

Tab. 5. Shelf life of yogurts without and with addition of pinhão seed coat extract.

Parameter	Standard yogurt		Yogurt with extract	
	0 day	30 days	0 day	30 days
Phenolics [g·kg^{-1}]	12.8 ± 0.8 ^{Ab}	11.4 ± 0.5 ^{Ab}	24 ± 2 ^{Ba}	33.4 ± 0.8 ^{Aa}
Flavonoids [g·kg^{-1}]	322 ± 42 ^{Aa}	352 ± 30 ^{Aa}	382 ± 9 ^{Aa}	272 ± 5 ^{Bb}
AA_{ABTS} [μmol·kg^{-1}]	6 968 ± 85 ^{Bb}	7 710 ± 190 ^{Bb}	8 162 ± 629 ^{Ba}	13 250 ± 200 ^{Aa}
AA_{DPPH} [μmol·kg^{-1}]	762 ± 30 ^{Ab}	760 ± 20 ^{Aa}	1 645 ± 143 ^{Aa}	1 810 ± 180 ^{Ab}
AA_{FRAP} [μmol·kg^{-1}]	0 ± 0 ^{b}	0 ± 0 ^{Aa}	1 132 ± 32 ^{Aa}	0 ± 0 ^{Ba}
L^*	96.38 ± 0.02 ^{Aa}	93.87 ± 0.02 ^{Ba}	93.11 ± 0.01 ^{Ab}	90.88 ± 0.01 ^{Bb}
a^*	–1.29 ± 0.03 ^{Aa}	–1.47 ± 0.02 ^{Ba}	1.15 ± 0.03 ^{Ab}	1.13 ± 0.03 ^{Ab}
b^*	10.88 ± 0.03 ^{Aa}	10.62 ± 0.03 ^{Aa}	9.56 ± 0.03 ^{Bb}	9.63 ± 0.03 ^{Ab}
ΔE	–	2.52 ± 0.02	4.28 ± 0.03	2.23 ± 0.01

Different capital letters in superscript indicate statistically significant differences over time by the paired T-test ($p < 0.05$). Different lowercase letters in superscript indicate statistically significant differences on each day by Tukey's post-hoc t-test ($p < 0.05$).
Values are expressed on a dry basis for pinhão seed coat. Content of phenolics is expressed as gallic acid equivalents. Content of flavonoids is expressed as catechin equivalents. Antioxidant activity is expressed as micromoles of Trolox equivalents.
AA_{ABTS} – antioxidant activity determined by ABTS assay, AA_{DPPH} – antioxidant activity determined by DPPH assay, AA_{FRAP} – antioxidant activity determined by FRAP assay.
L^* – degree of brightness (0 – black, 100 – white), a^* – degree on green to red colour gradient ($-a^*$ – green colour, $+a^*$ – red colour), b^* – degree on blue to yellow colour gradient ($-b^*$ – blue colour, $+b^*$ – yellow colour), ΔE – colour variation.

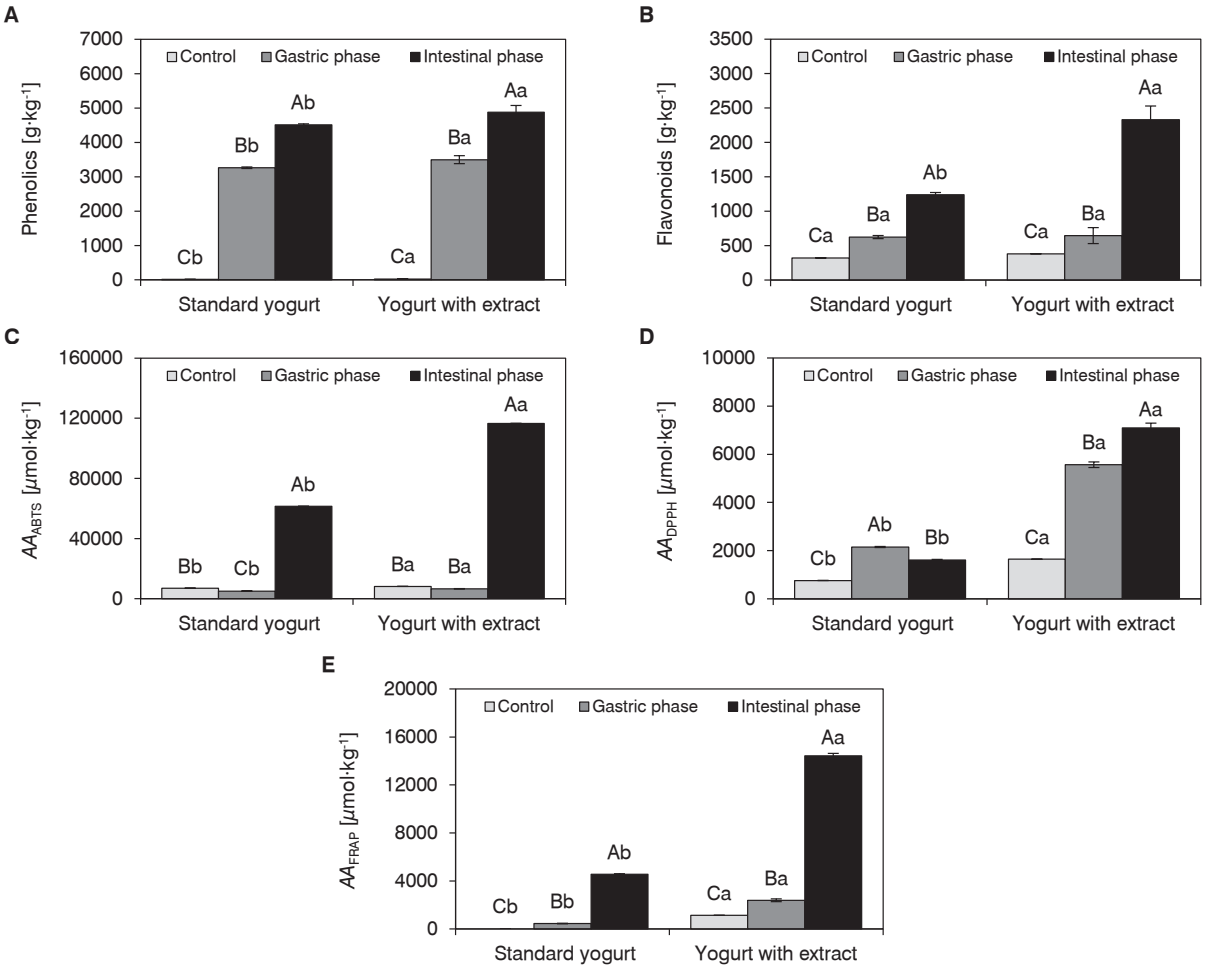

Fig. 5. Bioaccessibility of bioactive compounds from yogurts added with pinhão seed coat extract.

A – phenolics, B – flavonoids, C – antioxidant activity determined by ABTS assay, D – antioxidant activity determined by DPPH assay, E – antioxidant activity determined by FRAP assay,
Different capital letters in superscript indicate statistically significant differences between samples by Tukey's test ($p < 0.05$). Different lowercase letters in superscript indicate statistically significant differences at each digestion stage (control, gastric and intestinal) by *T*-test ($p < 0.05$).
Content of phenolics is expressed as gallic acid equivalents. Content of flavonoids is expressed as catechin equivalents. Antioxidant activity is expressed as micromoles of Trolox equivalents.
AA_{ABTS} – antioxidant activity determined by ABTS assay, AA_{DPPH} – antioxidant activity determined by DPPH assay, AA_{FRAP} – antioxidant activity determined by FRAP assay.

tive compounds content at timepoint 0 and by 70–190 % after 30 days.

The addition of extracts to yogurt made it darker, redder and less yellow. There were significant variations between the formulations ($p < 0.05$) and over time ($p < 0.05$), except for a^* of yogurt with the extract. Compounds that present red colour for the extracts were stable. The variation $\Delta E > 2$ between formulations was expected, but the variation ΔE in yogurt over storage time was approximately 2 and not noticeable by the human eye, which requires $\Delta E > 2$.

Our findings are in line with the potential application of phenolic compounds as fortification agents in functional food production, specifically in fermented dairy products [21]. However, the practical use of these compounds is often restricted due to their undesirable bitter or astringent taste and limited bioavailability [22].

Digestibility of the compounds in yogurts

Digestibility results for the bioactive compounds are shown in Fig. 5 and, in detail, in Tab. 6. The addition of the extract to the yogurt contributed to a significant increase ($p < 0.05$) of bioactive compounds, with mean values of approximately 61 % in control (undigested), around 135 % in the gastric phase and approximately

149 % in the intestinal phase. After digestion, yogurts exhibited high in vitro bioaccessibility of bioactive compounds and high antioxidant activity, being higher in the intestinal phase ($p < 0.05$). The most significant increase was in phenolic compounds, with values after gastrointestinal digestion between 34 600 % and 60 700 % of the control. Flavonoids were between 579 % and 779 %; and the antioxidant activity between 493 % and 1 501 %. The results obtained showed that digestion increased the in vitro bioaccessibility of bioactive compounds and, consequently, increased the antioxidant activity.

The results emphasize the significance of conducting in vitro digestion assays. The undigested sample displayed relatively low levels of bioactive compounds, whereas a substantial increase in digestion was observed in the yogurt enriched with the extract. These findings underscore the importance of simulating the digestion process to assess the enhanced release and availability of bioactive compounds, highlighting their potential impact on overall nutritional quality and health benefits.

Digestibility variations were also reported in previous studies [9]. This can be explained by the physico-chemical changes in the gastrointestinal tract, as temperature, pH and enzymes affect the bioavailability of antioxidant compounds [23]. Furthermore, polyphenols are sensitive to alkaline conditions, where a proportion of the compounds can be transformed into different structural forms with different chemical properties and biological activity [24]. The observed in vitro bioaccessibility of standard yogurt can be attributed to whey proteins, particularly α-lactoalbumin, which exhibits a notable antioxidant capacity. Importantly, the potential of α-lactoalbumin is not only preserved but also enhanced during the digestion process. Additionally, it should be noted that previous studies indicated variations in the digestion models of milk proteins between infants and adults, with factors such as life stage and gut location influencing the digestion process. These factors emphasize the complex interplay between protein digestion and various physiological factors, underscoring the need for further investigation [25].

CONCLUSIONS

The study of the extraction methods showed that the extract obtained by Soxhlet extraction had the highest content of bioactive compounds and antioxidant activity, followed by S and I extractions. The extract obtained by S extraction was incorporated in yogurt, making it reddish and

Tab. 6. Bioaccessibility of yogurts added with pinhão seed coat extract in relation to bioactive compounds and the antioxidant potential.

	Standard yogurt			Yogurt with extract		
Parameters	Control	Gastric phase	Intestinal phase	Control	Gastric phase	Intestinal phase
Phenolics [g·kg^{-1}]	12.8 ± 0.8 ^{Cb}	3 262.0 ± 22.6 ^{Bb}	4 508.3 ± 31.2 ^{Ab}	24.1 ± 1.7 ^{Ca}	3 496.7 ± 116.1 ^{Ba}	4 875.0 ± 199.3 ^{Aa}
Flavonoids [g·kg^{-1}]	322.0 ± 42.2 ^{Ca}	624.5 ± 20.5 ^{Ba}	1 240.1 ± 50.6 ^{Ab}	381.9 ± 9.2 ^{Ca}	646.0 ± 46.8 ^{Ba}	2 329.7 ± 156.1 ^{Aa}
AA_{ABTS} [μmol·kg^{-1}]	6 967.8 ± 85.0 ^{Bb}	5 031.0 ± 535.8 ^{Cb}	61 437.0 ± 363.0 ^{Ab}	8 162.0 ± 629.0 ^{Ba}	6 604.0 ± 4 425.0 ^{Ba}	11 695.5 ± 964.0 ^{Aa}
AA_{DPPH} [μmol·kg^{-1}]	762.0 ± 29.7 ^{Cb}	21510. ± 206.2 ^{Ab}	1 606.7 ± 53.3 ^{Bb}	1 645.0 ± 143.0 ^{Ca}	5 568.1 ± 123.2 ^{Ba}	7 095.5 ± 89.3 ^{Aa}
AA_{FRAP} [μmol·kg^{-1}]	0.0 ± 0.0 ^{Cb}	449.3 ± 35.0 ^{Bb}	4 565.5 ± 513.1 ^{Ab}	1 132.1 ± 32.0 ^{Ca}	2 389.4 ± 88.8 ^{Ba}	14 431.1 ± 381.0 ^{Aa}

Different capital letters in superscript indicate statistically significant differences between samples by Tukey's test ($p < 0.05$). Different lowercase letters in superscript indicate statistically significant differences at each digestion stage (control, gastric and intestinal) by *T*-test ($p < 0.05$).
Content of phenolics is expressed as gallic acid equivalents. Content of flavonoids is expressed as catechin equivalents. Antioxidant activity is expressed as micromoles of Trolox equivalents.
AA_{ABTS} – antioxidant activity determined by ABTS assay, AA_{DPPH} – antioxidant activity determined by DPPH assay, AA_{FRAP} – antioxidant activity determined by FRAP assay.

attractive, increasing the content of bioactive compounds in their shelf life from 20 % to 190 %. Still, yogurts had greater in vitro bioaccessibility than standard yogurt after digestion regarding phenolics (34 600 % and 60 700 %), flavonoids (579 % and 779 %) and antioxidant activity (493 % and 1 501 %). These results are significant for two main reasons. Firstly, the inclusion of phytosterols in the product ensures a safety margin of lethality below 50 % for *A. salina*, indicating its low toxicity. This finding highlights the potential of the product as safe for consumption. Secondly, the presence of phytosterols introduces a novel product to the market, offering unique benefits and appealing to consumers seeking fortified food options. Overall, these results have both safety and market relevance, positioning the product as promising to the industry.

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