Colorimetric analysis of *Hibiscus* beverages and their potential antioxidant properties

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Running head: Color-composition of *Hibiscus* beverages

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Abstract

In food industry, roselle beverages and by-products could be a functional ingredient since is an excellent source of bioactive compounds with improved functional. Roselle is a plant that is recognized by their anthocyanins content, its importance as food coloring and

as a source of antioxidant compounds in the food industry. The aimed is the study of the color, as indicative of the anthocyanins composition, of *Hibiscus* beverages prepared with different particle sizes flours. Tristimulus Colorimetry was applied to characterize the beverages color. Color measurements were made by transmission spectrophotometry, anthocyanins quantification was determined by HPLC, and antioxidant potential was evaluated with ABTS and FRAP methods. Positive correlations among pigments composition and color parameters were found, showing that anthocyanins content, antioxidant capacity, C^*_{ab} and h_{ab} values increased in relation with smallest particle size of flours. The obtained mathematical models could be an important tool to be used in the food industry to characterized roselle pigments or functional compounds with potential health benefits.

Introduction

H. sabdariffa L., an attractive plant, believed to be native to Africa and cultivated in tropical areas as Sudan, Eastern Taiwan, Thailand and México. In last decades, it has gained an important position in the food industry because its high anthocyanins content, related to the persistent red calyx of its flowers as the major component, and its sour taste, which is commonly used in the preparation of cold and hot beverages. Also, it has been used as a food colorant and active ingredient to develop food with some health benefits [1].

Recently in Mexico, chalices with different degrees of pigmentation have been cultivated from traditional plant breeding with important content of anthocyanins (>3g/100g) [1, 2] in comparison with other cultivars [3,4]. Volatile [5], phenolic compounds and antioxidant capacity [2] are characteristics that have been studied from *Hibiscus* Mexican cultivars. Camelo-Méndez *et al.* [2] reported that the degree of

pigmentation of chalices was highly correlated with the anthocyanin content and hence *in vitro* antioxidant capacity ($r>0.8$, $p<0.05$). Despite these results, is limited the literature about the relation between their pigment content and the color properties of *Hibiscus* by-products.

Tristimulus colorimetry has been widely applied to food and food products with different purposes, such as the assessment of color changes during food processing [6], to determinate anthocyanins in wine [7], strawberries [8] and exotic fruits such as: Jaboticaba [9], gulupa [10] and tamarillo [11]. In despite of the relationships between individual anthocyanins and the color parameters in derived *Hibiscus*, products have not been studied exhaustively. Recently, mathematical models of color-composition relationship using digital image analysis of chalices have been proposed, these models could provide an assessment of total anthocyanins content of *Hibiscus* chalices with different degrees of pigmentation [2].

The aim of this study was the use of colorimetric assessment by instrumental analysis of *Hibiscus* beverage prepared with different size particles to estimate the anthocyanin composition with antioxidant potential properties and to evaluate the individual contribution of anthocyanins to the color of the product.

Material and methods

Plant Material

Roselle chalices were obtained from breeding programs and named according to pigmentation degree (Negra, Sudan and Rosa). Dried calyces were lyophilized and pulverized in a food processor (Sunbeam, 220 watts motor, Kitchen Aid, Canada), then

samples were sifted and separated in five groups: 1) flours with particle size greater than 710μm, 2) flours with particle size between 710-550μm, 3) flours with particle size between 550-355μm, 4) flours with particle size between 355- 250μm and 5) flours with particle size less than 250μm. Sample characteristics and storage conditions were previously described [2, 5].

Beverage preparation

Beverage was prepared following a popular Mexican procedure [2, 12]. Briefly, 5 grams of sample were decocted in 100 ml of water for 5 min. Then beverage was filtered and kept in refrigeration for posterior analysis.

Spectrophotometric color measurement

A Hewlett-Packard UV-Vis HP8453 spectrophotometers (Palo Alto, CA, USA) were used to carry out color measurements of all beverages. The whole visible spectrum (380-770 nm) was recorded at constant intervals ($\Delta\lambda = 2$ nm), using 2 mm path length glass cells and distilled water as reference. The CIELAB parameters (L^*, a^*, b^*, C^*_{ab} and h_{ab}) were determined using the original software CromaLab® [13], following the Commission Internationale de l'Éclairage (CIE) recommendations [14]: the CIE 1976 10º Standard Observer and the Standard Illuminant D65 [15]. Also, color differences were calculated as the Euclidean distances between pairs of points in the three-dimensional space defined by L^*, a^* and b^* according to the following formula:

$$\Delta E^*_{ab} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

HPLC analysis of anthocyanins

Anthocyanins determination was carried out following the methodology previously used [2]. These compounds were separated using a Zorbax C18 column (250 x 4.6 mm, 5 μm particle size) maintained at 38 °C. A gradient of the mobile phase A (trifluoroacetic acid, 0.1%), and acetonitrile as solvent B, were used. The elution profile was as follows: 0-15.62 min 90% A - 10% B; 15.62-26.04 min 85% A - 15% B; 26.04-46.88 min 82% A - 18% B; 46.88-52.08 min 70% A - 30% B; 52.08-60 min 65% A - 35% B; 60-65 min 90% A -10% B. The flow rate was 0.8 mL/min, and the injection volume was 50 μL. The wavelength of detection was 525 nm. The identification of anthocyanin was achieved by comparison between the retention time and those of the available pure standards of our data library.

Antioxidant capacity

Antioxidant capacity was evaluated by FRAP and ABTS assays according to Benzie and Strain [16] and Re *et al.* [17], respectively. Different dilutions of each extract were assayed and the results were obtained by interpolating the absorbance on a calibration curve obtained with Trolox (30-1000μM). Three independent experiments in triplicate were performed for each assayed extracts and results were expressed as Trolox-equivalent antioxidant capacity (TEAC; mmols of Trolox with the same antioxidant capacity as 100 g of dry matter).

Statistical analysis

Statgraphics Plus version 5.1® (Manugistics, Inc., Rockville, MA, USA) was used for the statistical treatment of the data. One-way analysis of variance (ANOVA) was employed to establish significant differences between samples. Correlations between anthocyanins content and color parameters were studied by both, simple and multiple

regression analysis. Multiple regression analyses were carried out by means of partial least squares (PLS). In all cases, statistically significant level was considered at $p < 0.05$.

Results and discussion

Chromatographic determination of anthocyanins

Hibiscus beverages showed the same chromatographic profile, regardless the size of particle used for beverage elaboration, where two peaks could be identified on the basis of the retention times and UV-vis spectra. However, quantitatively, the results show that the use of minor size particle had a positive effect on the extraction of anthocyanins during the process (Table 1). Cyanidin 3-sambubioside (Cy-3-Sa) and delphinidin-3-sambubioside (Dp-3-Sa) were the two anthocyanins identified in *Hibiscus* beverages. Where Dp-3-Sa represented more than the 60% of the total anthocyanin content.

Anthocyanin concentration of *Hibiscus* beverages showed to be inversely depended on the particle size of powders: higher pigment content in beverage prepared with flour with minor particle size ($p<0.05$). Using powders with minor size particle of 710 μm, total anthocyanin content was higher, which shows that a minimum reduction on the particle size leads to an important increase in the amount of anthocyanins content in the beverages (Table 1). Using *Hibiscus* flours with minor particle size to 250μm, the anthocyanin content was obtained up to three more times than the concentrations reported in our previously results using the same cultivars [2]. Mexican *Hibiscus* cultivars obtained from breeding programs presented higher anthocyanin content than other cultivars [3, 4, 12]. This increase on the extraction of bioactive compounds could be an important feature for the elaboration of derived *Hibiscus* products, since extracts of this plant have present positive biological activities [1].

Color characterization

The definition of the color by tristimulus colorimetry enables to establish objectively the chromatic characteristics of *Hibiscus* beverages and their variability. The color points represented in the CIELAB diagram as well as their lightness are shown in Figure 1. It can be noticed that practically all of samples are located in the first quadrant (positive a^* and b^*) of the (a^*b^*)-plane, corresponding to reddish region. The (a^*b^*) and L^* color representation allow to observe a clear trend in the evolution of color by the diminution of particle size of *Hibiscus* flours. In general, color parameters were significantly different ($p<0.05$) between beverages prepared with flours with minor particle size than 710 μm, and ones prepared with flours with major particle size (Online Rosurce 1). C^*_{ab} and h_{ab} parameters showed an increase up to 20 and 3 CIELAB units, respectively, while L^* decreases significantly ($p<0.05$), in the process of preparing the beverage, as the particle size was decreased were obtained beverages with more saturated red color, which is consistent with a higher concentration of anthocyanins. Also, color changes (ΔE^*_{ab}) between beverages prepared with different particle size were evaluated. *Hibiscus* beverages presented ΔE^*_{ab} higher values than 3 CIELAB units that can be perceived by the human eye [18], a noticeable influence of the reduction size particle on the color of *Hibiscus* beverages were found.

Antioxidant capacity

The antioxidant capacity of *Hibiscus* beverages prepared with flour of different particle size was measured by ABTS and FRAPS assays (Figure 2). Beverages prepared with Negra cultivar presented the greatest antioxidant capacity in both assays, followed by beverages prepared with Sudan and Rosa cultivars. These results are in conformity with total anthocyanin content determined by HPLC, which was significantly correlated with ABTS (r = 0.69, $p < 0.01$) and FRAP (r = 0.96, $p < 0.01$) values, as shown by regression

analysis. Different authors also reported significant correlation between antioxidant activity and phenolic composition content in *Hibiscus* by-products and extracts [2, 19]. The effect of the size particle of *Hibiscus* flours, presented a significant increase ($p<0.05$) in their antioxidant capacity of beverages. Beverages prepared with flours with minor particle size of 250μm presented an increase of the antioxidant capacity almost in double values for both assays.

Color-composition relationships

In this study, we established correlations between the chemical characteristics and color parameters in *Hibiscus* beverages. As a first stage, the relation between the color parameters and the anthocyanins content was explored by means of simple correlations (Table 2). Significant correlations ($p<0.01$) were found between the colorimetric parameters and pigment content. Anthocyanin levels (individual and total content) were negatively correlated with L^* while the other color parameters (a^*, b^* C^*_{ab} and h_{ab}) were positively correlated. However, to define the color of any object completely by means of Tristimulus Colorimetry, the scalar coordinates L^*, a^*, b^*, or L^* and the angular coordinates (C^*_{ab} and h_{ab}) have to be considered together, due to the tridimensional nature of color [7, 20]. To achieve a more accurate evaluation of the relationship between color and anthocyanins content, two correlation studies were performed.

Then, individual and total anthocyanins content were considered as a dependent variable and color parameters L^*, a^*, b^* and L^*, C^*_{ab}, h_{ab} were considered as predictor or independent variables. This analysis allows to generate equations to calculate the anthocyanin composition in *Hibiscus* beverages prepared with flours of different particle size as a function of color, based on scalar and polar color parameters (Table 3). The correlations obtained were better than those obtained by the simple regression analysis,

which was expectable as more variables are considered and a better prediction is therefore achieved. By comparing these regression coefficients to those in Table 2, it can be readily concluded that this statistical approach enhances considerably the prediction of anthocyanin levels in *Hibiscus* beverages from color data.

The antioxidant activity of *Hibiscus* products is mainly due to phenolic compounds such as phenolic and benzoic acids, flavonoids and anthocyanins, among others. Anthocyanins have been recognized as the main responsible for the antioxidant capacity of *Hibiscus* products due to their redox properties and it high ratio with the other compounds [2], also to be responsible of red brilliant color of *Hibiscus* beverages.

A multiple regression was used to predict the antioxidant capacity (free radical inhibition and reducing power) in *Hibiscus* beverages from color parameters (Table 4). These prediction models had acceptable correlation values ($r>0.89$). Although the antioxidant capacity - color relation models in the literature are limited, in this study we proposed equations for the quick estimation of the antioxidant capacity (of two methods) in the *Hibiscus* beverages with high anthocyanin content.

Statistical analysis (ANOVA) showed that there is a statistically significant relationship ($p<0.01$) for each generated model. In addition, the values of the correlation coefficients obtained were acceptable for predicting the antioxidant capacity of Roselle beverages using color parameters obtained by transmission spectrophotometry measurement. The differences between the correlation values of the models for the ABTS and FRAP methods is due to the affinity or polarity of the molecules with the development of the method, the FRAP model presented higher *r* values because this method is more

suitable for aqueous extract, while ABTS method has been widely used for lipophilic compounds.

The models proposed in this study can be useful tools to the prediction of anthocyanins content and antioxidant capacity in *Hibiscus* beverages and by-products and their potential use in food and pharmaceutical industry because their nutraceutical properties.

Conclusions

The results obtained in this study indicate that it seems feasible to estimate the individual and total anthocyanin content as well as antioxidant capacity in *Hibiscus* beverages prepared with flours of different particle size by means of color parameters, applying Tristimulus Colorimetry and multivariate statistics. These equations could be used by food industry and quality control of development of *Hibiscus* beverages, although further studies should be carried out to extend the applicability of Tristimulus Colorimetry to estimate the anthocyanin content and antioxidant capacity properties in industrial production of *Hibiscus* beverages.

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Table 1. Anthocyanins content in *Hibiscus* beverages (mg/100mL)

Anthocyanin	Size particle	*Hibiscus* cultivar		
		Negra	Sudan	Rosa
Dp-3-Sa	x>710 μm	57.42 ± 5.90^{d}	60.76 ± 5.60^{d}	6.53 ± 1.01^{c}
	710>x>550 μm	137.42 ± 5.93^{d}	144.31 ± 19.48^{c}	15.15 ± 1.09 ^{b}
	550>x>355 μm	180.16 ± 7.87^{b}	196.88 ± 14.87^{b}	24.47 ± 3.49 ^{a}
	355>x>250 μm	192.65 ± 2.91^{a}	197.42 ± 14.35^{b}	25.13 ± 0.53 ^{a}
	x<250 μm	202.09 ± 6.92^{a}	224.96 ± 2.18^{a}	27.46 ± 5.06 ^{a}
Cy-3-Sa	x>710 μm	36.38 ± 5.47^{d}	26.52 ± 4.15^{d}	0.97 ± 0.38 ^{c}
	710>x>550 μm	66.03 ±6.35^{c}	63.25 ± 7.19^{c}	8.50 ± 1.09^{b}
	550>x>355 μm	86.31 ± 3.42^{b}	76.48 ± 5.44^{b}	12.07 ± 2.89^{a,b}
	355>x>250 μm	89.57 ± 3.37^{a,b}	81.22 ± 5.97^{a,b}	13.91 ± 4.80^{a}
	x<250 μm	96.58 ± 1.08^{a}	88.71 ± 1.66^{a}	14.26 ± 0.28^{a}
Sum_{ANTs}	x>710 μm	93.79 ± 11.34^{d}	87.28 ± 9.74^{d}	9.47 ± 1.47^{c}
	710>x>550 μm	188.43 ± 8.85^{c}	207.56 ± 26.61^{c}	21.68 ± 2.10^{b}
	550>x>355 μm	269.73 ± 11.23^{b}	273.36 ± 20.31^{b}	36.54 ± 6.38^{a}
	355>x>250 μm	278.96 ± 3.10^{b}	278.64 ± 20.32^{b}	39.38 ± 0.69^{a}
	x<250 μm	298.67 ± 6.44^{a}	313.68 ± 2.23^{a}	41.37 ± 9.84^{a}

Data expressed as mean ± SD (n=6). Different letters in the same column indicate significant differences, least significant differences (LSD) test.

Dp-3-Sa: Delphinidin-3-sambubioside, Cy-3-Sa: Cyanidin-3-sambubioside, Sum_{ANTs}: sum of anthocyanin

Table 2. Summary of simple regression analyses between color parameters and anthocyanin content.

	L^*	a^*	b^*	C^*_{ab}	h_{ab}
Cy-3-Sa	-0.983	0.970	0.892	0.970	0.868
Dp-3-Sa	-0.987	0.985	0.916	0.986	0.886
Sum_{ANTs}	-0.981	0.970	0.895	0.970	0.869

Dp-3-Sa: Delphinidin-3-sambubioside, Cy-3-Sa: Cyanidin-3-sambubioside, Sum_{ANTs}: sum of anthocyanins

Table 3. Regression equations for the anthocyanins estimation in *Hibiscus* beverages as a function of CIELAB (L^*, a^* and b^*; L^*, C^*_{ab} and h_{ab}) color parameters

Dependent variable	Model	r
Delphinidin-3-sambubioside	$Dp3Sa = 2614.48 - 26.58L^* - 8.84a^* + 1.63b^*$	0.98
	$Dp3Sa = 2588.13 - 26.34L^* - 8.58C^*_{ab} + 0.51h_{ab}$	0.98
Cyanidin-3-sambubioside	$Cy3Sa = 272.85 - 2.97L^* + 0.83a^* + 0.80b^*$	0.97
	$Cy3Sa = 261.60 - 2.86L^* + 0.97C^*_{ab} - 0.18h_{ab}$	0.97
Total anthocyanin content	$Sum_{ANTs} = 3314.38 - 33.78L^* - 10.33a^* + 3.15b^*$	0.97
	$Sum_{ANTs} = 3269.54 - 33.37L^* - 9.89C^*_{ab} + 0.93h_{ab}$	0.97

Dp3Sa: Delphinidin-3-sambubioside, Cy3Sa: Cyanidin-3-sambubioside, Sum_{ANTs}: sum of anthocyanins

Table 4. Regression equations for antioxidant capacity estimation in *Hibiscus* beverages as a function of CIELAB (L^*, a^* and b^*; L^*, C^*_{ab} and h_{ab}) color parameters

Dependent variable	Model	r
Free radical inhibition (ABTS)	$ABTS = -2606.20 + 26.04L^* + 15.31a^* + 8.52b^*$	0.89
	$ABTS = -2640.13 + 26.32L^* + 15.59C^*_{ab} + 6.31h_{ab}$	0.89
Reducing power (FRAP)	$FRAP = 179.87 - 1.58L^* + 0.95a^* + 4.93b^*$	0.97
	$FRAP = 159.10 - 1.40L^* + 0.99C^*_{ab} + 5.42h_{ab}$	0.97

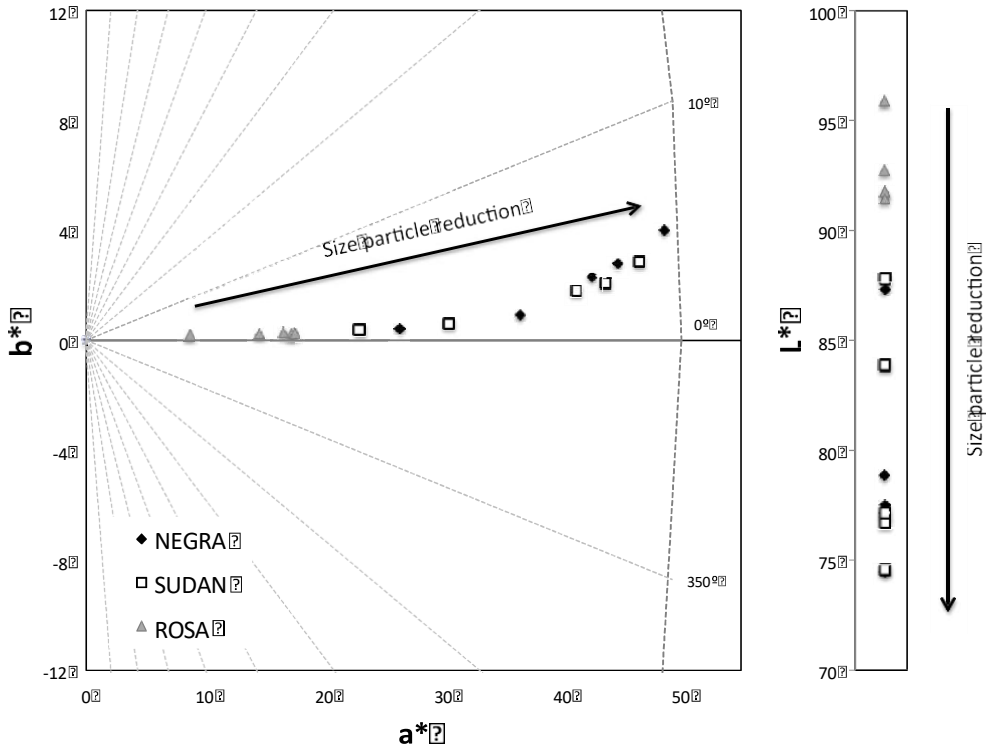

Figure 1. CIELAB color space (a^*b^*)-plane and lightness (L^*) of *Hibiscus* beverages.

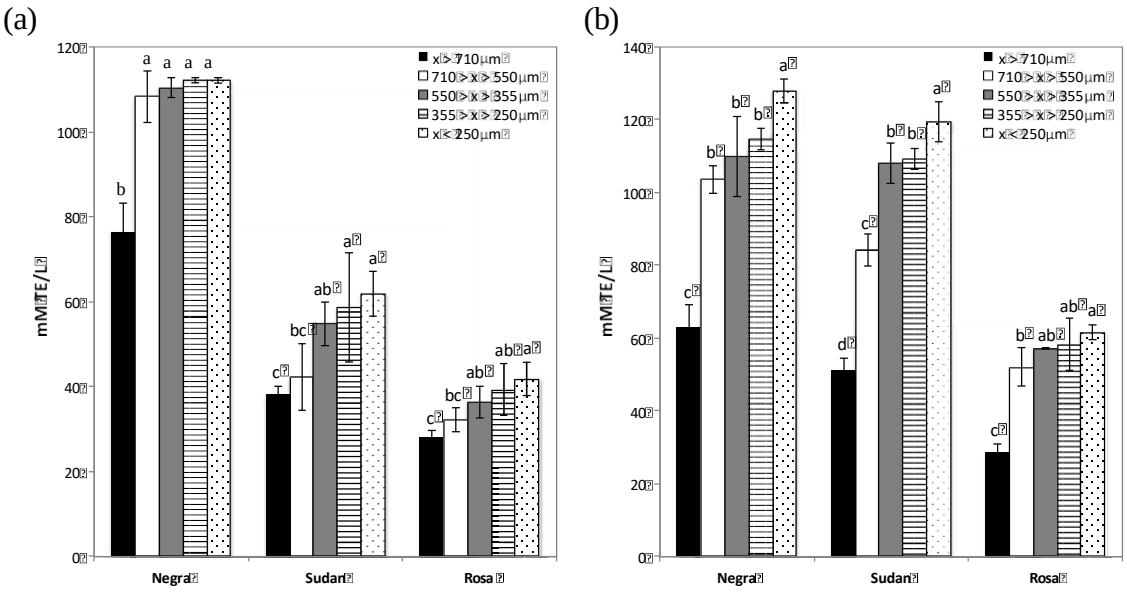

Figure 2. Antioxidant activity of *Hibiscus* beverages by (a) ABTS and (b) FRAP assay.

Online Resource 1. Color parameters (CIELAB units) of *Hibiscus* beverages prepared with different size particles.

Hibiscus cultivar	Size particle	Color parameter					
		L^*	a^*	b^*	C^*_{ab}	h_{ab}	ΔE^*_{ab}
Negra	x>710 µm	87.34 ± 1.34^{a}	26.29 ± 2.77^{c}	0.43 ± 0.12^{d}	26.29 ± 2.77^{c}	0.50 ± 0.43^{d}	-
	710>x>550 µm	78.86 ± 0.50^{b}	41.97 ± 1.05^{b}	2.22 ± 0.16^{c}	42.03 ± 1.06^{b}	3.02 ± 0.15^{c}	17.92
	550>x>355 µm	77.49 ± 0.52^{c}	42.41 ± 2.31^{b}	2.30 ± 0.42^{c}	42.47 ± 2.33^{b}	3.10 ± 0.41^{b,c}	18.98
	355>x>250 µm	77.17 ± 0.23^{c}	44.55 ± 0.22^{b}	2.79 ± 0.15^{b}	44.64 ± 0.23^{b}	3.59 ± 0.17^{b}	22.95
	x<250 µm	74.75 ± 0.36^{d}	48.50 ± 0.34^{a}	4.01 ± 0.26^{a}	48.67 ± 0.35^{a}	4.73 ± 0.27^{a}	25.78
Sudan	x>710 µm	87.82 ± 0.60^{a}	22.91 ± 1.28^{c}	0.41 ± 0.17^{c}	22.91 ± 1.28^{c}	1.02 ± 0.45^{c}	-
	710>x>550 µm	83.87 ± 3.17^{b}	30.47 ± 6.65^{b}	0.62 ± 0.30^{c}	30.47 ± 6.66^{b}	1.12 ± 0.33^{c}	8.53
	550>x>355 µm	77.17 ± 1.48^{c}	41.10 ± 0.41^{a}	1.84 ± 0.11^{b}	41.14 ± 0.41^{a}	2.57 ± 0.14^{b}	22.13
	355>x>250 µm	76.74 ± 1.03^{c}	43.56 ± 1.93^{a}	2.21 ± 0.37^{b}	43.62 ± 1.94^{a}	2.89 ± 0.36^{b}	23.50
	x<250 µm	74.56 ± 0.29^{c}	46.36 ± 1.04^{a}	2.87 ± 0.08^{a}	46.44 ± 1.04^{a}	3.55 ± 0.06^{a}	27.05
Rosa	x>710 µm	95.87 ± 0.33^{a}	8.59 ± 0.70^{c}	0.20 ± 0.03^{a}	8.59 ± 0.70^{c}	0.78 ± 0.30^{b}	-

710>x>550 µm	92.76 ± 0.61[b]	14.46 ± 0.42[b]	0.23 ± 0.05[a]	14.46 ± 0.42[b]	0.86 ± 0.15[b]	6.64
550>x>355 µm	91.78 ± 1.14[b]	16.56 ± 2.20[a,b]	0.24 ± 0.11[a]	16.56 ± 2.21[a,b]	0.93 ± 0.16[b]	8.96
355>x>250 µm	91.55 ± 1.11[b]	17.17 ± 2.10[a]	0.26 ± 0.05[a]	17.17 ± 2.10[a]	1.01 ± 0.17[a,b]	9.61
x<250 µm	91.44 ± 0.08[b]	17.44 ± 0.14[a]	0.30 ± 0.08[a]	17.45 ± 0.14[a]	1.35 ± 0.29[a]	11.21

Data expressed as mean ± SD (n=6). Different letters in the same column for each *Hibiscus* beverage indicate significant differences, least significant differences (LSD) test.

Conflicts of interes statment

Manuscript entitled: Colorimetric analysis of Hibiscus **beverages and their potential antioxidant properties**

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We certify that there is no actual or potential conflict of interest in relation of this article

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