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Formulation and characterization of an optimized functional beverage from hibiscus (*Hibiscus sabdariffa* L.) and green tea (*Camellia sinensis* L.)

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Abstract

Hibiscus sabdariffa and *Camellia sinensis* are traditionally consumed as beverages and are good sources of health-promoting phenolic compounds. The objective of this work was to use response surface methodology to develop an optimized functional beverage with high total phenolic content, antioxidant capacity, and acceptable for potential consumers. Optimum infusion conditions were 4.9 g of hibiscus calyces or *C. sinensis* leaves/100 ml of water at 26 °C for 291 min. These conditions yielded a total phenolic content of 14.80 ± 1.4 and 33.02 ± 0.34 mg gallic acid equivalents/100 ml for hibiscus and green tea, respectively. The optimized beverages were combined in a 7:3 (hibiscus:green tea, v/v) ratio; a consumer preference test showed that this combination had an acceptable taste according to untrained panelists. A chromatographic analysis showed that this formulation contained flavonoids, phenolic acids, and anthocyanins as its main components. Our data suggested that hibiscus and green tea phenolic compounds were efficiently extracted using near-ambient temperature water for prolonged times, contrary to routine methods (high temperature, short time). Our method also preserved antioxidant capacity, possibly by avoiding chemical changes/degradation due to high temperatures. This process can be used to produce organoleptically acceptable functional beverages that deliver a varied phenolic compound profile to the consumer.

Keywords

Beverages, food process modeling, bioactive compounds, sensory analysis

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INTRODUCTION

As consumers have become increasingly aware that their dietary habits exert important effects on their health, the demand for products that can preserve it has increased, for example the high prevalence of chronic noncommunicable diseases correlates with the intake of sugar-rich foods and drinks, such as soft drinks (Rivera et al., 2008). Because of this, the American Diabetes Association has issued recommendations against consuming them in large amounts, suggesting that healthier options be consumed instead, such as water or herbal (Bleich and Wang, 2011) infusions. Functional beverages (FBs) are thus currently

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being developed as a consequence of these recommendations and to satisfy the in-market demand for them. A functional product can be found in its natural state, or it can be processed by various technologies to improve one or more of its characteristics in order to become health promoting, such as increased phytochemical content or bioactivities related to them (Corbo et al., 2014). FBs can be made from one or more vegetable ingredients, which results in a product with high bioactive concentration, such as phenolic compounds (PCs). Positive effects of PCs have been documented during digestion throughout the length of the human gastrointestinal tract and on various systems, organs, and tissues, suggesting that their presence on an edible product is highly valued (Del Corno et al., 2016; Dominguez-Avila et al., 2017; Lao et al., 2017; Santangelo et al., 2016; Zhang and Tsao, 2016; Zulueta et al., 2013). In addition to their health effects, consumers also prefer items that have good organoleptic qualities, which incentivize continued research to improve or develop new products that satisfy the palate of established consumers, while also drawing new ones in. In the case of vegetable-based FBs, the same bioactive compounds that exert health benefits can also produce unappealing flavors if present at high concentrations, which make it necessary to strike a balance between bioactivity and organoleptic qualities.

Hibiscus (*Hibiscus sabdariffa* L.) calyces and *Camellia sinensis* L. leaves have been traditionally used to prepare beverages (hibiscus infusions and green tea, respectively). Both contain PCs that confer the health benefits and organoleptic qualities that distinguish each of them. Hibiscus contains anthocyanins, such as cyanidin, cyanidin-3-sambubioside, delphinidin, delphinidin-3-sambubioside, and malvidin (Da-Costa-Rocha et al., 2014; De Moura et al., 2018). Biological effects of hibiscus consumption include an improved antioxidant status, decreased blood pressure, anticancer effects, etc. (Frank et al., 2012; McKay et al., 2010; Wu et al., 2016). Green tea contains phenolic acids (i.e. gallic acid) and flavonoids (i.e. catechins) that are responsible for various health effects, for example protection against cardiovascular disease and some cancers (Bravi et al., 2017; Oyama et al., 2017; Sampath et al., 2017). Reports that document the bioactivity of green tea compounds are abundant due to its worldwide popularity, while those that focus on hibiscus are less numerous since its consumption is not as widespread. Therefore, an FB prepared from both of them could be a source of PCs with varied chemical structures that exert complementary or synergistic bioactivities (Hidalgo et al., 2010), while also having good sensory characteristics that appeal to a broad number of consumers in different regions of the world.

Hibiscus infusions or green tea are generally prepared by soaking hibiscus calyces or *C. sinensis* leaves in hot water for approximately 5 min and filtering. This procedure is done empirically; however, it is important to consider standardizing every variable possible when preparing functional products, in order to maximize PC extraction and AC. Response surface methodology (RSM) can be used to determine the effects of the different variables that have an impact on the final product. For example, Wong et al. (2003) performed an optimization with the aid of RSM to improve anthocyanin and ascorbic acid extraction from hibiscus, evaluating extraction time (30–300 min) and water temperature (30–90 °C). Others have used RSM to standardize the production of different beverages such as blackberry juice, carrot juice, and coffee (Ahn et al., 2017; Cervantes-Elizarraras et al., 2017; Ferrario et al., 2017).

When preparing an infusion, leaves-to-water ratio, water temperature and infusion time are critical variables that, if carefully optimized, will produce an FB of consistently high quality and organoleptic characteristics. As previously stated, PCs are the main bioactives, thus, a high PC concentration would be desirable. But PCs can also alter sensory attributes, imparting bitter or astringent tastes that not all consumers prefer. Some authors report that beverages with high phenolic content often have a bitter taste (Bechoff et al., 2014; Jaeger et al., 2009). Narukawa et al. (2010) report that a high catechin concentration is responsible for unpleasant tastes in some beverages, especially those containing green tea. In addition, it has also been reported that acid, bitter, or astringent tastes are mostly tolerated by men (usually over 30 years old), as compared to women (Monteiro et al., 2017). Using water at higher temperatures and/or a longer infusion time increases the rate and amount of PCs that can be extracted; nevertheless, PCs are also thermo-sensitive, and their AC and bioactivities may be compromised if their chemical structure degrades during extraction. Our objective was to develop an FB based on hibiscus and green tea with high PC content, high AC, and acceptable sensory characteristics that would increase the likelihood of regular consumption.

MATERIALS AND METHODS

Plant material and reagents

Dry calyces of *H. sabdariffa* cv. Criolla and dry leaves of *C. sinensis* cv. Sencha were obtained from a local supermarket in Hermosillo, Mexico. All reagents and analytical standards for the identification and quantification of PCs and anthocyanins ($\geq$ 95%, HPLC grade) were purchased from Sigma-Aldrich (St Louis, MO, USA). Solvents were HPLC grade (JT Baker, Mexico City).

Individual beverage preparation

Individual hibiscus and green tea beverages were prepared with different calyces or leaves-to-water ratios (X_1), water temperatures (X_2), and infusion times (X_3). Dry hibiscus calyces and dry *C. sinensis* leaves were weighted in an analytical balance (AE160, Mettler Toledo, Inc.) and placed in glass containers that had 100 ml of purified water at the desired temperature to obtain different calyces- or leaves-to-water ratios (g/100 ml). Figure 1 depicts the experimental process.

Total phenolic content (TPC)

Folin–Ciocalteu colorimetric assay was used to determine TPC (Singleton and Rossi, 1965). Results are expressed as mg gallic acid equivalents (GAEs)/100 ml.

Identification and quantification of individual PCs and anthocyanins

PCs were chromatographically identified and quantified on the combined FB. A 10 ml aliquot of the samples was added to 7 ml of a solution of 90% methanol and 10% butylated hydroxytoluene (2 g/l)/acetic acid (85%) solution. The mixture was sonicated for 30 min and centrifuged (10,000 *g*, 15 min). Free PCs were analyzed in the supernatant.

In order to identify and quantify chemically bonded PCs, alkaline and acid hydrolyses were sequentially performed on the pellet obtained after the previous centrifugation step, as previously reported (Mattila and Kumpulainen, 2002).

Extracted PCs were analyzed in an ultra-performance liquid chromatography (UPLC) system with diode array detector (DAD) (ACQUITY, Waters Corp.) as previously reported (Velderrain-Rodríguez et al., 2018). PCs were separated in a BEH C18 column (3.0 mm × 100 mm, 5 µm, Waters.), at 60 °C. Mobile phases were 0.5% formic acid (A) and methanol (B), as follows: 80% A 20% B, 0–5 min; 55% A 45% B, 5–12 min; 0% A 100% B, 12–25 min; 60% A 40% B, 25–26 min; and 80% A 20% B, 26–30 min. Flow rate was 0.4 ml/min, and a volume of 1 µl was injected. Eluted compounds were identified by comparing their retention times and absorption spectra with those of commercial standards, and

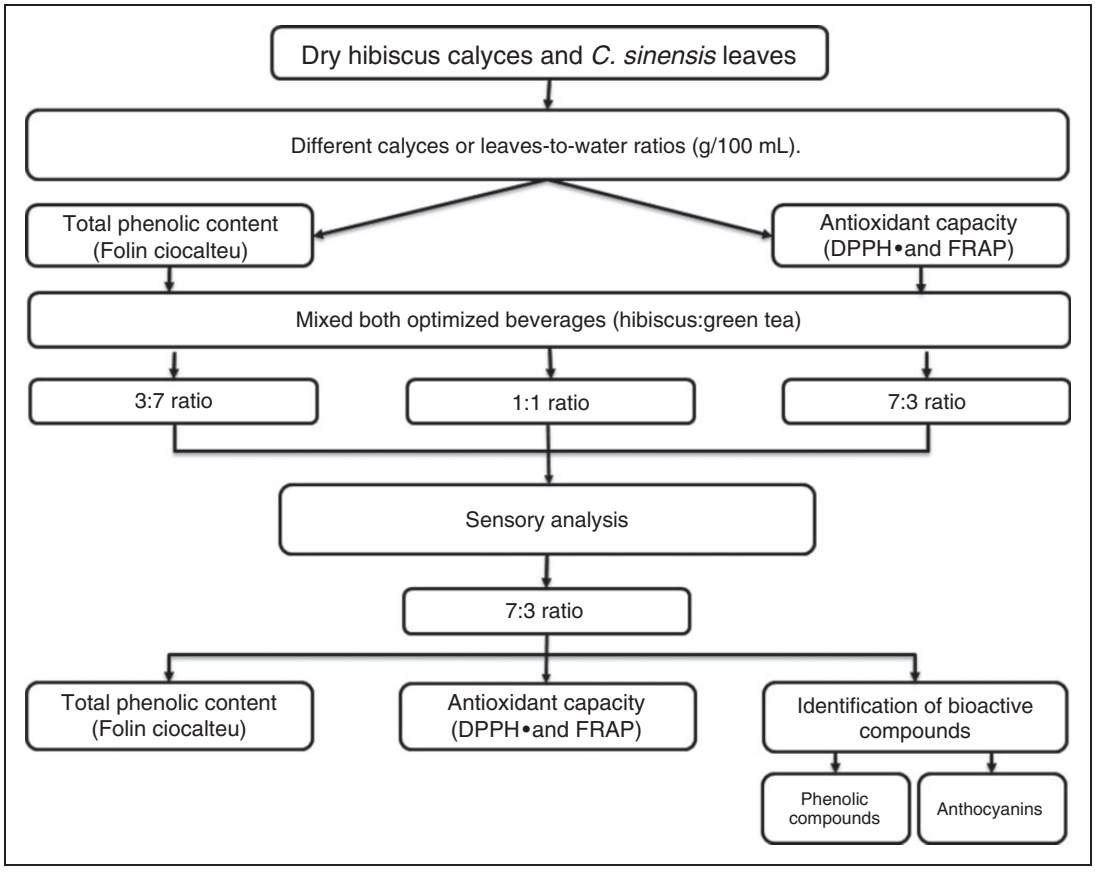

Figure 1. General diagram of the methodology used. DPPH•: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing antioxidant power.

were quantified using standard curves prepared from the same standards.

Anthocyanin analysis was performed as described by He et al. (2016), with some modifications. UPLC-DAD was used as follows: column was kept at 35 °C, run time was 8 min and mobile phases were 2.0% formic acid (A) and acetonitrile (B), using the following gradient: 98% A 2% B, 0–1 min; 84% A 16% B, 1–3 min; 80% A 20% B, 3–5 min; 88% A 12% B, 5–6 min; and 98% A 2% B, 6–8 min.

Antioxidant capacity (AC)

Two different methods were used to determine AC, 2,2-diphenyl-1-picrylhydrazyl (DPPH•) assay and ferric reducing antioxidant power (FRAP) assay. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as standard, and results are expressed as mg Trolox equivalents (TEs)/100 ml.

DPPH• assay was performed according to Quirós-Sauceda et al. (2014) and FRAP assay was performed as described by Benzie and Strain (1999).

Experimental design and statistical analysis

A central composite design was used to optimize calyces or leaves-to-water ratio (X_1: 1–20 g/100 ml), water temperature (X_2: 7–100 °C), and infusion time (X_3: 1–1200 min) of hibiscus and green tea beverages. Coded and uncoded levels of the independent variables

Table 1. Coded and levels of the central composite design used to optimize functional hibiscus and green tea beverages

	Factor levels				
Independent factors	−1.682	−1	0	1	1.682
X_1: leaves-to-water ratio (g/100 ml)	1	4.9	10.5	16.2	20
X_2: temperature (°C)	7	26	53.5	82	100
X_3: time (min)	60	291	630	969	1200

Table 2. Runs of the central composite design (CCD) used to optimize functional hibiscus and green tea beverages

Run	Coded levels	X_1: leaves-to-water ratio (g/100 ml)	X_2: temperature (°C)	X_3: time (min)
1	000	10.5	53.5	630
2	+−+	16.2	26	969
3	A00	20	53.5	630
4	000	10.5	53.5	630
5	000	10.5	53.5	630
6	−−+	4.9	26	969
7	0a0	10.5	7	630
8	−+−	4.9	82	291
9	−−−	4.9	26	291
10	000	10.5	53.5	630
11	+−−	16.2	26	291
12	A00	1	53.5	630
13	0A0	10.5	100	630
14	000	10.5	53.5	630
15	+++	16.2	82	969
16	++−	16.2	82	291
17	−++	4.9	82	969
18	000	10.5	53.5	630
19	00A	10.5	53.5	1200
20	00a	10.5	53.5	60

Factorial (+,−), axial (A, a), and central (0) runs.

are given in Table 1. The range of each variable was selected based on previous work by Alarcon-Aguilar et al. (2007), Mozaffari-Khosravi et al. (2009), and Sabzghabaee et al. (2013). Table 2 shows experimental design used and 20 experimental runs performed. Three response variables were evaluated, TPC (Y_1), DPPH• (Y_2), and FRAP (Y_3). Data were fitted into a second-order polynomial equation.

Statistical significance was evaluated by an analysis of variance, confirmed by an *F*-test and results were considered significant when $p<0.05$. Coefficient of determination (R^2) was also evaluated to determine the suitability of the model. Graphical and numerical optimizations were used to find the optimum levels of independent variables. Additional confirmation experiments were subsequently conducted to verify the optimal conditions. Finally, a hypothesis testing (Student's *t*-test) was carried out on the JMP™ software package (SAS Institute Inc.) to validate our mathematical model.

Formulation and consumer preference test of the combined FB

Once hibiscus and green tea beverages were individually optimized, they were combined to produce a hibiscus–green tea FB. Three formulations were prepared by mixing both optimized beverages in a 1:1 ratio (v/v), and two additional mixtures in a 3:7 and 7:3 (hibiscus:green tea, v/v) ratio. Sugar was added to all beverages (5 g/l). A consumer preference test was performed with untrained panelists (n=110). The participants in this test were men (44%) and women (56%) between 22 and 45 years old; they were graduate students and laboratory technicians from the Research Center for Food and Development (in Northern Mexico), and all were habitual consumers of teas and infusions. In this test, the panelists order a series of samples according to personal appreciation or preference (Pedrero et al., 1989). The preference test was performed in two stages: in the first stage, participants were asked to rank all

Table 3. Experimental values of the optimization of a functional hibiscus beverage using a central composite design (CCD)

	Coded variables			Experimental values		
Experimental run	X_1	X_2	X_3	TPC (mg GAE/100 ml)	DPPH• (mg TE/100 ml)	FRAP (mg TE/100 ml)
1	−1	−1	−1	14.80	25.95	61.91
2	1	−1	−1	10.23	19.81	50.58
3	−1	1	−1	21.47	11.37	68.76
4	1	1	−1	13.77	0.52	15.14
5	−1	−1	1	16.08	28.03	105.80
6	1	−1	1	11.66	4.50	37.46
7	−1	1	1	12.48	7.83	15.74
8	1	1	1	12.36	1.62	35.91
9	−1.682	0	0	5.48	0.18	12.62
10	1.682	0	0	11.21	8.93	20.69
11	0	−1.682	0	14.15	2.94	2.98
12	0	1.682	0	16.43	5.76	72.20
13	0	0	−1.682	11.94	18.88	53.46
14	0	0	1.682	15.51	18.19	51.35
15	0	0	0	8.73	10.68	36.42
16	0	0	0	12.10	9.34	23.79
17	0	0	0	13.19	5.55	20.96
18	0	0	0	11.08	5.68	22.34
19	0	0	0	15.58	7.25	15.62
20	0	0	0	12.18	22.37	50.52

DPPH•: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing antioxidant power; GAE: gallic acid equivalent; TE: Trolox equivalent; TPC: total phenolic content.

X_1: leaves-to-water ratio (g/100 ml), X_2: temperature (°C), X_3: time (min). Values are the average of three replicates.

three FBs in a scale of 1–3, from least to most liked, respectively. After results were analyzed, the least liked sample was discarded, and the test was repeated with the same participants choosing from the two most liked samples, giving them a score of either 1 (preferred less) or 2 (preferred more). A rank ordering test was performed on the data.

RESULTS AND DISCUSSION

Hibiscus beverage

Table 3 shows TPC and AC of 20 experimental runs that were used to optimize the hibiscus FB. TPC values ranged from 5.48 to 21.47 mg GAE/100 ml; highest value was found under the experimental conditions of $X_1 = 4.9$ g/100 ml, $X_2 = 82\,^{\circ}$C, and $X_3 = 291$ min. Most papers that analyze hibiscus calyces report values ranging from 20 to 64 mg GAE/ml using different solvents, while purified water was used in the present work. For example, Sirag et al. (2014) obtained 41.07 mg GAE/ml from Sudanese hibiscus using ethanol as solvent. Extractions with acidified methanol have been reported to yield 20.6 mg GAE/ml, from hibiscus cv. Criolla from Mexico (Sáyago-Ayerdi Sonia et al., 2013). In the case of AC, DPPH• values were higher than those of FRAP, and a wider range was found in the latter. Results ranged from 0.18 to 28.03 mg TE/100 ml and 2.98–105.80 mg TE/100 ml for DPPH• and FRAP, respectively. Maximum response was found when experimental conditions were $X_1 = 4.9$ g/100 ml, $X_2 = 26\,^{\circ}$C, and $X_3 = 969$ min for both assays. These data suggest that near-ambient temperatures and prolonged times (approximately 16 h) result in a high AC, even if PCs are extracted using high temperatures for shorter periods of time. Infusions are usually prepared in short periods of time, using water at a temperature of approximately 80 °C, but because of thermal sensitivity of the anthocyanins, these conditions may induce some changes to their molecular structure, which results in low AC values. It has been reported that higher temperature does not affect anthocyanin extraction (Cisse et al., 2012a); however, high temperatures may induce chalcone formation. Cisse et al. (2012b) have proposed that the mechanism to produce chalcones is due to ring B opening, and that these changes alter anthocyanin color and stability.

It should also be remarked that both AC methods showed consistent results using conditions that are comparable to those from other authors. For example, Wong et al. (2003) showed that optimal conditions to extract anthocyanins and ascorbic acid from hibiscus juice were 60 °C for 210 min.

Experimental data obtained were fitted into a second-order equation using a multiple regression analysis. This yielded adjusted models for TPC, DPPH•,

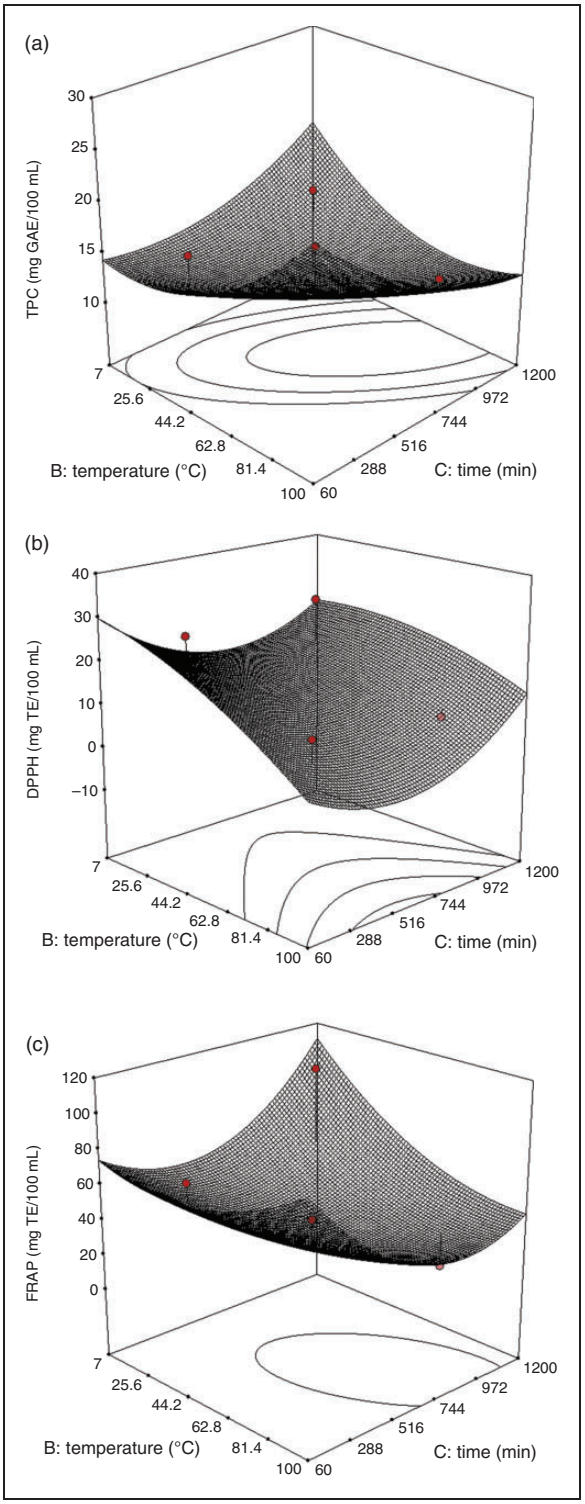

Figure 2. Response surface graphs used to produce an optimized hibiscus beverage. Calyces or leaves-to-water ratio was fixed at 4.9 g/100 ml, and the combined effect of water temperature and infusion time is shown 5 on (a) TPC, and AC by (b) the DPPH• assay and (c) by the FRAP assay. DPPH•: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing antioxidant power; GAE: gallic acid equivalent; TE: Trolox equivalent; TPC: total phenolic content.

and FRAP, which are shown in equations (1) to (3), respectively. Terms with positive values increase the response, while negative values decrease it (Martins et al., 2013). The interaction between X_1 and X_3 (calyces or leaves-to-water ratio and time) had negative coefficients on all equations, suggesting that it would decrease the value of the three responses

$$TPC = 11.75 - 1.17X_1 - 1.07X_2 + 1.03X_3 + 2.13X_1X_2 - 1.02X_1X_3 - 0.70X_2X_3 + 0.52{X_1}^2 + 0.19{X_2}^2 + 0.68{X_3}^2 \quad (1)$$

$$DPPH = 7.72 + 2.06X_1 + 0.79X_2 - 4.71X_3 - 1.34X_1X_2 - 170.73X_1X_3 - 167.79X_2X_3 - 0.81{X_1}^2 - 1.21{X_2}^2 + 3.83{X_3}^2 \quad (2)$$

$$FRAP = 11.22 - 14.75X_1 + 8.55X_2 + 5.50X_3 + 2.36X_1X_2 - 1.42X_1X_3 + 2.25X_2X_3 + 2.25{X_1}^2 + 2.36{X_2}^2 - 0.45{X_3}^2 \quad (3)$$

Coefficients of determination (R^2) were 0.97, 0.94, and 0.98 for equations (1) to (3), respectively, demonstrating that 94–98% of the response is attributable to experimental factors studied, and that the model was significant ($p<0.05$), which suggest that the models are reliable. In addition, *F* values were 12.27 for equation (1) (TPC), 8.10 for equation (2) (DPPH), and 42.85 for equation (3) (FRAP) ($p<0.05$).

Figure 2 shows the response surface plots obtained for the optimization of the hibiscus FB. The combined effect of temperature and time on TPC (Figure 2(a)) shows that TPC increases with time, reaching its maximum value at approximately 24 h, but water temperature must be low. Other authors have reported that

Table 4. Experimental values of the optimization of a functional green tea beverage using a central composite design (CCD)

	Coded variables			Experimental values		
Experimental run	X_1	X_2	X_3	TPC (mg GAE/100 ml)	DPPH• (mg TE/100 ml)	FRAP (mg TE/100 ml)
1	−1	−1	−1	33.02	93.81	236.32
2	1	−1	−1	24.89	79.11	188.08
3	−1	1	−1	32.48	75.52	198.06
4	1	1	−1	2.13	6.92	0.82
5	−1	−1	1	66.77	143.42	14.14
6	1	−1	1	11.34	21.68	3.51
7	−1	1	1	11.76	42.54	71.89
8	1	1	1	9.14	27.58	12.69
9	−1.682	0	0	53.73	13.13	180.62
10	1.682	0	0	8.12	59.40	0.77
11	0	−1.682	0	23.21	70.77	153.52
12	0	1.682	0	15.79	28.47	7.51
13	0	0	−1.682	20.56	75.24	133.75
14	0	0	1.682	24.49	72.32	130.82
15	0	0	0	20.44	48.81	107.54
16	0	0	0	24.24	110.89	4.83
17	0	0	0	36.76	133.22	6.61
18	0	0	0	35.99	130.50	7.52
19	0	0	0	26.71	131.55	6.61
20	0	0	0	25.19	133.22	7.47

DPPH•: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing antioxidant power; GAE: gallic acid equivalent; TE: Trolox equivalent; TPC: total phenolic content.
X_1: leaves-to-water ratio (g/100 ml), X_2: temperature (°C), X_3: time (min). Values are the average of three replicates.

hibiscus bioactives degrade at temperatures of 70–90 °C (Domínguez López et al., 2008; Patras et al., 2009). According to our findings, infusing calyces in water at near-ambient temperatures for long periods of time releases high amounts of hibiscus PCs.

The combined effects of temperature and time on DPPH values (Figure 2(b)) show that low temperatures and short times result in the highest AC values for this method. When considering the FRAP assay, the combined effects of temperature and time (Figure 2(c)) show that the highest values are found when the lowest temperature and longest times are used. Ramirez-Rodrigues et al. (2011) reported that high temperatures (90 °C) and prolonged extraction times (≥240 min) promote hibiscus PC degradation. Others report that high temperatures improve PC solubility, but these conditions also induce their degradation and may result in low AC values (Pinelo et al., 2005; Ramirez-Rodrigues et al., 2011; Spigno and Faveri, 2007). Our results suggest that hibiscus PCs are extracted better under low temperatures which also preserves their AC. This is likely because temperature-induced chemical changes (such as chalcone formation) are minimized. In addition to temperature, other chemical changes are induced by prolonged extraction times; for example Spigno and Faveri (2007) propose that polymerization reactions can form new compounds with varying AC.

Green tea beverage

Table 4 shows TPC and AC of 20 experimental runs that were used to optimize the green tea FB. TPC values ranged from 2.13 to 66.77 mg GAE/100 ml, highest value was found under experimental conditions of $X_1 = 4.9$ g/100 ml, $X_2 = 26$ °C, and $X_3 = 969$ min. TPC results reported here differ to those reported by others; for example Carloni et al. (2013) and Muniandy et al. (2016) report 40.82–547.81 mg GAE/ml. Others report that PCs in green tea vary according to cultivar used, leaf size, pre- and post-harvest conditions, storage conditions, drying and fermentation, among other factors, suggesting that the differences found between our results and those previously reported can be attributed to these variables. In the case of AC, values ranged from 6.92 to 143.42 mg TE/100 ml and 0.77 to 236.32 mg TE/100 ml for DPPH• and FRAP, respectively. Highest AC was found when $X_1 = 4.9$ g/100 ml, $X_2 = 26$ °C, and $X_3 = 969$ min for DPPH•, and $X_1 = 4.9$ g/100 ml, $X_2 = 26$ °C, and $X_3 = 291$ min for FRAP. This shows that the highest values of TPC and DPPH• were found under identical conditions. In the case of FRAP, X_1 (calyces or leaves-to-water ratio) and X_2 (temperature) also yielded the highest values FRAP values, but required shorter times. Adjusted models

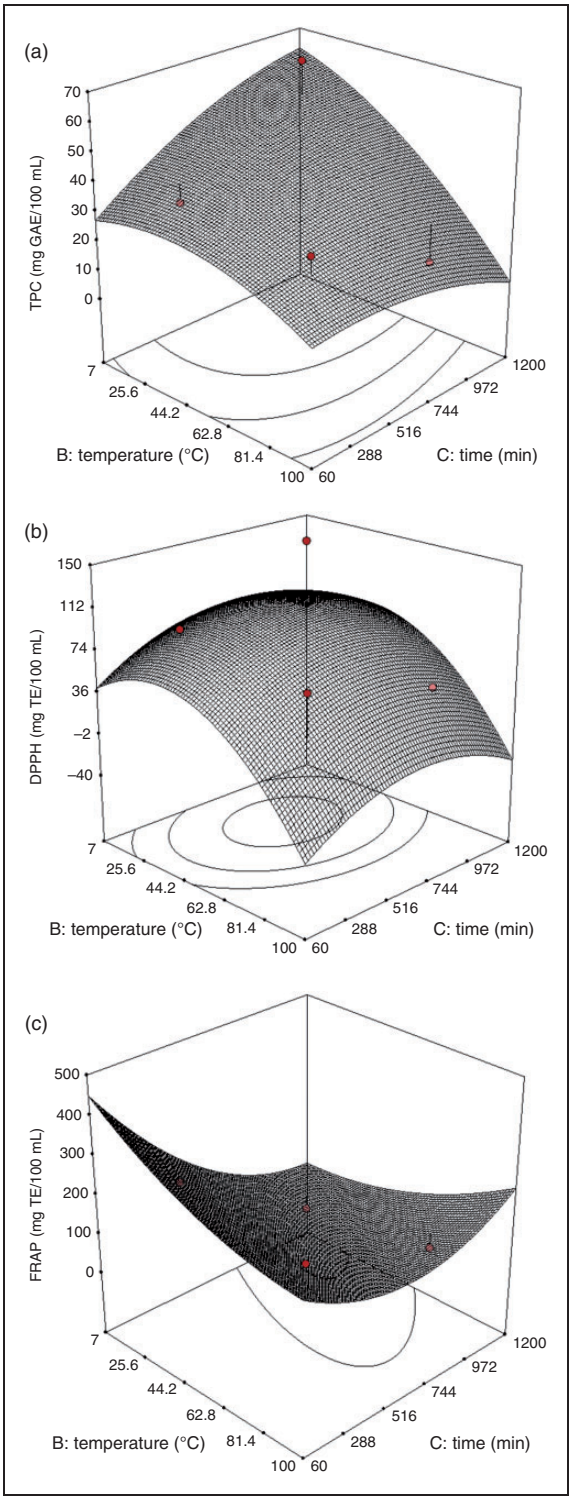

Figure 3. Response surface graphs used to produce an optimized green tea beverage. Calyces or leaves-to-water ratio was fixed at 4.9 g/100 ml, and the combined effect of water temperature and infusion time is shown on (a) TPC, and AC by (b) the DPPH• assay and (c) by the FRAP assay. DPPH•: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing antioxidant power; GAE: gallic acid equivalent; TE: Trolox equivalent; TPC: total phenolic content.

for TPC, DPPH•, and FRAP are shown in equations (4) to (6), respectively

$$TPC = 28.09 - 4.90X_1 - 2.84X_2 - 3.13X_3 + 10.60X_1X_2 - 9.25X_1X_3 - 11.07X_2X_3 + 6.54{X_1}^2 - 2.83{X_2}^2 - 1.69{X_3}^2 \quad (4)$$

$$DPPH = 127.74 - 0.25X_1 - 8.79X_2 - 12.17X_3 - 6.84X_1X_2 + 6.57X_1X_3 + 12.61X_2X_3 - 32.45{X_1}^2 - 28.27{X_2}^2 - 19.09{X_3}^2 \quad (5)$$

$$FRAP = 6.46 - 55.54X_1 - 36.10X_2 - 31.58X_3 - 35.50X_1X_2 + 32.78X_1X_3 + 47.18X_2X_3 + 19.86{X_1}^2 + 22.04{X_2}^2 + 39.82{X_3}^2 \quad (6)$$

Interestingly, linear terms (X_1, X_2, and X_3) had negative coefficients on all three equations, indicating that they would decrease the response. This suggests that in order for the response to increase, the most relevant factors are the interactions between independent variables. Specific interactions are key for each equation, but they vary by model because no interaction had consistent positive (or negative) coefficients on all equations, regardless, the models were statistically significant ($p < 0.05$). Lack of adjustment shows values of $p > 0.05$, indicating that the contribution of the models is significant. These models showed *F* values of 6.25 for TPC, 5.00 and 4.71 for DPPH and FRAP, respectively ($p < 0.05$). Significant interactions between extraction temperatures and times have been reported when preparing infusions from other plants, like *Rubus coreanus* Miq., where temperature and time also play an important role on final PC concentration (Ku and Mun, 2008).

Figure 3 shows response surface plots obtained for the optimization of green tea FB. Combined effect of temperature and time on TPC (Figure 3(a)) shows that highest values were obtained when using the lowest temperatures and longest times. This trend is similar to the one found for hibiscus, which suggests that PCs are slowly released from the matrix during hours of infusion. Regarding AC, DPPH• response (Figure 3(b)) is optimal if extreme values are avoided, and intermediate values of each of factor are used instead. That is, using water at 40–80 °C for 400–800 min would yield the highest DPPH• values. When using FRAP method (Figure 3(c)), short times and low temperatures yield high AC values.

Combined FB optimization

Optimal TPC and AC values of hibiscus and green tea beverages were determined using the desirability function. Results show that $X_1 = 4.9$ g/100 ml, $X_2 = 26$ °C, and $X_3 = 291$ min are optimal conditions for both beverages. Table 5 lists the predicted and experimental values of TPC and AC, as determined using the previously mentioned conditions, and the experimental values of the combined beverage. Predicted TPC values were statistically similar ($p > 0.05$) to experimental values of the hibiscus beverage. Experimental DPPH• values of the hibiscus beverage were significantly higher ($p < 0.05$) than those predicted, and experimental FRAP values were significantly lower ($p < 0.05$) than those predicted. All predicted and experimental data of the green tea beverage were statistically similar ($p > 0.05$).

When both optimized beverages were combined, TPC was higher ($p < 0.05$) than that of hibiscus and

Table 5. Predicted and experimental values of the response variables for the optimization of a combined hibiscus and green tea functional beverage

	TPC		DPPH•		FRAP	
Beverage	PV	EV	PV	EV	PV	EV
Hibiscus	14.78	14.80 ± 1.41b	24.69	61.91 ± 0.76c	55.06	25.95 ± 0.63*c
Green tea	31.23	33.02 ± 0.34a	86.69	93.81 ± 0.04b	242.97	236.30 ± 14.70a
Combined		34.07 ± 0.71a		175.33 ± 6.14a		169.74 ± 4.43b

DPPH•: 2,2-diphenyl-1-picrylhydrazyl; EV: experimental value; FRAP: ferric reducing antioxidant power; PV: predicted value; TPC: total phenolic content.

Optimal conditions were $X_1 = 4.9$ g/100 ml, $X_2 = 26$ °C, and $X_3 = 291$ min. The experimental values are expressed as mean ± standard deviation (SD), n=3. An asterisk (*) indicates statistically significant differences ($p < 0.05$) between predicted and actual value. Different superscript letters indicate significant differences ($p < 0.05$) between the experimental values of the individual beverages and the combined beverage.

similar to green tea ($p > 0.05$). It is likely that the major quantified PCs of the combined beverage were those of green tea (catechins) and that hibiscus anthocyanins were minimally reactive to the Folin–Ciocalteu reagent.

AC data of the combined beverage had interesting tendencies, where a synergistic effect was found on the DPPH• method, and an antagonistic effect on the FRAP method. According to these data, PCs found on both beverages are highly reactive toward the DPPH• radical and can efficiently neutralize it. Conversely, hibiscus anthocyanins appear to hinder electron transfer from the green tea catechins to the TPTZ:Fe^{+2} complex of the FRAP assay. Previous studies have shown that the FRAP method is suitable to measure AC activity of anthocyanins from hibiscus (Sáyago-Ayerdi Sonia et al., 2013; Tsai et al., 2002). AC of beverages with high PC content may result from a synergy between them, as other authors suggest (Nanasombat et al., 2015). This synergism is often proposed, because AC of a mixture of PCs is usually greater than the sum of each individual compound (Blasa et al., 2010). Our results suggest that synergic/antagonistic effects are assay dependent. The exact cause of this antagonism was not studied, and more specific analyses are required to conclusively establish the molecular bases of this phenomenon.

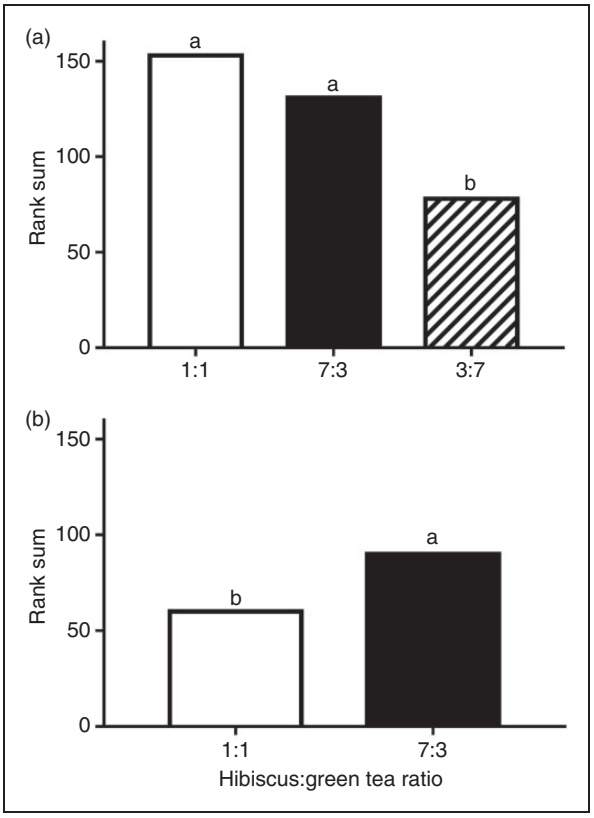

Figure 4. Sensorial evaluation by a panel of volunteers ($n = 110$) of a combined hibiscus:green tea FB using three ratios. Different letters indicate significant differences between samples. (a) First trial where three formulations were evaluated and (b) second trial where two formulations were evaluated.

Consumer preference test

Delivering the highest amounts of bioactive compounds is among the goals of FBs, but it is also important to consider that the product must have appealing sensory qualities that ensure repeated consumption. High amounts of hibiscus calyces or *C. sinensis* leaves modify the flavor of the infusions, because PCs and organic acids present impart a bitter and/or astringent sensation (Narukawa et al., 2010; Scharbert and Hofmann, 2005; Wong et al., 2003; Yu et al., 2014). In order to obtain a product of good sensory characteristics, the previously optimized FBs were mixed in varying ratios of 1:1, 7:3, or 3:7 (v/v) of hibiscus and green tea, respectively, and a untrained panel ($n = 110$) ranked them according to their personal preference. Results of sensory evaluation are presented in Figure 4.

Untrained panelists were initially given all three combinations (Figure 4(a)), and the 1:1 and 7:3 (hibiscus:green tea) formulations were similarly ranked ($p > 0.05$), while demonstrating a noticeable and significant dislike ($p < 0.05$) when green tea was the major component. It has been reported that catechins in general are among the molecules responsible for the astringency of teas (Narukawa et al., 2010; Scharbert and Hofmann, 2005).

A second sensory evaluation was performed, taking into account the results of the initial analysis. The least liked formulation of the first trial was discarded, and the same panelists chose between the most liked formulations (Figure 4(b)). The 7:3 (hibiscus:green tea) formulation was ranked significantly higher than the 1:1 formulation. According to these data, consumers preferred the taste of hibiscus over that of green tea. Hibiscus has a characteristic acid taste, that is related to its anthocyanin content (Bechoff et al., 2014), but apparently, the unappealing sensory characteristics of green tea overwhelmed those of hibiscus. Feedback given was generally favorable for the 7:3 formulation; some reported perceiving a slightly acidic and sometimes bitter taste, but it was not strong enough for them to negatively evaluate it. Some authors report that beverages made with fruits with high PC content usually have bitter, astringent, and acidic flavors associated with the various PCs present (Jaeger et al., 2009; Lawless et al., 2012).

Chromatographic analysis

Because the 7:3 (hibiscus:green tea) formulation was the preferred option, a detailed chromatographic

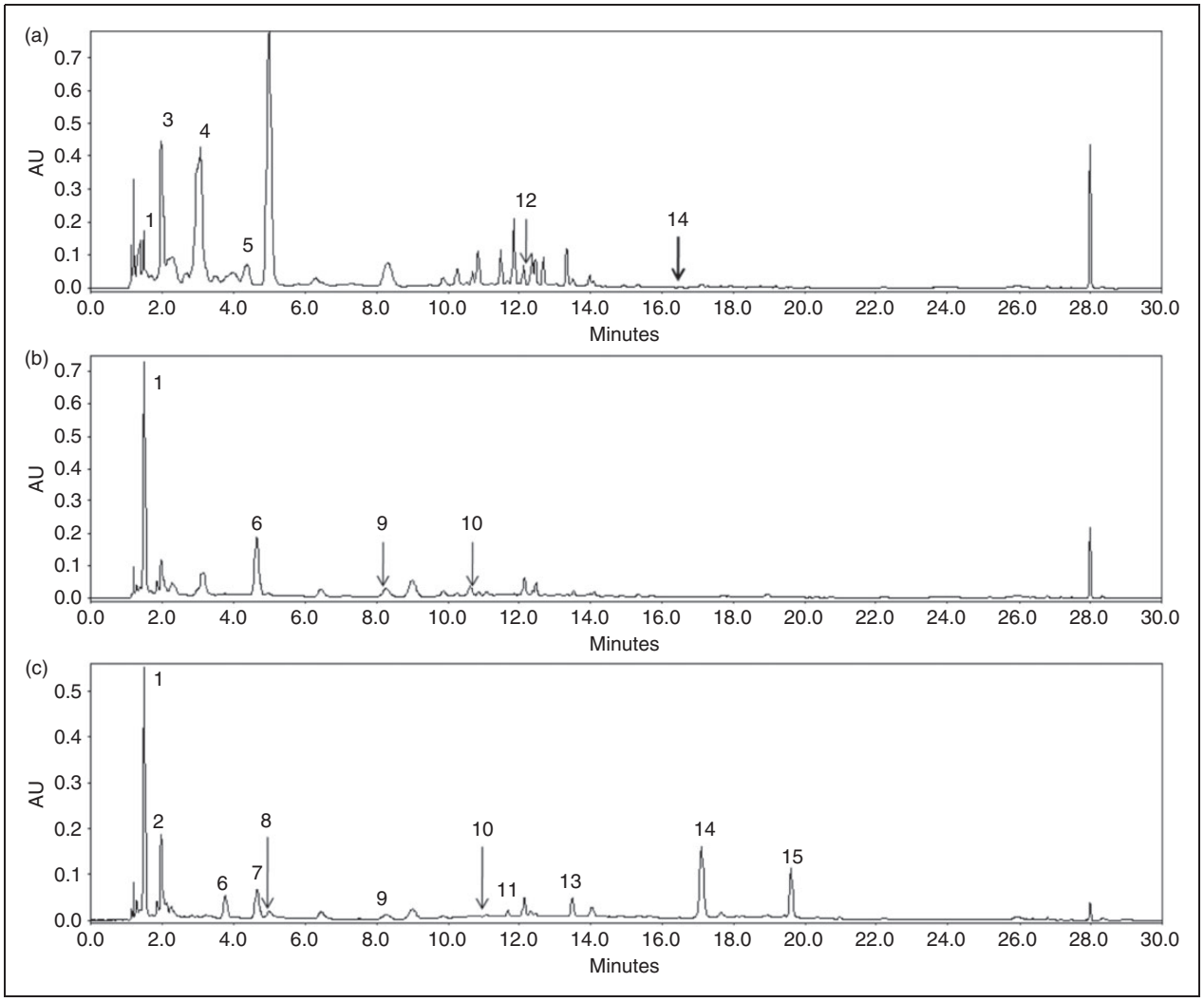

Figure 5. Representative UPLC-DAD chromatogram used to identify and quantify PCs present in a combined hibiscus:green tea (7:3) FB. (a) Free PCs, (b) PCs released after an alkaline hydrolysis, and (c) PCs released after an acid hydrolysis. 1—gallic acid, 2—protocatechuic acid, 3—catechin, 4—chlorogenic acid, 5—epicatechin, 6—caffeic acid, 7—vanillic acid, 8—syringic acid, 9—*p*-coumaric acid, 10—ferulic acid, 11—ellagic acid, 12—rutin, 13—myricetin, 14—quercetin, 15—kaempferol.

analysis was performed on it. Figure 5 shows a representative chromatogram, where a total of 15 compounds were identified, specifically, flavonoids and phenolic acids. Figure 6 shows a representative chromatogram, where two anthocyanins were identified and quantified. Retention times of all identified compounds are listed in Table 6, in addition to the specific fraction where they were found, and their total concentration. The strongest signals were detected on the free fraction, but only six molecules were present. The use of two sequential hydrolyses increased the number of quantifiable compounds, particularly, the alkaline treatment.

Most PCs found in the FB belong to compounds usually identified in hibiscus drinks, this makes sense because hibiscus was the main component used to prepare it. Gallic acid was the major PC, followed by catechin and epicatechin. Gallic acid is ubiquitously found in plants and was the only molecule present in all analyzed fractions. Catechin and epicatechin are characteristically found in teas; galloylated catechins are also known, and various bioactivities have been reported (Kobayashi et al., 2016). In a paper by Ifie et al. (2018), the presence of some of these compounds has been reported, although in higher concentrations than those found in our FB. Regarding anthocyanins, delphinidin was found in an approximately three-fold higher concentration than cyanidin. Our results of anthocyanin quantification are similar to those of Sindi et al. (2014), which were performed in aqueous extracts. They found four anthocyanins, all of them

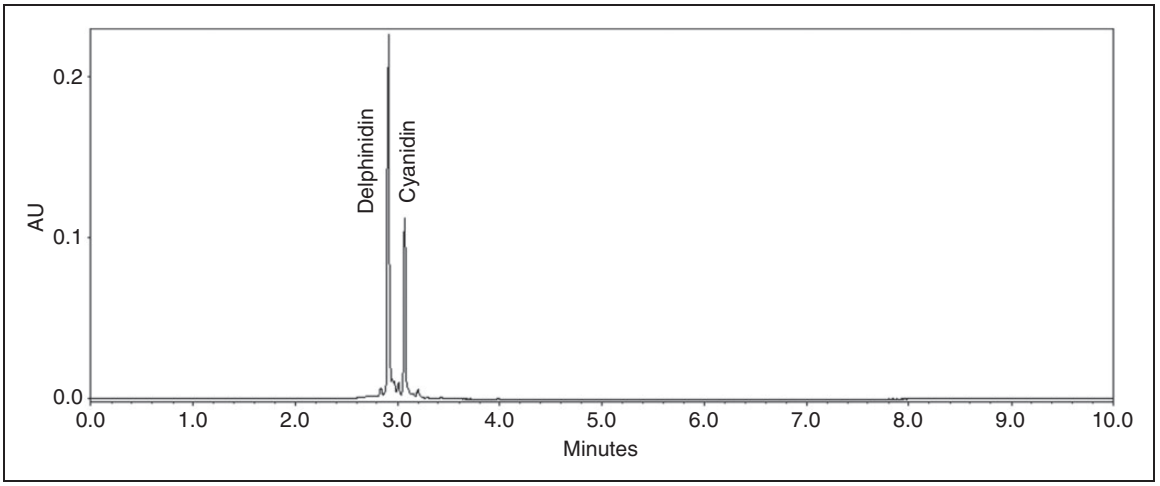

Figure 6. Representative UPLC-DAD chromatogram used to identify and quantify anthocyanins present in a combined hibiscus:green tea (7:3) FB.

Table 6. Phenolic compounds (PCs) identified and quantified on a combined hibiscus:green tea (7:3) functional beverage

Phenolic compounds (PCs)	Retention time (min)	Free compound	Alkaline-released	Acid-released	Concentration (mg/100 ml)
Gallic acid	1.483	✓	✓	✓	10.38 ± 0.00
Protocatechuic acid	2.209	–	–	✓	0.37 ± 0.00
Catechin	2.211	✓	–	–	5.85 ± 0.01
Chlorogenic acid	3.249	✓	–	–	0.64 ± 0.04
Epicatechin	4.060	✓	–	–	5.91 ± 0.05
Caffeic acid	4.436	–	✓	✓	5.72 ± 0.00
Vanillic acid	5.096	–	–	✓	0.04 ± 0.00
Syringic acid	5.998	–	–	✓	0.27 ± 0.00
p-coumaric acid	8.524	–	✓	✓	4.74 ± 0.02
Ferulic acid	10.775	–	✓	✓	0.98 ± 0.04
Ellagic acid	11.896	–	–	✓	0.21 ± 0.05
Rutin	12.210	✓	✓	–	0.76 ± 0.00
Myricetin	13.717	–	–	✓	0.36 ± 0.00
Quercetin	16.710	✓	–	✓	1.54 ± 0.00
Kaempferol	19.366	–	–	✓	0.48 ± 0.00
Anthocyanins					
Delphinidin chloride	2.909				13.22 ± 0.16
Cyanidin chloride	3.069				3.92 ± 0.03
					392.90 ± 3.45

Data are expressed as mean ± standard deviation (SD), n = 3.

isomers of the compounds reported in the present work. These compounds are characteristic of hibiscus (De Moura et al., 2018) and are responsible for its organoleptic properties, such as color and taste. It has been reported that the color of hibiscus extracts can be affected according to extraction conditions (such as water temperature), thermal treatments used for its conservation, as well as storage conditions (Cisse et al., 2012b). In addition, Bechoff et al. (2014) positively correlated the taste of hibiscus drinks with anthocyanin content and concluded that these chemical attributes are related to consumer acceptance.

These parameters can improve the methods used to develop new beverages or edible products.

CONCLUSION

Hibiscus and green tea were used to develop FBs. Calyces or leaves-to-water ratio, water temperature, and infusion time were optimized with RSM. Optimal conditions were 4.9 g/100 ml of water, at 26 °C for 291 min. Optimized beverages were combined and showed a synergistic effect on AC when measured with the DPPH• method, and an antagonistic effect when measured with the FRAP assay. A sensory evaluation showed that the beverage prepared with a 7:3 ratio (v/v) of hibiscus and green was preferred over those with different ratios (1:1 and 3:7). Phenolic profile contained flavonoids, phenolic acids, and anthocyanins. Further experiments are needed to confirm the in vivo antioxidant properties or other bioactivities, using an animal model or human clinical trials.

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