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Thermal Stability of Anthocyanin Extract of *Hibiscus sabdariffa* L. in the Presence of β-Cyclodextrin

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The thermal stability of anthocyanin extract isolated from the dry calyces of *Hibiscus sabdariffa* L. was studied over the temperature range 60−90 °C in aqueous solutions in the presence or absence of β-cyclodextrin (β-CD). The results indicated that the thermal degradation of anthocyanins followed first-order reaction kinetics. The temperature-dependent degradation was adequately modeled by the Arrhenius equation, and the activation energy for the degradation of *H. sabdariffa* L. anthocyanins during heating was found to be ∼54 kJ/mol. In the presence of β-CD, anthocyanins degraded at a decreased rate, evidently due to their complexation with β-CD, having the same activation energy. The formation of complexes in solution was confirmed by nuclear magnetic resonance studies of β-CD solutions in the presence of the extract. Moreover, differential scanning calorimetry revealed that the inclusion complex of *H. sabdariffa* L. extract with β-CD in the solid state was more stable against oxidation as compared to the free extract, as the complex remained intact at temperatures 100−250 °C where the free extract was oxidized. The results obtained clearly indicated that the presence of β-CD improved the thermal stability of nutraceutical antioxidants present in *H. sabdariffa* L. extract, both in solution and in solid state.

KEYWORDS: Hibiscus extract; cyclodextrin; nuclear magnetic resonance; inclusion complex; natural antioxidants; anthocyanins

INTRODUCTION

Hibiscus sabdariffa L. (roselle) is an annual shrub cultivated in most cases to consume its aqueous calyx extract tea for therapeutic reasons. A roselle infusion, commonly mixed with some sweetener, is an intensely red transparent liquid that exhibits a very low pH and an astringent flavor (*1*). The importance of the roselle calyx resides mainly in its anthocyanin content (2.52 g/100 g) (*2*). The extract of red calyx mainly contains two anthocyanins, delphinidin-3-sambubioside and cyanidin-3-sambubioside (**Figure 1**) (*3*).

The pharmacological actions of the calyx extracts include strong in vitro and in vivo antioxidant activities (*4, 5*). On the basis of animal models, it has been suggested that the extract from the dried calyx of roselle can be used to inhibit the oxidation of low-density lipoproteins and to prevent various types of hyperlipidemia (*6, 7*). It also exhibits antihypertensive and antipyretic activities as well as cardioprotective effects (*8*), reduces serum cholesterol (*4, 8*), and protects the primary hepatocytes against oxidative stress (*9*). A strong antihypertensive action has been demonstrated in humans (*10*). Therefore, the roselle calyx could be considered as a functional food or as

	MW	R_1	R_2
1:	597.3	OH	sambubiose
2:	581.2	H	sambubiose
3:	625.2	OH	rhamnose-ferulic acid

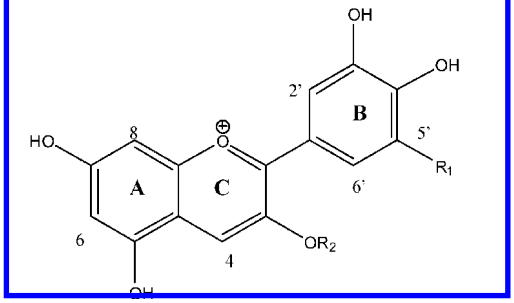

Figure 1. Anthocyanins of *H. sabdariffa* L. identified by liquid chromatography−mass spectrometry (LC-MS).

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a source of nutraceutical constituents, as daily consumption of its extract can be beneficial to human health (*1*).

Anthocyanins are not stable, and their degradation can be affected by many factors, such as temperature, oxygen, enzyme, light, and time, during processing and storage (*9*). Previous investigations emphasized the effect of temperature and time on anthocyanin degradation (*9, 11, 12*). However, the degradation kinetics of the roselle extract in the presence of β-cyclodextrin (β-CD) has not been previously reported.

Cyclodextrins (CDs), formed by the enzymic modification of starch (*13*), are cyclic D-glucopyranose oligomers having six, seven, or eight glucose units linked by 1,4-α-glucosidic bonds, termed as α-, β-, and γ-CDs, respectively. The hollow molecular shape allows them to form inclusion complexes with a wide variety of organic compounds, which enter partly or entirely into their relatively hydrophobic cavities, simultaneously expelling the few high-energy water molecules from the interior. The cavity size of the CDs offers selectivity for the complexation of guest molecules (*14*). The physical, chemical, and biological properties of molecules, which are encapsulated by CDs, may be thus modified drastically (*14, 15*). The encapsulation may lead to dissolution rate enhancement, increased membrane permeability, and bioavailability of low solubility nutraceuticals. CDs may also act as flavor carriers and provide protection against oxidation, light-induced decompositions, and heat-induced changes. Moreover, CDs may prolong the shelf life of food products and mask or reduce undesired taste and odor (*16*).

During the cooking process, the chemical composition of the calyx and consequently its antioxidant properties may change. However, until now, no methods of general application have been proposed to increase the stability of anthocyanins during thermal treatment. In this study, the stability of anthocyanins of *H. sabdariffa* L. extract was increased in aqueous solutions in the presence of β-CD due to complexation. In the solid state, the constituents of roselle were protected against oxidation as determined by oxidative differential scanning calorimetry (DSC). The encapsulated *H. sabdariffa* L. extract in β-CD could result in a product that can be used readily as a food ingredient, in a better way than *H. sabdariffa* L. extract alone, due to its protection from heat and oxidation.

MATERIALS AND METHODS

Samples, Standards, and Solvents. β-CD was purchased from Aldrich (Steinheim, Germany). *H. sabdariffa* L. extract was provided by the Department of Food Quality and Chemistry of Natural Products, Mediterranean Agronomic Institute of Chania (M.A.I.Ch.), using a previously published extraction procedure (*3*). Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Aldrich. Methanol, high-performance liquid chromatography (HPLC) grade, was obtained from Merck (Darmstadt, Germany).

LC-MS. A Finnigan MAT Spectra System P4000 pump coupled with a UV6000LP diode array detector and a Finnigan AQA mass spectrometer were used. Analyses were carried out on a Superspher RP-18, 125 mm × 2 mm, 4 mm column (Macherey-Nagel, Germany), protected by a guard column packed with the same material, and maintained at 40 °C. Samples were analyzed by employing electrospray ionization (ESI) at the positive mode, with acquisition set at 12 eV, capillary voltage of 4.90 kV, source voltage of 50 V, detector voltage of 650 V, and probe temperature of 450 °C. For the development of the chromatograms, an acetic acid (2.5%) (A)−MeOH (B) gradient was used for 45 min. The gradient used was as follows: 0−5 min, isocratically 100% A; 5−40 min, gradient from 100 to 0% A (100% B); 40−45 min, isocratically 100% B. The flow rate was 0.33 mL/min, and monitoring of the eluent was performed at 520 nm.

Gas Chromatography−Mass Spectrometry (GC-MS) of *H. sabdariffa* L. Extract. An Agilent HP series GC 6890N (Wallborn, Germany), coupled with a HP 5973 MS detector (EI, 70 eV), split-splitless injector, and an HP 7683 autosampler were used. Prior to GC analysis, the hibiscus extract was diluted 1:100 v/v in methanol, and 0.05 mL was mixed with internal standard 3-(4-hydroxyphenyl)-1-propanol (50 μL, 19.2 μg/mL), evaporated to dryness under nitrogen, and derivatized by the addition of 250 μL of BSTFA at 70 °C for 20 min. An aliquot (1 μL) of derivatized sample was injected into the gas chromatograph at a split ratio 1:20. Analysis of the sample was achieved using an HP-5 MS capillary column (5% phenyl−95% methyl siloxane, 30 m × 0.25 mm × 250 μm). The chromatographic conditions applied are described elsewhere (*17*). A selective ion monitoring (SIM) GC-MS method was applied for detection of 25 target polyphenolic compounds. Detection of polyphenols was based on the ±0.05 RT presence of target and qualifier ions of the standard polyphenols at the predetermined ratios. Target and qualifier ions for 25 polyphenolic compounds and the internal standard are described elsewhere (*17*). Linearity was obtained for all target compounds detected in samples in the range of quantitation limit and up to 20 times higher concentrations of each compound.

Thermal Degradation Studies. The thermal degradation of anthocyanins extracted from *H. sabdariffa* was studied in a citrate buffer solution (pH 4, which is typical to products that contain anthocyanins) at temperatures of 60−90 °C. Aliquots of 1 mL of the solution (0.01% w/v, prepared by 5 mg of extract dissolved in 50 mLof deionized water) were placed into Eppendorf tubes already equilibrated in a thermostatic water bath at 60, 70, 80, and 90 °C. The concentration of anthocyanins in the solution was about 45 μM. At predetermined intervals, sample tubes were removed from the water bath and rapidly cooled at −40 °C. Before analysis, each sample was allowed to come to room temperature (23−25 °C). The anthocyanin content of the samples was measured spectrophotometrically (Specord 20, Analytikjena, Jena, Germany). The thermal degradation of anthocyanins in the presence of β-CD was studied by diluting β-CD, at a molar ratio of hibiscus anthocyanins/β-CD 1:1, in the initial buffer solution, followed by the same thermal treatment and analysis as previously described.

Determination of Anthocyanins. The total anthocyanin content was determined by using the pH-differential method described by Lee, Durst, and Wrolstad (*18*), using two buffer systems: potassium chloride buffer, pH 1.0 (0.025 M), and sodium acetate buffer, pH 4.5 (0.4 M). Aliquots (0.2 mL) of the thermally treated samples were mixed with 0.8 mL of the corresponding buffer, and the absorbance was measured at 510 and 700 nm. Total anthocyanins were calculated using the following equation:

$$\text{total anthocyanins (mg/L)} = A \cdot \text{MW} \cdot \text{DF} \cdot 1000/(\varepsilon \cdot 1) \quad (1)$$

where $A = (A_{510} - A_{700})_{\text{pH=1.0}} - (A_{510} - A_{700})_{\text{pH=4.5}}$, MW = molecular weight, DF = dilution factor, 1 = path length in cm, ϵ = 26900 molar extinction coefficient in L/mol/cm for cyanidin-3-glucoside, and 1000 = conversion from g to mg.

Complexation with β-CD. The hibiscus extract (0.037 g) was dissolved in 10 mL of aqueous solution of β-CD (16 mM) at an approximate molar ratio of 1:2 (calculations were based on the molecular weight of two major anthocyanins, since these compounds were the main constituents of the hibiscus extract) and left under stirring for 24 h at room temperature. Subsequently, the mixture was frozen at −40 °C for 24 h and then lyophilized in a freeze dryer (Telstar, Cryodos, Terrassa, Spain).

Study of the Decomposition of Hibiscus Extract and Its β-CD Complex by DSC. DSC studies using DSC equipment (DSC-6, Perkin-Elmer, Boston, MA) were performed to evaluate the stability against thermal oxidation of the samples. Approximately 2 mg of extract or a quantity of the complex of hibiscus extract with β-CD containing the same amount of extract was placed in aluminum pans with a hole in their lid. The specimens were heated from room temperature to 40 °C in oxygen atmosphere at a heating rate of 90 °C/min. Samples remained at 40 °C for 1 min, to ensure an homogeneous temperature distribution within the sample, and then were heated up to 360 °C at a heating rate of 10 C°/min.

Nuclear Magnetic Resonance (NMR) Spectroscopy. ^{1}H NMR spectra were acquired on a Bruker Avance DRX spectrometer at 500

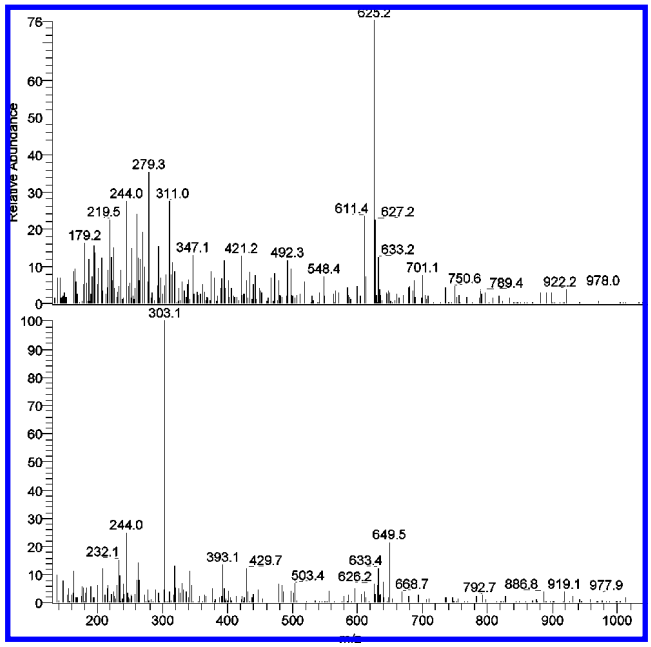
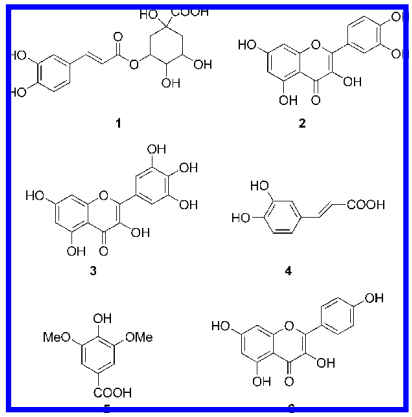


Figure 2. Positive mode ESI mass spectrum of the newly identified minor (7%) anthocyanin component. Upper and lower spectra were obtained at 12 and 50 eV, respectively.

Table 1. Polyphenolic and Terpenic Acids Species in the *H. sabdariffa* L. Extract Identified and Quantified by GC-MS



compound	(mg/g *H. sabdariffa* L. extract)
chlorogenic acid (**1**)	46.00
quercetin (**2**)	8.71
myricetin (**3**)	5.21
caffeic acid (**4**)	1.79
syringic acid (**5**)	1.61
campherol (**6**)	1.44
gallic acid	0.71
sinapic acid	0.49
ursolic acid	0.20
ferulic acid	0.19
p-coumaric acid	0.16
protocatechuic acid	0.14
epicatechin	0.13

MHz in unbuffered D_2O solutions at 298 K. For the analysis of the dry extract alone, extract (3 mg) was dissolved in D_2O (1 mL), resulting in a completely clear dark red solution (pH 3.1). For the complexation studies, 5 mg of dry extract was added (roughly calculated 1 equiv) to a solution (1 mL) of β-CD in D_2O (10 mM), without change of color. The one-dimensional spectra of both solutions were run with FID resolution of 0.18 Hz/point. The residual HDO line had a line width at half-height of 2.59 Hz. Two-dimensional (2D) ROESY spectra were acquired at 298 K with presaturation of the residual water resonance and a mixing (spin-lock) time of 350 ms at a field of ~2 kHz, using the TPPI method, using a 1024 K time domain in F2 (FID resolution 5.87 Hz) and 460 experiments in F1. Processing was carried out with zero-filling to 2K in both dimensions using sine (F2) and qsine (F1) window functions, respectively.

Statistical Analysis. All DSC experiments were duplicated. All analyses were performed in triplicate ($n = 3$). The results presented are the average of the obtained values. Data analysis was carried out using Microsoft Excel.

RESULTS AND DISCUSSION

Anthocyanins and Polyphenols in *H. sabdariffa* L. Extracts. Based upon the absorbance at 510 nm, the average concentraction of anthocyanins in hibiscus extract was found 275 mg per gram of extract. The presence of chlorogenic acid in the extract (copigmentation) is not anticipated to affect the stability, as was shown recently in copigmentation studies (*19*). LC-MS analysis was used in order to determine anthocyanins, the major constituents that are present in *H. Sabdariffa L.* extract. Identification and quantification of anthocyanins by LC-MS showed the existence of two major anthocyanins with retention times of 10.39 and 11.67 min respectively. Based on mass spectrometric data these anthocyanins were identified as delphinidin 3-O-(2''-xylosyl)-glucoside **1** (**Figure 1**) and cyanidin 3-O-(2''-xylosyl)-glucoside **2** (**Figure 1**) with molecular weights 597.3 and 581.2, respectively. These results were in agreement with those previously reported (*3*). The two predominant anthocyanins comprise 64% (176 mg/g extract) and 25% (68 mg/g extract), respectively, of the total anthocyanins in *H. Sabdariffa L.* extract. Moreover, a third anthocyanin with a retention time of 13.32 min, comprising 7% of the total anthocyanins of the extract was identified. The mass spectrum of this minor component showed the base peak at m/z=625.2, when the ESI MS spectrum was measured at low voltage (**Figure 2**). This peak was not observed at high voltage, whereas the base peak appeared at m/z=303.1. The latter corresponds to the delphinidin aglycon. The peak at m/z=625.2 can thus be the molecular ion peak and can be tentative assigned to delphinidin-3-(feruloyl)rhamnoside. Wu and Prior (*20*) reported that ferulic acid is one of the five aromatic acids that can be found in plant anthocyanins. Furthermore rhamnose is one of the most common sugars that can be present in the chemical structure of a plant anthocyanin. The polyphenol content of the extract was also determined by GC/MS (**Table 1**). Based on the results of GC/MS analysis the *H. Sabdariffa* L. extract was found to contain chlorogenic acid, quercetin, myricetin, caffeic acid, syringic acid, campherol, gallic acid, sinapic acid, ursolic acid, ferulic acid, p-coumaric acid, protocatechuic acid and epicatechin.

Thermal Degradation Kinetics. The relative concentrations of anthocyanins in solutions during incubation at 60°, 70°, 80°, 90 °C and in solutions containing β-CD at a mole ratio 1:1 (calculations were based on the molecular weight of delphinidin-3-sambubioside and cyanidin-3-sambubioside, since these compounds were the main constituents of the roselle extract) are plotted versus time (**Figure 3**).The linear relations apparently indicate that the degradation of anthocyanins follows a first-order reaction kinetics both in aqueous solutions and in aqueous solutions containing the β-CD. Previous studies showed that the degradation of anthocyanins in aqueous solutions (*9, 11, 12, 19*) as well as during dry heating (*21*) also follows a first order degradation kinetics (*9, 11, 12, 19*). The reaction rate constants (k) and half-lives ($t_{1/2}$, the time needed for 50% degradation of anthocyanins), were calculated by the following equations:

$$\ln(C/C_0) = -kt \quad (2)$$

$$t_{1/2} = \ln 0.5/k \quad (3)$$

Where $C/C_0 = A/A_0$, A_0 is the initial absorbance of diluted extract and A is the absorbance value after t min incubation at a given temperature (*22*).

As expected, the degradation of anthocyanins increased with increased heating temperature and time. The rate of degradation decreased in the presence of β-CD as it can be seen by the values of the degradation rate constant in **Table 2**. Stabilization of anthocyanins was possible due to the complexation with β-CD, as CDs may protect nutraceutical molecules containing phenolic groups (*23, 24*). β-CD protects sensitive molecules either by providing a basic or hydrophobic environment. The degradation studies were carried out in acidic pH, so the protection was offered by the hydrophobic cavity of β-CD. Charged species are energetically unstable in hydrophobic environments so complexation generally decelerates chemical degradations, in which a highly charged intermediate is involved in the transition state (*25*). Comparison of $t_{1/2}$ values (**Table 2**) revealed that anthocyanins were more resistant in the presence of β-CD, as the presence of β-CD nearly doubled the half-time values.

The Arrhenius model was applied to describe the temperature dependence of anthocyanins degradation.

$$k = k_0 \cdot e^{-Ea/RT} \quad (4)$$

The activation energies of degradation in the absence or in the presence of β-CD, as calculated by the Arrhenius plot, were nearly the same 54.05 and 54.02 kJ·mole^{-1}, respectively. Similar values have been reported for the degradation of pure anthocyanins during dry heating (*21*). It is possible that the degradation mechanism of anthocyanins inside the β-CD and that of the free anthocyanins are the same. These E_a values were lower than values reported for juices or concentrates containing anthocyanins (*11*). The degradation of anthocyanins occurred at a faster rate in a concentrate than in a buffer solution. Evidently, when a product is concentrated, the reacting molecules become closer, thus the rate of the chemical reaction accelerates (*11*).

In **Figure 4** the DSC oxidation curves of hibiscus extract and of the inclusion complex of hibiscus extract with β-CD as a function of temperature and time are given. An exotherm was initiated at 210 °C for hibiscus extract related to the oxidation anthocyanins. The exothermic peak was not present in the DSC scan of the complex hibiscus extract/β-CD, which means that the anthocyanins, the major constituents of hibiscus extract, were protected from oxidation due to complexation with β-CD.

NMR Studies on the *H. sabdariffa* L. Extract and Evidence for Complexation in Solution via Inclusion and External Interactions. The clear dark red solution of the extract in deuterated water was mildly acidic (pH 3.1), similar to the solution used for the thermal degradation studies (pH 4), therefore citrate or acetate buffers were not used to avoid undesired residual signals. The ^{1}H spectra, examined after the completion of the 2D experiments (15−24 h) were unchanged. The ^{1}H NMR spectrum of the extract (**Figure 5**) revealed

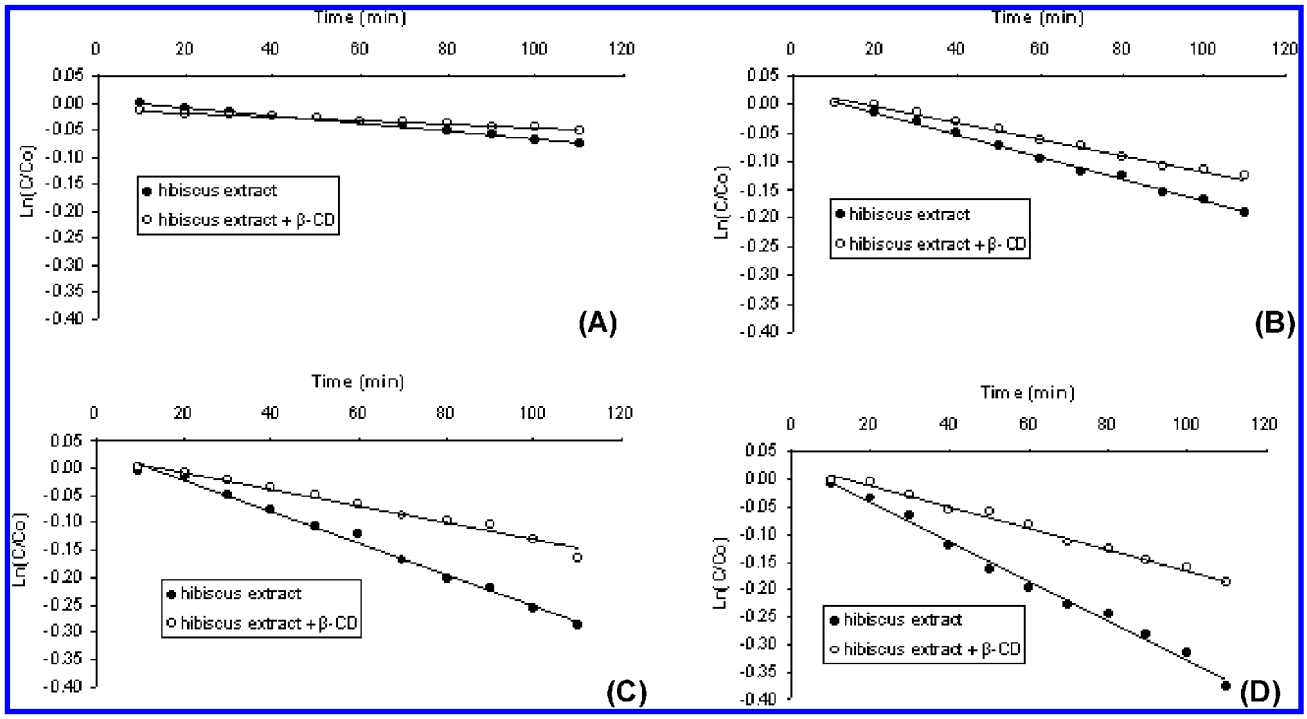


Figure 3. Degradation of anthocyanins in aqueous solutions and in aqueous solutions containing β-CD at temperatures of (**A**) 60, (**B**) 70, (**C**) 80, and (**D**) 90 °C.

Table 2. Degradation Kinetics Parameters of Anthocyanins Extract or Anthocyanins Extract + β-CD

	k ($\times 10^{-3}$ min^{-1})		$t_{1/2}$ (h)		R^2	
temperature (°C)	free anthocyanins	anthocyanins + β-CD	free anthocyanins	anthocyanins + β-CD	free anthocyanins	anthocyanins + β-CD
60	0.7	0.3	16.50	38.51	0.9893	0.9877
70	1.9	1.4	6.08	8.25	0.9957	0.9896
80	2.9	1.6	3.98	7.22	0.9918	0.9770
90	3.6	1.9	3.21	6.08	0.9915	0.9905

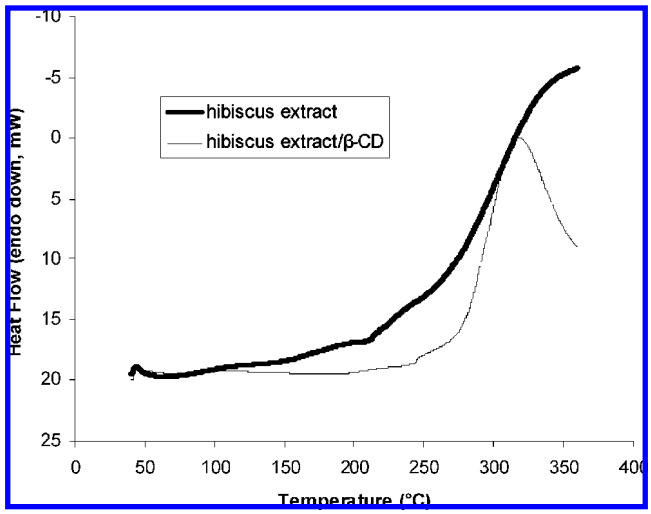


Figure 4. DSC thermograms of *H. sabdariffa* L. extract and its complex with β-CD under oxidative conditions.

resonances in the entire ppm range, i.e. aromatic and double bond area of (8.5−6.0 ppm), carbohydrate area (5.4−3.0 ppm), and aliphatic area (2.5−0.5 ppm). Aromatic signals were expected for the LC-identified delphinidin and cyanidin cores as well as the GC-identified hydroxylated cinnamic acid derivatives (chlorogenic, caffeic, ferulic, e.t.c acids) and polyphenols (quercetin, myricetin, e.t.c.). Carbohydrate signals were expected from the results of LC analysis (sambubiose of **1** and **2**, proposed rhamnose for **3**), whereas the presence of free mono- or disaccharides cannot be excluded. Among the various monosaccharides reported (*20*) as associated with anthocyanins, the only one that displays signals in the aliphatic region is rhamnose (CH_3 group at ~1.2 ppm). The equilibrium forms of anthocyanins present (*26*) at this pH are the flavylium cation red form, the hydrated hemiacetals and the colorless chalcone forms, and the blue quinonoidal base form. The vast majority of the reported NMR spectra of anthocyanins are in trifluoroacetic acid/methanol (*27*−*29*) and trifluoroacetic acid/DMSO (*30*) or trifluoroacetic acid/D_2O (*31*). Apparently at very low pH the flavylium cation prevails in the equilibrium mixture and the spectra are simplified. On the other hand, it is known that in natural aqueous solutions additional association phenomena are encountered (*32*) such as self-association of anthocyanins (usually at concentrations 10^{-2} or 10^{-3} M), formation of molecular complexes with other natural components (e.g., the molecular complex malvidin/chlorogenic acid, K_{assoc} = 350 M^{-1}) or complexes with metal ions. It follows, therefore, that the present solution with a 6.1 mM concentration of extract, intermediate pH and existence of all components identified in LC- and GC-MS analyses as well as free sugars or other hydroxyphenols, constitute a very complicated condition. Based on a previously shown trend (*31*) of pelargonidin aromatic protons to significantly shift to lower frequencies on going from TFA/methanol to TFA/D_2O, and specifically the H4 peak to move from 9.10 ppm to 8.31 ppm, a rough assignment of the observed peaks to types of structures is attempted. Thus, the high frequency signal at 8.48 ppm, attached to a carbon atom at 132.5 ppm (via HSQC 2D correlation) can be attributed to H4 (*30*) of ring C (**Figure 1**) of the flavylium cation of the two delphinidin components. The H4 of the cyanidin core also appears at high frequency (*28*) and is apparently hidden under the delphinidin H4 signal at 8.48 ppm, revealing itself only in the presence of excess of β-CD, as observed in **Figure 8**B below. The ratio $H4_{delphinidines}$:$H4_{cyanidine}$ is ~2.9 by integration [the LC ratio of ([**1**]+[**3**])/[**2**] =2.84]. In the 2D ROESY

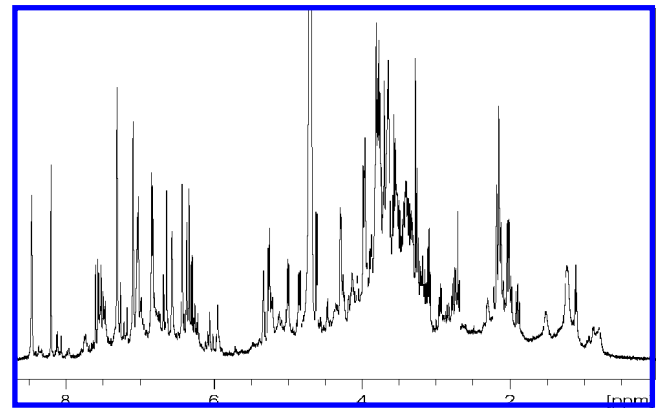


Figure 5. Full ^{1}H NMR spectrum (500 MHz) of *H. sabdariffa* L. extract in D_2O (pH 3.1).

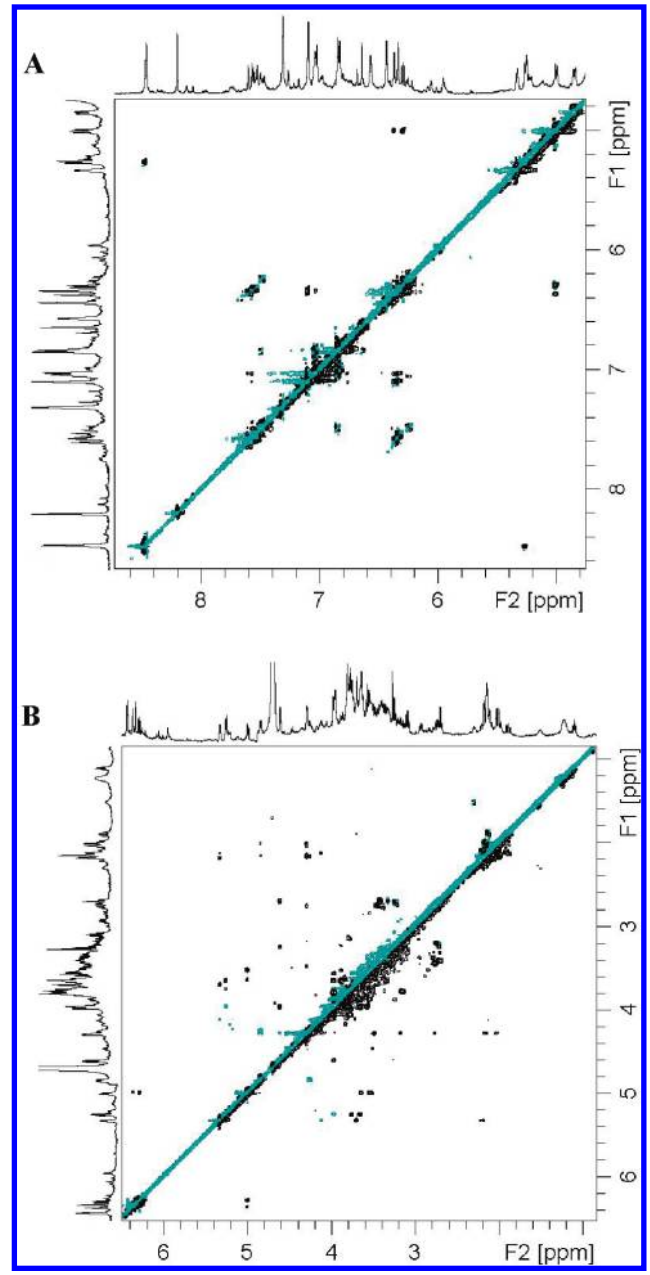


Figure 6. 2D ROESY spectra of the *H. sabdariffa* L. extract in D_2O at 298 K: (**A**) aromatic region and (**B**) aliphatic region.

spectrum the signal of H4 displays noe cross-peak with a presumably sugar-type anomeric H1″ doublet (J = 8 Hz, **Figure**

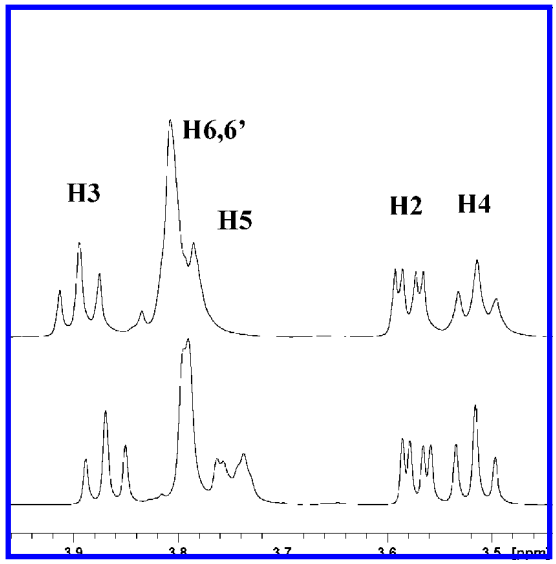


Figure 7. Partial ^{1}H NMR spectra (500 MHz) of (**a**) β-CD alone (10 mM) and (**b**) in the presence of *H. sabdariffa* L. extract (5 mg/mL, ~1 equiv) in D_2O at 300 K.

6A) at 5.26 ppm, verifying the presence of a sugar at position 3 of ring C, this type of interaction indeed observed in similar systems (e.g.flavylium cation of malvidin 3-*O*-β-glucopyranoside (*27*). No exchange cross peaks (of same phase as the diagonal) was observed between the H4 of flavylium ion and the assumed hemiacetal forms at 7.0−6.0 ppm, opposite to previously observed in malvidin 3-*O*-β-glucopyranoside methanolic solution (*27*). However, exchange peaks do exist between signals in the region 5.2−4.8 ppm with signals in the region 4.2−3.9 ppm (**Figure 6**B). Exchange of H1″ (glucose anomeric proton) between flavylium and hemiacetal forms (*27*), could account for this observation, however this is not supported by observation of exchange in other peaks, therefore they could arise from α- to β- anomeric exchange, possibly of free sugars. The signal at 8.20 ppm could arise from H6′ of ring B, however it could not be correlated with any carbon [expected C6′ between 120 and 105 ppm] (*30*) in a 2D HSQC experiment. The region 8.0−6.0 ppm is filled by the signals of protons of all hydroxyphenolic components as well as double bonds (**Figure 6**A). Next, signals characteristic of chlorogenic acid (2.3 −1.8 ppm) are observed at a ratio chlorogenic acid: anthocyanins greater than 2:1. Finally, signals in the region 2.5−0.7 ppm (**Figure 6**B) indicate the presence of at least two rhamnose residues (two nearly overlapped triplets, α- and β-anomers, at 1.23 and 1.10 ppm), verifying the presence of delphinidin **3**, maybe along with free rhamnose.

Addition of dry hibiscus extract to a solution of β-CD in D_2O, at a molar ratio 1:1, at 298 K immediately induced chemical shift changes of the cyclodextin cavity hydrogens ($\Delta\delta H_3$=10.0 Hz, $\Delta\delta H_{6,6'}$=5.1 Hz, $\Delta\delta H_5$=18.5 Hz) (**Figure 7**) but not of the outer protons H2, H4, a direct evidence of molecular inclusion of one or more of the components of the extract. Regarding the extract's signals, many were differentiated from their initial positions thus again signifying interactions with β-CD. No loss of the red color was observed. Analysis of the 2D ROESY spectrum of the extract alone (**Figure 8A**) and in the presence of β-CD (**Figure 8B**) confirms the presence of many cross-peaks due to intermolecular interactions of the cavity with the aromatic and double bond hydrogens (7.8−6.0 ppm). However, the protons at ~8.5 attributed to the flavylium part do not display any interactions; thus, ring A is not included. It is reported in the literature (*33*) that whereas γ-CD can include

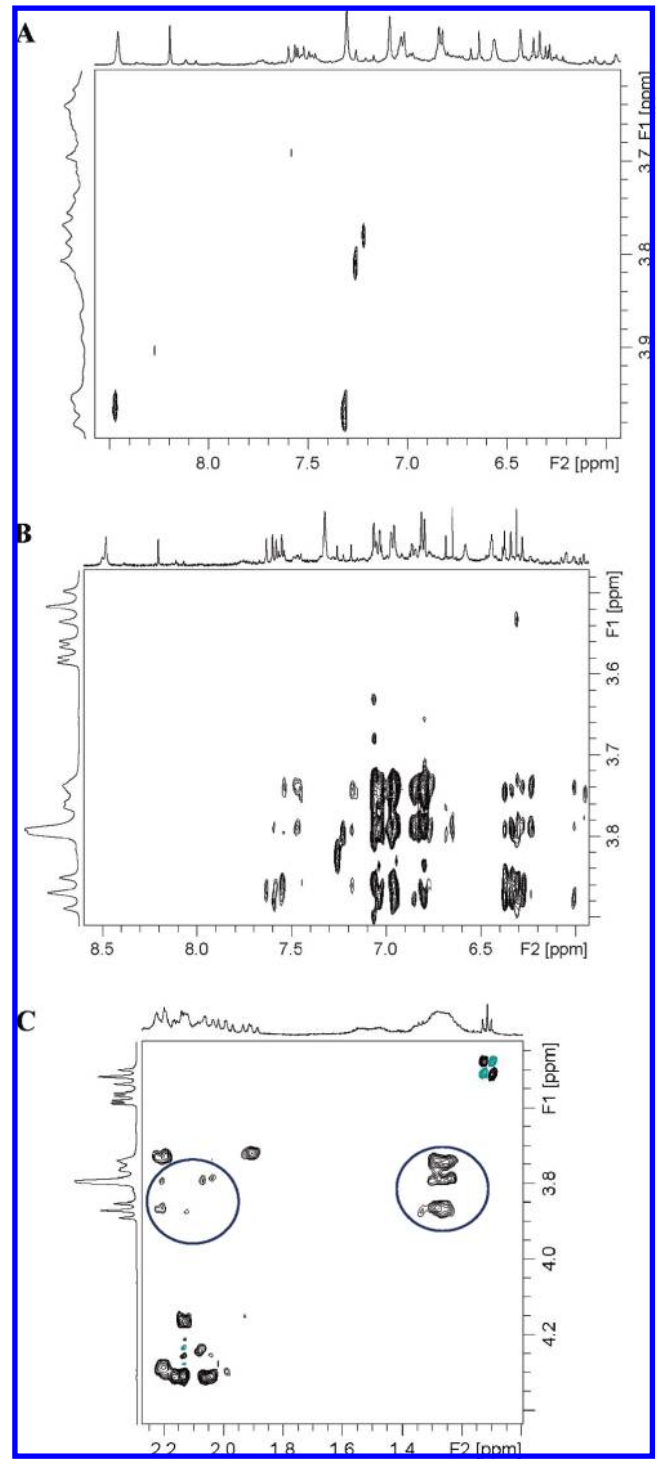


Figure 8. Selected interactions of 2D ROESY spectra in D_2O at 298 K (**A**) of the *H. sabdariffa* L. extract alone, (**B**) of the *H. sabdariffa* L. extract (aromatic region) with β-CD, and (**C**) of the *H. sabdariffa* L. extract (aliphatic region) with β-CD.

the flavylium part of pelargonidin, β-CD cannot, readily engulfing only the *p*-hydroxyphenyl part (ring B), and both CDs protect pelargonidin from discoloration at pH 1−4. Therefore, only rings B of the components in the present extract can be considered for inclusion. We have previously shown that oleuropein (*24*) readily inserts its 3,4-dihydroxyphenylethyl part in the β-CD cavity, whereas others have shown that epigallocatechin gallate forms an inclusion complex via its dihydroxybenzopyran A/C rings (*34*), whereas the trihydroxy B and B′ rings do not seem to be included inside the β-CD cavity. On

the other hand, it is known (*35*) that ferulic acid forms an inclusion complex with γ-CD, whereas chlorogenic acid, quercetin, and rutin form complexes with β-CD (*36*−*38*). Furthermore, the inclusion of the noncolored forms of several anthocyanins (R_1=R_2=H or R_1=H, R_2=OH) in β-CD has been evoked to account for the observed loss of color (anticopigmentation) in slightly neutral aqueous solutions (*26*). During the current experiments, no change of color was observed (in the extract alone or in the presence of β-CD); thus, the participation of the hemiacetal forms in the inclusion cannot be concluded. The dihydroxyphenyl moiety that is prone to inclusion, that is, of cyanidine **2**, is the only part able to form an inclusion complex. Thus, the interactions that we observe must primarily arise from inclusion of the aromatic part of chlorogenic acid (abundant in the extract solution, as shown by the NMR integration), quercetin, and similar molecules (**Table 1**) and, to a small extent, from inclusion of ring B of **2**, which constitutes only a 25% of the anthocyanin mixture. Interestingly, the interactions between the β-CD peak area and the aliphatic signal area show (**Figure 8C**) inclusion of rhamnose methyl groups (~1.3 ppm) and also inclusion of the H2,2′ (weak, 2.2−2.0 ppm) of chlorogenic acid. This last observation shows clearly that the CD host is located close and interacts externally with the anthocyanins via nonspecific interactions, including in the cavity whatever small part is available in the surroundings. Inclusion, external association, and accruing beneficial effects (protection from hydrolysis, increase of solubility) have been reported previously (*39*−*41*). This explains the significant protection toward degradation observed in the present study, in spite of the fact that inclusion of the anthocyanin aglycon parts does not seem to be the dominating event. Therefore, oxidation involving dihydroxyphenyl rings or degradation of the anthocyanins can be minimized by both inclusion and external interactions, as shown by the result of UV−vis spectroscpy and the DSC results. Anthocyanin degradation is generally accepted to be initiated by hydration at the 2-position (ring C) and subsequent cleavage of the flavonoid skeleton. Although the interactions of β-CD with the anthocyanin molecule might be neither strong nor direct, steric phenomena could be implicated in preventing extended hydration and thus degradation (*42*).

Conclusions. Anthocyanins are protected from thermal degradation by the presence of β-CD as shown by thermal stability studies. The addition of β-CD in aqueous solutions of anthocyanins is recommended to minimize anthocyanin degradation due to temperature when consuming hot beverages or in the production of functional foods containing anthocyanins. Moreover, the solid complex of roselle extract/β-CD can be used either to fortify foods or as a food supplement.

ABBREVIATIONS USED

CD, cyclodextrin; β-CD, β-cyclodextrin; NMR, nuclear magnetic resonance; BSTFA, bis-(trimethylsilyl)trifluoroacetamide; GC-MS, gas chromatography−mass spectrometry; LC-MS, liquid chromatography−mass spectrometry; DSC, differential scanning calorimetry.

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