SUPPLEMENTARY MATERIAL

Optimization of spray drying conditions to microencapsulate cupuassu (*Theobroma grandiflorum*) seed by-product extract

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ABSTRACT

Cupuassu (*Theobroma grandiflorum* Schum.) is a popular Amazonian fruit because of its intense aroma and nutritional value. Its lipid fraction is alternatively used in cosmetics, but the resulting by-product still contains proteins and nutraceuticals that may be exploited industrially. To preserve active principles and ensure their controlled release, they were microencapsulated by spray drying. Influence of spray-drying conditions on microencapsulation of cupuassu seed by-product extract was investigated according to a 3^3-Box Behnken factorial design, selecting inlet temperature, maltodextrin concentration and feed flowrate as independent variables, and total polyphenol and flavonoid contents, antiradical power, yields of drying and microencapsulation as responses. Fitting the results by second-order equations and modelling by Response Surface Methodology allowed predicting optimum conditions. Epicatechin and glycosylated quercetin were the major microencapsulated flavonoids. Microparticles showed satisfactory antiradical power and stability at 5°C or under simulated gastrointestinal conditions, thus they may be used to formulate new foods or pharmaceuticals.

Keywords

Cupuassu; *Theobroma grandiflorum*; seed by-product; microencapsulation; spray drying; antioxidant activity.

Experimental

Chemicals and reagents

Chemicals used in this study were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fluka Chemika (Milwaukee, WI, USA). All the standards were purchased from Sigma.

Extract preparation

Cupuassu seeds were roasted at 60°C for 45 min and subsequently pressed to remove the oil for industrial use. The remaining material was dehydrated, pulverized and extracted with 70% (v/v) ethanolic solution until complete removal of polyphenols. The by-product was then extracted according to the percolation process (Farmacopeia Brasileira 1959), filtered and concentrated in a rotary evaporator, model 400 (Laborota, Heidolph, Germany), under low pressure and controlled temperature (40 ± 2°C). The crude extract thus obtained was kept under refrigeration (-18°C) and protected from light until analyses.

Microencapsulation of the extract by spray drying

Crude extract was dissolved in 50 mL of 50% (w/v) ethanolic solution until complete homogenization; 100, 150 or 200 g/L maltodextrin (MD) solutions were added to obtain liquid extracts containing 5.0, 7.5 or 10.0% (w/v) MD. These solutions were fed into a Mini Spray Dryer, model B-290 (Büchi, Flawil, Switzerland), with 0.5 mm-nozzle diameter, drying air flow of 32.5 m^3/h and aspirator rate of 80%. The inlet temperature (IT) range was selected according to preliminary experiments, those of MD concentration and feed flowrate (FF) based on the literature available so far (Fazaeli et al. 2012; Paini et al. 2015), while the outlet temperature (OT) was recorded after each run. Cupuassu seed by-product microcapsules (CSWM) were weighed and stored in desiccator in closed dark vessels before analyses.

Experimental design

To optimize the conditions of crude extract microencapsulation, tests were carried out according to a 3^3-Box Behnken factorial design (BBD), while Response Surface Methodology (RSM) was used to check the agreement between experimental results and predicted values, considering linear, quadratic and interaction effects. Three levels (-1, 0, +1) were selected for each independent variable, namely IT (150, 160, 170°C), MD concentration (5.0, 7.5 and 10.0% w/v) and FF (5.0, 7.5, 10.0 mL/min), hence giving a total of 15 experiments, including three replicates of the central point. The experimental data of six responses, namely microencapsulated total polyphenols (TP) content, microencapsulated total flavonoids (TF) content, antiradical power (AP) according to the 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) method, yield of drying, TP microencapsulation yield and TF microencapsulation yield, were fitted by second-order polynomial equations expressed in general form as follows:

$$y_k = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij}\ x_i\ x_j \qquad (1)$$

where, y_k (k = 1, 2, 3, 4, 5 and 6) are the predicted responses, β_0 is the intercept coefficient, β_i are the linear terms, β_{ii} are the quadratic terms and β_{ij} the interaction ones, while x_i and x_j are the actual values of the three independent variables (x_1, x_2 and x_3 for IT, FF and MD concentration, respectively).

Analytical methods

Phenolic compounds were extracted from the core and surface (SP) of cupuassu seed waste microcapsules (CSWM) according to Robert et al. (2010) with adaptations. Briefly, 2.0 mL of a methanol/acetic acid/water solution (50:8:42 v/v/v) were added to 0.4 g of CSWM under vortexing for 1 min, and the suspension was kept in an ultrasonic bath, model UP100H (Hielscher Ultrasonics, Teltow, Germany), for 20 min to make the

disruption of particles easier. After centrifugation for 15 min at 7500 rpm, the supernatant was filtered through membranes with 0.45 µm-pore diameter (Millipore, Bedford, MA, USA). Similarly, in the case of SP, 0.4 g of CSWM was dispersed in 2.0 mL of ethanol and methanol (1:1 v/v) mixture. The mixture was vortexed for 1 min and then filtered as mentioned above.

TP content either of the extract or microparticles was determined by the Folin-Ciocalteu assay as described by Aliakbarian et al. (2011), using a UV-Vis spectrophotometer, model Lambda 25 (Perkin Elmer, Wellesley, MA, USA), at a wavelength of 725 nm. TP content was expressed in milligrams of gallic acid equivalents per gram of dry weight (mg_{GAE}/g_{DW}) or powder (mg_{GAE}/g_{DP}). The calibration curve was made with standard methanolic solutions of gallic acid in the concentration range 6.25–100 mg/mL.

TF content, either of the extract or microparticles, was determined colorimetrically according to Aliakbarian et al. (2011), and expressed in micrograms of catechin equivalents per gram of dry weight (mg_{CE}/g_{DW}) or powder (mg_{CE}/g_{DP}). The absorbance of the mixture was determined at 510 nm using the same spectrophotometer as above. The calibration curve was made with standard methanolic solutions of catechin in the concentration range 0.01–0.50 mg/mL.

AP either of the extract or microcapsules was determined by the ABTS method (Re et al. 1999) with modifications. Aliquots (50 µL) of the CSWM suspension obtained after disruption of particles as described above were diluted and added to 1.0 mL of $ABTS^{\cdot+}$ solution, and the absorbance of samples was read after 2 min of reaction at 734 nm. AP was calculated using a standard 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) curve (expressed in µg/L). Results were expressed in mg of Trolox equivalent per 100 g of dry weight ($mg_{TEAC}/100g_{DW}$) or powder ($mg_{TEAC}/100g_{DP}$).

Drying yield

Drying yield (Y_D) was the ratio of the powder weight obtained by spray drying to the initial weight of all components before drying and expressed as a percentage.

Microencapsulation yields and efficiencies

Microencapsulation yield was calculated as the ratio of bioactive compound (TP or TF) content of microcapsules to that of the extract and expressed as a percentage, according to the equation:

$$Y_{\text{TP (or TF)}} = \frac{\text{TP (or TF) content of CSWM}}{\text{TP (or TF) content of the extract}} \text{ x } 100 \qquad (2)$$

Surface percentage of bioactive compounds (TP or TF) was expressed as percentage of its content on CSWM surface in relation to the total one:

$$SP_{\text{TP (or TF)}} = \left(\frac{\text{TP (or TF) content on CSWM surface}}{\text{total TP (or TF) content of CSWM}}\right) \text{x } 100 \qquad (3)$$

Microencapsulation efficiency (ME) of bioactive compound (TP or TF), which is useful to evaluate its degradation during spray drying, was calculated as:

$$ME_{\text{TP (or TF)}} = 100 - SP_{\text{TP (or TF)}} \quad (4)$$

and expressed as a percentage.

Moisture content, Zeta potential and microstructure of particles

CSWM were dried at 105°C until constant weight, and moisture content was determined based on the weight loss between before and after drying (AOAC 2005).

Polydispersity index, particle size and Zeta potential were determined by means of a Zetasizer equipment, model NanoZS (Malvern Instruments, Malvern, UK), using DTS 1070 cuvettes. For this purpose, 100 mg of each sample were solubilized in 5.0 mL of ultrapure water and diluted up to 1:100 (v/v).

CSWM microstructure was observed by a scanning electron microscope, model SEM 515 (Philips, Eindhoven, The Netherlands). Small amounts of powders were coated with a 30 nm-thick gold layer and observed in secondary electrons (5.0 kV) at 500 or 1000 magnification. Diameters of particles were measured on SEM images using the Sigma Scan Pro 5 software (Systat Software Inc., San Jose, CA, USA).

Water solubility index, water absorption index and swelling capacity

Water solubility (*WSI*) and water absorption (*WAI*) indexes were determined, according to Ahmed et al. (2010) with adaptations, on 0.5 g aliquots of CSWM suspended in 6.0 mL of water. They were calculated according to the equations:

$$WSI = (DW_{sup}/DW_{part}) \times 100 \quad (5)$$

$$WAI = PW/DW_{part} \quad (6)$$

where DW_{sup} is the dry weight of supernatant, DW_{part} the initial dry weight of microparticles and PW the weight of pellet after centrifugation.

Swelling capacity (*SC*) was calculated by the equation (Lai & Cheng 2004):

$$SC = \frac{PW}{DW_{\text{part}}\,(100-WSI)} \quad (7)$$

Quantification of phenolic compounds

The main polyphenols contained in crude extract and microcapsules obtained under predicted optimal conditions were quantified by HPLC. For this purpose, solutions of standard compounds were prepared at a concentration of 0.5 mg/mL, except for glycosylated quercetin (0.1 mg/mL). Before the analyses, solutions of standard compounds were diluted (1:2) with methanol, while the microcapsule samples were filtered through membranes with 0.22 μm-pore diameter and injected directly into the system. Analyses were performed using a HPLC system, model 1100 (Agilent, Palo Alto, CA, USA), coupled with a diode array detector (DAD), model 1260 Infinity (Agilent), and equipped with a C18 reverse phase Eclipse plus column (4.5 x 250 mm) packed with 5.0 μm-diameter particles (Agilent). Samples (20 μL) were analysed at a constant flowrate of 1.0 mL/min and column temperature of 30°C. Mobile phase was a gradient system, with mobile phase A (methanol/acetonitrile,

1:1) and B (1% acetic acid in water), varying the latter for 0-5 min to 100%, 10 min to 95%, 30 min to 70%, 40 min to 60%, 45 min to 52%, 55 min to 30%, 60-65 min to 0%. Chromatographic peaks of analytes were detected at 280 nm for all phenolics and 369 nm only for quercetin. Then they were confirmed by comparing their retention times and UV spectra with those of reference standards. Quantification was carried out by integration of peaks using the external standard method.

Resistance of CSWM antiradical power to in vitro simulated gastrointestinal conditions

CSWM produced under predicted optimal conditions were submitted to simulation of gastrointestinal environment with the purpose of evaluating the chemical resistance of their AP. Thus, 10 millilitres of each dilution of 0.1 g/mL of CSWM in NaCl 0.5% (w/v) were transferred to three sterile vials, and the pH adjusted to 2.1 with 1.0 M HCl. Pepsin and lipase were added to the solutions up to concentrations of 3.0 and 0.9 mg/L, respectively. Flasks were incubated for 2 h at 37°C at 150 rpm in vortex (Heraeus Electro-Nite, Organo, MB, Italy), simulating the gastric phase (SGP). In the next step, the pH of samples was raised to 4.5 using an alkaline solution (150 mL of 1.0 M NaOH and 14 g of $NaH_2PO_4.2H_2O$) and distilled water up to 1.0 L. Bile and pancreatin were then added up to concentrations of 10.0 and 1.0 g/L, respectively. Samples were incubated at 37°C for 2 h under stirring, leading to the simulated enteric phase 1 (SEP1). In the last step, pH was raised to 6.9 using the same alkaline solution. Bile and pancreatin were adjusted to maintain their concentrations at 10.0 and 1.0 g/L, respectively, and the samples were incubated again at under the same conditions, thus leading to simulated enteric phase 2 (SEP2) and reaching 6 h of assay (Bedani et al. 2013). CSWM, maintained under conditions simulating the gastrointestinal ones, were analysed in aliquots collected from triplicate samples after 2, 4 and 6 h. For each aliquot referring to simulations of SGP, SEP1 and SEP2, TP content and AP were determined as early described.

Stability tests

CSWM produced under predicted optimal conditions were stored at three different temperatures (5, 25 and 45°C) in the absence of light for 120 days. During storage, microcapsules were kept in sealed Falcon tubes under dry conditions to avoid any effect of moisture on TP stability. TP content and AP of samples were determined at different storage times.

Statistical analysis

To minimize the experimental error, each run was performed in triplicate, while the central point in three triplicate runs (13, 14 and 15). Analysis of the second-order models was based on the most significant terms ($p < 0.05$ for *t*-test), and reparametrized models were evaluated for their fitness by analysis of variance (ANOVA) to verify their suitability and reliability in prediction. The "Design Expert" software trial version 7.1 (Stat-Ease, Minneapolis, MN, USA) was employed for the regression analysis and graphical optimization, while the adequacy of models was based on the coefficient of determination (R^2). The model of each response was expressed in terms of actual variables, and non-significant effects ($p > 0.05$) were maintained to preserve model hierarchy. The means of all other results were analysed by Tukey's post hoc test. For numerical optimization, whereas all independent variables were selected within their respective set ranges, the same weight was attributed to responses.

References

Ahmed M, Akter MS, Lee -JC, Eun J-B. 2010. Encapsulation by spray drying of bioactive components, physicochemical and morphological properties from purple sweet potato. LWT-Food Sci Technol. 43:1307-1312.

Aliakbarian B, Casazza AA, Perego P. 2011. Valorization of olive oil solid waste using high pressure-high temperature reactor. Food Chem. 128:704–710.

AOAC. 2005. Official methods of analysis. Washington: Association of Official Analytical Chemists.

Bedani R, Rossi EA, Saad SMI. 2013. Impact of inulin and okara on *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* Bb-12 viability in a fermented soy product and probiotic survival under in vitro simulated gastrointestinal conditions. Food Microbiol. 34:382–389.

Farmacopeia Brasileira. 1959. volume 2. Brasilia, Brazil: Agência Nacional de Vigilância Sanitária.

Fazaeli M, Emam-Djomeh Z, Ashtari AK, Omid M. 2012. Effect of spray drying conditions and feed composition on the physical properties of black mulberry juice powder. Food Bioprod Process. 90:667–675.

Lai H-M, Cheng H-H. 2004. Properties of pregelatinized rice flour made by hot air or gum puffing. Int J Food Sci Technol. 39:201-212.

Paini M, Aliakbarian B, Casazza AA, Lagazzo A, Botter R, Perego P. 2015. Microencapsulation of phenolic compounds from olive pomace using spray drying: a study of operative parameters. LWT-Food Sci Technol. 62:177–186.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med. 26:1231–1237.

Robert P, Gorena T, Romero N, Sepulveda E, Chavez J, Saenz C. 2010. Encapsulation of polyphenols and anthocyanins from pomegranate (*Punica granatum*) by spray drying. Int J Food Sci Technol. 45:1386–1394.

Table S1. Regression coefficients of second-order models (equation 1) describing the influence of inlet temperature (x_1, °C), extract flowrate (x_2, mL/min) and maltodextrin concentration (x_3, %) on total polyphenols (TP, y_1) and flavonoids (TF, y_2) contents of cupuassu seed waste microcapsules, their antiradical power according the ABTS method (AP, y_3), yield of drying (Y_D, y_4), TP (Y_{TP}, y_5) and TF (Y_{TF}, y_6) microencapsulation yields.

Regression coefficient	y_1 (mg_{GAE}/g_{DP})	y_2 (mg_{CE}/g_{DP})	y_3 ($mg_{TEAC}/100g_{DP}$)	y_4 (%)	y_5 (%)	y_6 (%)
β_0	-415.1012[b]	-78.8990[b]	-519.7804	103.0946[b]	-959.9732	-179.8458[c]
β_1	4.6978	0.7372	8.5539	-1.1950	9.2699	-0.9382
β_2	11.7842	4.5872[c]	-10.1582	0.6565	40.57883	42.6526[b]
β_3	5.9463[a]	3.3625[a]	-19.5621[c]	-0.4160[a]	32.6370	37.3221
β_{12}	-0.0586	-0.0267	0.1028	-0.0078	-0.2142	-0.2425
β_{13}	-0.1096[c]	-0.0430	0.0466	0.0070	-0.3274[c]	-0.2953[c]
β_{23}	0.1597	0.2052[c]	-0.7112	-0.0624	0.1324	0.8341
β_{11}	-0.0106	-4.9×10^{-4}	-0.0307	0.0038	-0.0157	0.0169
β_{22}	-0.2486	-0.1553	-0.0908	0.0605	-0.5369	-0.9198
β_{33}	0.5146[b]	0.0705	1.0006[c]	0.0625	1.1561[c]	0.2209

[a] significant at 0.1% ($p < 0.001$); [b]significant at 1% ($p < 0.01$); [c] significant at 5% ($p < 0.05$).

Table S2. Results of ANOVA applied to the regression models (equation 1) of Table S1.

	DF[a]	Sum of squares					
		TP content[b] (mg_{GAE}/g_{DP})	TF content[c] (mg_{CE}/g_{DP})	AP[d] ($mg_{TEAC}/100g_{DP}$)	Y_D[e] (%)	Y_{TP}[f] (%)	Y_{TF}[g] (%)
Model	9	457.77	73.98	632.30	72.53	801.26	1473.97
Lack of fit	3	5.89	2.12	69.06	0.92	74.10	76.51
Pure error	2	5.54	1.94	27.15	1.07	41.28	57.25
R^2		0.9756	0.9479	0.8679	0.9733	0.8741	0.9168

[a] DF = degree of freedom; [b] TP content = total polyphenols content of cupuassu seed waste microcapsules; [c] TF content = total flavonoids content of cupuassu seed waste microcapsules; [d] AP = antiradical power according to the ABTS method; [e] Y_D = yield of drying; [f] Y_{TP} = TP microencapsulation yield; [g] Y_{TF} = TF microencapsulation yield.

Table S3. Main phenolic compounds identified and quantified by HPLC in cupuassu seeds by-product crude extract and microcapsules obtained by spray drying using maltodextrin as coating agent.

		Crude extract		CSWM	
Compound	λ(nm)	Rt (min)	Concentration ($mg/100g_{DW}$)	Rt (min)	Concentration ($mg/100g_{DP}$)
Gallic acid	280	8.8	5.68	8.2	2.21
Quercetin	369	40.78	5.79	n.i.	n.d.
Glycosylated quercetin	280	35.6	28.01	35.8	39.83
Protocatechuic acid	280	17.32	33.36	17.2	15.49
p-Coumaric acid	280	29.5	1.26	29.4	2.21
Epicatechin	280	25.9	20.74	25.6	39.83
Epigallocatechin gallate	280	25.45	6.81	25.2	8.85

CSWM = cupuassu seed by-product microparticles; Rt = retention time; DW = dry mass basis; n.i. = not identified; n.d. = not determined.

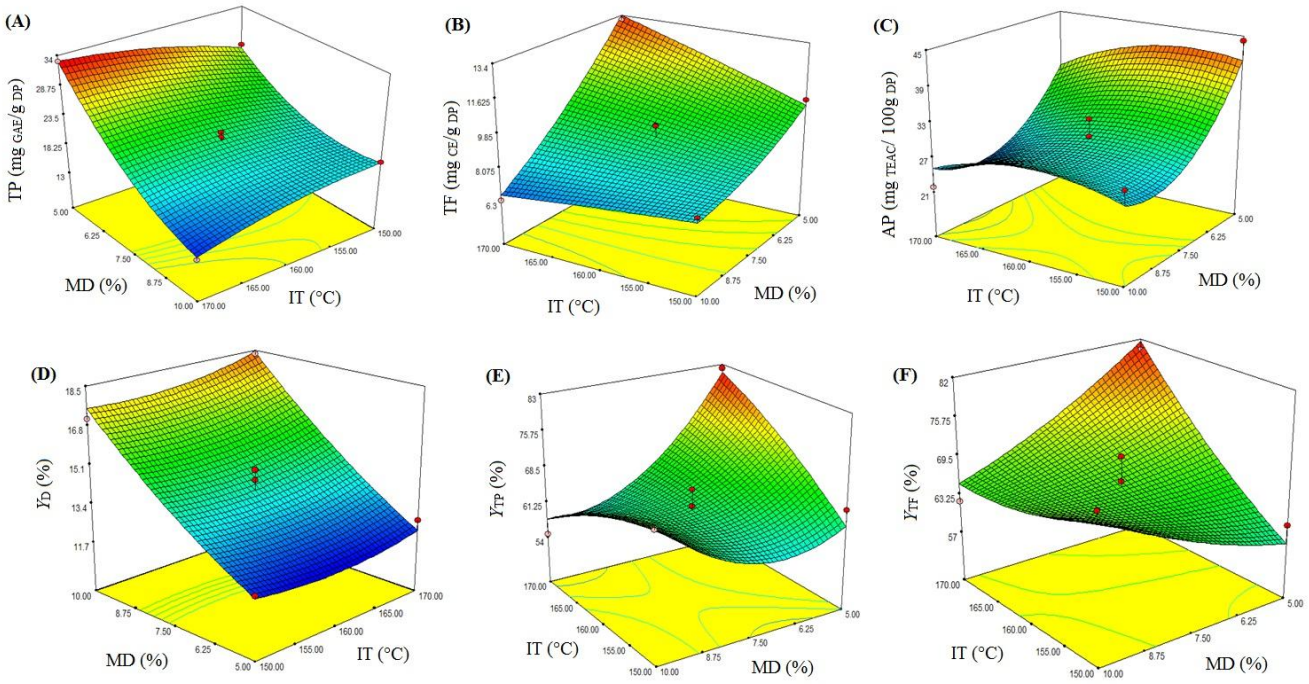

Figure S1. 3D Response Surfaces of microencapsulated cupuassu seed by-product extract: (A) total polyphenols (TP) content; (B) total flavonoids (TF) content; (C) antiradical power (AP); (D) yield of drying (Y_D); (E) total polyphenols microencapsulation yield (Y_{TP}) and (F) total flavonoids microencapsulation yield (Y_{TF}). IT = inlet temperature, MD = maltodextrin concentration.