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Stability and degradation kinetics of crude anthocyanin extracts from *H. sabdariffa*

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Abstract

Hibiscus sabdariffa is an under-utilized plant that has reported to have great potential in the food industry. The vibrant red pigment from the calyces indicate a source of anthocyanins. Anthocyanins would make ideal natural food colourants with additional nutritional benefits however stability is a hindering factor. Stability studies were the main focus of this study. Crude anthocyanins were extracted using four different solvent systems. The crude extracts were analysed under the following parameters; heat (50 and 80 °C), light (darkness and 20 W light) and pH (pH 1-9) stability. Degradation kinetic studies were done on thermally treated samples. Radical scavenging ability was thereafter calculated. Anthocyanidins were identified and quantified by HPLC coupled with a Diode Array Detector (DAD). Total phenolic content was determined with Folin – Ciocalteu's method. Approximately 87% of pigments were retained when heated at 50 °C while heating at 80 °C resulted in 61% pigment retention. The pH stability of samples incubated for 7 days indicated that crude anthocyanins degraded slower at acidic pH. Light stability showed slower degradation in dark incubated samples resulting in 84% pigment retention after a 10 day period. *H. sabdariffa* shows potential for the application of a food in products such as jelly and yoghurt.

Keywords: *anthocyanins; degradation kinetics; Folin-Ciocalteu; Hibiscus sabdariffa.*

Practical application: A natural red food colourant from anthocyanins from *H. sabadriffa*.

1 Introduction

Due to the number of illnesses and diseases, that has plagued the world, consumers are becoming more aware of the products that they consume. The appearance of foods are essential, as food has to look almost equally as desirable as it taste (Delgado-Vargas & Paredes-Lopez, 2002; Selim et al., 2008). Substituting synthetic pigments with natural pigments from edible sources would be advantageous as they would not requires the various rigorous toxicological analysis which can be time consuming and expensive as well as fulfilling a consumer requirement of a natural pigment (Markakis, 1982; Hendry & Houghton, 1996). Anthocyanins are among the most important water soluble plant pigment found in higher plants. They contribute to the vibrant red, blue, purple, violet and orange colours of various plants. They can be found in numerous plant species such as red grapes, berries, purple fleshed sweet potato, radish and red cabbage (Kerio et al., 2012; Castañeda-Ovando et al., 2009; Gradinaru et al., 2003). There are more than 500 different anthocyanins and 23 anthocyanidins. However, there are six most common anthocyanins found in vascular plants or higher plants, namely, delphinidin, malvidin, cyanidin, pelargonidin, peonidin and petunidin (Castañeda-Ovando et al., 2009). Anthocyanidins are available singly in certain fruits such as cyanidin in apples and figs or delphinidin in eggplant and pomegranate (Andres-Bello et al., 2013; Wang et al., 2013).

Anthocyanin pigments make a good source for a natural food colourant however, they are known to be very unstable. Factors that most commonly affect the stability are pH, temperature, light and storage. Additional benefits of using anthocyanins as food colourants is the biological roles that it plays such as antioxidant activity (Amr & Al-Tamimi, 2007). Ensuring the chemical stability of anthocyanins has become a focal point in recent studies, as there is an abundance of potential industrial applications. A major benefit would be to substitute synthetic colourants and dye and replace them with stable anthocyanins (Rein, 2005).

Hibiscus sabdariffa is an annual herbaceous shrub from the *Malvaceae* family (Mahadevan et al., 2009), and is cultivated in both tropical and sub-tropical regions around the world. The plant is described as being red stemmed with serrated leaves and red calyces. Although *H. sabdariffa* is termed an under-utilized crop, it is used commonly in in households for its traditional medicinal properties and produced into various edible products such as jam, jelly and tea (Da-Costa-Rocha et al., 2014). *H. sabdariffa* is known for its various pharmaceutical, nutritional and traditional medicinal properties and has said to be a rich source of anthocyanins (Patel, 2014; Mahadevan et al., 2009). The aim of this present study was to determine the stability parameters of the crude anthocyanins present in *H. sabdariffa*.

2 Materials and methods

2.1 Plant material

H. sabdariffa was collected by plant taxonomist Prof H. Baijnath from different locations in and around Durban, Kwazulu Natal, South Africa during the period of May - June 2014. Voucher

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specimens were deposited at the Ward Herbarium (UDW) – (University of Kwazulu Natal, Durban, South Africa – Voucher Number: S. Sipahli 1). The fresh *H. sabdariffa* calyces were removed and dried at 40 °C for 48 h in a convection oven, once completely dry the material was stored in Amber Schott bottles at room temperature until required.

2.2 Pigment extraction

Four different solvents systems were used for the extraction of the crude pigments including hydrochloric acid (37%): ethanol (1:99, v: v), formic acid (85%): methanol (3:97, v: v), citric acid (1 M): methanol (3:97, v: v) and methanol: acetic acid: water (25:1:24 v: v: v). All solvents used were of analytical grade. The method by Amr & Al-Tamimi (2007) was used with modifications. A 2 g amount of dried calyces was added to 100 ml of the given solvent system. The flasks were then incubated at 25°C at 150 rpm for 2 hours after which the supernatant was filtered and the residue re-extracted once more. The combined supernatant was concentrated using a rotary evaporator (Buchi RE Rotoevaporator including a Buchi 461 water bath) at 35/40°C at 60 rpm.

2.3 Stability studies

Heat stability

Heat stability of crude extracts were determined according to Amr & Al-Tamimi (2007) with modifications. An aliquot of 0.1 mL of extract was made up to 25 mL with 0.1 M (pH 3.5) citrate phosphate buffer. Samples were heated at 50 °C and 80 °C respectively for 6 hours. Absorbance was read hourly at 520 nm using a UV/Visible spectrophotometer (Varion Cary IE). Percentage retention of anthocyanins was calculated using Equation 1:

$$Pigment\ retention\ (\%) = \frac{\text{Absorbance after heating}}{\text{Absorbance before heating}} \times 100 \quad (1)$$

pH stability

The method by Selim et al. (2008) was used with modifications. Buffers ranging from pH 1 to pH 9 were prepared according to DeLloyd (2000). A 2 mL aliquot of crude extract was made up to 20 mL with desired buffer. The test tubes were covered with aluminium foil to prevent light entry. The samples were stored at 25 °C for 2 weeks. Absorbance was read at 520 nm after 0 and 48 h, 7 and 14 days.

Light stability

Light stability of the four extracts were analysed according to method set out by Amr & Al-Tamimi (2007). Extracts were prepared in equal concentrations in citrate phosphate buffer (pH 3.5) in screw top test tubes. One set of samples were incubated under 20 W fluorescent light in a closed chamber at room temperature and the other set of samples were incubated under dark conditions at room temperature. Absorbance was read at 520 nm. Readings were taken at 0 and 24 h, thereafter every 2 h for 10 days.

2.4 Degradation kinetics

Thermal stability

The thermal stability of crude extracts were determined using the method by Li et al. (2014) with modifications. An amount of 50 mg/L of extract was made up to 7 mL with 0.1 M citric acid-sodium citrate (pH 3.0). The test tubes were covered with aluminium foil. Tubes were heated from 70 to 90 °C at 5 °C increments. Thereafter they were cooled to ± 25 °C and absorbance was read at 527 nm. The reaction rate constant (k) and half-life ($t_{1/2}$) was calculated using Equations 2 and 3 respectively.

$$k = -In_t^{\left(\frac{Ct}{Co}\right)} \quad (2)$$

Where C_t is the absorbance after heating and C_o is the absorbance before heating and t is time (h).

$$t(1/2) = -\ln 0.5 \times k - 1 \quad (3)$$

DPPH radical scavenging ability

The radical scavenging ability of the thermal treated crude extracts were analysed using the method by Li et al. (2014). An aliquot of 2 mL 1 mM DPPH (prepared in ethanol) was added to thermal treated samples. Test tubes were shaken vigorously and stored in the dark for 30 minutes at room temperature (25 °C). After which, absorbance was read at 517 nm. The control was carried out by replacing the extract with ethanol. Radical scavenging ability was calculated using Equation 4.

$$DPPH\ radical\ scavenging\ ability(\%) = \frac{1 - \text{Sample absorbance}}{1 - \text{Control absorbance}} \times 100 \quad (4)$$

2.5 Total phenolic content

The total phenolic content of each of the crude extracts were determined by Folin – Ciocalteu's method. Briefly, 0.05 mL of extract and 0.45 mL distilled water was mixed with 2.5 mL of 1:10 2 N Folin – Ciocalteu's phenolic reagent (Sigma Aldrich). Thereafter 2 mL of 7.5% (w/v) sodium carbonate was added. Samples were heated at 50 °C for 5 minutes after which absorbance was read at 760 nm with a UV/vis spectrophotometer (Varion Cary IE). Total phenolic content was estimated according to a Gallic acid standard curve (20-100 mg/mL), results were expressed as milligrams of Gallic acid equivalent (GAE) per 100 g of dry weight (Kim et al., 2009).

2.6 Identification and quantification

Four major anthocyanidins were identified and quantified from the crude extracts according to the method by Ruiz et al. (2013) with modifications. A Shimadzu HPLC system equipped with a diode array detector, column heater and automatic injection. Chromographic separation was carried out on a C18 column (LiChroCART 250 × 4 mm, 5 um) at 40 °C. Two solvents were prepared i.e. solvent A water/acetonitrile/formic acid (87:3:10% v/v/v) and solvent B water/acetonitrile/formic acid (40:50:10% v/v/v). The flow rate was 0.4 ml/min and injection volume was 10 uL. The gradient program was set from 6% of solvent B and increase exponentially over the 22 minute

runtime. PDA detection wavelength was recorded at 520 nm. Identification and quantification were carried out by comparing retention values of the standards.

Anthocyanidin standards, delphinidin chloride, malvidin chloride, cyanidin chloride and pelargonidin chloride, were reconstituted with mobile phase A and concentrations of 20, 40, 60, 80 and 100 ug/ml were used.

2.7 Data analysis

All experiments were carried out in triplicate. Data was expressed as mean ± standard deviation. Differences were evaluated by one-way and two-way analysis of variance (GraphPad Prism Version 6.01). Significance was accepted at $p < 0.05$.

3 Results and discussions

3.1 Stability studies

Heat stability

The four extracts were treated to 80 and 50 °C for 6 hours respectively. A general decline was noted in pigment retention of the samples. However there was not a vast difference in retention between the 80 and 50 °C treatments (Figure 1), this could indicate imply that most of the pigments were degraded at 50 °C and no further degradation could occur (Amr & Al-Tamimi, 2007). Comparatively there was significant ($p < 0.05$) difference in retention between 1 and 6 hours by HCl extracts. While acetic acid and citric acid extracts showed a slight, however steady decrease of pigment retention over time at both 50 and 80 °C heat treatment. Pigment retention of formic acid extract showed to degrade the most at both temperature treatments as significant ($p < 0.05$) difference was observed over the time interval. Acetic acid solvent system showed the most consistency of pigment retention at both temperatures. The rate of anthocyanin degradation when heated increases because of reacting molecules that come closer when the extract is concentrated (Kirca et al., 2007). Colour changes were also noted upon heating. In all of the samples, the colour decreased in intensity. A bleaching effect can be noted because the equilibrium is changing toward the uncoloured forms. The flavonoid structure is open to form a chalcone (an aromatic ketone and an enone that forms the central core of the variety of important biological compounds) which is further degraded to form brown products (Francis & Markakis, 1989). Heat stability could possibly be improved by increasing the anthocyanin concentration, removal of oxygen and he inactivation of enzymes (Hellstrom et al., 2013).

pH stability

Anthocyanins are known to favour acidic conditions (Selim et al., 2008). Higher pigment retention values could be observed at pH 1 and 2 and this was where the anthocyanin colour was most intense and vibrant among this pH range. The results that were obtained were in keeping with this statement as most of the results are at their highest pigment retention at lower pH as seen in Figure 2. However, Ozela et al. (2007) indicated that pigments were more stable at pH 5.0 and 6.0 than 4.0, this could also explain the reason for pigment retention in HCl containing extracts at 48 h, Formic acid containing extracts at 48 h and 7 days and acetic acid containing extracts at 0h. Pigment retention of HCl and citric acid acidified solvents had greater significant ($p < 0.05$) difference between 24 h and 7 day across the pH spectrum (pH 1-9) as compared to formic acid and acetic acid acidified solvents. However, the general increasing trend of degradation confirms that crude pigments from *H. sabdariffa* are more stable at lower pH values. The colour stability of anthocyanins can be dependent on pH and the anthocyanin structure (Cabrita et al., 2000; Kirca et al., 2007). Colour changes occurred in acetic acid and formic acid containing solvent systems after 7 days of incubation at room temperature. Brown precipitate was found in samples at pH 6-9 after 7 days of incubation. The colour changes of anthocyanins are more significant in the alkaline region because of their instability. A reason for low pigment retention can be explained by the chemistry of anthocyanins, the red flavylium cation concentration in acid medium and its possible interaction with existing co-pigments affect the adsorption properties of anthocyanin solutions thereby creating a much more stable

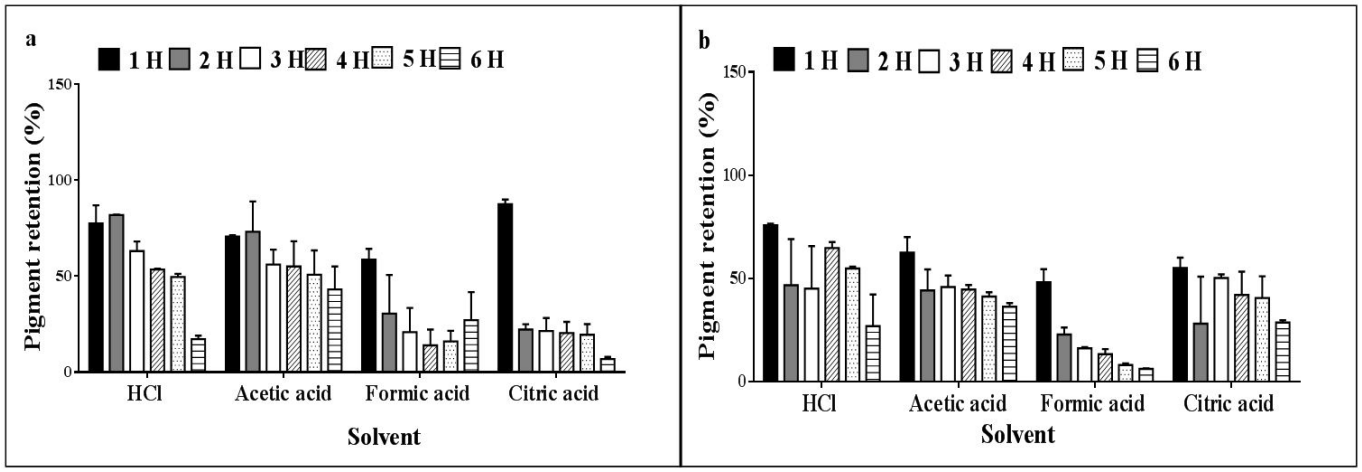

Figure 1. Effect of heat on stability of *H. sabdariffa* anthocyanins extracted by different solvent systems [a: 80 °C and b: 50 °C]. Values represent mean ± standard deviation of replicate readings (n = 3).

extract as well as protecting the colour attribute of the solution (Oancea & Drăghici, 2013).

Light stability

Light stability of anthocyanins is an important aspect as it aids in storage conditions. HCl samples appeared to be the most as the least amount of pigment degradation over time, pigment retention ranged from 97-66% in light treated samples while dark treated samples ranged from 96-68% (Figure 3). Significant ($p < 0.05$) decrease was observed over 6 h and 10 days in acetic acid and citric acid light treated samples however acetic acid showed no significant ($p < 0.05$) decrease in dark treated samples. According to Amr & Al-Tamimi (2007), their samples retained 84% of the pigment which was treated to dark conditions for 10 days.

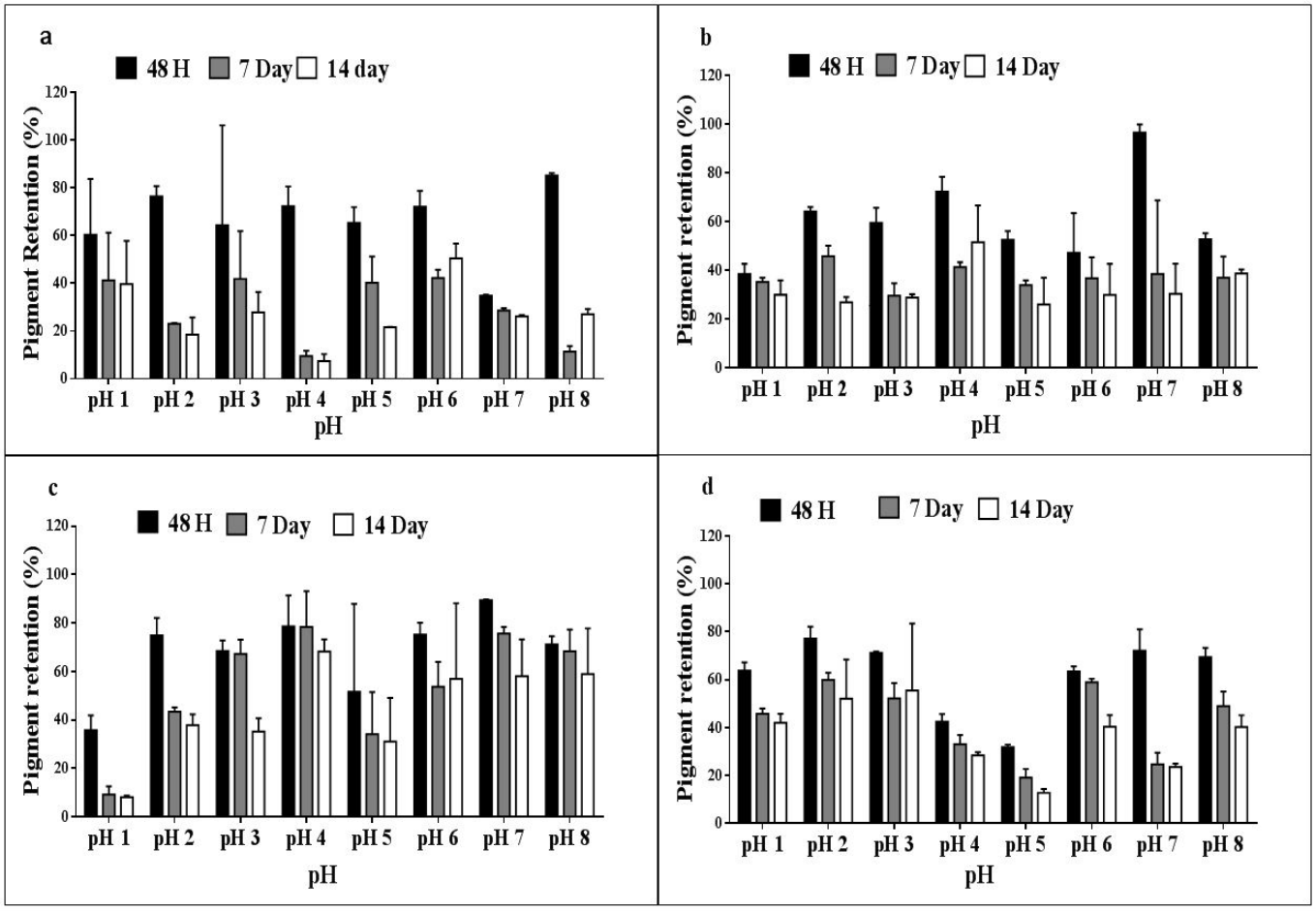

Figure 2. Effect of pH on stability of *H. sabdariffa* anthocyanins extracted by different solvent system [a: HCl and ethanol; b: Acetic acid and Methanol; c: Formic acid and methanol; d: Citric acid and methanol]. Values represent mean ± standard deviation of replicate readings (n = 3).

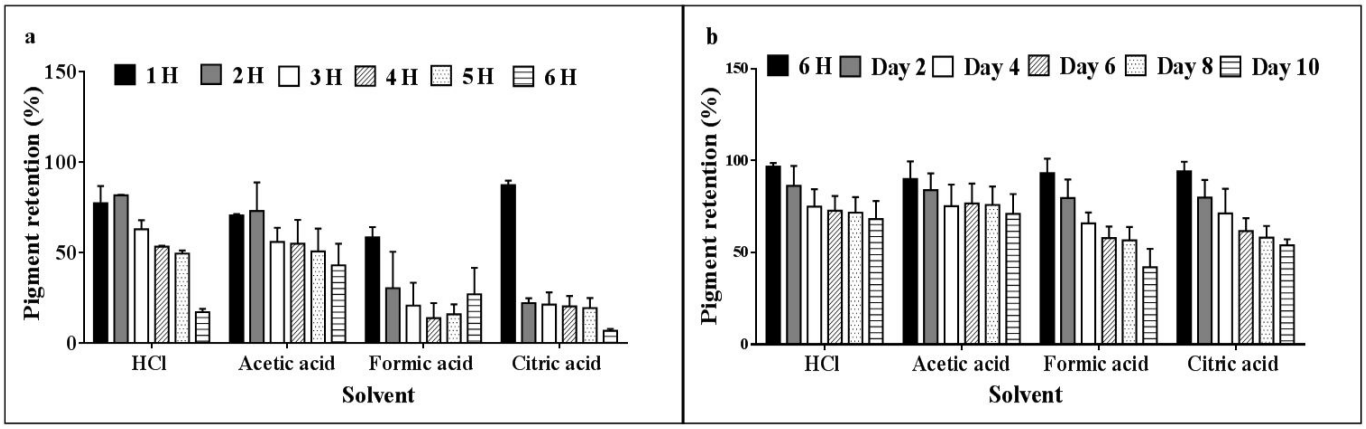

Figure 3. Effect of light on stability of *H. sabdariffa* anthocyanins extracted by different solvent system [a: Light conditions; b: Dark conditions]. Each column represents mean ± SD (n = 3).

3.2 Degradation kinetics

Thermal stability

The four extracts were tested for their thermal stability after which the rate constant in order to calculate the half-life of the extracts at each temperature treatment, as depicted in Table 1. Overall, the half-life ranged between 10 to 26 hours between the solvent systems. HCl containing solvent system showed a slow decrease in half-life, a 22% decrease was observed between 70 and 85 °C. Formic acid samples decreased by 35% between 70 and 80 °C however an 11% increase in half-life was observed at 85 °C. Citric acid samples followed a similar trend as formic acid samples. Between 70 and 80 °C, half-life decreased by 44% which was followed by an increase of 60% at 85 °C. A gradual decrease in half-life was seen in acetic acid containing solvent systems followed by an increase of 23% between 80 and 85 °C. The colour of these extracts changed over the heating period from the vibrant red to a dull brown. Acetic acid and HCl extracts changed colour slightly however the red colour was retained. Formic acid and citric acid samples lost most of its red pigment and was replaced by a dull brown colour.

DPPH radical scavenging ability

The method of scavenging DPPH is based on the reduction of DPPH ethanol solution in the presence of a hydrogen donating antioxidant, the reaction therefore resulting in the formation of the non-radical form DPPH-H (Li et al., 2014). DPPH radical scavenging activity was quantified in terms of percentage of a pre-formed free radical by antioxidants in each of the samples (Figure 4). Anthocyanins did not experience extreme degradation however; a very slight increase in radical scavenging ability at 85 °C was noted. Yue & Xu (2008) noted an increase in radical scavenging activity at 80 and 120 °C. This could be due to the release of degradation products during heating that had antioxidant capability. A study conducted on anthocyanins extracted from litchi observed that the antioxidant activity was heightened when the temperature was increased up to 45 °C thereby suggesting that antioxidant activity could be maintained with controlled temperature, this could be due to an effected created by the combination of non-enzymatic reactions and anthocyanin stability (Ruenroengklin et al., 2008).

3.3 Total phenol content

The total phenolic content was determined by Folin- Ciocalteu's method. The mechanism employs the transfer of electrons from phenolic compounds to the Folin- Ciocalteu's reagent in alkaline medium (Fu et al., 2011). The total phenol content of the extracts ranged from 54.42-55.03 mg/g GAE (Table 2). Although each of the extraction solvents were different, the plant extracted was the same, therefore there was no significant difference observed between the four solvents. No significant ($p < 0.05$) difference was observed by formic acid acidified solvent in relation to HCl acidified solvent. The results that were obtained were in keeping with literature as fruits such as apple, litchi and navel oranges were reported to have similar total phenolic content values (Fu et al., 2011). Sindi et al. (2014), reported to have lower phenolic content

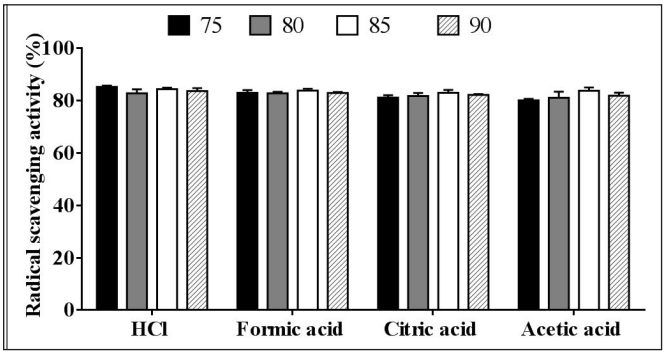

Figure 4. DPPH radical scavenging activity of *H. sabdariffa* anthocyanins subjected to thermal treatment at 70, 75, 80, 85 and 90 °C respectively. Values represent mean ± standard deviation of replicate readings (n = 3).

Table 1. Rate constant (k) and half-life (t1/2) for thermal degradation of *H. sabdariffa* anthocyanins extracted by different solvent systems.

Solvent system	Temperature (°C)	k	$t_{1/2}$ (h)
HCl + EtOH	70	0.0007 ± 0.0003	22.0 ± 4.0
	75	0.0005 ± 0.0000	18.0 ± 9.0
	80	0.0004 ± 0.0001	19.0 ± 9.0
	85	0.0004 ± 0.0001	18.0 ± 9.0
Formic acid + MeOH	70	0.0009 ± 0.0001	26.0 ± 0.3
	75	0.0006 ± 0.0007	17.0 ± 9.0
	80	0.0006 ± 0.0005	17.0 ± 7.0
	85	0.0007 ± 0.0006	19.0 ± 9.0
Citric acid + MeOH	70	0.0011 ± 0.0008	18.0 ± 0.2
	75	0.0011 ± 0.0008	12.0 ± 6.0
	80	0.0013 ± 0.0003	10.0 ± 1.0
	85	0.0012 ± 0.0008	16.0 ± 0.3
Acetic acid + water + MeOH	70	0.0009 ± 0.0006	19.0 ± 2.0
	75	0.0007 ± 0.0001	17.0 ± 0.4
	80	0.0007 ± 0.0004	13.0 ± 3.0
	85	0.0005 ± 0.0004	16.0 ± 0.1

Values represent mean ± standard deviation of replicate readings (n = 3).

Table 2. Total phenolic content of each of *H. sabdariffa* crude extracts extracted by different solvent systems.

Solvent	mg/g GAE
HCl	54.0 ± 0.2
Acetic acid	55.0 ± 0.0
Formic acid	55.0 ± 0.0
Citric acid	55.0 ± 0.1

Values represent mean ± standard deviation of replicate readings (n = 3).

Table 3. Identification and quantification of *H. sabdariffa* anthocyanins extracted by different solvent systems.

Standard (mg/100 g) / Solvent	Delphinidin chloride	Malvidin chloride	Cyanidin chloride	Pelargonidin chloride
HCl	69.0 ± 1.0	12.0 ± 0.2	77.0 ± 3.0	12.2 ± 2.0
Acetic acid	116.0 ± 0.1	ND	20.0 ± 1.0	ND
Formic acid	415.0 ± 4.0	16.0 ± 1.0	77.0 ± 1.0	ND
Citric acid	402.0 ± 2.0	15.0 ± 0.1	62.0 ± 1.0	ND

Values represent mean ± standard deviation of replicate readings (n = 3). ND represents an amount was not detected.

from *H. sabdariffa* anthocyanins extracted from methanol while Sirag et al. (2014), found 41 mg/g GAE total phenolic content. Abou-Arab et al. (2011), evaluated the total phenolic content of *H. sabdariffa* extracted by HCl acidified ethanol, the authors found 42.00 mg/g GAE.

3.4 Identification and quantification

The anthocyanidins were identified and quantified by HPLC-DAD from crude anthocyanin extracts. Four anthocyanidin standards, delphinidin chloride, malvidin chloride, cyanidin chloride and pelargonidin chloride were evaluated. Identification was carried out by comparing retention times of the standards while quantification was calculated from the calibration curves. Yue & Xu (2008) reported that delphinidin and cyanindin contribute to approximately 80% of the total anthocyanins present in a plant. The results obtained from *H. sabdariffa* extracts indicates that these compounds occupy between 85 and 100% of the total anthocyanins (Table 3). Delphinidin and cyanidin are known to contribute largely to the bioactive compound of anthocyanins and have higher bioavailability as compared to the other anthocyanins (Yue & Xu, 2008). Sindi et al. (2014) studied aqueous and methanolic extracts of *H. sabdariffa*. The authors reported delphinidin and cyaniding 3 – sambubiosides were the major compounds. Absorption peaks appeared at 330 nm of the UV–vis characteristics of the major anthocyanins, which indicated that the anthocyanins are acylated. Acylated anthocyanins suggest that the anthocyanins are mono- or di- acylated forms. A high amount of acylated anthocyanins shows high stability thereby confirming the industrial use as a natural food colourant (Fan et al., 2008).

4 Conclusion

In this study, the stability and degradation kinetics of anthocyanin, crude extracts from *H. sabdariffa* were determined. HCl acidified ethanol was selected as the solvent for anthocyanin extraction as it fulfils food safety requirements as well as allows for good anthocyanin stability when subjected to pH, heat and light. Degradation kinetics revealed that anthocyanins could be heated to temperatures up to 70 °C with antioxidant contents decreasing only gradually. Anthocyanins from *H. sabdariffa* can be used as an alternative to synthetic food colourants with nutraceutical properties imparted from the anthocyanins.

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