Chapter 5.

EFFECT OF PHYSICOCHEMICAL TREATMENTS ON EXTRACTED PIGMENT
5.1. Summary

Stability of betalains in *Rivina humilis* berry juice (RBJ) was studied at different pH, temperature, under nitrogen atmosphere, in presence of metals and stabilising agents (ascorbic acid and gallic acid). *Rivina* berry betalains were comparatively more stable at pH 5, low temperature (5°C), and under nitrogen atmosphere in dark. RBJ betacyanins degraded upto 95% and 96% on treatment for 36 min at 90°C, and storage for 48 days at 25°C, respectively, whereas only 15% pigment was lost on storage at 5°C over a period of 90 days. Presence of ascorbic acid protected betalains from degradation at 25°C and 90°C, whereas it did not show significant protection at 5°C. Effect of presence of inorganic selenium (Se⁴⁺), Cu²⁺, and Zn²⁺ with ascorbic acid (0.25% and 0.5%, w/v) on stability of RBJ betacyanins was studied. A similar set of samples in the absence of ascorbic acid was concurrently analysed. During heating at 90°C, Se⁴⁺ (upto 40 µg/mL) had mild bleaching effect on RBJ betacyanins, whereas Zn²⁺ and Cu²⁺ (upto 40 µg/mL) degraded 33% and 96% of the pigments, respectively, compared to control. Ascorbic acid protected the pigments from metal–induced bleaching and stabilising effect of ascorbic acid at 0.25% (w/v) was significantly higher than 0.5% ascorbic acid (w/v). Presence of ascorbic acid (0.25%, w/v)+Se⁴⁺ (40 µg/mL) enhanced the half–life time of betacyanins at 90°C by five–fold. Betaxanthins degraded rapidly in all the samples. Ascorbic acid (0.25%, w/v)+Se⁴⁺ (40 µg/mL) regenerated RBJ betacyanins efficiently after thermal destruction, on storage at 5°C. Samples containing ascorbic acid (0.25%, w/v)+Se⁴⁺ (40 µg/mL) produced an orange tinge (probably, due to complex formation) resulting in the lowest values of colour parameters (Hunter’s *Lab*) compared to other samples. Also, HPLC analysis revealed that there was bathochromic shift in the $\lambda_{\text{max}}$ of betanin and isobetanin in these samples suggesting complex formation. Based on these results, thermal processing of betalains containing juice may be carried out in presence of ascorbic acid (0.25%, w/v)+Se⁴⁺ (40 µg/mL) and stored at 5°C for maximum regeneration of pigments. Since betalains have high tinctorial value, the juice may be diluted before use in order to minimise selenium level.
Stability

5.2. Introduction

Betalains are nitrogen–containing heterocyclic pigments accumulated in vacuoles. They have betalamic acid as chromophore and are sub-divided into betacyanins, which are cyclo-DOPA derivatives of betalamic acid that may be glucosylated and further acylated, and betaxanthins, which are amino or amine derivatives of betalamic acid. Betalains are relatively more stable at pH 4–6 range and storage at 4°C significantly reduces pigment loss. Hence, betalain pigments are used as colourant in frozen foods, low temperature dairy products and short–shelf life foods. Unstable nature of betalains in light, heat, high pH (> 6), on contact with air and metals such as Fe^{3+}, Fe^{2+}, Cu^{2+}, Cu^{+}, Sn^{2+}, Al^{3+}, Hg^{2+}, Cr^{3+} has restricted them from extensive applications in foods (reviewed by Herbach et al., 2006b). Betalains could be stabilised to certain extent by ascorbic acid (AA), isoscorbic acid, β-cyclodextrin, glucose–oxidase, and other preservatives, whereas organic acids such as lactic acid, acetic acid, and antioxidant phenolic compounds have no stabilisation effect on the pigments (Herbach et al., 2006b). β-Cyclodextrin was found to stabilise betalains (Drunkler et al., 2006) through formation of 1:1 inclusion complex (Norasiha et al., 2009). Recently, maltodextrin has been also shown to stabilise indicaxanthin, a type of betaxanthin, after microencapsulation (Gandia-Herrero et al., 2010). Apart from antioxidants, and inclusion complexes or microencapsulation, metal chelating agent EDTA also has been shown to stabilise betalains through prevention of metal–induced betanin degradation by forming EDTA–metal complex (Attoe and von Elbe, 1985).

Although selenium (Se) is a component of selenoproteins such as glutathione peroxidases with in vivo antioxidant properties (Rotruck et al., 1973), high levels of Se is known to be toxic. AA is water soluble antioxidant which can be regenerated in vivo through reduction of its oxidised forms (dehydroascorbic acid and the ascorbate free radical), mediated by glutathione (May et al., 2001). Since Se acts synergistically with AA in preventing carcinogenesis (Gonzalez, 1990), adequate levels of Se and AA appears to be important for our body. AA stabilises betalains in vitro (Han et al., 1998; Herbach et al., 2006a; Mößhammer et al., 2007; Woo et al., 2011) but, in vivo, it is known to reduce dietary inorganic Se to less absorbed form (i.e., elemental selenium) in the body (Gonzalez, 1990; Jacques-Silva et al., 2001). Therefore, the effect of Se on betalains stability and the cumulative effect of Se and AA on betalains need to be studied.

Regeneration ability of betalains after thermal destruction in presence of antioxidants is a unique property. Limited reports on regeneration of betalains indicate that the phenomenon has not received due attention (Han et al., 1998; Herbach et al., 2006a).

R. humilis berries accumulate betalain pigments (Chapter 3 and 4) and pigment rich berry extracts have been shown to possess antioxidant properties in vitro (Chapter 3). Since
betalains are poorly stable, to increase their uses, stabilisation is important. For this, preservatives such as AA, isoascorbic acid are commonly used. Betalain-metal complex formation also may stabilise the pigments. Preliminary studies were conducted with respect to the effect of pH, temperature and nitrogen flushing on Rivina berry betalains stability. Further studies were focussed on the effect of AA and Se on chemical stability, colourant properties, and regeneration of betalains in R. humilis berry juice after thermal processing and storage. In order to bridge the gap in AA concentration (0.1 and 1.0%, w/v) (Herbach et al., 2006a; Woo et al., 2011) reported earlier for betalains stabilisation and regeneration, intermediate AA concentrations (0.25 and 0.5%, w/v) were used in this study. For comparative understanding of the observed changes in presence of Se, betalain-complex forming (Cu) and non-complex forming (Zn) metals, both of which are otherwise known to bleach betalains, have been also included in this study.

5.3. Materials and methods

5.3.1. Preliminary stability studies

Ripened berries (~40 days after anthesis) of R. humilis (red variety) were collected during September–November 2009 from shady areas of the environs of Central Food Technological Research Institute, Mysore (India) located geographically between 12° 18´ 26” North latitude and 76° 38´ 59” East longitude. The berries were deseeded manually and pulp was crushed and extracted with MeOH/H₂O (6/4, v/v). Betacyanins and betaxanthins contents were quantified using molar extinction co–efficients of betanin (60000 L.M⁻¹.cm⁻¹) and vulgaxanthin I (48000 L.M⁻¹.cm⁻¹) in water in an equation reported earlier (see Materials and methods, Chapter 3). The extract was concentrated under reduced pressure and resuspended in different buffer solutions (maintained at pH 2, 4, 5, 6, 8, and 10) to get a final concentration of 1 mg betalains in 10 mL. The solutions were filled in glass vials ca. 5 mL, tightly capped and held at 25°C for 30 days. Every 5 days vials were withdrawn for spectrophotometer reading. The pigment retention was calculated and expressed as percentage of the pigment content on day 0. The batches of vials containing berry extracts (1 mg betalains in 10 mL) were also stored at 5°C (dark), 25°C (dark) and 25°C under light [12 h photoperiod, intensity of 45 μMm⁻² s⁻¹, maintained using fluorescent tubes (Philips India Ltd, Mumbai, India)] with or without nitrogen gas flushing. Vials were withdrawn every week and pigment retention was calculated. To see the effect of ascorbic acid and gallic acid as stabilisers, batches of vials were exposed to 60°C in dark and every two hours samples were withdrawn to calculate pigment retention compared to that of initial sample (before heating).

5.3.2. Chemicals, source of berry and juice extraction

Chemicals used in this study were of analytical grade (see Materials and methods of Chapetr 3 and 4). R. humilis (red variety) were collected during September–November 2010
from shady areas of the environs of Central Food Technological Research Institute, Mysore (India). The berries were squeezed manually to get 67-70 mL juice/100 g.

5.3.3. Analysis of physicochemical properties of *R. humilis* berry juice

Betacyanins and betaxanthins contents in *Rivina* berry juice (RBJ) were quantified as mentioned in section 5.3.1. Total carbohydrates (phenol sulphuric acid method), phenols (Folin-Ciocalteau method), total soluble solids (TSS) and titrable acidity in RBJ were analysed following standard protocol (see Materials and methods, Chapter 4). Colour of RBJ was measured following *Lab* method (Hunter and Harold, 1987) in the visible range (380–700 nm) using barium sulfate as standard white. Approximately 4 mL sample in glass vial was placed on sample port and reading was recorded using the operating conditions described earlier (Khan et al., 2011b). Chroma (*C*) and hue (*h*) values were calculated as reported earlier (Khan et al., 2011b). RBJ pH and density were measured at 26±0.5°C.

5.3.4. Sample preparation

RBJ was diluted 125 times with triple distilled water, then filtered through Whatman no. 1, 0.45 μm, and 0.22 μm membranes, in succession, using a membrane filtration unit (Tarsons India Pvt. Ltd, Bengaluru, India) before adding into screw–capped glass vials of ca. 5 mL to formulate 4 mL of samples:

- I, control (RBJ diluted with water);
- II, RBJ diluted with water containing AA (0.25%, w/v);
- III, RBJ diluted with water containing AA (0.5%, w/v);
- IV, Sample I added with 10 μg Se⁴⁺/mL;
- V, Sample I added with 40 μg Se⁴⁺/mL;
- VI, Sample II added with 40 μg Se⁴⁺/mL;
- VII, Sample III added with 10 μg Se⁴⁺/mL;
- VIII, Sample III added with 40 μg Se⁴⁺/mL;

In the similar way, samples containing copper (Cu²⁺) and zinc (Zn²⁺) were also formulated in presence of AA (0.5%, w/v). Metals used in this study were of atomic absorption spectroscopy–grade solutions in diluted nitric acid (Sisco Research Laboratory, Mumbai, India). Final pH of the samples was adjusted to 5 using 0.5N NaOH or 2N HCl to pH 5 (Reynoso et al., 1997; Herbach et al., 2006a). The initial optical density of control sample was approx. 1.0 at 535 nm which corresponds to *λ*ₘₐₓ of betanin.

5.3.5. Thermal stability study of betalains in presence of metals

Thermal treatment of all samples (mentioned in the previous section) was carried out in a multi–chamber digital water bath (Daihan Labtech, Seoul, South Korea) at 90 ± 1°C for 3, 6, 12, 24 and 36 min in dark. Samples were cooled immediately under tap water and pigment remaining (%), compared to that of control before treatment, was calculated using
optical density at 477 nm, and 535 nm (corresponding to absorption of betaxanthins and betacyanins, respectively) recorded against water (pH 5) using a spectrophotometer (Model-UV 160 A, M/s Shimadzu Corp., Kyoto, Japan). Results were expressed as half-life time \( T_{1/2} \) of betacyanins, which was derived from the degradation kinetics using Microcal Origin 6.0 software (M/s Microcal Software Inc., Northampton, MA, USA). Results were also expressed as percentage of betacyanins content at zero time. Colour profile \( (L, a \text{ and } b \text{ values}) \) was measured without further delay as described in the previous section.

5.3.6. Storage stability study of betalains

Samples containing AA (0.25 or 0.5%, w/v), Se\(^{4+}\) (40 \( \mu \)g/mL), AA (0.25 or 0.5%, w/v)+Se\(^{4+}\) (40 \( \mu \)g/mL), and control were stored in dark at 25±1°C after blanching treatment at 90°C for 3 min. Samples stored at 25±1°C were withdrawn every 8 days for recording optical density to calculate the remaining betaxanthins and betacyanins. The results were expressed as percentage of their content in the control samples on day 0. Colour profile was recorded immediately after that. After about 30 days of storage, some of the samples developed mild precipitation (dark brown colour) which was removed through centrifugation (8000 \( \times \)g, 15 min, 4°C) prior to analysis. Samples containing AA (0.25 or 0.5%, w/v)+Se\(^{4+}\) (40 \( \mu \)g/mL) formed violet–red coloured colloidal particles after 30 days of storage that settled at bottom. This phenomenon resulted in slight reduction in optical density of those samples particularly in colour measurement.

Samples were also stored in dark at 5±1°C. These samples were withdrawn every 15 days for analysis of betacyanins and colour profile. Owing to the aseptic processing condition and temperature of storage (5±1°C), there was neither contamination nor precipitation.

5.3.7. Stability study of purified betacyanins

Betacyanins were partially purified from RBJ as described in Materials and methods of Chapter 3 of this thesis. Purified betacyanins were diluted to get an optical density of approx. 1.0 at 535 nm. Stability of purified betacyanins was studied in the following samples (4 mL each):

- I, control (diluted partially purified betacyanins with water);
- II, sample I added with AA (0.5%, w/v);
- III, sample I added with Se\(^{4+}\) (40 \( \mu \)g/mL);
- IV, sample I added with AA (0.5%, w/v)+Se\(^{4+}\) (40 \( \mu \)g/mL).

Stability of pigment during thermal (90°C) treatment, storage at 25°C and 5°C was studied using spectrophotometer readings recorded at different time intervals as described in section 5.3.5.

5.3.8. Regeneration studies of betalains

Samples I, II, III, V, VI and VIII (see section 5.3.4) were treated for 3 min in dark at
90±1°C and, after cooling immediately, stored at 5±1°C in dark. After 24 h of cold storage, the optical densities corresponding to betaxanthins and betacyanins were recorded as described in section 5.3.5. The results were expressed as percentage of their contents in the untreated control on day 0. This cycle of heat treatment, cold storage and pigment analysis was repeated for the next six days. This was done to study the regeneration of betalain pigments after intermittent exposure to heat in presence and absence of AA (0.25 or 0.5%, w/v) with or without Se⁴⁺ (40 μg/mL).

Samples I, II, III, V, VI and VIII (see section 5.3.4) were also treated in dark at 90±1°C for 24 min continuously, cooled immediately, one set of samples were analysed for thermal degradation of pigment, and another set of samples were stored at 5±1°C in dark for 7 days. On day 7, betaxanthins and betacyanins contents in stored samples were analysed as described in section 5.3.5 and compared with that of before and after 24 min thermal treatment on day 0. Results were expressed as percentage of betaxanthins and betacyanins contents before heat treatment on day 0.

5.3.9. HPLC analysis of betalains

Betalains in RBJ were analysed and identified as described (Chapter 3). Betanin and isobetanin peaks (detected at 535 nm) were identified by comparing their retention times with that of a reference sample prepared from red beet juice (in which betanin was the major peak) and earlier report on pigment identification in Rivina berries (see Results and discussion, Chapter 3).

5.3.10. Statistical analysis

All the analyses were carried out using analytical triplicates which were treated concurrently under the same conditions. Results are presented as mean ± standard deviation (SD, n=3). Data analysis was done using Two-Way ANOVA and values were considered significant at P < 0.05.

5.4. Results and discussion

5.4.1. Preliminary stability studies

In preliminary study (Fig. 5.1), pH 5 was found to be optimum for stability of betalains in Rivina berry extract. Pigment loss was very less upto 20 days at pH < 4, whereas there was intense browning since day 0 at pH > 6. This observation was supported by earlier reports on stability of betalains at pH 5 (Harivaindaran et al., 2008; Woo et al., 2011).

Pigment retention was more at 5°C in dark after flushing with nitrogen, whereas in the absence of nitrogen flushing pigment retention was comparatively less (Fig. 5.2A). Least stability was observed when the extracts were exposed to air, light and maintained at room temperature (Fig. 5.2B). Even in the absence of nitrogen flushing, at refrigerated temperatures
**Stability**

Figure 5.1. Stability of *Rivina humilis* extract (containing 1 mg betalains in 10 mL) held at 25°C in different pH (2, 4, 5, 6, 8, and 10; left to right). First row of samples were photographed on day 0 and following rows represent the batch of samples withdrawn every five days thereafter. After 25 days, samples at pH 4 and 5 retained 44.4 and 45.7% of initial betalains content, respectively.

The retention of betalains of *Rivina* berries was about 28% at the end of 8 weeks of storage, whereas at ambient temperature pigment completely lost at the end of 8 weeks. In nitrogen flushed samples, pigment retention at 5°C was twice that of 25°C (in light) at the end of 8 weeks. When samples were stored in dark at 25°C, pigment retention improved significantly in both nitrogen gas flushed and without nitrogen gas flushing (Fig. 5.2C) compared to that of 25°C in light. When the samples were heated at 60°C in dark, half of the pigments degraded in 2 h (Fig. 5.2D), whereas addition of ascorbic acid stabilised the pigments (only ~30% pigment lost in 12 h). From the data, it was evident that ascorbic acid at 0.5% (w/v) was better stabiliser than ascorbic acid at 0.1% (w/v). A phenolic antioxidant gallic acid was also studied for its role as stabiliser but, it was observed that ascorbic acid was comparatively more...
Stability

Effective stabiliser (Fig. 5.2D). Similar observations have been reported earlier (reviewed by Herbach et al., 2006b; Azeredo, 2009). Our observations after addition of gallic acid were consistent with earlier report that phenolic acids have no stabilising effect on betalains (Attoe and von Elbe, 1985). Based on these observations, further studies were focussed on betalains stability in presence of metals, with/without ascorbic acid (0.25 and 0.5%, w/v) at three different temperatures signifying refrigerated (5°C), ambient (25°C) and thermal processing (90°C) conditions.

![Figure 5.2. Effect of nitrogen flushing on betalains pigment stability. Rivina humilis berry extracts containing 1 mg betalains in 10 mL MeOH/H₂O (pH 5) was held at 5°C in dark (A), 25°C in light (B) and dark (C) and 60°C in dark (D). Values are mean ± SD of three concurrent samples. AA- ascorbic acid, GA- gallic acid.](image)

5.4.2. Physicochemical properties of Rivina berry juice

Table 5.1 presents data on physicochemical properties of RBJ. In this study, it was observed that betaxanthins content was higher than that of betacyanins. In many of the
betalains sources, betaxanthins to betacyanins ratio is less than 1. For example, red beet and cactus pear fruit have betaxanthins to betacyanins ratios of 0.58–0.60 (Gasztonyi et al., 2001), and 0.63–1.5 (Chavez-Santoscoy et al., 2009), respectively, on fresh weight basis. Since the ratio of absorbance of betaxanthins to betacyanins was more than 1, colour shade of RBJ was yellow–red.

Table 5.1. Physicochemical parameters of Rivina humilis berry juice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betacyanins (mg/100 mL)</td>
<td>155.5 ± 7.5</td>
</tr>
<tr>
<td>Betaxanthins (mg/100 mL)</td>
<td>209.7 ± 12.2</td>
</tr>
<tr>
<td>(^a)TSS (°Brix)</td>
<td>15.0 ± 0.3</td>
</tr>
<tr>
<td>(^a)pH</td>
<td>6.3 ± 0.05</td>
</tr>
<tr>
<td>Colour</td>
<td></td>
</tr>
<tr>
<td>(^b)L</td>
<td>5.3 ± 0.15</td>
</tr>
<tr>
<td>(^b)a</td>
<td>6.18 ± 0.56</td>
</tr>
<tr>
<td>(^b)b</td>
<td>2.0 ± 0.19</td>
</tr>
<tr>
<td>(^b)ΔE</td>
<td>85.44 ± 0.2</td>
</tr>
<tr>
<td>(^b)C</td>
<td>6.5 ± 0.59</td>
</tr>
<tr>
<td>(^b)h</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>(^a)Density (g/mL)</td>
<td>1.08 ± 0.01</td>
</tr>
<tr>
<td>Titrable acidity (g CAE/L)</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Total carbohydrates (g/100 mL)</td>
<td>2.8 ± 0.16</td>
</tr>
<tr>
<td>Total phenols (mg GAE/100 mL)</td>
<td>741.5 ± 52.3</td>
</tr>
</tbody>
</table>

TSS- total soluble solids, CAE- citric acid equivalent, GAE- gallic acid equivalent, ΔE- colour difference. \(^a\)Values were measured at 26±0.5°C. \(^b\)Values are expressed in Hunter Lab method. Values are mean ± SD (n=3).

TSS includes mainly sugars and other soluble components such as proteins. pH and acidity indicate the content of organic acids in the juice. Total carbohydrates include simple and complex sugars, their derivatives, including methyl esters with free reducing groups. Since betalains also contribute to total phenols (Wu et al., 2006), its content was high in RBJ.

### 5.4.3. Effect of metals on betacyanins stability in presence or absence of ascorbic acid

Effect of Cu and Zn on betalains stability has been well documented (Pasch and Elbe, 1979). Hence, these two metals were considered for a comparative understanding of the effect of antioxidant metal, Se, on betalains stability. Ascorbic acid was used in this study as it is a popular preservative of betalains and based on results of preliminary studies. In the following
sections, the results pertaining to betacyanins only have been presented as betaxanthins were relatively unstable and showed poor regeneration. Moreover, optical density recorded at 477 nm gives mixed value of betaxanthins+betacyanins, not betaxanthins alone, whereas at 535 nm only betacyanins absorb.

Table 5.2 reports half–life time of betacyanins during thermal treatment in presence of Se, Zn and Cu (10 or 40 $\mu$g/mL) with or without AA (0.25 or 0.5%, w/v).

<table>
<thead>
<tr>
<th>Sample</th>
<th>$T_{1/2}$ (min) in absence of AA</th>
<th>$T_{1/2}$ (min) in presence of AA (0.5 g/100 mL)</th>
<th>$T_{1/2}$ (min) in presence of AA (0.25 g/100 mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.8 ± 0.1</td>
<td>12.8 ± 0.5*</td>
<td>13.5 ± 0.4*</td>
</tr>
<tr>
<td>Se (10 $\mu$g/mL)</td>
<td>8.4 ± 0.05</td>
<td>14.8 ± 0.6</td>
<td>na</td>
</tr>
<tr>
<td>Se (40 $\mu$g/mL)</td>
<td>8.5 ± 0.15</td>
<td>39.6 ± 1.2**</td>
<td>54.8 ± 2.1**</td>
</tr>
<tr>
<td>Zn (10 $\mu$g/mL)</td>
<td>7.4 ± 0.17*</td>
<td>12.5 ± 0.4</td>
<td>na</td>
</tr>
<tr>
<td>Zn (40 $\mu$g/mL)</td>
<td>6.6 ± 0.07*</td>
<td>8.4 ± 0.1*</td>
<td>na</td>
</tr>
<tr>
<td>Cu (10 $\mu$g/mL)</td>
<td>3.5 ± 0.04*</td>
<td>11.2 ± 0.3</td>
<td>na</td>
</tr>
<tr>
<td>Cu (40 $\mu$g/mL)</td>
<td>0.44 ± 0.02**</td>
<td>0.64 ± 0.04**</td>
<td>na</td>
</tr>
</tbody>
</table>

AA- ascorbic acid, na- not analysed.

*P < 0.05, **P < 0.001 compared to control in the absence of ascorbic acid.

#P < 0.01, ##P < 0.001 compared to control in the presence of ascorbic acid.

Compared to control ($T_{1/2}$ 9.8 min), Se (10 or 40 $\mu$g/mL) showed mild bleaching effect on betacyanins ($T_{1/2}$ 8.4 or 8.5 min), whereas Zn (10 or 40 $\mu$g/mL) significantly reduced $T_{1/2}$ of the pigments to 7.4 or 6.6 min and Cu (40 $\mu$g/mL) showed the maximum bleaching effect by reducing the $T_{1/2}$ to just 0.44 min. Presence of AA (0.25 or 0.5%, w/v) significantly ($P < 0.05$) enhanced $T_{1/2}$ of betacyanins to 13.5 or 12.8 min during thermal treatment. Compared to control (containing 0.5% AA, w/v), AA (0.5%, w/v) combined with Se, Zn and Cu (10 $\mu$g/mL) did not have significant difference in $T_{1/2}$ of betacyanins, while AA (0.5%, w/v)+Zn or Cu (40 $\mu$g/mL) had significant ($P < 0.01$) bleaching effect on betacyanins. AA (0.5%, w/v)+Se (40 $\mu$g/mL) increased half–life time of betacyanins four–fold compared to control, whereas AA (0.25%, w/v)+Se (40 $\mu$g/mL) enhanced more than five–fold. It was clear from Table 5.2 that Se (40 $\mu$g/mL) was most effective enhancer of half–life time of betacyanins during thermal treatment in presence of AA (0.25%, w/v). It appears that presence of both Se and AA
produced a synergistic stabilizing effect. Similar observations on protective effect of AA in presence of iron or chromium were reported (Reynoso et al., 1997). The stabilising effect could be also due to formation of a relatively more stable betalain complex with Se in presence of AA. This observation is important particularly because ascorbic acid has been known to combat toxicity of heavy metals such as Cu, Zn and Se (Fox, 1975). Moreover, AA has been shown to ameliorate organoselenium toxicity in mice (Jacques-Silva et al., 2001). Further, reports say that Se could be supplemented (up to 200 µg.d¹), without any toxic effects, while immune responses get enhanced, bacterial and viral infections are reduced, and heart diseases, cancers (such as breast, prostate, lung, and liver cancers), Alzheimer’s and associated dementias are reduced (Spallholz et al., 2001; Ellis and Salt, 2003). Based on these reports, the effect of Se and AA on stability of betalains and its nutritional and bioavailability implications need to be investigated. Since AA (0.25%, w/v)+Se (40 µg/mL) increased half–time of betacyanins to more than five–fold of control (Table 5.2), Se (40 µg/mL) was considered for further study.

5.4.4. Stability of betalains in presence of selenium and ascorbic acid: effect of temperature

Fig. 5.3A shows that after 36 min of exposure at 90°C, control and Se (40 µg/mL) containing samples retained about 4% and 5% betacyanins, respectively, whereas only AA (0.25 or 0.5%, w/v) containing samples retained 36% or 43% betacyanins. Samples containing AA (0.25%, w/v)+Se showed 68% retention, which was not significantly higher than that of AA (0.5%, w/v)+Se (62%). All the samples showed degradation kinetics similar to that of first order reactions, except samples containing AA (0.25 or 0.5%, w/v)+Se, which may be due to formation of a relatively stable betalain-metal coordinate complex.
Stability

Figure 5.3. Betacyanins remaining in diluted *Rivina humilis* berry juice after treatment in dark at 90°C (A), during storage at 25°C (B), and at 5°C (C) in absence (control) and presence of ascorbic acid (0.25 and 0.5%, w/v) and selenium (40 μg/mL). Values are mean ± SD of triplicate samples. Diluted juice (control) (□), diluted juice containing ascorbic acid (0.25%, w/v) (○), ascorbic acid (0.5%, w/v) (△), selenium (40 μg/mL) (×), ascorbic acid (0.25%, w/v)+selenium (40 μg/mL) (△), ascorbic acid (0.5%, w/v)+selenium (40 μg/mL) (O).

Control and Se alone containing samples lost more than 90% pigments in 24 days at 25°C (Fig. 5.3B). Approximately 38% pigment retention, nine-fold that of control, was
Stability

observed in presence of AA (0.25%, w/v) and 24% retention in presence of AA (0.25%, w/v)+Se during storage at 25°C for 48 days. AA (0.25%, w/v) alone or combined with Se protected betacyanins significantly ($P < 0.01$) higher than AA (0.5%, w/v) alone or combined with Se that showed retention of only 15% pigments but, three–fold higher than that of control samples at the end of storage period. This could be attributed to pro–oxidant nature of higher AA concentration at the given storage temperature. None of the other samples showed significant difference in pigment retention compared to control at the end of storage period. Since the samples were subjected to blanching treatment at 90°C for 3 min before storage at 25°C, none of the samples showed linear degradation kinetics.

RBJ betacyanins was very stable at low temperature (5°C). Only 15% pigment was lost over a period of 90 days (Fig. 5.3C). AA (0.25 or 0.5%, w/v) alone did not show any protective effects on betacyanins compared to control samples, whereas AA (0.25%, w/v)+Se and AA (0.5%, w/v)+Se retained 98% and 83% pigments, respectively, up to 90 days. The results showed that when AA concentration was increased, even the presence of Se did not lead to protective effect on betacyanins. This could be ascribed to presence of high concentration of AA as it reduces betalain stability (Moßhammer et al., 2007; Woo et al., 2011), probably, due to pro–oxidant activity of hydrogen peroxide formed during degradation of AA to dehydro–ascorbic acid (Pasch and Elbe, 1979). From Fig. 5.3, it may be inferred that protection of betalains from degradation in presence of AA and Se is better at high temperature and reduces with decrease in temperature. This may be owing to interaction among betalains and Se resulting in formation of a relatively stable complex at high temperature, apparently facilitated by the presence of AA.

Se alone could not protect RBJ betacyanins in any of the conditions studied (Fig. 5.3). Protective effect of AA on betacyanins has been shown in earlier reports also (Han et al., 1998; Herbach et al., 2006a; Woo et al., 2011). In this study, in presence of AA (0.25 or 0.5%, w/v), there was comparable stabilisation effect on betalains at all the temperatures studied. This could mean that AA’s betalains stabilisation efficiency is maximum at 0.25% (w/v) specially at 25°C and 5°C.

5.4.5. Stability of purified betacyanins in presence of selenium and ascorbic acid

During thermal treatment of purified betacyanins, within 15 min almost 94% pigments were lost (Fig. 5.4A). Storage at 25°C resulted in reduction of 93% pigments in 16 days, whereas 34% pigment lost during 60 days at 5°C. AA (0.5%, w/v) and Se (40 μg/mL) protected betacyanins efficiently during thermal treatment and storage at ambient temperature (Fig. 5.4B). In presence of AA+Se, there was ten–fold increase in pigment retention at 90°C and 25°C, whereas at 5°C there was significant ($P < 0.05$) enhancement in pigment retention (73%) compared to control (Fig. 5.4C). Purified betacyanins degraded in first order reaction
Stability

kinetic during treatment at 90°C, and storage at 25°C (Fig. 5.4) as documented earlier (Han et al., 1998; Fernandez–Lopez and Almela, 2001; Castellar et al., 2003; Woo et al., 2011).

Figure 5.4. Purified betacyanins (from *Rivina humilis* berry) remaining after treatment in dark at 90°C (A), 25°C (B) and 5°C (C) in absence (control) and presence of ascorbic acid (0.5%, w/v) and selenium (40 µg/mL). Values are mean ± SD of triplicate samples. Diluted purified betacyanins (control) (□), diluted purified betacyanins containing ascorbic acid (0.5%, w/v) (◇), selenium (40 µg/mL) (△), ascorbic acid (0.5%, w/v)+selenium (40 µg/mL) (×).

Partially purified betacyanins were comparatively less stable at 90°C, 25°C and 5°C (Fig. 5.4) than RBJ betacyanins. This was observed earlier in case of purified betacyanin which was less stable compared to that of betacyanins in juice of red dragon fruit (Herbach et al., 2006a) and cactus pear fruit (Moßhammer et al., 2007). Se⁴⁺ significantly protected purified betacyanins compared to control in all the temperature conditions studied (Fig. 5.4A and B). It appears that the antioxidant nature of Se⁴⁺ was more pronounced on purified betacyanins than RBJ betacyanins.
Stability

5.4.6. Effect of ascorbic acid and selenium on colourant properties of betalains

Contrary to the reported changes in lightness (Moßhammer et al., 2007), Fig. 5.5A revealed that lightness increased significantly ($P < 0.05$) on thermal treatment. Control and Se$^{4+}$ alone containing samples had maximum $L$ value owing to loss of more pigments. Lightness of the samples tended to increase on thermal treatment at 90°C compared to that of initial value except, the samples containing AA (0.25 or 0.5%, w/v) alone or combined with Se, which showed the minimum $L$ value. As pigment degradation in all the samples was less during storage at 5°C, there was no significant change in lightness of the samples (Fig. 5.5B).
Stability

Figure 5.5. Lightness (A and B), chroma (C and D) and hue angle (E and F) profile of Rivina humilis berry juice during thermal treatment at 90°C and storage at 5°C, respectively, in absence (control) and presence of ascorbic acid (0.25 and 0.5%, w/v), and selenium (40 μg/mL). Values are means of triplicate analyses having standard deviation less than 10% of mean. For figure legends, refer Fig. 5.3.

Chroma values of control, AA (0.25 or 0.5%, w/v), and Se alone containing samples reduced marginally during thermal treatment while there was two to three–fold reduction in chroma of AA (0.25 or 0.5%, w/v)+Se⁴⁺ containing samples (Fig. 5.5C), owing to orange colour development. The reduction in chroma value on thermal exposure is consistent with earlier report (Herbach et al., 2006a; Moßhammer et al., 2007). During low temperature storage of the samples, there was no significant ($P < 0.05$) change in chroma value over a period of 90 days (Fig. 5.5D), owing to less pigment degradation. Hue angle of all samples increased two to three–fold in control, AA (0.25 or 0.5%, w/v), and Se⁴⁺ containing samples, whereas presence of AA (0.25 or 0.5%, w/v)+Se⁴⁺ slowed down the change in hue angle compared to control during thermal treatment (Fig. 5.5E). Increase in hue angle upon thermal treatment has been observed by other researchers as well (Herbach et al., 2006a; Moßhammer et al., 2007). During storage at low temperature, hue angle was not affected significantly ($P < 0.05$) (Fig. 5.5F). Similar to that of lightness and chroma, hue angle also did not change significantly on storage at 5°C. Colour characteristics presented in Fig. 5.5 indicated that during exposure of RBJ at 90°C in presence of AA (0.25 or 0.5%, w/v)+Se⁴⁺, there was appearance of orange red tinge, which was visibly different from that of control and other samples.

5.4.7. Regeneration of betacyanins

There was no regeneration of betacyanins in control and Se alone containing samples
Stability

on storage at 5°C after intermittent (Fig. 5.6A) and continuous (Fig. 5.6B) thermal treatment.

Figure 5.6. Betacyanins remaining in diluted *Rivina humilis* berry juice after repeated thermal treatment for 3 min at 90°C every 24 h followed by storage at 5°C in dark (A) and betacyanins remaining (B) after one-time thermal treatment for 24 min at 90°C followed by storage at 5°C in dark for seven days in absence (control) and presence of ascorbic acid (0.25 and 0.5%, w/v) and selenium (40 μg/mL). Values are mean ± SD of triplicate samples. For figure legends, refer Fig. 5.3.

Intermittently heated samples regenerated 90% and 80% betacyanins in presence of AA (0.25%, w/v) and AA (0.5%, w/v), whereas 100% and 88% regeneration was observed in presence of AA (0.25%, w/v)+Se⁴⁺ and AA (0.5%, w/v)+Se⁴⁺, respectively (Fig. 5.6A). This
Stability

data clearly showed that betacyanins regeneration was not significantly different in presence of AA with or without Se⁴⁺, however, it appeared that higher concentration of AA significantly reduced pigment regeneration. In contrast, continuously heated samples showed higher regeneration (89%), in presence of higher concentration of AA (0.5%, w/v), than in presence of AA (0.25%, w/v), which could regenerate 77% of the pigments (Fig. 5.6B). However, efficient regeneration (100%) of betacyanins was observed in samples containing AA (0.25%, w/v)+Se⁴⁺ or AA (0.5%, w/v)+Se⁴⁺. Results of RBJ betacyanins regeneration studies showed that neither betacyanins can regenerate spontaneously at low temperature after thermal treatment nor presence of Se⁴⁺ could facilitate pigment regeneration (Fig. 5.6). Moreover, level of pigment regeneration in presence of AA and Se⁴⁺ was affected by the treatment condition, i.e., intermittent heating (less regeneration) and continuous heating (efficient regeneration). Failure of betacyanins regeneration on cold storage after thermal exposure at 85°C and 100°C has been reported earlier (Han et al., 1998; Herbach et al., 2006a; Moßhammer et al., 2007). The regeneration of RBJ betacyanins in presence of AA (0.25%, w/v), in this study, was comparable to the regeneration in presence of 0.1% AA (w/v) (Herbach et al., 2006a). It was also observed that regeneration efficiency was significantly (P < 0.01) reduced on increasing the AA level from 0.25% to 0.5% (w/v). Efficient regeneration of betacyanins after intermittent and continuous treatment of RBJ at 90°C in presence of AA (0.25%, w/v)+Se⁴⁺ after storage at 5°C (Fig. 5.6A and B), may be due to regeneration of ascorbic acid from its degradation products in the presence of selenium. Regeneration of betacyanins has been reported even in presence of 40 mM AA (~ 0.7%, w/v) (Han et al., 1998).

However, in a recent report, no significant regeneration of purified betacyanins was observed on cold storage after thermal exposure, whereas crude extract of betacyanins (in juice) solution showed 75% regeneration when the samples were supplemented with 0.1% AA (w/v) (Herbach et al., 2006a). Additionally, the study reported that there was no improvement in regeneration efficiency on increasing AA level from 0.1% to 1.0% (w/v), rather there was a slump in regeneration. On the other hand, betaxanthins could not regenerate in any of the samples in this study (data not shown). Results reported by Moßhammer et al. (2007) supported the inability of betaxanthins regeneration.

5.4.8. Purified betacyanins profile as affected by thermal treatment in presence of selenium and ascorbic acid

Profile of purified betacyanins from RBJ before and after thermal treatment in presence of AA (0.25%, w/v)+Se⁴⁺ (40 μg/mL) (Fig. 5.7A) showed that the retention time of betanin and isobetanin peaks were shifted by more than 1 min after thermal treatment (Fig. 5.7B), and, also, storage at room temperature for about 3 weeks, probably, owing to blanching treatment for 3 min at 90°C before storage (data not shown). The spectra of betanin and isobetanin
peaks in AA (0.25 or 0.5%, w/v)+Se⁴⁺ (40 μg/mL) containing samples showed bathochromic shift resulting in maximum absorbance at 544 nm, whereas before thermal treatment betanin and isobetanin peaks showed λ_{max} at 535 nm. Bathochromic shift in betacyanins absorption has been ascribed to glucosylation at 6-O position of betanidin (Heuer et al., 1992), acylation with aromatic acids, deglycosylation, and betanin–metal complex formation (Attoe and von Elbe, 1985; Herbach et al., 2006b), which efficiently stabilised betanin. It is possible that the spectral changes observed in this study may be due to formation of betanin/isobetanin–Se coordinate complex in presence of AA. Heating might have played an important role in reversing the reduction of Se⁴⁺ to elemental form in the presence of AA, and thus enabling Se⁴⁺ to interact with betanin/isobetanin, probably, through coordinate bond with the free hydroxyl groups. Complex formation was further supported by the distinctive colourant properties of AA (0.25 or 0.5%, w/v)+Se⁴⁺ (40 μg/mL) containing samples after heat treatment (Fig. 5.5A, C and D). Further studies on this complex are warranted because it is possible that
bioavailability of Se from this complex may be higher than that of inorganic Se, as organic Se is absorbed better in the body (Ellis and Salt, 2003).