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Optimization of Aqueous Extraction of Anthocyanins from Hibiscus sabdariffa L. Calyces for Food Application

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Abstract
Aqueous extracts of Hibiscus sabdariffa calyces are worldwide used for the production of several products such as polyphenolic-antioxidant containing beverages. However, optima conditions for extraction of polyphenolics such as anthocyanins from this plant species were unknown. For this, the influence of particle size and calyx/water ratio on the anthocyanins extraction and biochemical composition during extraction were investigated using bright red and dark red Hibiscus sabdariffa calyces.Results showed that the pH of the bright red calyces was 2.24 ± 0.01 corresponding to 12.66 ± 0.15 g/100g dry weight (DW) titratable acidity equivalent of malic acid. The pH of the dark red calyces was 2.15 ± 0.01 corresponding to 15.44 ± 0.15 g/100g DW titratable acidity equivalent malic acid. Total sugars content was 3.24±0.04 and 3.13±0.06 g/100g DW for bright red and dark red, respectively. Proteins content ranged from 4.56 ± 0.04 to 6.96 ± 0.17 g/100g DW. The level of the total phenols ranged from 3.82 ± 0.33 to 5.22 ± 0.08 g/100g DW. Average anthocyanins content was 2.61 ± 0.11 and 2.98 ± 0.02 g/100g DW for dark red and bright red calyces, respectively. Calyces were found to show an antioxidant capacity of up to 300 µmolTrolox equivalent/100g. For all calyx/water ratios, the anthocyanins content increased with extraction time till 60 min and remained almost stable thereafter.The optimization of the aqueous extraction was obtained at the ratio 1/10 (calyx/water) for the production of roselle concentrated extract, using calyces size less than 250 µm,at 30°C, for one hour percolation.
Keywords: *Hibiscus sabdariffa* L, anthocyanins water, calyx/water ratio, particle size, optimization, extraction.

1. Introduction

Hibiscus sabdariffa L. is a tropical plant belonging to the Malvaceae family, known under common names as sorrel, karkade or Roselle. It is one of the most widely used plant as food, medicine and fodder in some African countries such as Burkina Faso, Senegal, Mali, Guinea and Sudan (Al-Wandawi *et al*. 1984; Abu-Tarboush *et al*. 1997; Wong *et al.* 2002; Hirunpanich *et al.* 2006; Cissé *et al.* 2009a). Roselle leaves are used to prepare sauces, whereas seeds are fermented and used as a thickening agent or flavour enhancer (Mounigan & Badrie 2007; Parkouda *et al.* 2008; Cissé *et al.* 2009a). Used as food or coloring compounds, calyces constitute the most valuable product of the plant for international market (Cissé *et al.* 2009a). Aqueous extracts of calyces are worldwide used as natural antioxidant-containing foods or beverages (D'Heureux-calix & Badrie 2004; Juliani *et al.* 2009). Phenolic compounds such as anthocyanins are interestingly reported to be the major sources of antioxidants in Roselle extracts (Tsai *et al.* 2002). Indeed, several investigations have reported that roselle calyces displayed high content in anthocyanins. Four major anthocyanins have been identified in the calyces of roselle, including delphinidin-3-sambubioside, cyaniding 3-sambubioside, delphinidin 3–glucoside and cyaniding 3–glucoside (Du and Francis 1973; Palé *et al.* 2004; Cissé *et al.* 2009b). These calyces also contained other important phenolic compound such as protocatechuic acid (Herrera-Arellano *et al.* 2004; Dickel *et al.* 2007). As stated for several polyphenols, they display diverse therapeutic properties such as antispasmodic, anthelmintic, anti-leukemia,bactericidal, antihypertensive effects and cardioprotective (Wong *et al.* 2002; Garcia *et al.* 2006; Hirunpanich *et al.* 2006; Prenesti *et al.* 2007; Cissé *et al.* 2009a). These biological properties are usually linked to antioxidant capacities of phenolic compounds. In addition, extracted phenolic compounds such anthocyanins from natural sources are currently used in the preparation of food supplements, functional food ingredients, food additives, pharmaceutical, cosmetic products, etc. Nevertheless, valorisation of the roselle by production of beverage is limited by the extraction conditions and instability of anthocyanins due to several physicochemical factors (Jackma *et al.* 1987; Mazza & Miniati 2000; Chen *et al.* 2005). Thus, upgrading the quality of the calyces extract with better functional properties will require several critical experiments including optimization of the aqueous extraction of anthocyanins. The objective of this contribution is to evaluate the influence of particle size and calyx/water ratio on anthocyanin aqueous extraction and biochemical composition during the production of the Roselle calyx juices.

2. Materials and methods

2.1. Samples preparation

Cultivars of roselle, bright and dark red calyces were obtained from the Western region of Burkina Faso. Prior to physicochemical characterization, dried calyces were ground using a porcelain mortar and pestle and then

packed into alimentary opaque bags for analysis.

2.2. Determination of physicochemical characteristics of calyx

Dry matter, protein, lipid and crude ash were determined according to official analysis methods (AOAC 2005). Quantitation of total carbohydrate was operated according to the phenol–sulphuric acid assay (AOAC 2005). The pH of extracts were measured with a glass-electrode pH meter (HI 8520, HANNA Instruments, France), directly in a mixture prepared with 10% (w/v) grounded calyces in distilled water. Total titratable acidity was determined by titration with 0.1 M NaOH to reach pH 8.1 and the results were expressed as malic acid equivalent (AFTER 2011).

The analysis of the phenol content was performed according to the Folin-Ciocalteu's method (Singleton *et al.* 1999) adapted to microtitration (Dicko *et al.* 2002). Briefly, 0.5 g dried calyx powder was suspended in 20 mL of acidified methanol (1%, v/v, HCl in methanol), for percolation. The mixture was stirred continuously for 20 min. The extract was filtered through a filter Whatman N°2 paper, in a volumetric flask and analyzed. After 30 min, the absorbance was measured at 760 nm using a multiwell plate spectrophotometer reader (Epoch, Biotek instrument Inc Highland park, USA). The results were expressed as grams of gallic acid equivalent per 100 gram on dry matter basis.

The same extract was used for quantitation of anthocyanins and for screening of antioxidant capacity. Total anthocyanin content was estimated by the pH differential method (Lee *et al.* 2005). Roughly, an aliquot of the extract was adjusted to pH 1.0 with potassium chloride buffer, pH 1.0 (0.025 M) and another aliquot to pH 4.5 with sodium acetate buffer, pH 4.5 (0.4 M). Absorbances were measured using a multiwell plate reader spectrophotometer (Epoch, Biotek instrument Inc Highland park, USA) at 510 nm and 700 nm against control blanks. Total anthocyanin content was calculated using the major anthocyanin of roselle calyces, delphinidine - 3 - sambubioside (MW = 577 g/mol) as standard. The molar extinction coefficient of delphinidine - 3 - sambubioside at 510 nm (pH 1) used for calculation was 26000 M-1cm-1(Cissé *et al.* 2009b).

The antioxidant capacity was evaluated using ABTS (2.2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) assay as described by Re *et al* (1999) and adapted to microtitration (Dicko et *al.* 2005). A stock solution of ABTS•+ radical was produced by the reaction of 7 mM ABTS with 2.45 mM potassium persulfate. This mixture was allowed to react in the dark at room temperature for 16 h before use. The stock ABTS•+ solution was daily diluted with 50 mM sodium phosphate buffer, pH 7.4, to give an absorbance between 0.7 and 0.9 at 734 nm. For the routine assay, 20 µL of diluted extract were mixed with 250 µL of ABTS•+ solution in multiwall plate and the absorbance was read after 30 min at 734 nm using a multiwall plate reader spectrophotometer. The control assays were performed by replacing the sample with distilled water or ABTS•+, with the same buffer. All tests were carried out in triplicate and results were expressed as µmoL Trolox per 100 grams on dry weight basis. All used reagents were of analytical grade.

2.3. Optimization of anthocyanin extraction

2.3.1. Extracts preparation

The scheme of extraction procedure is summarized in Figure 1. The extraction was performed at 30 ± 1°C. Different calyx/water ratios (w/v) were prepared: 1/5, 1/10, 1/15, 1/20 and 1/25. The kinetic study of the extraction of anthocyanin was carried out at 30 ± 1°C for different calyx/water ratios: 1/5, 1/20, for crushed calyces (CC) and 1/20 for whole calyces (WC). The ratios of 1/5 and 1/20 using crushed calyces (average diameter ≤ 250 microns) were used to assess the effect of the particle size of calyces during the extraction process. The ratio 1/20 with whole calyces was used as control. Crushed calyces and water were mixed in 200 ml glass bottles. For percolation, the bottles were regularly agitated manually each 10 min. To determine the effect of calyx/water ratio and the particle size on the extraction process, a mass ratio of calyx/water of 1/5 and 1/20 using whole calyces or crushed calyces was performed for 4 h. To determine the optimum calyx/water ratio in the extraction process, three different crushed calyx/water ratios including 1/10, 1/15 and 1/25 (w/v) were run for 1 h extraction time. At specific time intervals, one bottle was removed from the batch and the mixture was filtered through a filter Whatman paper N°2. The resulting aqueous extracts were either directly analyzed or stored at -30°C before analysis. Crushed calyces were obtained manually using a mortar and pestle. The particle sizes of diameter ≤ 250 microns were kept by sieving.

2.3.2. Extract analysis

All extracts were analyzed for pH, titratable acidity using standard methods (AFTER 2011). Total soluble solid was measured using a digital Atago refractometer (°Brix 0-32%, Hand-held, Japan) according to official methods (AOAC 2005). Anthocyanin concentration and the extraction yields of anthocyanins were also determined as described by Cissé *et al* (2012). The extraction yield of anthocyanin was expressed as a ratio between the anthocyanin concentration in the extract and the initial anthocyanin concentration in the calyces extracted by acidified methanol.

2.4. Statistical analysis

All experiments were done in triplicate. Data on the properties of calyces and the quality of aqueous extracts of roselle calyces were analyzed using XLSTAT "Pro. 7.5.2" database software. The Fisher and Newman-Keuls range tests were used to compare the differences among means.

3. Results and discussion

3.1. Physical characteristics and proximate composition

Some physicochemical parameters of roselle dried calyces are reported in Table 1.The titratable acidity contents were 12.66±0.15 and 15.44±0.15 g malic acid/100 g of dry matter for bright and dark red calyces, respectively. It appeared that the calyces of roselle contained high levels of organic acids. The pH values were 2.15±0.01 and 2.24±0.01 for the dark red and the bright red, respectively. Similar values have been reported for calyces of roselle from other countries (Wong *et al.* 2002, Cissé *et al.* 2009b).

Calyces had an average dry matter content of 88.79±0.08 and 87.23±0.13 for the bright red and dark red cultivars, respectively. This low water content of calyces could be correlated with water activity. Thus, dried roselle calyces should be stable product when maintained under good storage conditions.

The total proteins content were 4.56±0.04 and 3.13±0.06 g/100g DM for bright and dark red calyces, respectively. These values were lower than those reported in previous works (Babalola *et al.* 2001; Cissé 2010; Suliman *et al.* 2011). The values of total carbohydrates found in the present study are comparable of data found in literature (Morton & Roselle 1987; Wong *et al.* 2002). The crud lipid contents are similar to the values reported in previous studies (Cissé 2010). However, these levels are lower than those (2.1g/100g DM for the red calyces and 2.9 g/100g DM for the dark red calyces) reported by Babalola *et al* (2001).

Nevertheless, results obtained in the present study, with respect to proximate composition are overly comparable with previous reports (Wong *et al.* 2002; Cissé *et al.* 2009a). Results showed that, in general, there is a significant difference ($P < 0.05$) between cultivars. This difference could be explained by the variety of the roselle or can be influenced by the environment such as soil type, water photoperiod or sunlight intensity (Babalola *et al.* 2001).

Data on total phenol, anthocyanin and Trolox equivalents antioxidant capacity (TEAC) of roselle calyces are reported in Table 2. It appeared that bright red calyces showed higher total phenols and anthocyanin contents than dark red ones. However, there was no significant difference in antioxidant capacity for the two cultivars. Levels of total phenols are higher that reported (3.74 g/100g) for dark red variety in Senegal, Cissé (2010). These authors also found differences among varieties. Phenolic compounds are believed to be involved in plant growth and reproduction, protection against UV radiation, and resistance to pathogens and predators (Dicko *et al.* 2005). Thus levels of phenolic compounds are not only dependent on genotype but also on abiotic conditions such assoil composition, temperature, irrigation and photoperiodicity. Thus, there may be a great variability of these phytochemical compounds even for the same cultivar according to ecotypes. It could be interesting to notice that roselle calyces were found to have high content of total phenols than *Adansonia digitata* L. and *Tamarindus indica* L., two other polyphenolic-containing fruits consumed in Burkina Faso (Lamien-Meda *et al.* 2008).

The reported values for the anthocyanin contents (2.5 g/100g dry weight) were similar to those reported by Juliani *et al* (2009) for roselle. Comparing the anthocyanin content with other edible plants, it was found that roselle calyces displayed high levels of anthocyanin (Mazza & Miniati, 2000). TEAC of roselle calyces was higher than those of many fruits and vegetables consumed in Burkina Faso such as tamarind (220 µmol/100g), lemon (130 µmol/100g) and tomato (120 µmol/100g) (Bayili *et al.* 2011). Our findings showed that the roselle calyces are potential source of bioactive compounds and may contribute to their nutritional potential as edible fruit.

3.2. Optimization of the anthocyanin extraction

3.2.1. Extraction kinetic

Tables 3 showed the variation of pH, titratable acidity and the soluble extract of the roselle calyces according to the time and calyx/water ratios. All of extracts showed a low pH values, ranging from 2.09to 2.36 for various ratios of 1/5 CC, 1/20 CC and 1/20 WC. The titratable acidity on the other hand varied from 0.37 to 3.03 g/100 mL. Total soluble extracts were within the range of 0.20 to 8.40 °Brix. The calyx/water ratio of 1/5 CC showed the highest level of titratable acidity (3.03 ± 0.02) and total soluble extracts (8.8 ± 0.28) than the other ratios. Data showed that a reduction of calyx sizes increased total soluble extracts and titratable acidity. Reduction of the calyx sizes (average diameter ≤ 250 µm) brought the yield at 81% and 95% for total soluble extracts and titratable acidity, respectively, after 4h of extraction time using the ratio 1/20. The increase of acidity in the aqueous extracts may be governed by high levels of water soluble organic acids such as oxalic, tartaric, and malic acids, previously reported in roselle (Wong *et al.* 2002).

An increase of anthocyanins content as a function of extraction time is reported in figure 2. The ratio

1/5 CC showed the highest content of anthocyanins. As seen from the 1/5 CC extraction, the anthocyanin concentration increased at the onset of the extraction and reached the maximum value (2743 mg/L) at 30 min extraction and remained stable thereafter. Comparatively to the ratio 1/20 WC, the decrease in particle size, reduced the extraction time as showed the curve of ratio1/20 CC. Figure 3 shows that for the ratio 1/20 CC the maximum extraction yield is reached after 30 min extraction time meaning that the anthocyanin extraction is achieved after 30 minutes. The same effect was previously reported in solid-liquid extraction of antioxidants from grape byproducts (Pinelo *et al.* 2006).These authors suggested that, smaller particle size reduced the diffusion distance of the solute within the solid, thus increasing the extraction rate. In fact, solutes take a shorter time to reach the surface. Cissé et al. (2012) also reported similar results. In all cases, reduction of particle size should increase superficial are as available for mass transfer and then, increased extraction yield. The reduction of the particles size improved water diffusion, within the solid, and modified the apparent constant of equilibrium solid/liquid. Anthocyanin concentration and extraction yield of anthocyanin were significantly affected by the calyx/water ratio (Figures 2 and 3). Decreasing the calyx/water ratio (increasing water proportion) increased the anthocyanin extraction yield, but decreased the anthocyanin concentrations. Indeed, solids are completely submerged by the solvent in the ratio 1/20 CC. These conditions allowed to speed up the mass transfers between both phases (Cacace & Mazza, 2003a). Indeed, increasing of water level improve the concentration gradient and thus the rate of diffusion of the compounds from the solid to the solvent.

In order to optimize the calyx/water ratio, the extraction was performed at 30 ± 1°C with different ratios ranging from 1/5 to 1/25 using crushed calyces. Figures 4A and 4B showed the response plots for anthocyanin content and extraction yield of anthocyanins, respectively. The anthocyanin concentration obtained with the ratio 1/5 was 41%, 67%, 70% and 77% higher than the ratios of 1/10, 1/15, 1/20 and 1/25, respectively. Meanwhile, the extraction yield for the ratio 1/25 was 3% and 66% higher than ratios 1/20 and 1/5, respectively. Furthermore, as illustrated in figure 3B, there is no significant difference among the yield obtained with the ratio 1/10 and 1/15. In all the cases, an increase of the solvent proportion in the system improved the extraction yield. As the concentration of anthocyanins, increasing the amount of solvent in the system decreased the acidity and the proportion of soluble solids as evaluated by the Brix degrees (Figure 5A and 5B). Similar results were also reported on the extraction of anthocyanins from other sources such as blackcurrant (Cacace & Mazza, 2003b), *Melissa officinalis* L. leaves (Herodez *et al.*2003), grape pomace (Pinelo *et al.* 2005), and roselle calyces from Senegal (Cissé *et al.* 2012).

3.2.2. Optimum conditions

Several parameters may affect the anthocyanins diffusion, including extraction time, particle size and calyx/water ratio. Thus, the data showed that the extraction of anthocyanins was achieved as less as 1 h with calyces that were grounded to an average diameter ≤ 250 µm. The decrease of particle size to an average less than 250 µm strongly reduced the extraction time to 30 min. The main effect of the calyx/water ratio was to increase the extraction yields of anthocyanins up to a maximum corresponding to the lower calyx/water ratio. The choice of the ratio is made according to the desired end-product. In this study, the optimum levels of anthocyanins content and extraction yield of anthocyanins was achieved with the ratio 1/10. This ratio would be more suitable for the production of roselle concentrate. Nevertheless, for the preparation of roselle drinks where the yield of the extraction is the premium choice, the ratios 1/20 and 1/25 would be better for these products.

4. Conclusion

Optimization of the aqueous extraction of anthocyanins revealed that the calyx/water ratio and the size of the particles strongly affected the anthocyanin concentrations and the extraction yields. The particle size and calyx/water ratio affected either the extraction time or the extraction yield of anthocyanins, respectively. Results revealed that the red calyces are naturally acidic products and an excellent source of anthocyanins with several potential applications in the food, pharmaceutical and cosmetic industries. The acquired data can serve as a starting point to make powerful locally drinks prepared from dried calyces of *Hibiscus sabdariffa*.

Abbreviations

CC: crushed calyces
DW: dry weight
WC: whole calyces

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Table 1. Physical characteristics and Proximate composition of *Hibiscus sabdariffa* dried calyces

Characteristics	brightred calyx	darkred calyx
pH	2.24 ± 0.01^{a}	2.15 ± 0.01^{b}
[1]Titratable acidity	12.66 ± 0.15^{a}	15.44 ± 0.15^{b}
Dry matter	88.79 ± 0.08^{a}	87.23 ± 0.13^{b}
[2]Total protein	4.56 ± 0.04^{a}	6.96 ± 0.17^{b}
[2]Total lipids	0.60 ± 0.52^{a}	1.13 ± 0.04^{b}
[2]Crude ash	6.07 ± 0.05^{a}	7.89 ± 0.01^{b}
[2]Total carbohydrates	3.24 ± 0.04^{a}	3.13 ± 0.06^{b}

Values within each row with different letters are significantly different ($p < 0.05$). [1]Expressed as gram malic acid per 100g dry matter.[2]Expressed as gram per 100 grams dry matter basis (g/100g, DM).

Table 2. Total phenol content, anthocyanins, and antioxidant capacity of *Hibiscus sabdariffa* calyx cultivars

	Total phenols (g GEA/100g DM)[1]	Anthocyanins (g/100g DM)[2]	TEAC (µmol/100g)
Bright red calyx	5.22 ± 0.33a	2.98 ± 0.02a	300.13 ± 0.12a
Dark red calyx	3.82 ± 0.08b	2.61 ± 0.11b	300.02 ± 0.48a

Values within each row with different letters are significantly different ($p < 0.05$). [1]Reported as Gallic acid equivalents (GAE).[2]Reported as delphinidine-3 sambubioside. TEAC: Trolox equivalents antioxidant capacity. DM: dry matter.

Table 3. Variation of the pH, titratable acidity and total soluble solids of extracts according to time for different calyx-to-water ratios

Time (min)	Ratio 1/5 CC			Ratio 1/20 CC			Ratio 1/20 WC		
	pH	TA	TSS	pH	TA	TSS	pH	TA	TSS
10	2.21 ± 0.02	2.83 ± 0.02	8.40 ± 0.57	2.29 ± 0.01	0.81 ± 0.01	1.35 ± 0.07	2.36 ± 0.00	0.37 ± 0.03	0.20 ± 0.00
20	2.20 ± 0.02	2.84 ± 0.02	8.45 ± 0.64	2.29 ± 0.00	0.82 ± 0.01	1.65 ± 0.07	2.35 ± 0.01	0.41 ± 0.01	0.40 ± 0.00
30	2.19 ± 0.01	2.88 ± 0.01	8.60 ± 0.57	2.28 ± 0.01	0.82 ± 0.00	1.70 ± 0.14	2.33 ± 0.01	0.52 ± 0.02	0.60 ± 0.00
40	2.18 ± 0.00	2.93 ± 0.01	8.70 ± 0.42	2.27 ± 0.01	0.83 ± 0.00	1.80 ± 0.00	2.31 ± 0.00	0.59 ± 0.04	0.90 ± 0.14
50	2.18 ± 0.01	2.95 ± 0.01	8.80 ± 0.28	2.27 ± 0.00	0.83 ± 0.00	1.90 ± 0.00	2.31 ± 0.01	0.65 ± 0.01	1.00 ± 0.00
60	2.16 ± 0.01	2.96 ± 0.01	8.80 ± 0.28	2.26 ± 0.01	0.84 ± 0.00	2.00 ± 0.00	2.29 ± 0.01	0.67 ± 0.02	1.10 ± 0.14
90	2.14 ± 0.03	3.00 ± 0.01	8.80 ± 0.28	2.26 ± 0.01	0.84 ± 0.00	2.00 ± 0.00	2.28 ± 0.01	0.73 ± 0.01	1.30 ± 0.14
120	2.15 ± 0.05	3.00 ± 0.01	8.80 ± 0.28	2.25 ± 0.02	0.84 ± 0.00	2.00 ± 0.00	2.27 ± 0.01	0.74 ± 0.00	1.60 ± 0.00
150	2.12 ± 0.05	3.01 ± 0.01	8.80 ± 0.28	2.25 ± 0.01	0.84 ± 0.01	2.15 ± 0.21	2.26 ± 0.01	0.80 ± 0.03	1.60 ± 0.00
180	2.12 ± 0.04	3.02 ± 0.01	8.80 ± 0.28	2.24 ± 0.00	0.85 ± 0.00	2.20 ± 0.28	2.25 ± 0.00	0.80 ± 0.03	1.70 ± 0.14
210	2.10 ± 0.06	3.02 ± 0.01	8.80 ± 0.28	2.24 ± 0.01	0.85 ± 0.00	2.20 ± 0.28	2.25 ± 0.01	0.81 ± 0.02	1.80 ± 0.00
240	2.09 ± 0.05	3.03 ± 0.02	8.80 ± 0.28	2.23 ± 0.00	0.85 ± 0.01	2.20 ± 0.28	2.25 ± 0.01	0.81 ± 0.02	1.80±0.0 0

TA: titratable acidity, TSS: total soluble solid, CC: crushed calyces, WC: whole calyces

Figures and captions

Figure 1. Process of aqueous extracts obtained from *Hibiscus sabdariffa* calyces

Figure 2. Anthocyanin concentrations as a function of time, at 30°C for different calyx/water ratios. CC: crushed calyces, WC: whole calyces.

Figure 3. Extraction yields of anthocyanins as function of time at 30°C for different calyx/water ratios. CC: crushed calyces, WC: whole calycesFigure4. Anthocyanins content (A) and extraction yields of anthocyanins (B) as function of calyx/water ratio at 30°C with an average particle size ≤ 250 µm.

Figure 5. Anthocyanins content (A) and extraction yields of anthocyanins (B) as function of calyx/water ratio at 30°C with an average particle size ≤ 250 µm.

Figure 6. Evolution of titratable acidity (A) and proportion of soluble solids (B) according to calyx/water ratios at 30°C with an average particle size ≤ 250 µm.

Figure 1.

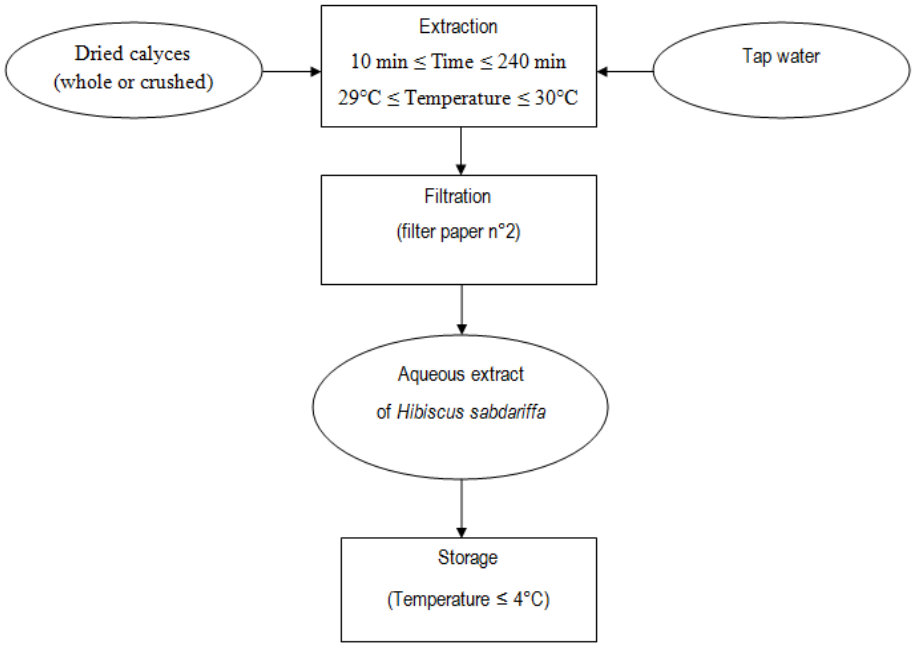

Figure 2.

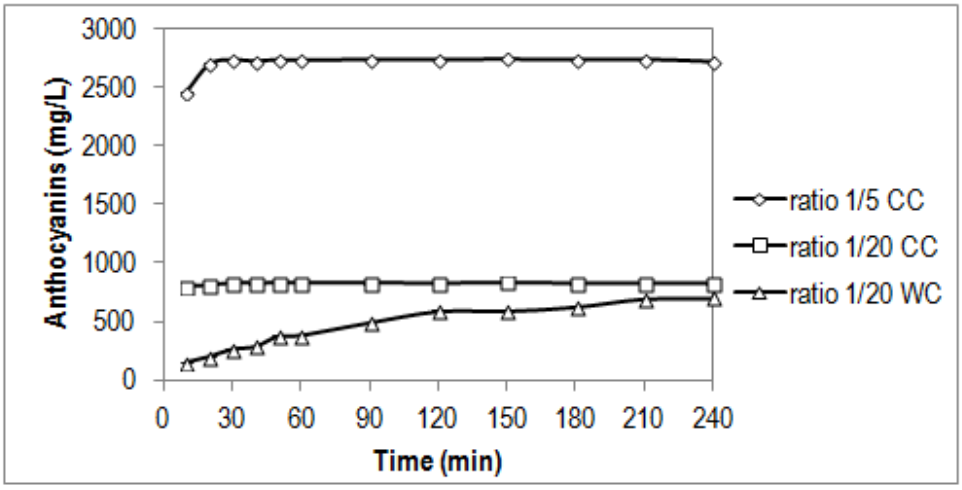

Figure 3.

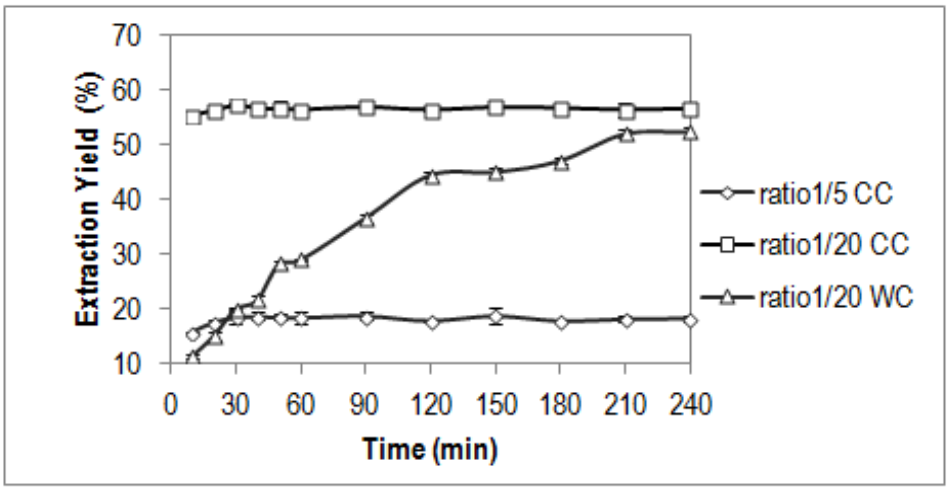

Figure 4.

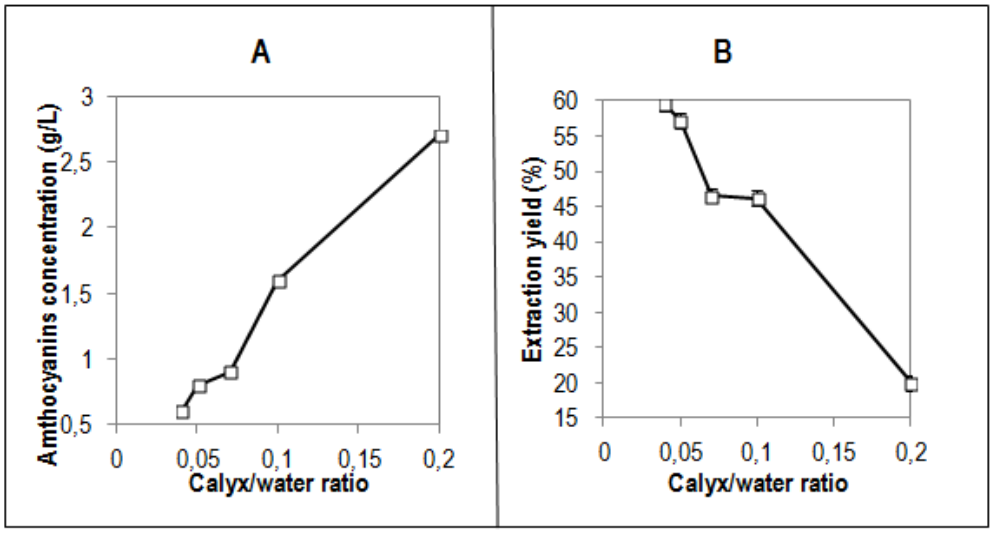

Figure 5.