Anthocyanin Pigments: Stability, Availability, and Biotransformation in the Gastrointestinal Tract

M. Mónica Giusti
Department of Food Science & Technology
Ohio State University, Columbus, OH 43210 USA

Basics about anthocyanins
- Natural pigments (blue, purple, red)
- Potent antioxidants (1-4 times > vitamin E)
- 637 found in nature (Andersen and Jordheim, 2008)
- Structure and color change with pH

Anthocyanin chemical structure

Flavonoids

C6-C3-C6 skeleton

Anthocyanin

Aglycone

Glycosylation

Acylation

anthocyanin

Value of anthocyanins
- Research – health benefits
  - Prevention of cardiovascular diseases (Day et al. 1997)
  - Relief of oxidative stress (Ramirez-Tortosa et al. 2001)
  - Anti-cancer (Reen et al. 2006; Harris et al. 2001)
  - Prevention of obesity (Kwon et al. 2007; Tsuda 2008)

Anthocyanin chemical structure

C6-C3-C6 skeleton

Different aglycones found in nature
- Each Aglycone has a characteristic color and spectra

<table>
<thead>
<tr>
<th>Aglycon</th>
<th>R1</th>
<th>R2</th>
<th>λ_max (nm) visible / color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pelargonidin</td>
<td>H</td>
<td>H</td>
<td>494 nm / orange</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>OH</td>
<td>H</td>
<td>506 nm / orange-red</td>
</tr>
<tr>
<td>Purpureidin</td>
<td>OMe</td>
<td>H</td>
<td>506 nm / orange-red</td>
</tr>
<tr>
<td>Delphinidin</td>
<td>OH</td>
<td>OH</td>
<td>508 nm / bluish-red</td>
</tr>
<tr>
<td>Petunidin</td>
<td>OMe</td>
<td>OH</td>
<td>510 nm / bluish-red</td>
</tr>
<tr>
<td>Malvidin</td>
<td>OMe</td>
<td>OMe</td>
<td>510 nm / bluish-red</td>
</tr>
</tbody>
</table>
In nature, anthocyanins are always glycosylated and can be acylated... with acids attached to the sugars.

Anthocyanin sources

Berries: Simple pigments

Other Sources: Complex acylated pigments

HPLC anthocyanin profiles

- Anthocyanin profiles can vary a lot among plants
- Chokeberry: simple profile, one aglycone (cy), 4 different sugars
- Bilberry: 5 different aglycones, 3 different sugars
- Grape: One sugar: glucose, 5 different aglycones, acylation

Comparing different anthocyanin pigment profiles

Science of Foods for Health

There is evidence that anthocyanins may contribute to the protective effect of fruits and vegetables.

Anthocyanins can be used as food colorants... of added value
Anthocyanins are poorly absorbed

- Anthocyanins are widely distributed in nature and in foods
- High daily intake (100+ mg/day)
- HOWEVER… levels of anthocyanins found in plasma are very low
- Less than 1% of the dietary intake is absorbed
- So… can compounds that are not even absorbed have any impact on health?

In vitro tests: Anthocyanins and colon cancer protection

Science of Foods for Health

Cells lining the intestine can be affected by compounds entering the blood as well as by compounds that are not absorbed due to direct contact.

What is colorectal cancer?

- Epithelial cell layer of the colon, rectum, and appendix
- Five stages
- Symptoms
  - Unexplained weight loss
  - Fatigue
  - Anemia
  - Change in bowel habits
  - Liver metastasis
- Anthocyanins in direct contact with the GI tract

Who does colorectal cancer affect?

- Third most prevalent cancer in the Western World
- 7% lifetime risk of developing
- Risk factors
  - Age
  - Heredity
  - Diet
  - Smoking
  - Alcohol
  - Physical inactivity

Anthocyanin extract preparation

- Plant Material
- Acetone-Chloroform Extraction
- C18 Semi-purification
- Fractionated w/ Ethyl Acetate
- Rotoevaporated
- Lyophilized
- Acylated Anthocyanin

- Saponified w/ KOH
- Rotoevaporated
- Lyophilized
- Saponified Anthocyanins

HT-29 Cell treatment

- HT-29 cells seeded at 10,000 cells/mL
- 24 hrs
- HT-29 cells treated with anthocyanin extracts
- 48 hrs
- SRB Assay
- Plate Reader @ 490 nm
Inhibitory effects of fractionations of chokeberry on HT-29 colon cells

\[
\% \text{ Growth Inhibition} = 100 - \frac{(T_{trt} - T_0) \times 100}{(T_{ctr} - T_0)}
\]

Where:
- \(T_0\) is time zero
- \(T_{trt}\) is the absorbance with treatment at 72 hrs
- \(T_{ctr}\) is the absorbance with out treatment at 72 hrs

\(GI_{50}\) : amount of extract required to inhibit 50% growth

Fractionation of anthocyanins

- Different solvents used to separate anthocyanin fraction from other phenolics fraction
- Very high efficiency and low cost
- More than 95% pure

The \(GI_{50}\) of anthocyanin-rich extracts from different natural sources

Zhao et al., 2005

Jing et al., 2008
Inhibitory effects of fractionations of chokeberry on HT-29 colon cells

**Background Information**

- ARE: Anthocyanin-rich extract
- AP: ACN+OPF
- ACN: Anthocyanin fraction
- OPF: Other phenolics fraction

**Combination Index: Interaction Between Anthocyanins and other Phenols**

It is used to determine if the compounds tested work more efficiently alone or combined.

\[ CI_i = \frac{[\text{ACN}_i]}{[\text{ACN}_0]} + \frac{[\text{OPF}_i]}{[\text{OPF}_0]} \]

<table>
<thead>
<tr>
<th>Source</th>
<th>Combination Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI(25)</td>
<td>GI(50)</td>
</tr>
<tr>
<td>Anthocyanins + Other Phenols</td>
<td>1.222</td>
</tr>
</tbody>
</table>

**Combination Effect**

- Additive
- Additive
- Additive

**Our cell line studies showed that...**

- All AREs inhibited growth of HT29 colon cancer cells, with little effect on NCM460 non-tumorogenic cells.
- Data suggests that the protective effect of specific anthocyanins or extracts may depend on the chemical structure of anthocyanins present.

**What questions should we ask...?**

- Are these compounds actually reaching the tissue?
- And if so... are these the actual forms of the compounds that reach the tissue (or have they been transformed?)
- Are they present in concentrations that are high enough to make any impact?
- Are the effects observed really due to the compounds of interest? Or are there other potentially bioactive compounds?
- How will the compounds impact cancer cells... and how will they impact normal cells?
- Will these results be reproduced in an in-vivo system?

**Animal models: Anthocyanin absorption and chemoprotection**

- Animal trial: 11 F344 rats / treatment
- 15 wk ARE treatments: 4g anthocyanin/kg diet
- Animal trial: 11 F344 rats / treatment
- 15 wk ARE treatments: 4g anthocyanin/kg diet
- AOM injection (20 mg/kg body wt)
- Urine, feces
- Colon, Plasma
Anthocyanins in Plasma (520 nm)

- 0.1 to 1 μg/mL plasma
- Major peaks detected
- Acylated anthocyanin detected
- Anthocyanin metabolites

Anthocyanin metabolites in urine

Urine

Chokeberry

Bilberry

Grape

Anthocyanin methylation vs glucuronidation

Cy-3-glu M+ 449 (449 –> 287)

Methylation

Pn-3-glu M+ 463 (463 –> 301)

Glucuronidation

Cy-3-glucuronide M+ 463 (463 –> 287)

Methylated anthocyanin were found in urine

Urine

Chokeberry

ARE

Presence of intact anthocyanins and metabolites in plasma and urine, demonstrating absorption.

Anthocyanin chemical structure may affect anthocyanins absorption and excretion.

Our Animal Studies Show...

- Anthocyanin levels in feces correlated with inhibition of early cancer lesions, suggesting unabsorbed anthocyanins may be chemoprotective.

Effect of AREs on Aberrant Crypt Foci

- Anthocyanin levels in feces correlated with inhibition of early cancer lesions, suggesting unabsorbed anthocyanins may be chemoprotective.

He et al., 2006

Lala et al. 2006. Nutr Cancer 54(1)
The GI<sub>50</sub> of Anthocyanin-rich Extracts from Different Natural Sources

<table>
<thead>
<tr>
<th>Subset I</th>
<th>Subset II</th>
<th>Subset III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purple</td>
<td>Black</td>
<td>Red</td>
</tr>
<tr>
<td>13.8</td>
<td>31.2</td>
<td>68.5</td>
</tr>
<tr>
<td>Bioavailability</td>
<td>Anthocyanins in the GIT</td>
<td></td>
</tr>
<tr>
<td>0.1 mg/mL</td>
<td>Anthocyanins in the GIT</td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal lumen</td>
<td>Anthocyanins in the GIT</td>
<td></td>
</tr>
<tr>
<td>70-200 µg/mL in Feces</td>
<td>Anthocyanins in the GIT</td>
<td></td>
</tr>
<tr>
<td>0.1 mg/mL in plasma</td>
<td>Anthocyanins in the GIT</td>
<td></td>
</tr>
</tbody>
</table>

Jing et al., 2008

**Experimental Design**
- Black raspberry anthocyanin-rich extracts and lyophilized were used in feeding
- 6 male Fischer 344 rats (11 wks of age) per group
- Single dose of 12 ± 3 mg anthocyanins in 0.1% citric acid solution by stomach tube after 24 hr fasting

**Anthocyanins in the GIT**
- Anthocyanins in the small intestine tissue linearly decreased with 1%/ of 120 min.
- Anthocyanins in the small intestinal content reached maximum at 120 min before decreasing.
- Anthocyanins recovered from gastric and small intestinal lumen accounted for 75-79% of the administered dose between 30 and 120 min.

**Anthocyanin impact on GIT**
- Importance in the GIT
  - Improve lumen condition
  - Protect epithelial cells
  - Dose dependent effects
- Stability in the GIT
  - In vivo evidence is currently scarce

**Anthocyanins Profile Change**
- Cyanidin aglycon was found in the gastric and intestinal contents.
  - Attributed to acid hydrolysis in the stomach and β-glucosidase activity in the small intestine – membrane bound (1)
- Relative proportion of Cy-3-glu decreased in the small intestine

**Anthocyanins in Stomach and Small Intestine**
- Anthocyanins in the small intestinal tissue also reached maximum at 120 min before decreasing.
- The small intestine tissue took up 7.5% of the administered anthocyanins, very high compared to absorption into the plasma levels.
### Bladder Urine Anthocyanins

Urine anthocyanin profile closely reflected that in the lumen of absorption sites.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Cy-3-glu% in urine</th>
<th>Cy-3-glu% in GIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>9.3</td>
<td>8.8a</td>
</tr>
<tr>
<td>60</td>
<td>8.6</td>
<td>6.3b</td>
</tr>
<tr>
<td>120</td>
<td>7.4</td>
<td>6.2</td>
</tr>
<tr>
<td>180</td>
<td>6.4</td>
<td>6.2</td>
</tr>
</tbody>
</table>

### Anthocyanin-Protein Complex in Stomach Tissue

- Stomach tissue extracts exhibited red color that decreased over time.
- Spectral data obtained by altering the pH of the solution confirmed the presence of anthocyanins, suggesting binding to other compounds, possibly a protein transporter for anthocyanins (Passamonti, S. et al. 2003).

### Enzyme Activity in the GIT

LPH substrate specificity on the 5 anthocyanin glucosides in blueberry.

### Some Concluding Remarks

- Anthocyanins are abundant in nature, and could be incorporated into foods as food colorants with added value.
- Not always we require absorption of compounds in order to exert a protective action.
- The activity of phytochemicals in the body will be affected by other components present in the food and compounds present in the GIT.
- Some compounds can act synergistically, in an additive way or can inhibit each other’s action.
What do you think???

- Do you think anthocyanins can provide protection against colon cancer?
- Could a compound that is not absorbed into the plasma have an impact on health?
- How and or why could the chemical structure affect the bioactivity of the compounds?
- What are some important questions to ask when working with bioactive components using in-vitro experiments?
- How much can we conclude from an animal model test?
- What would be some advantages and disadvantages of using an in-vitro test vs an animal test vs a clinical trial?

Acknowledgements

- Collaborators:
  - OSU: Joshua Bomser, Mark Failla, Steven Schwartz, Laura Kresty.
  - MD: Berna Magnuson
  - China: P. Jing

- Students and Research Assistants
  - Pu Jing, Jian He, Taylor Wallace, Kristin Keatley, Lucy Zhao, Minnie Malik, Geeta Lala, Qingguo Tian.

- Artemis International, Inc / Polyphenolics, Inc. / GlobeNatural International / Agricomseeds

- Research supported by USDA-NRI competitive grants and OARDC