ANTHOCYANIN CONTENT OF TWO HIBISCUS SABDARIFFA CULTIVARS GROWN IN SENEGAL

C. BEYE[1,2*], S. HILIGSMANN[2], L. S. TOUNKARA[1] AND P. THONART[2]

[1]Institut de Technologie Alimentaire (ITA), Route Maristes, BP 2765, Dakar, Sénégal.

[2]Wallon Center of Industrial Biology (CWBI), University of Liège, Boulevard du Rectorat, 29, B 40 - P 70, 4000 Liège, Belgium.

*Corresponding author. E-mail : cbeye@ita.sn. Tel : + 221 338 590 753. Fax : + 221 338 322 408.

ABSTRACT

Anthocyanin content of *Hibiscus sabdariffa* calyces was determined to compare two cultivars from Senegal called Koor and Vimto. Results showed a significant difference in terms of total anthocyanin content (TA) and relative abundance (RA) of anthocyanin species. Values of TA for Vimto were 3-fold higher than Koor's. Two minor anthocyanins, delphinidine-3-glucoside and cyanidine-3-glucoside, not frequently mentioned in literature, were detected in both cultivars. Cyanidin-3-glucoside in particular was present only in Koor calyces. The influence of agronomic conditions on TA and RA was highlighted.

Keywords : Anthocyanin, Chemotaxonomic markers, Cultivar diversity

RESUME

CONTENU EN ANTHOCYANES DE DEUX CULTIVARS D'HIBISCUS SABDARIFFA CULTIVES AU SENEGAL

*La concentration en anthocyanes d'*Hibiscus sabdariffa *a été déterminée dans le but de comparer deux cultivars provenant du Sénégal appelés Koor et Vimto. Les résultats montrent une différence significative entre les deux cultivars du point de vue de la concentration totale et du point de vue de la concentration relative des différents types d'anthocyanes détectés dans les calices. La concentration en anthocyanes du cultivar Vimto est trois fois supérieure à la concentration trouvée dans le cultivar Koor. Deux anthocyanes mineurs, la delphinidine-3-glucoside et la cyanidine-3-glucoside qui ne sont pas fréquemment citées dans la littérature ont été détectées dans les deux cultivars. La cyanidine-3-glucoside était présente uniquement dans le cultivar Koor. L'influence des conditions de culture sur la concentration en anthocyanes a été mise en exergue.*

Mots clés *: Anthocyanes, marqueurs chimiotaxonomiques, diversité des cultivars*

INTRODUCTION

Hibiscus sabdariffa var. sabdariffa (called *H. sabdariffa* hereafter) from malvaceae family is mostly cultivated for its calyces which are traditionally used in many countries (India, Thailand, Nigeria, Soudan, Senegal) to make soft drinks.

In Senegal the main commercial cultivars of red *H. sabdariffa* called « Vimto » originate from Sudanese (Cissé *et al*., 2008) and « Koor » varieties are native from Senegal. They are often mixed to take advantage of each other's particularity: Koor is tastier probably because of its high organic acid content, while dark red calyces of Vimto are used to give an intense red color to drinks.

Besides color of calyces, the various phenotypes *H. sabdariffa* differ mostly by the color of stems and flowers that can be either red or green, with color changes during maturation. Various *H. sabdariffa* cultivars have been previously characterized by their agro-morphological characteristics (Stevels, 1990 ; Sié *et al*., 2009). In addition, agro-morphological differentiation of various cultivars has been successfully correlated to genetic differences (Torres-Morán *et al*., 2011).

The typical red color of *H. sabdariffa* calyces is given by anthocyanins that are present in high quantity. Anthocyanins are secondary metabolites found in all plant species, mainly in flowers, but also in leaves or seeds with a specific quantitative and qualitative distribution. Due to this specificity, they are often used as

chemotaxonomic markers (Stintzing *et al.*, 2004 ; Andersen *et al.*, 2005). However, a biochemical differentiation based on anthocyanin concentration is not yet made.

The general objective was to assess the nutritional value of *H. subdariffa* varieties. More specifically, the study aimed at evidencing specific traits characterizing the two *H. sabdariffa* cultivars grown in Senegal based on their anthocyanin content.

MATERIAL AND METHODS

MATERIAL

All solvents and chemicals (Aceton, formic acid, chloroform, and acetonitril) were analytical grade, purchased from VWR International (Leuven, Belgium).

The High Performance Liquid Chromatography (HPLC) equipment used was an Agilent 1100 series HPLC system (Agilent Technologies, Massy, France). Separation was carried out on a LiChrospher® RP-18 column (5 ìm, 4.6 x 250 mm) according to Beye *et al.* (2013).

All samples of calyces of cultivars *H. sabdariffa* Vimto and Koor varieties were collected from three farmers around a village in the Louga region (North-west of Senegal) involved in a certification process as organic *H. sabdariffa* producers. Farmers were previously trained for good farming practices, received certified seeds and applied a specified production system.

METHODS

Dried calyces of Koor and Vimto were harvested from plantations grown by farmers from the same village. Dried calyces of both cultivars were received from these three different farmers in 25kg bags. Anthocyanins were extracted using a method slightly modified from Ordaz-Galindo *et al.* (1999). The extraction of *H. sabdariffa* anthocyanins was performed. HPLC analysis of anthocyanins was carried out on both cultivars and their contents compared. Extraction was carried out at room temperature to prevent deacylation or release of the aglycone group that might occur at high acid content and elevated temperature conditions as it was evidenced by few authors (Revilla *et al.*, 1998 ; Da Costa *et al.*, 2000).

H. sabdariffa samples were ground in a stainless steel blender (Waring, Marne la vallée, France). About 2.5 grams of sample were accurately weighed in an Erlenmeyer flask with 30 mL acetone. The flasks were stirred for 60 min. The acetone fraction was collected and 30 mL of 70/30 acetone/acidified water (4 % formic acid) mixture were added to the flask and stirred for 60 min. The latest operation was repeated five times and the five acetone/water fractions were collected and pooled. Five extractions were necessary to achieve a colorless supernatant. An aliquot of the acetone/water anthocyanin extract was submitted to a liquid-liquid extraction with 3 volumes of chloroform in a separating funnel. An aliquot of the chloroform fraction was collected and solvents evaporated under vacuum at 40°C in a Büchirotavapor.

Identification was made using standards of Delphinidin-3-sambubioside (D3S), cyanidine-3-sambubioside (C3S), cyanidine-3-glucoside (C3G) and delphinidine-3-glucoside (D3G) (Polyphenols Laboratories AS, Sandnes, Norway). D3S and D3G were quantified using a calibration curve for D3S standards in a concentration range of 25 - 2500 mg/l. C3S and C3G were quantified using a calibration curve for C3S standards in a concentration range of 25 - 2500 mg/l. Anthocyanin concentration was expressed in mg anthocyanin per gram of dry calyces.

Relative abundance of the major anthocyanins (D3S and C3S), defined as the ratio of their individual concentrations to the sum of their concentrations was calculated.

STATISTICAL ANALYSIS

The « farmer » parameter, i.e. the variability due to agricultural operating conditions was considered in this study as a global parameter including the soil, climatic conditions and agricultural techniques.

The data were subjected to analysis of variance to determine differences, with Duncan's multiple range tests used to separate the means at 5 % confidence level. Two-way ANOVA was performed on the experimental data in both cultivar and farmer factors.

RESULTS

Delphinidine-3-sambubioside (D3S) (peak 1) and cyanindin-3-sambubioside (C3S) (peak 3) were quantitatively the main anthocyanins identified in both cultivars (Figure 1).

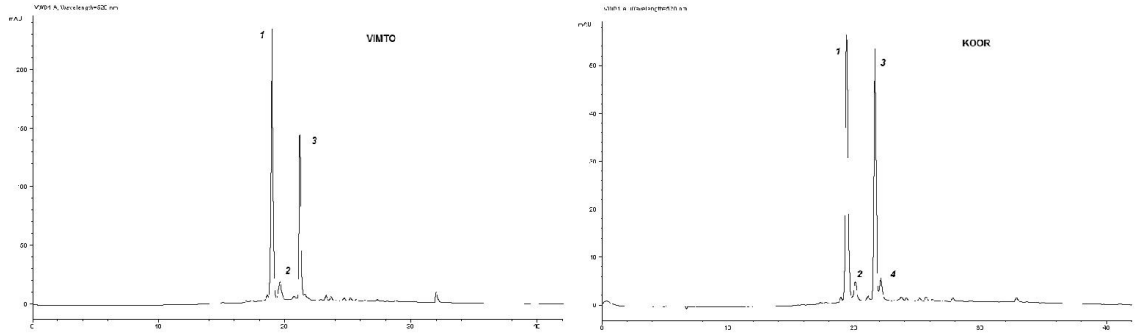

Figure 1 : HPLC chromatogram of anthocyanins from h. sabdariffa calyces extracts.

Chromatographie par HPLC des extrais de calices H. sabdariffa.

Legend : Vimto and Koor are local names of h. sabdariffa cultivars. Peaks were identified as (1) delphinidine-3-sambubioside, (2) delphinidine-3-glucoside, (3) cyanidin-3-sambubioside, (4) cyanidin-3-glucoside.

Légende Vimto et Koor sont les noms locaux des deux cultivars étudiés. Les pics identifies sont les suivants : (1) delphinidine-3-sambubioside, (2) delphinidine-3-glucoside, (3) cyanidin-3-sambubioside, (4) cyanidin-3-glucoside

Two minor anthocyanins, delphinidine-3-glucoside and cyanidine-3-glucoside were detected in both cultivars : Peak 2, identified as delphinidine-3-glucoside (D3G) was found in both cultivars at concentrations ranging from 1.0 to 1.8 mg/g.

Peak 4 (C3G) was not detected in Vimto samples but was detected in all Koor samples at an average concentration of 0.3mg/g.

Table 1 shows anthocyanin content (mg/g of calyces) of two cultivars (Vimto and Koor) of red *H. sabdariffa* var. *sabdariffa.* The results show that the specific anthocyanin are Delphinidine-3-sambubioside (D3S), Delphinidine-3-glucoside (D3G), Cyanindin-3-sambubioside (C3S) and Cyanindin-3- glucoside (C3G) respectively.

Table 1 : Anthocyanin content (mg/g of calyces) of two cultivars (Vimto and Koor) of red *H. sabdariffa* var. *sabdariffa.* All results are expressed on a dry weight bases. Mean of 4 determinations ± sdv.

Contenu en anthocyanes de deux cultivars d'H. sabdariffa. Tous les résultats sont exprimés par rapport au poids sec des échantillons de calice. Moyenne de 4 déterminations ± écart type.

Cultivar	D3S (mg/g)	D3G (mg/g)	C3S (mg/g)	C3G (mg/g)	Total anthocyanins (TA in mg/g)
Vimto	9.19 ± 2.08	1.38 ± 0.37	4.72 ± 1. 05	0.00	15.29 ± 3.47
Koor	3.54 ± 0.29	0.47 ± 0.05	2.52 ± 0.24	0.34 ± 0.02	6.88 ± 0.45

Delphinidine-3-sambubioside (D3S), Delphinidine-3-glucoside (D3G), Cyanindin-3-sambubioside (C3S) and Cyanindin-3- glucoside (C3G).

In addition to the qualitative difference, a quantitative difference could also be noticed between these two *H. sabdariffa* cultivars. Vimto samples had an average TA value (15.3mg/g) significantly higher ($p < 0.05$) than Koor (6.9 mg/g) with D3S as the most abundant in both.

The results of relative abundance of the major anthocyanins (D3S and C3S), defined as the ratio of their individual concentrations to the sum of their concentrations, were presented in Table 2. Results show a significant difference ($p < 0.05$) between Koor and Vimto, the proportion of D3S (66.0 %) being significantly higher ($p < 0 .05$) in Vimto calyces.

TABLE 2 : Relative abundance (ra) of *H. sabdariffa* major anthocyanins : Delphinidine-3-sambubuoside (D3S) and Cyanidine-3-sambubioside (C3S). Samples are calyces of two red *H. sabdariffa* var. *sabdariffa* cultivars (Vimto and Koor).

Abondance relative des deux principaux anthocyanes d'H. sabdariffa : Delphinidine-3-sambubuoside (D3S) et Cyanidine-3-sambubioside (C3S). Les échantillons de calices proviennent de deux cultivars appelés Vimto et Koor.

Cultivar	D3S ± RSD	C3S ± RSD
Vimto	66.0 ± 0.7	34.0 ± 1.4
Koor	58.4 ± 4.5	41.6 ± 6.3

DISCUSSION

Usually, anthocyanins are extracted with acidified water or polar solvents (Lee *et al.,* 1992; Sukwattanasinit *et al.*, 2007; Castañeda-Ovando *et al.*, 2009) leading to non-specific extraction of all compounds soluble in those conditions. In this study, acetone/acidified water was used as it is recommended when samples contain high amounts of pectin (Garcia-Viguera *et al.*, 1998), which is the case of *H. sabdariffa* calyces (Muller *et al.*, 1992). The extraction procedure allowed a complete extraction of anthocyanins while avoiding some interfering compounds such as soluble polysaccharides or proteins contained in the calyces. The subsequent liquid/liquid separation procedure allowed further purification of anthocyanins. These technical approaches should reduce the influence of environmental conditions and agricultural practices on plant's characteristics (Torres-Moran *et al*., 2011). For the HPLC analysis, samples were maintained in acidic medium as anthocyanins are majorly in their flavylium form at low pH values (96 % at pH = 1.5) allowing a maximum absorbance of anthocyanins at 520 nm. For this reason, the column used in the HPLC analysis was chosen for its ability to operate in wide range of pH including acidic region.

Comparison of total anthocyanin (TA) content showed a significant difference between Vimto calyces (15.5 mg/g) and Koor calyces (6.8 mg/g). The anthocyanins of *H. sabdariffa* calyces have already been studied by other authors who reported values from 0.02 mg/g to 25 mg/g (Du *et al.*, 1973 ; Badreldin *et al.*, 2005 ; Christian *et al.*, 2009). These results are rather different from a quantitative standpoint and can be hardly compared to our results as both samples (cultivar is not always specified) and extraction methods were different. In addition, two anthocyanins not always mentioned by authors were identified : Peak 2, identified as delphinidine-3-glucoside (D3G) was found in both cultivars at concentrations ranging from 1.0 to 1.8 mg/g. It was formerly reported by Sukwattanasinit *et al*. (2007) in *H. sabdariffa* samples from Thailand and by Juliani *et al.* (2009) in samples from Senegal.

A high variability was noticed in Vimto samples, with values ranging from 12.5 to 19.8 mg/g depending on the farmer. While values for Koor expressed a restricted variability with values ranging from 6.4 to 7.5 mg/g. Results of 2-way ANOVA performed on the experimental data showed a significant effect of both factors (cultivar and farmer) on TA. Such variability was also noticed by Khafaga *et al.* (1980) in five *H. sabdariffa* cultivars, including a sample from Senegal, where TA values of 17 mg/g to 25 mg/g were reported. Authors noticed that maximum anthocyanin concentration was reached 30 to 50 days after flowering, depending on the cultivar. For this reason, since *H. sabdariffa* flowers do not ripen at the same time, the harvest of calyces should be performed gradually (from bottom to top of the plant) as suggested by various authors (Plotto, 1999 ; Mohamed *et al.,* 2012) in order to achieve the highest concentration and a uniform quality in terms of anthocyanin content.

This suggests that recommended agricultural and harvesting practices may have been applied in a different manner by farmers. The recommended gradual harvesting procedure, being tedious and time consuming, may not be applied strictly by farmers. Indeed, in Senegal calyces are traditionally collected 2 to 3 weeks after flowering (Cissé *et al.*, 2008) by harvesting the whole plants at once or after flower has dropped (Juliani *et al.*, 2009).

In addition, Vimto and Koor have different growing cycles ; Vimto, 140 days and Koor, 120 days (Cissé *et al.*, 2008), hence, should be cultivated and harvested distinctively.

Relative abundance (RA) of major anthocyanins seems to be specific to each cultivar. For Vimto D3S accounted for 66.0 % of TA while in Koor, the same compound accounted for 58.4 % of TA. Analysis of variance showed significant difference of anthocyanin's RA due to both cultivar and field factors.

The interaction of these two factors was significant showing the effect of the cultivation conditions on anthocyanins' relative abundance. Both factors (TA content and RA) appear to be specific to each *H. sabdariffa* cultivar and could be used as distinctive criteria. Similarly, anthocyanin concentration and relative distribution have been proven to be specific to each cultivar in studies carried out on other vegetal species such as potato (Lachman *et al*. 2009 ; Ieri *et al*., 2011), grape (Flamini *et al*., 2013) or basil (Flanigan *et al*., 2014).

CONCLUSION

The results of this study showed a significant difference between the main cultivars of *H. sabdariffa* in terms of total anthocyanin and relative abundance. Anthocyanin content of Vimto was 3-fold higher than Koor cultivar. Two minor anthocyanins (D3G and C3G) that are not frequently mentioned in the literature were detected in both cultivars with a specific distribution depending on the cultivar. Cyanidin-3-glucoside in particular was present only in the Koor cultivar. Relative abundance of the major anthocyanins along with total anthocyanin content could be valuably used as distinctive parameters between cultivars. They could also be used as indicators of farmer's respect of agricultural and post-harvest handling recommendations.

ACKNOWLEDGEMENT

We acknowledge financial support of the Walloon Region (Belgium) within the framework of the bilateral cooperation programs between Wallonie - Bruxelles - International (Belgium) and Senegal and the Centre d'Etude Régionale pour l'Amélioration de l'Adaptation à la Sécheresse (CERAAS) through The West Africa Agricultural Productivity Program (WAAPP).

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