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

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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
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)

- Food industry
  - Natural colorant
  - Added value ingredient
  - Good image
Flavonoids

C6-C3-C6 skeleton

anthocyanin
Each Aglycone has a characteristic color and spectra.

<table>
<thead>
<tr>
<th>Aglycon</th>
<th>R1</th>
<th>R2</th>
<th>( \lambda_{\text{max}} ) (nm) visible / color</th>
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<tbody>
<tr>
<td>Pelargonidin</td>
<td>H</td>
<td>H</td>
<td>494 nm / orange</td>
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<tr>
<td>Cyanidin</td>
<td>OH</td>
<td>H</td>
<td>506 nm / orange-red</td>
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<tr>
<td>Peonidin</td>
<td>OMe</td>
<td>H</td>
<td>506 nm / orange-red</td>
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<tr>
<td>Delphinidin</td>
<td>OH</td>
<td>OH</td>
<td>508 nm / bluish-red</td>
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<tr>
<td>Petunidin</td>
<td>OMe</td>
<td>OH</td>
<td>508 nm / bluish-red</td>
</tr>
<tr>
<td>Malvidin</td>
<td>OMe</td>
<td>OMe</td>
<td>510 nm / bluish-red</td>
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In nature, anthocyanins are always glycosylated and can be acylated...

... with acids attached to the sugars

Aliphatic acid (malonic acid)  Cinnamic acid (p-coumaric acid)
Anthocyanin sources

Berries: Simple pigments

Other Sources: Complex acylated pigments
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

Chokeberry

Del-3-Gal
Cy-3-Gal
Cy-3-Arab
Cy-3-Glu
Cy-3-xyI

Bilberry

Del-3-Gal
Del-3-Glu
Del-3-Ara
Del-3-Gal
Cya-3-Glu
Cya-3-Ara
Peo-3-glu
Pet-3-glu
Mal-3-gl
Mal-3-Ara

Grape

Del-3-glu
Cya-3-gl
Pet-3-gl
Peo-3-gl
Mal-3-gl
Mal-3-Ara

Acylated anthocyanins

5  10  15  20  25  30 minutes
Anthocyanins can be used as food colorants ... of added value
There is evidence that anthocyanins may contribute to the protective effect of fruits and vegetables.
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

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

American Cancer Society
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

American Cancer Society
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
HT-29 Cell treatment procedure

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<tr>
<th>B</th>
<th>C</th>
<th>100ug</th>
<th>50ug</th>
<th>25ug</th>
<th>12.5ug</th>
<th>100ug</th>
<th>50ug</th>
<th>25ug</th>
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Blank
Control
Unused

Anthocyanin Treatment Concentrations

Al t d
Si f i d
Percent inhibition of HT-29 Cells

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

Where:
- \( T_0 \): time zero
- \( T_{\text{trt}} \): absorbance with treatment at 72 hrs
- \( T_{\text{ctr}} \): absorbance without treatment at 72 hrs

**\( \text{GI}_{50} \)**: amount of extract required to inhibit 50% growth
Inhibitory effects of fractionations of chokeberry on HT-29 colon cells

Zhao et al., 2005
Fractionation of anthocyanins

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

- **Purple corn**
- **Chokeberry**
- **Bilberry**
- **Purple carrot**
- **Grape**
- **Elderberry**
- **Red radish**

**In vitro model:**
- HT29 cell line
- SRB test
- GI50

**Experimental setup:**
- **SPE**
- Semi-purification
- **Fractionation** (C18 SPE)

**Extract fractions:**
- **ARE:** anthocyanin-rich extract
- **ACN:** anthocyanin fraction
- **OPF:** other phenols fraction
The GI$_{50}$ of anthocyanin-rich extracts from different natural sources

<table>
<thead>
<tr>
<th>Subset I</th>
<th>Subset II</th>
<th>Subset III</th>
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<tbody>
<tr>
<td>Purple corn</td>
<td>13.8</td>
<td>107.7</td>
</tr>
<tr>
<td>Chokeberry</td>
<td>31.2</td>
<td>68.5</td>
</tr>
<tr>
<td>Bilberry</td>
<td>32.2</td>
<td>71.2</td>
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<tr>
<td>Purple carrot</td>
<td>68.5</td>
<td>107.7</td>
</tr>
<tr>
<td>Grape</td>
<td>71.2</td>
<td>130.3</td>
</tr>
<tr>
<td>Radish</td>
<td>107.7</td>
<td></td>
</tr>
<tr>
<td>Elderberry</td>
<td>130.3</td>
<td></td>
</tr>
</tbody>
</table>

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

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

Growth Inhibition (% control)

Dose (μg/mL)

Jing et al., 2008
Combination Index: Interaction Between Anthocyanins and other Phenols

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

\[ CI_i = \frac{[ACN_0]}{[ACN_i]} + \frac{[OPF_0]}{[OPF_i]} \]

<table>
<thead>
<tr>
<th>Source</th>
<th>Combination Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthocyanins + Other Phenols</td>
<td>[ GI_{25} \ 1.222</td>
</tr>
</tbody>
</table>

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.

Malik et al., Nutr Cancer. 2003
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?
Anthocyanin absorption and chemoprevention in an animal model
Animal trial: 11 F344 rats / treatment

15 wk ARE treatments: 4g anthocyanin/kg diet

- Week 1: AOM injection (20 mg/kg body wt)
- Week 14: Urine, feces
- Week 15: 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

Cy-3-Gal

Cy-3-Arab

Cy-3-Gal

Cy-3-Glu

Cy-3-Arab

Cy-3-xyl

Chokeberry

ARE
Anthocyanin methylation vs glucuronidation

Cy-3-glu $M^+ 449$
(449 $\rightarrow$ 287)

Methylation

Glucuronidation

Pn-3-glu $M^+ 463$
(463 $\rightarrow$ 301)

Cy-3-glucuronide $M^+ 463$
(463 $\rightarrow$ 287)
Methylated anthocyanin were found in urine

Urine

~ 35% methylated pigments

Chokeberry ARE
Our Animal Studies Show...

- Presence of intact anthocyanins and metabolites in plasma and urine, demonstrating absorption.
- Anthocyanin chemical structure may affect anthocyanins absorption and excretion.

He et al., 2006
Anthocyanin levels in feces correlated with inhibition of early cancer lesions, suggesting unabsorbed anthocyanins may be chemoprotective.
The GI$_{50}$ of Anthocyanin-rich Extracts from Different Natural Sources

![Bar chart showing GI$_{50}$ values for different anthocyanin-rich extracts.]

- **Subset I**: Purple corn: 13.8 µg/mL, Chokeberry: 31.2 µg/mL, Bilberry: 32.2 µg/mL
- **Subset II**: Purple carrot: 68.5 µg/mL, Grape: 71.2 µg/mL
- **Subset III**: Radish: 107.7 µg/mL, Elderberry: 130.3 µg/mL

**Bioavailability**

- GI$_{50}$ (<1.3 µg/mL in plasma)
- 700~2000 µg/g in Feces (GI tract)

*Jing et al., 2008*
Anthocyanin impact on GIT

- Importance in the GIT
  - Improve lumen condition
  - Protect epithelial cells
  - Dose dependant effects

- Stability in the GIT
  - *In vivo* evidence is currently scarce

### Anthocyanin-rich diets vs. rat colon cancer development

![Graph showing the impact of anthocyanins on various colon cancer development markers.](Source: Lala et al. 2006)
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

Treatments

Control (0.1% citric acid)

Bladder urine
Gastric and small intestinal contents
Gastric and small intestinal tissues

HPLC-PDA-MS
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 (?

Relative proportion of Cy-3-glu decreased in the small intestine.
Anthocyanins in Stomach and Small Intestine

- Anthocyanins in the gastric content linearly decreased with $t_{\frac{1}{2}}$ 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.
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.
Stomach tissue extracts exhibited red color that decreased over time. However, free anthocyanins were not detected by HPLC.

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

The Effect of GIT Enzymes
Some concluding remarks

- Results obtained in in-vitro and animal experiments were in agreement, suggesting a protective effect of anthocyanins against colon cancer.

- Anthocyanins were relatively stable in the gastric and small intestinal lumens of fasted rats in contrast to reports from some in vitro studies.

- A significant portion of anthocyanins was taken up into the GIT tissues, but neither extensively absorbed or retained. This suggests that anthocyanins may have in-situ protective effects in this tissue.

- The chemical structure of the anthocyanin molecule will impact its stability to the conditions of the GIT, and therefore, its chances to interact with the GIT lining tissues.

- Anthocyanins could be incorporated into foods as food colorants with added value.
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.
In the news…

**Study: Dark-Colored Fruits and Veggies Fight Cancer**

**Tuesday, August 21, 2007**

**FOX NEWS**

The darker the berry, the sweeter the juice, so goes the saying.

But it turns out the darker berry or plum or grape, for that matter, the stronger the cancer-fighting properties.

Researchers conducting a recent study found that the compounds that give some fruits and vegetables their rich colors are powerful cancer deterrents.

Evidence from laboratory experiments on rats and on human colon cancer cells also suggest that anthocyanins, the compounds that give color to most red, purple and blue fruits and vegetables, also slow the growth of colon cancer cells.

"These foods contain many compounds, and we're just starting to figure out what they are and which ones provide the best health benefits," said Monica Giusti, the lead author of the study and an assistant professor of food science at Ohio State University, in a news release.

The findings, which Giusti presented August 19 at the national meeting of the American Chemical Society in Boston, also bring scientists a step closer to figuring out what gives fruits and vegetables their cancer-fighting properties.

Giusti and her colleagues found that in some cases, altering the structure of anthocyanin molecules made these compounds more potent anti-cancer agents.

In their studies on human colon cancer cells grown in laboratory dishes, the researchers tested the anti-cancer effects of anthocyanins and identified the optimal location of a nitro group on the anthocyanin's molecular structure to achieve this effect.
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