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# Thermal stability of *Hibiscus sabdariffa* L. anthocyanins in solution and in solid state: effects of copigmentation and glass transition

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**Abstract**

Kinetic studies on thermal stability of anthocyanins isolated from the dry calyces of *Hibiscus sabdariffa* L. (roselle) were carried out in aqueous solutions (55–98 °C), either as free or copigmented anthocyanins with chlorogenic acid, and in the dry state as free anthocyanins or co-lyophilized with an amorphous polysaccharide (pullulan) and stored in different relative humidity environments (water activities 0.33, 0.53, 0.75 and 0.84) at 40 °C. The rate constants for degradation were obtained from first-order reaction kinetic plots. The degradation kinetics of individual anthocyanin components in solution, as assessed by HPLC, followed an Arrhenius-type response with respect to temperature; activation energies, $E_a$, varied between 13.3 and 15.1 kcal/mol. Copigmentation of anthocyanins with chlorogenic acid did not seem to improve their stability in solution. In the dry state, the degradation rate constants increased with the water activity, particularly above 0.53. In the freeze-dried pullulan–anthocyanin mixtures, the polysaccharide matrix delayed colour degradation compared to the free anthocyanin preparations by 1.5–1.8 times. The degradation kinetics of anthocyanins did not show any dependence on the molecular mobility of the system, as it relates to the glass–rubber transition ($T_g$) detectable by calorimetry. Anthocyanin degradation occurred, even at sub-$T_g$ temperatures of the amorphous matrices, whereas no changes in the rate constants were observed in the vicinity of the glass transition; the plot of $(\ln k)^{-1}$ against $(T-T_g)$ was linear with all data fitting into a common line as predicted by the Williams–Landel–Ferry (WLF) equation. Both free and co-lyophilized with pullulan Hibiscus anthocyanins exhibited good antiradical activity throughout storage in all humidity environments studied, despite of a substantial loss in colour intensity.


*Keywords:* Anthocyanins; *Hibiscus sabdariffa*; Thermal stability; Copigmentation; Glass transition; Kinetics; Calorimetry; HPLC

## 1. Introduction

Anthocyanins, the biggest group of water-soluble natural pigments of plants, are responsible for the attractive colours of flowers, fruits (particularly berries) and vegetables, contributing largely to the aesthetic quality of plant-derived products. These polyphenolic substances are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavilium salts. Glycosylation and acylation of the aglycone moieties (mainly six anthocyanidins: pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin) by different sugars and acids, at different positions, account for the broad structural diversity of these pigments. In plants, anthocyanins may enhance their resistance to insect attack (Strack & Wray, 1993). Available evidence also suggests that this group of phytochemicals could exhibit multiple biological effects, e.g. antioxidant-antiradical activity, antiinflamatory action, inhibition of blood platelet aggregation and antimicrobial activity, treatment of diabetic retinopathy and prevention of cholesterol-induced atherosclerosis (Clifford, 2000; Espin, Soler-Rivas, Wichers, & Garcia-Viguera, 2000; Mazza & Miniati, 1993; Wang, Cao, & Prior, 1997).

It has been early recognized that anthocyanin-rich plant extracts might have potential as natural food colorants, especially if suitable purified and stable materi-

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als become commercially available (Francis, 1975). A major impediment to the use of these natural colorants is their inherent instability, either in simple aqueous solutions or in complex food formulations. Anthocyanins exhibit greater stability under acidic conditions, but under normal processing and storage conditions readily convert to colourless derivatives and subsequently to insoluble brown pigments. A number of factors influence anthocyanin stability, including pH, heat-humidity, light, oxygen, enzymes, as well as the presence of ascorbic acid, sugars, sulfur dioxide or sulfite salts, metal ions and copigments (Francis, 1989; Jackman, Yada, Tung, & Speers, 1987).

Aqueous extracts from the dry calyces of *Hibiscus sabdariffa* L., variety *sabdariffa* (ruber), a tropical annual shrub known as roselle or karkade, contain two main anthocyanins: delphinidin-3-sambubioside or delphinidin-3-xylosylglucoside or hibiscin and cyanidin-3-sambubioside or cyanidin-3-xylosylglucoside or gossypicyanin, and two minor anthocyanins, delphinidin-3-glucoside and cyanidin-3-glucoside (Du & Francis, 1973). The dry calyces of *H. sabdariffa* yield as much as 1.5% (w/w d.b.) pigment that has transmission spectral features very similar to those of Red No 2 (amaranth) (Francis, 1975). Esselen and Sammy (1973, 1975) have first attempted to study the stability of *H. sabdariffa* anthocyanins in different food formulations (jellies, drinks, carbonated beverages, freeze-dried powders). Clydesdale, Main, and Francis (1979) have also studied the stability of Hibiscus anthocyanins in dry pack foods (a beverage mix and a gelatin dessert), while Pouget Lejeune, Vennat, and Pourrat (1990) examined the effects of different chemical compounds (ascorbic acid, BHA, propyl gallate, disodium EDTA, sodium sulfite) on *H. sabdariffa* anthocyanin stability.

Copigmentation is a phenomenon widely seen in plant tissues and their aqueous extracts. Molecules acting as copigments, such as flavonoids, alkaloids, organic acids, usually have no colour by themselves, but when added to an anthocyanin solution, they greatly enhance the colour of the solution (Mazza & Brouillard, 1990). The studies of Maccarone, Maccarone, and Rapisarda (1985, 1987) and Teh and Francis (1988) have supported the view that copigmentation and self-association influence colour intensity and stability of the anthocyanins. However, to our knowledge, there have been no kinetic studies on thermal degradation of copigmented anthocyanins in solution. Such information would be relevant to thermally processed food products containing these pigments. Moreover, for low- and intermediate-moisture content foods, the physical state of the product (glassy or rubbery state) has been claimed to be a major determinant of the diffusion rates of reactants, thus affecting the rate of deteriorative reactions in foods including color degradation (Levine & Slade, 1992; Serris & Biliaderis, 2001; Slade & Levine, 1991; Tsimidou & Biliaderis, 1997). Early studies on caking and colour fading-browning reactions of *H. sabdariffa* powders have revealed an influence of humidity on colour stability, due to water sorption and enhancement of reaction rates (Al-Kahtani & Hassan, 1990; Clydesdale, Main, Francis, & Hayes, 1979). Several studies which have recently dealt with encapsulated natural colorants, have led to the conclusion that encapsulation significantly reduces the degradation rate of the core material and thereby improves the shelf life of the colorant in different humidity environments (Beatus, Raziel, Rosenberg, & Kopelman, 1985; Desobry, Netto, & Labuza, 1997, Selim, Tsimidou, & Biliaderis, 2000; Wagner & Warthesen, 1995). However, there is a need to relate the chemical and physical changes of such materials to the water-mediated plasticization and the concomitant increase in molecular mobility of the system.

The aim of the present study was to investigate the stability of freeze-dried anthocyanins from *H. sabdariffa* under varying water activity conditions, in either free form or co-lyophilized (encapsulated) with pullulan, as well as to study the degradation kinetics of copigmented anthocyanins in solution under varying temperature conditions, using chlorogenic acid as a copigment. A further objective was to explore whether the degradation rates in the freeze-dried preparations of anthocyanins are related to the molecular mobility associated with the glass transition of the material. The last but not the least objective was to examine how the antiradical activities of anthocyanins are influenced during storage of the low-moisture systems. The kinetic studies reported herein could be useful in establishing appropriate processing and storage protocols to reduce pigment degradation in food products containing these natural colorants.

## 2. Materials and methods

### 2.1. Samples and chemicals

Dried calyces of Hibiscus were purchased from a local market in Cairo, Egypt. Cyanidin-3-rutinoside was supplied by Sigma (St. Louis, MO, USA). Pullulan IP 20 was kindly donated by Hayashibara Biochemical Lab. Inc. (Okayama, Japan). Chlorogenic acid and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) were purchased from Aldrich (Steinheim, Germany). All other reagents (methanol, formic acid, citric acid, disodium phosphate, as well as the salts used to create different relative humidity environments), were of analytical grade and obtained from Aldrich, Sigma, Fluka (Buchs, Switzerland), Riedel-de Haën (Seelze, Germany) or Merck (Darmstadt, Germany). The deionized water used in this study was prepared with a Labconco Water Pro™ system (Kansas City, Missouri, USA).

### 2.2. Methods

#### 2.2.1. Extraction—anthocyanin analysis

The extraction procedure was similar to the procedure adopted by Espin et al. (2000). Dried calyces of Hibiscus were powdered and extracted with 3% formic acid in methanol for 24 h at 4 °C. The extraction procedure was repeated three times, until the extract was colourless. The extracts were combined and filtered through a filter paper, and the methanol was removed under reduced pressure with a rotary evaporator (Heidolph VV 2011), keeping the temperature of the water bath below 40 °C. Following evaporation, the residue was redissolved in acidified water (3% formic acid). The aqueous solution was adsorbed onto activated $C_{18}$ Sep-Pak cartridges (Waters Associates, Milford, MA). The cartridges were first washed with 3% formic acid and the pigments were eluted with 3% formic acid in methanol. The methanolic extract was concentrated to dryness leaving a red residue, which was dissolved in diethyl ether. The extract collected after evaporation of the diethyl ether was kept at −18 °C.

Qualitative and quantitative analyses of the extract were carried out by high performance liquid chromatography (HPLC) using a Liquid Chromatograph 1090 series II (Hewlett-Packard GmbH, Waldbronn, Germany) equipped with autoinjector and a UV-visible diode array detector. Hibiscus anthocyanins were characterized by chromatographic comparisons with standards (retention time, UV-Vis spectral features). Their relative concentrations were determined from the respective peak areas (absorbance at 520 nm) of the chromatograms, using cyanidin-3-rutinoside as a standard.

#### 2.2.2. Degradation kinetics of copigmented anthocyanins in solution

A solution of $2\times10^{-4}$ M Hibiscus anthocyanins ($1.6\times10^{-4}$ M of Dp-3-sambubioside and $0.6\times10^{-4}$ M of Cy-3-sambubioside), prepared in citrate buffer (0.2 M, pH 3.6) was split into two halves: the first was used as a control and, to the other half, the copigment (chlorogenic acid) was added at a molar ratio of 40:1 (copigment: pigment). Falcon vials, filled with 20 ml of anthocyanin solution, were stored at different temperatures (55, 70, 85 and 98 °C) in thermostatted water baths. Sampling was done periodically and the samples were subsequently left for 6 h at room temperature to allow the anthocyanin mixture to re-equilibrate before HPLC analysis. For HPLC analysis, the samples were filtered through Acrodisc filters, 0.45 μ. Measurements were carried out at least in duplicate and first order reaction rate constants ($k_s$) and half-life periods (time to reduce the concentration by one-half, $T_{1/2}$) were calculated using a first-order reaction kinetic model. The HPLC data allowed kinetic calculations for each of the main anthocyanins of Hibiscus extract (Dp-3-sambubioside and Cy-3-sambubioside), as well as for total anthocyanins, by taking into account all eluted anthocyanin compounds of the Hibiscus extract.

#### 2.2.3. Co-lyophilization of anthocyanins with pullulan

Pullulan (9.0 g) was dissolved in citrate buffer (0.2 M, pH 4.6, 150 ml) with 450 mg of anthocyanin extract, under stirring for 30 min. Aliquots (5 ml) were transferred in semi-transparent plastic containers and frozen in liquid nitrogen before freeze-drying. All freeze-dried (encapsulated) samples were kept at −18 °C until used.

#### 2.2.4. Kinetic studies of degradation of freeze-dried anthocyanins

Different relative humidity environments were obtained, using saturated solutions of $MgCl_2{\cdot}6H_2O$, $Mg(NO_3)_2{\cdot}6H_2O$, NaCl, KCl, which provide water activity ($a_w \sim p/p_o$ or 'relative vapour pressure of water') levels of 0.33, 0.53, 0.75 and 0.84, respectively (Labuza, 1984). Control samples (non-encapsulated Hibiscus extracts) and encapsulated anthocyanins were kept in various $a_w$ conditions at 40 °C under light exposure (12 fluorescent lamps, 36 W each) in a CDR Crisagis incubator (Athens, Greece). Degradation of anthocyanins was followed by periodic absorbance measurements (within 1–16 days of storage) of the reconstituted anthocyanins in 5 ml of 3% formic acid in water, followed by the addition of 45 ml methanol (to precipitate the polysaccharide), using a UV-visible diode array spectrophotometer HP 8452A. Percentage colour conservation was determined by the absorbance measurements, $A = A_{\lambda max} - A_{\lambda min}$ (Pouget et al., 1990). The starting optical density was taken as 100, and the percentage of remaining colour was plotted versus time. Measurements were carried out in triplicate and first order reaction rate constants ($k_s$) and half-life periods ($T_{1/2}$) were calculated using a first-order reaction kinetic model.

#### 2.2.5. Differential scanning calorimetry of freeze-dried anthocyanins

Differential scanning calorimetry (Polymer Labs Ltd, Epsom, UK) was used to determine the glass transition temperature, $T_g$ (midpoint temperature of endothermic baseline shift) of the Hibiscus anthocyanin extract, free or co-lyophilized with pullulan. The freeze-dried samples were stored over $P_2O_5$ at a temperature below their $T_g$. Portions of these materials (10–15 mg) were placed in Mettler medium-pressure stainless-steel pans (ME 29990, Mettler-Toledo AG Greinfensee, Switzerland) and rehumidified at various relative humidities over saturated salt solutions in vacuum desiccators at 25 °C; triplicate samples were tested at each relative humidity environment. The pans were then hermetically sealed and analyzed by DSC. The samples were scanned over

the glass transition region at 5 °C/min under continuous nitrogen flushing; samples were scanned twice to eliminate hysteresis effects of thermal relaxation, typical of aged glasses (Roos & Karel, 1991). Temperature and heat flow calibration of the calorimeter were carried out as described by Biliaderis, Lazaridou, and Arvanitoyannis (1999). The moisture content of the samples was determined by oven-drying at 105 °C (control samples) and 130 °C (encapsulated samples) for 1 h. The data were fit to the Gordon–Taylor model (Gordon & Taylor, 1952):

$$T_g = \frac{w_1 T_{g1} + K w_2 T_{g2}}{w_1 + K w_2}$$

where $w_1$ = dry solids, $T_{g1}$ = glass transition temperature of the sample at zero moisture content, $w_2$ = moisture content; $T_{g2}$ = glass transition temperature for glassy water, and $K$ = a constant related to the strength of polymer–diluent interaction (the larger the $K$, the greater the plasticization effect). A $T_{g2}$ of −138 °C was used for water (Sugisaki, Suga, & Seki, 1968). The Gordon–Taylor (G-T) plots were used to estimate the $T_g$ values for all hydrated materials using their respective moisture content values.

### 2.2.6. Temperature dependence of reaction rate constants

The dependence of reaction rate constants on temperature was modelled using the Arrhenius equation and the Williams–Landel–Ferry (WLF) kinetic model (Williams, Landel, & Ferry, 1955). According to the Arrhenius equation, a linear relationship exists between $\ln k$ and $1/T$:

$$k = k_0 \exp(-E_a/RT)$$

where $R$ is the gas constant and $E_a$ is the activation energy.

Instead, the WLF equation has been found useful for predicting the temperature dependence of relaxation times of mechanical changes, including viscosity. Accordingly, the plot of $\ln k^{-1}$ against $T - T_g$ gives a linear response as depicted by the following model:

$$\ln(k_{ref}/k) = -C_1(T - T_{ref})/[C_2 + (T - T_{ref})]$$

where $T_g$ is used as the reference temperature ($T_{ref}$) (Roos & Karel, 1991; Slade & Levine, 1991).

### 2.2.7. Antiradical activity assay

The antiradical activity of the anthocyanin samples was determined according to the procedure described by Brand-Williams, Cuvelier, and Berset (1995). Experiments were performed on freshly prepared solutions of DPPH$^{\cdot}$ and all the spectrophotometric data were acquired using the HP 8452A diode-array spectrophotometer and a 10 mm glass cuvette. From the reconstituted solutions of anthocyanins, different dilutions in methanol were made (1.5; 3.0; 5.0; 7.5 and 10.0 in 10 ml volumetric flasks). Aliquots of 0.1 ml of each dilution were added to 3.9 ml of DPPH$^{\cdot}$ solution ($3.0\times10^{-5}$ M in methanol). The decrease in absorbance was determined at 515 nm when the reaction reached a plateau (24 h). The DPPH$^{\bullet}$ concentration ($C_{DPPH}$) in the reaction medium was calculated from the following calibration curve, as determined by linear regression: $A_{515nm} = 26.247\ C_{DPPH}\ (mg/ml) + 6.813\times10^{-3}$, where $r^2 > 0.98$. For each concentration tested, the percentage of DPPH remaining at steady state was calculated as follows: % $DPPH_{rem} = [DPPH]_T/[DPPH]_{TC}$, where $T$ is the time necessary to reach the steady state and $[DPPH]_{TC}$ is the DPPH concentration of the control sample (0.1 ml MeOH in 3.9 ml of $3\times10^{-5}$ M DPPH) at steady state. These values were plotted vs. mg antioxidant/mg DPPH to obtain the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% ($EC_{50}$). For comparison purposes, the term of $1/EC_{50}$ or antiradical power (ARP) was used; the larger the ARP, the higher the antioxidant activity (Brand-Williams et al., 1995).

### 2.2.8. Statistical analysis

Linear regression analysis was used to obtain the degradation rate constants ($k$) for all materials studied. Standard errors of the rate constants $s_k$ were also calculated and significant differences among the rate constants were identified using the $t$-test (95% confidence level) and $(n_1-2+n_2-2)$ degrees of freedom (Steel & Torrie, 1980).

## 3. Results and discussion

### 3.1. Degradation kinetics of free and copigmented anthocyanins in solution

Anthocyanins may exhibit different colours, depending on their structure (e.g. glycosylation, acylation), pH and the presence and concentration of copigments. At a given pH, an equilibrium exists between four different anthocyanin/aglycone structures: a blue quinoidal (anhydro) base (A), a red flavylium cation ($AH^+$) and the colorless carbinol pseudobase (B) and chalcone (C). Under neutral or slightly acidic conditions, the anthocyanins exist predominantly in their colourless forms, due to the instability of the anhydrobase. The rate of anthocyanin degradation has long been known to be pH dependent; e.g. lowering the pH in the range of 5.0–1.0 resulted in a significant retention of anthocyanins in

strawberry juice (Meschter, 1953). The stability of these pigments at low pH is largely attributed to the higher concentration of the flavylium cation. Stabilization of the coloured species, especially the quinoidal base (A), could be further conferred through intermolecular (e.g. flavonoids, polyphenols) and intramolecular (e.g. presence of acyl groups on sugar moieties of the anthocyanin molecule itself) copigmentation (Brouillard & Dangles, 1994; Jackman et al., 1987). Copigmentation is a hydrophobically-driven association of an anthocyanin chromophore with the planar electronically saturated part of the copigment; i.e. van der Waals interactions and hydrophobic effects in the aqueous medium result in a large '$\pi-\pi$' stacking of anthocyanin and copigment molecules. This association leads to an absorbance increase in the visible range (hyperhromic effect) and a shift of the $\lambda_{max}$ toward higher wavelengths (bathochromic effect).

Studies by Maccarone et al. (1985) and Teh and Francis (1988) showed that copigmentation can influence both colour intensity and anthocyanin stability in solution during storage. Moreover, the work of Miniati, Damiani, and Mazza (1992) suggested that the structure of the copigment as well as temperature may have an influence on anthocyanin stability; e.g. gallic acid stabilized the colour at two temperatures examined in their studies (5 and 20 °C), quercetin increased colour stability at 5 °C but decreased it at 20 °C, and catechin decreased the colour stability at both storage temperatures. On the other hand, there are no data in the literature concerning the stability of anthocyanins in the presence of copigments at high temperatures, such as those encountered during thermal processing of foods. The effect of chlorogenic acid (5-*O*-caffeoylquinic acid), a widespread, natural, colourless phenolic compound, and known to be a good copigment, on the thermal stability of individual Hibiscus anthocyanins in solution was thus examined in the present study at four different temperatures (55, 70, 85, 98 °C). Some representative kinetic plots from the HPLC data are illustrated in Fig. 1. In general, linear relationships were observed between [ln(peak area)] and time for both control (free) and copigmented samples, implying first order reaction kinetics for pigment degradation. Similar kinetic responses for anthocyanins have been reported by other authors (Daravingas & Cain, 1968; Markakis Livingstone, & Fellers, 1957; Meschter, 1953; Segal & Negutz, 1969; Tanchev, 1983). The rate constant values, standard errors of the slope ($s_k$), half-life periods ($T_{1/2}$) and coefficients of determination at each temperature are summarized in Table 1; significant differences between the rate constants are also presented. The results of Table 1 show that there were no major differences in the degradation rates between the copigmented and free anthocyanins, examined either as individual components (Dp-3-sambubioside, Cy-3-sambubioside) or as total anthocyanins. According to Brouillard and Dangles (1994), pigment–copigment complexes become less stable with increase in temperature and the solvation effects on the flavylium ions are expected to play a dominant role; i.e. flavylium ions become more liable first to non-covalent hydration and then to covalent hydration, leading to the colourless species of carbinol pseudobase and chalcone. As the temperature is raised, competition between hydration and copigmentation turns against copigmentation and this may explain the lack of any major effect of the copigment on anthocyanin stability during heating, compared to the free Hibiscus colorant extracts. Temperature had a major influence on the degradation kinetics (Table 1); plots of $\ln k$ against $1/T$ ($K^{-1}$) gave straight lines for each of the Hibiscus anthocyanins (Fig. 2). The temperature dependence of reaction rate constants thus followed the Arrhenius relationship, typical of many deteriorative processes in food materials. Activation energies ($E_a$), derived from the slopes of the lines of Fig. 2, ranged between 13.3 and 15.1 kcal/mol. These values are in close agreement with those reported for strawberry anthocyanins (~16 kcal/mol, 20–100 °C; Lund, 1975) and anthocyanin colour loss for a variety of fruits stored at 10, 20, 30 and 40 °C (~17 kcal/mol, 10–40 °C; Tanchev, 1983). However, Tanchev (1983) reported an $E_a$ of ~23 kcal/mol for anthocyanin colour loss in various fruits heated at higher processing temperatures (78, 88, 98 and 108 °C). With respect to $E_a$, there seemed to be no significant differences for anthocyanin degradation between the copigmented and free Hibiscus anthocyanin samples (Fig. 2).

### 3.2. Degradation kinetics of free and encapsulated anthocyanins in the dry state

Colour stability in the dry state was assessed from absorbance measurements of the reconstituted (in solution) samples, $A = A_{\lambda max} - A_{\lambda min}$, where $\lambda_{min} = 440$ nm (Pouget et al., 1990); the maximum wavelength is dependent on the pH of the solution (Daravingas & Cain, 1968) and for the reconstituted solutions of the freeze-dried powders a $\lambda_{max}$ of 535 nm was obtained. The protective effect of the encapsulating pullulan matrix on Hibiscus anthocyanins was evaluated at four $a_w$ levels (0.33, 0.53, 0.75 and 0.84) under light exposure at 40 °C. Some results from these kinetic experiments are illustrated in Fig. 3. Linear relationships were observed from the plots of $[\ln(A_{535} - A_{440})]$ vs. time, implying first order reaction responses for colour degradation. These results are in disagreement with the findings of Erlandson and Wrolstad (1972) where degradation of strawberry anthocyanins at limited water concentrations did not seem to follow first order kinetics. However, in the latter study, instead of a purified anthocyanin extract, the whole fruit (freeze-dried ground strawberry puree) was

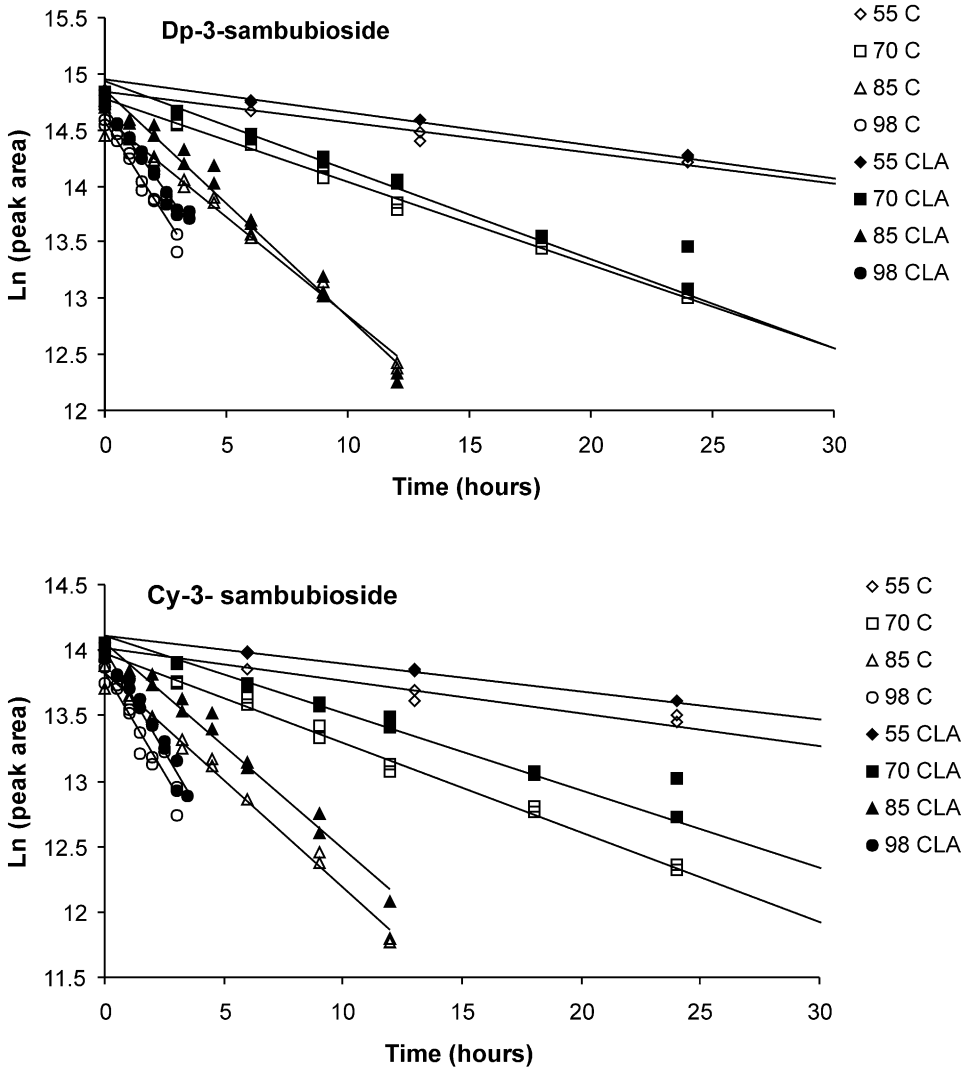


Fig. 1. First-order kinetic plots for *Hibiscus sabdariffa* L. anthocyanins, delphinidin-3-sambubioside and cyanidin-3-sambubioside degradation in solution without (C) or with chlorogenic acid (CLA) added as a copigment at different temperatures.

utilized and colour measurements were performed at a single wavelength (510 nm).

According to Erlandson and Wrolstad (1972), water availability is important for anthocyanin breakdown. In the present study, there was an increase in the rate constant ($k$) and a corresponding decline in the half-life values with increasing moisture content (Table 2); this was more pronounced above the zone assigned as the intermediate-moisture regime. These findings are in agreement with the observation of Erlandson and Wrolstad (1972) who showed anthocyanins from freeze-dried strawberries to be relatively stable in low moisture environments as opposed to a reconstituted beverage. Markakis et al. (1957) have postulated two hydrolytic mechanisms of degradation at limited water concentrations, one being hydrolysis of the glycosidic linkage to yield unstable aglycone and the other involving opening of the pyrilium ring to form a substituted chalcone and finally degradation products; the latter is consistent with the view that heating favours the formation of the chalcone structure. It is also assumed that further degradation of the primary anthocyanin breakdown products (most of which are colourless) leads to formation of brown polymeric compounds.

Oxygen and heat have been reported as the most important factors affecting the destruction of anthocyanins (Jackman & Smith, 1992). Oxygen may cause oxidative degradation of anthocyanins directly and/or indirectly, via oxidized constituents, to yield colourless or brown-coloured pigments, e.g. oxidation of o-dihydroxyphenols to quinones and subsequent reaction between quinones and anthocyanins. In the driest environment used in this work, the degradation rate constants were about the same for both free and encapsulated anthocyanins while, for samples stored at higher relative humidity levels, the free anthocyanins showed faster degradation than the pullulan-anthocyanin co-lyophilized materials (~1.5–1.8 times). Because of its

Table 1
Degradation reaction rate constants ($k$) and standard errors of the slope ($s_k$)[a], half-life periods ($T_{1/2}$) and correlation coefficients (in parentheses)[b] for the Hibiscus anthocyanins stored at different temperatures, in solution without (control) or with chlorogenic acid (CLA) added as a copigment

| Sample | $K\times10^3\pm s_k\times10^3$ ($h^{-1}$) | | |
|---|---|---|---|
| | Half-life period, $T_{1/2}$ (h) | | |
| | Dp-3-sambubioside | Cy-3-sambubioside | Total anthocyanins |
| **55 °C** | | | |
| Control (C) | 27.3±0.7a1<br>$T_{1/2}=25.4$<br>(0.99, $n=16$) | 24.9±0.8a1<br>$T_{1/2}=27.8$<br>(0.98, $n=16$) | 27.3±0.7a1<br>$T_{1/2}=25.4$<br>(0.99, $n=16$) |
| CLA | 29.5±0.7b1<br>$T_{1/2}=23.5$<br>(0.99, $n=16$) | 21.3±0.6b1<br>$T_{1/2}=32.58$<br>(0.99, $n=16$) | 26.9±0.6a1<br>$T_{1/2}=25.8$<br>(0.99, $n=16$) |
| **70 °C** | | | |
| Control (C) | 74.3±1.1a2<br>$T_{1/2}=9.3$<br>(0.99, $n=16$) | 68.6±1.0a2<br>$T_{1/2}=10.1$<br>(0.99, $n=16$) | 74.1±1.1a2<br>$T_{1/2}=9.4$<br>(0.99, $n=16$) |
| CLA | 79.6±3.29a2<br>$T_{1/2}=8.7$<br>(0.97, $n=16$) | 59.2±2.4b2<br>$T_{1/2}=11.7$<br>(0.97, $n=16$) | 72.3±2.7a2<br>$T_{1/2}=9.6$<br>(0.98, $n=16$) |
| **85 °C** | | | |
| Control (C) | 177±4.8a3<br>$T_{1/2}=3.9$<br>(0.99, $n=16$) | 164±4.0a3<br>$T_{1/2}=4.2$<br>(0.99, $n=16$) | 176±4.5a3<br>$T_{1/2}=3.9$<br>(0.99, $n=16$) |
| CLA | 203±7.6b3<br>$T_{1/2}=3.4$<br>(0.98, $n=16$) | 157±5.5a3<br>$T_{1/2}=4.4$<br>(0.98, $n=16$) | 188±6.1a3<br>$T_{1/2}=3.7$<br>(0.98, $n=16$) |
| **98 °C** | | | |
| Control (C) | 334±24.1a4<br>$T_{1/2}=2.1$<br>(0.94, $n=14$) | 306.3±27.9a4<br>$T_{1/2}=2.2$<br>(0.90, $n=14$) | 306±27.9a4<br>$T_{1/2}=2.2$<br>(0.99, $n=16$) |
| CLA | 296±11.7a4<br>$T_{1/2}=2.3$<br>(0.98, $n=16$) | 308±14.9a4<br>$T_{1/2}=2.2$<br>(0.97, $n=16$) | 308±9.6a4<br>$T_{1/2}=2.2$<br>(0.99, $n=16$) |

[a] Different letters following the mean values of rate constants show significant differences between $k$ values of the control and copigmented samples at a certain temperature ($P<0.05$). Different numbers following the mean values of rate constants show significant differences for one medium kept under different temperature conditions ($P<0.05$).
[b] The number of samples used for linear regression is given by $n$.

Table 2
Degradation reaction rate constants ($k$) and standard errors of the slope ($s_k$)[a], half-life periods ($T_{1/2}$) and correlation coefficients (in parentheses)[b] for Hibiscus anthocyanins, free or encapsulated in pullulan, stored at 40 °C, under light exposure and different water activity levels

| Sample | $K\times10^3\pm s_k\times10^3$ ($d^{-1}$) | | | |
|---|---|---|---|---|
| | Half-life period $T_{1/2}$ (d) | | | |
| | $a_w=0.33$ | $a_w=0.53$ | $a_w=0.75$ | $a_w=0.84$ |
| Free anthocyanins | 2.2±0.3a1<br>$T1/2=317.9$<br>(0.75, $n=21$) | 23.2±1.2a2<br>$T1/2=29.8$<br>(0.93, $n=29$) | 145.2±8.0a3<br>$T1/2=4.7$<br>(0.95, $n=17$) | 174.6±8.0a4<br>$T1/2=3.9$<br>(0.94, $n=29$) |
| Pullulan-anthocyanins | 2.2±0.4a1<br>$T1/2=320.8$<br>(0.58, $n=18$) | 12.7±0.7b2<br>$T1/2=54.6$<br>(0.92, $n=29$) | 83.3±4.1b3<br>$T1/2=8.3$<br>(0.95, $n=24$) | 115.3±5.5b4<br>$T1/2=6.0$<br>(0.95, $n=27$) |

[a] Different letters show significant differences between the control and encapsulated samples for a given environment ($P<0.05$). Different numbers following the mean values of rate constants show significant differences for the same carrier in different $a_w$ environments ($P<0.05$).
[b] The number of samples used for linear regression is given by $n$.

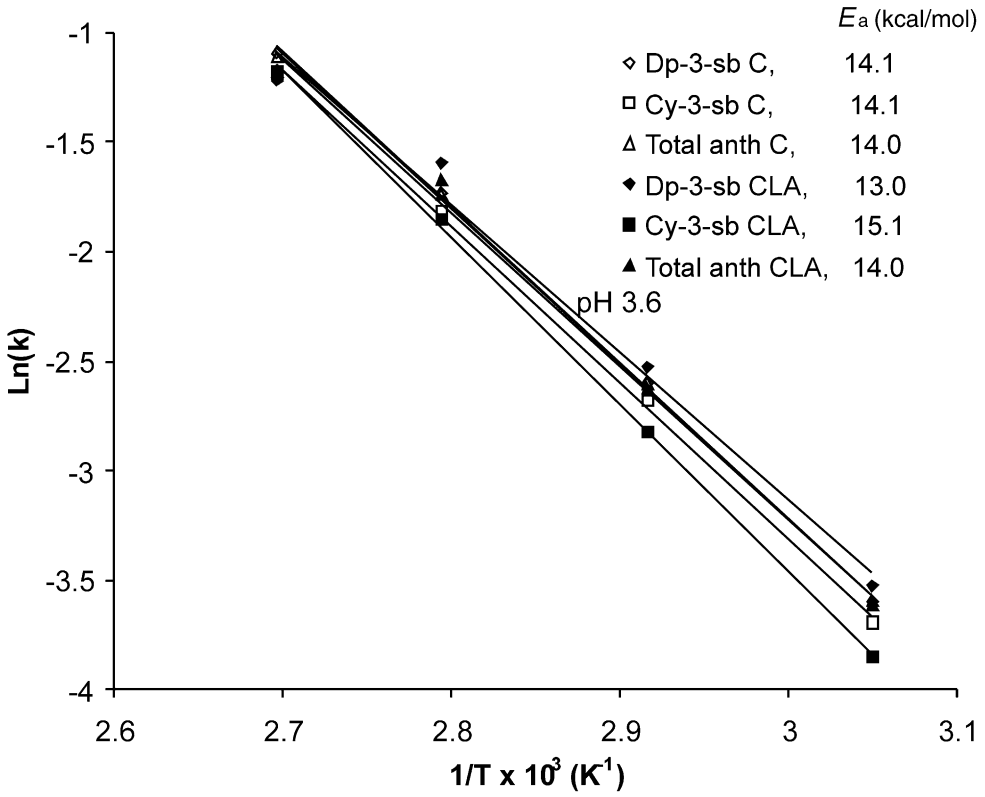


Fig. 2. Arrhenius plots of degradation rate constants for Hibiscus anthocyanins; estimates of activation energies ($E_a$) are also given.

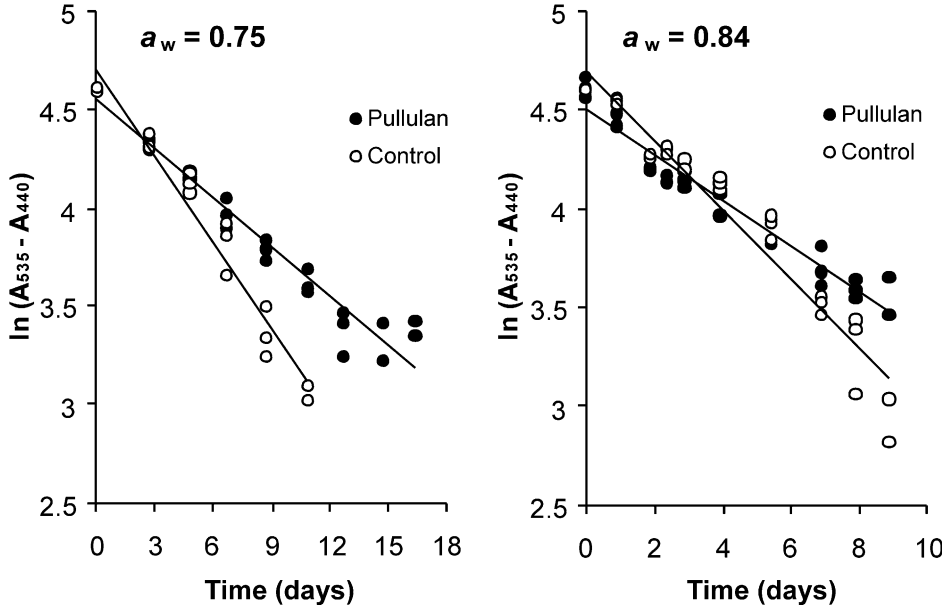


Fig. 3. First-order degradation plots for freeze-dried Hibiscus anthocyanins, free (control) or co-lyophilized with pullulan, at 40 °C and two water activity environments.

good film barrier properties (impermeability to oxygen) and the ability to remain highly amorphous under all storage conditions ($a_w$, temperature), pullulan as an encapsulating agent was shown to greatly improve the stability of other water-soluble pigments (e.g. crocins) against oxidation, decreasing the degradation rate constant up to 20 times (Selim et al., 2000). Instead, encapsulation of Hibiscus anthocyanins in pullulan by freeze-drying seemed to be less effective in improving stability of these pigments (Table 2). This is probably due to

differences in the degradation mechanism between anthocyanins (sensitivity to non-covalent and covalent hydration) and other type of pigments. According to Rosenberg and co-workers (Moreau & Rosenberg, 1998, 1999; Sheu & Rosenberg, 1998), the nature of the pigment (core) as well as the chemistry and physical properties of the wall material (pullulan), are important stability determinants of encapsulated systems.

### 3.3. The glass–rubber transition and degradation kinetics of Hibiscus anthocyanins

In recent years, mainly due to the pioneering work of Slade and Levine (Levine & Slade 1986, 1992; Slade & Levine, 1988a, 1988b, 1991; Slade, Levine, & Finley, 1989), the concept of glass–rubber transition and its implication for stability and properties of low-moisture foods and biomaterials has been largely advanced. Although the chemical changes in dry products are very slow, there is evidence that various deteriorative processes are accelerated if the dried products are stored at temperatures above their $T_g$; in contrast, translational mobility and diffusion are essentially restricted in a glassy solid (Slade & Levine, 1991). As the $T_g$ is highly sensitive to water content, the moisture-dependence of food product stability has been attributed to changes in molecular mobility, as controlled by the glass transition. Therefore, the $T_g$ is often considered as a reference temperature: below $T_g$, the food is expected to be stable, whereas above this temperature, the difference between storage temperature and glass transition temperature ($T-T_g$) is assumed to control the rate of physical, chemical and enzymic changes in the product. Moreover, processes that are diffusion-limited are expected to conform to WLF kinetics rather than to the Arrhenius formalism; in this respect, the WLF equation specifies a much stronger temperature-dependence of reaction rates than the Arrhenius relationship. However, conflicts exist as to whether only diffusion-limited processes (typically those of low activation energies, 2–6 kcal/mol) are controlled by the glass transition and whether translational diffusion coefficients, especially of small molecules, remain at quite a high level in a glassy matrix that is relatively 'non-dense', permitting molecular collisions between reactants and the reaction to occur even at sub-$T_g$ temperatures (Bell & Hageman, 1994; Comyns, 1985; Fennema, 1996; Karel, 1993; Orlien, Andersen, Sinkko, & Skibsted, 2000; Schebor, Buera, Karel, & Chirife, 1999).

Fig. 4 shows the DSC traces of the freeze-dried Hibiscus anthocyanins, free or co-lyophilized with pullulan, at different water contents. The observed endothermal baseline shifts, typical of the glass transition, indicate the progressive lowering of $T_g$ with increasing amounts of water. The $T_g$-water content relationships (Gordon–Taylor plots) presented in Fig. 5 more clearly reveal the sensitivity of the amorphous materials to water-plasticization. The $T_g$ curve of the free Hibiscus anthocyanins is shifted to lower temperatures (~40–50 °C) than the pullulan-anthocyanin samples, reflecting the average molecular size differences between the two systems. Estimated values of $T_{g1}$ for the dried products are included in the inset of Fig. 5 along with the $k$ values of the G-T model. The freeze-dried extracts of Hibiscus anthocyanins, because of their lower $T_g$ than the encapsulated product, would be expected to enter the rubbery domain at a much lower water content assuming a constant storage temperature. Fig. 6 presents the dependence of anthocyanin degradation rate as a function of $\Delta T = T - T_g$. It is clear from this plot that anthocyanin degradation greatly increases above the temperature vicinity of the glass transition for both materials. However, it is interesting to note that pigment degradation does occur, even at temperatures below the glass transition. These findings concur with data from recent studies which have shown that some chemical reactions (ascorbic acid oxidation, aspartame hydrolysis, non-enzymic browning, lipid oxidation) still proceed at substantial rates in the glassy state (Bell & Hageman, 1994; Karmas, Buera, & Karel, 1992; Nelson, 1993; Orlien et al., 2000; Schebor et al., 1999), failing to show the stability of the 'glassy system' as often claimed in the early literature on glass–rubber transitions of food materials (Levine & Slade, 1992; Slade & Levine, 1991; Slade, Levine, & Finley, 1989). The occurrence of anthocyanin degradation at temperatures far below the $T_g$ for both free and encapsulated systems (Fig. 6) strongly points to the possibility of some sort of reactant mobility in the glassy state and further indicates the insufficiency of using the calorimetrically-determined $T_g$ as an absolute threshold of stability. Macroscopic heterogeneities in the glassy matrix, non-homogeneous distribution of water, and phase separation phenomena (demixing of reactants and inert matrix) are also likely to influence the apparent reaction rates and further explain why reactions do not cease below the measured $T_g$.

A plot of experimental values of $1/k$ against $T-T_g$ showed a linear response with all data falling into a single line. This trend complies with the WLF relationship for the temperature dependence of reaction rates in the rubbery domain. Interestingly, there was no major change in the temperature-dependence of the degradation rate constants for all samples in the vicinity of the glass transition zone. It is worth noting here that in similar kinetic studies on the applicability of the WLF equation to nonenzymic browning (Roos & Himberg, 1994), oxidation of crocin carotenoids (Tsimidou & Biliaderis, 1997) and degradation of encapsulated beetroot pigment (Serris & Biliaderis, 2001), using several storage temperatures, the reaction rates did not seem to fit into a common line based on the WLF formalism.

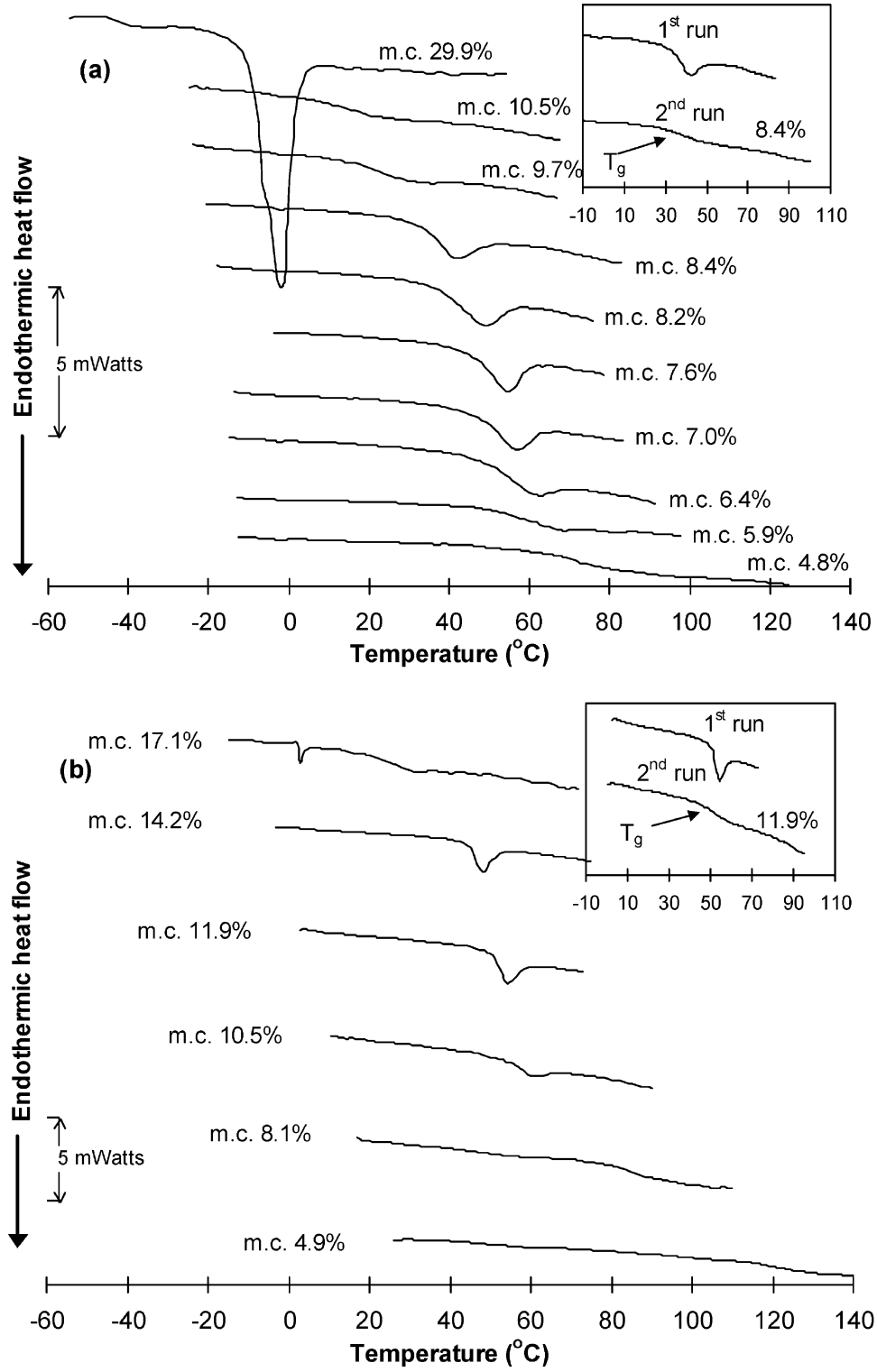


Fig. 4. DSC thermal scans of freeze-dried Hibiscus anthocyanins, free (a) or co-lyophilized with pullulan (b), at different moisture contents, showing the glass–rubber transition; the insets show the 1st (pronounced enthalpy relaxation peak) and rescans (2nd run) of the same sample.

### *3.4. Storage effect on antiradical activity of dry anthocyanin preparations*

The antiradical activity of the dry anthocyanin preparations (free and pullulan-encapsulated) was tested throughout storage at different $a_w$ environments. Anthocyanins, as other flavonoids and related phenolics of higher plants, are well known scavengers of free radicals. In this context, the antioxidant and antiradical activities of these compounds have been implicated in

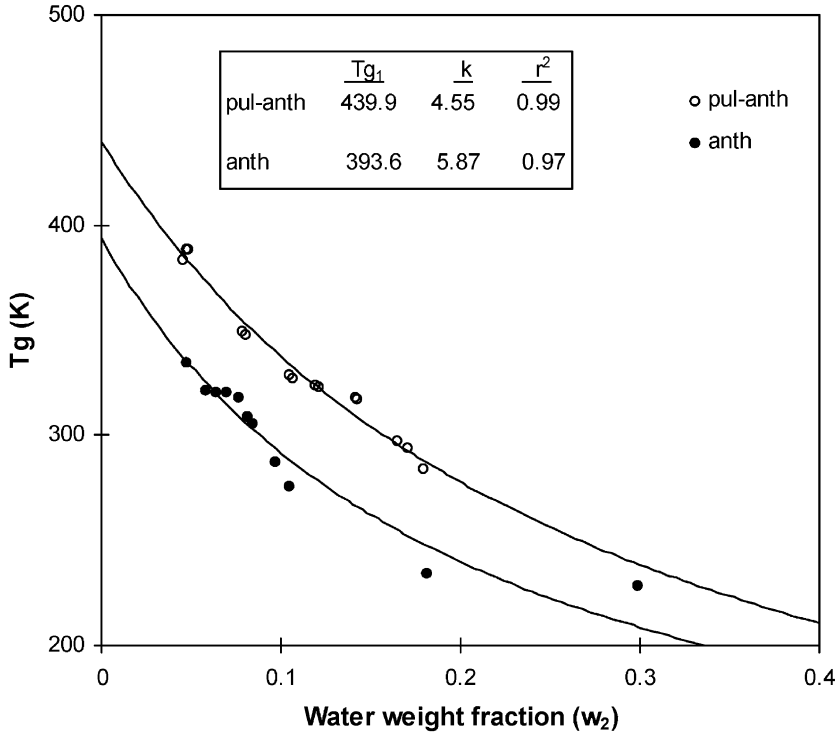


Fig. 5. Gordon–Taylor plots ($T_g$ against water weight fraction, $w_2$) for free and co-lyophilized (with pullulan) Hibiscus anthocyanin extracts. The $T_g$ data are those obtained from the DSC rescans of samples adjusted at different moisture contents; in the inset, the G-T parameters were estimated from statistical optimisation (best fit) of the lines.

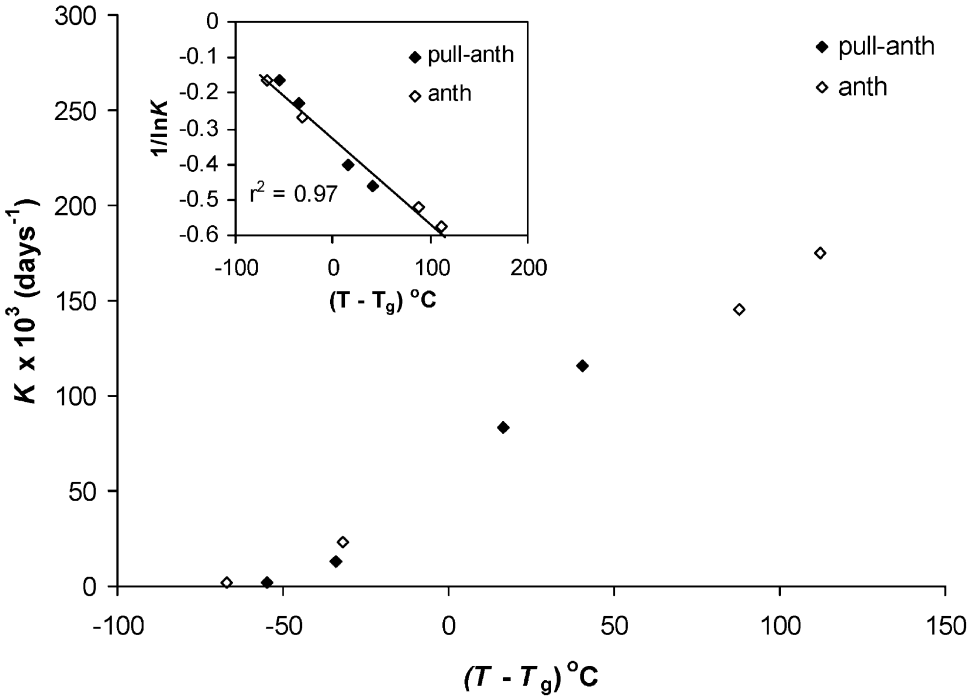


Fig. 6. Williams–Landel–Ferry (WLF) plot, illustrating the dependence of degradation rates, ($k$) and $(\ln k)^{-1}$ (inset), on $\Delta T = T - T_g$ for free and co-lyophilized (with pullulan) Hibiscus anthocyanins stored at 40 °C.

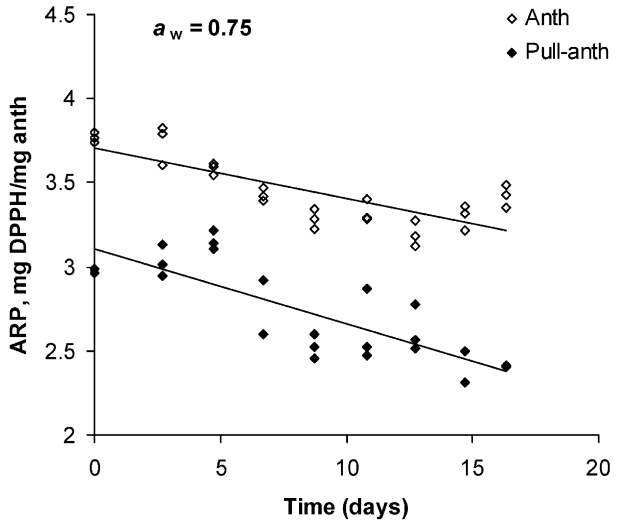


Fig. 7. Antiradical activity of freeze-dried Hibiscus anthocyanins (free and co-lyophilized with pullulan) during storage at $a_w = 0.75$.

Table 3
Average antiradical capacity (ARP) of free and encapsulated anthocyanins in a pullulan matrix at the beginning and at the end of the specified (in parenthesis) storage period

| $a_w$ | ARP[a], mg DPPH/mg of Hibiscus extract | | | |
|---|---|---|---|---|
| | Anthocyanins | | Pullulan-anthocyanins | |
| | Initial | Final (days) | Initial | Final (days) |
| 0.33 | 3.5±0.2 | 4.2±0.3 (210) | 3.1±0.2 | 3.7±0.2 (210) |
| 0.53 | 3.4±0.2 | 3.5±0.2 (75) | 3.0±0.3 | 2.9±0.3 (75) |
| 0.75 | 3.7±0.1 | 3.3±0.1 (17) | 3.1±0. 1 | 2.5±0.1 (17) |
| 0.84 | 3.8±0.1 | 3.6±0.1 (9) | 3.2±0.2 | 2.9±0.3 (9) |

[a] Values are given as average±standard deviation.

the protective effect of vegetable-/fruit-rich diets against coronary diseases (Hertog, Feskens, Hollman, Katan, & Kromhout, 1993). The results of Fig. 7, as well as the summarized data of the initial and final (after a specified storage period) antiradical capacity values presented in Table 3, clearly indicate that, despite colour fading, the breakdown products of anthocyanins still exhibit significant antiradical power. These results suggest that anthocyanins present in food products may continue to provide their beneficial health effects (antioxidants) even after some colour loss has occurred during processing and storage.

## 4. Conclusions

In this study, the thermal stability of isolated anthocyanins from dry calyces of *H. sabdariffa* was examined in aqueous solutions, with or without the presence of chlorogenic acid as a copigment, and in the dry state during storage at different relative humidity environments. Copigmentation did not seem to alter the degradation kinetics of individual anthocyanin components or total anthocyanins of the Hibiscus pigment extracts in the temperature range 55–98 °C. Instead, the degradation kinetics of dry anthocyanin preparations was largely affected by hydration, showing a gradual increase in the rate with increasing water activity. The co-lyophilized pullulan-anthocyanins showed a slightly improved stability at all relative humidity environments compared with the free anthocyanin extract. The temperature-dependence of the anthocyanin degradation rates in all freeze-dried materials seemed to follow the WLF kinetic model. However, the calorimetrically-determined $T_g$ of the preparations cannot be considered as an absolute threshold temperature for pigment stability. Degradation of anthocyanins, occurred even at sub-$T_g$ temperatures, for all samples, implying significant reactant mobility in the glassy state. Based on the findings of this work, further studies would be necessary for the determination of appropriate processing and formulation protocols that could lead to a more efficient utilization of these pigments in actual food products.

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