Pigmented Tannin: Structural Elucidation by a Complimentary Suite of Mass Spectrometric Techniques

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Andrew L. Waterhouse
Carlito B. Lebrilla
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Production

**White**
- Vineyard
- Crush
- Press
- Fermentation
- Settling
- Stabilization
- Aging
  - Barrel
  - Bottle

**Red**
- Vineyard
- Crush
- Fermentation
- Press
- Settling
- Stabilization
- Aging
  - Barrel
  - Bottle

- All Color comes from the Skin
- We extract phenolics, tannin, pigments from the skins and seeds
Control Points - Red

• Extraction/Maceration
  – Anthocyanins
  – Tannins
  – Cinnamates

• Retention
  – Oxidation
  – Precipitation

• Modification
  – Pyrano ring closures
  – Adducts
  – Bridging – Acetaldehyde and others
Total Mass of Solutes

Red Wine Composition, Minor Components

- Acetaldehyde
- Volatile Acidity
- Sugar
- Phenols
- Minerals *
- Esters
- Amino acids
- Acid
- Higher Alcohols
- Sorbitol & Mannitol
- Sulfites
- Glycerol
Structures

- Anthocyanins
- Catechin
- Cinnamates

- Ferulic Acid
- Cinnamic Acid
- Malvidin Aglycon
- Catechin
Compounds

TA type

A-T type

\[ \text{Compounds} \]
Impact of Pigmented Tannin

- Primary Quality Parameters
  - Flavor
  - Aroma
  - Texture
  - Color

- Softens astringency, alters flavor perception\(^1\)

- Responsible for persistent color\(^2\)
  - Monomeric anthocyanin and copigmentation almost disappears within 2 years
Challenges

- Diversity of molecular structures$^{3,4}$
  - Expanded Sensory Properties
  - Challenging Chemistry

- Hundred year problem
- Ribéreau-Gayon
## Compounds by Class

<table>
<thead>
<tr>
<th>Number of Compounds in Database by Class (Total 4218)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pyrananthocyanins</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>A-T/T-A Condensation Products</strong></td>
</tr>
<tr>
<td>(Single Subunit composition up to Decamer)</td>
</tr>
<tr>
<td><strong>Aldehyde Bridged</strong></td>
</tr>
<tr>
<td><strong>Anthocyanin Oligomers</strong></td>
</tr>
<tr>
<td><strong>Xanthilium Type</strong></td>
</tr>
</tbody>
</table>
Isomers

<table>
<thead>
<tr>
<th>Number of molecular formulas</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 isomer</td>
<td>1527</td>
</tr>
<tr>
<td>2 isomers</td>
<td>497</td>
</tr>
<tr>
<td>3 isomers</td>
<td>284</td>
</tr>
<tr>
<td>4 isomers</td>
<td>64</td>
</tr>
<tr>
<td>5 isomers</td>
<td>22</td>
</tr>
<tr>
<td>6 isomers</td>
<td>17</td>
</tr>
<tr>
<td>7 isomers</td>
<td>16</td>
</tr>
<tr>
<td>8 isomers</td>
<td>21</td>
</tr>
<tr>
<td>9 isomers</td>
<td>3</td>
</tr>
<tr>
<td>10 isomers</td>
<td>2</td>
</tr>
<tr>
<td>11 isomers</td>
<td>1</td>
</tr>
<tr>
<td>12 isomers</td>
<td>1</td>
</tr>
<tr>
<td>13 isomers</td>
<td>1</td>
</tr>
<tr>
<td>14 isomers</td>
<td>1</td>
</tr>
</tbody>
</table>
Objectives

• Identification of polymeric pigments from new wine and wine during aging.
  – Analysis of the components of these mixtures utilizing the mass spectrometry techniques, Q-TOF, MALDI-FT ICR, ESI-QQQ-FTICR, ESI-QQQ/DAD and HRxx
  – Structural Identification by means of MS data using standard and customized de-convolution algorithms.
Necessary MS Attributes

FT-ICR
- High Resolution
- High Mass Accuracy
- High Sensitivity
- Discrimination of Compounds
- Simultaneous analysis

QTOF
- Tandem MS
- High Sensitivity
- Collision Induced Dissociation (CID)
- High Selectivity (Q)
  - Mass Selectivity for fragmentation
  - Eliminate coeluting ions
- Accurate Analyzer (TOF)
# Illustration of mass error

## Single Quadrupole Analyzer
- Absolute Error ±0.2 m/z

## ICR
- Absolute Error < ±0.001 m/z

<table>
<thead>
<tr>
<th></th>
<th>Cyanidin Aglycon</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Precise Mass:</strong></td>
<td>287.05556307013</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Example Mass</strong></td>
<td>287.0559</td>
<td>0.0003</td>
<td>1.2</td>
<td></td>
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<tr>
<td><strong>Mass Error (ppm)</strong></td>
<td>287.16</td>
<td>0.10</td>
<td>348.4</td>
<td></td>
</tr>
<tr>
<td><strong>FTICR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Quadrupole</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Discriminating between Molecules

Two Pigmented Tannin molecules with the same nominal mass can be differentiated

<table>
<thead>
<tr>
<th>Ion m/z = 715.0989</th>
<th>1 ppm</th>
<th>5 ppm</th>
<th>10 ppm</th>
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</thead>
<tbody>
<tr>
<td>C13H22N12O23</td>
<td>C46H18O9</td>
<td>C35H18N6O12</td>
<td></td>
</tr>
<tr>
<td>C11H10N26O13</td>
<td>C17H26N6O25</td>
<td>C21H30O27</td>
<td></td>
</tr>
<tr>
<td>C28H26O22</td>
<td>C15H14N20O15</td>
<td>C19H18N14O17</td>
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</tr>
<tr>
<td>C26H14N14O12</td>
<td>C30H18N8O14</td>
<td>C17H6N28O7</td>
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<tr>
<td>C41H18N2O11</td>
<td>C28H6N22O4</td>
<td>C34H22N2O16</td>
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</tr>
<tr>
<td></td>
<td>...</td>
<td>...</td>
<td></td>
</tr>
</tbody>
</table>

Possible Compounds

<table>
<thead>
<tr>
<th>Accurate Mass</th>
<th>Absolute Mass Difference (amu)</th>
<th>Relative Mass Difference (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malvidin-3-O-Glucoside acetaldehyde adduct (Vitisin B)</td>
<td>517.1346</td>
<td>0.0364</td>
</tr>
<tr>
<td>Cyanidin-3-O-Glucoside vinylformic acid adduct</td>
<td>517.0982</td>
<td></td>
</tr>
</tbody>
</table>
Strategy

Low Pressure Liquid Chromatography

H$_2$O/MeOH/Acetone on Sephadex LH20

Proanthocyanidin Preparation

Hydroxycinnamic acid/Na Matrix

H$_2$O/Acetone +0.1%FA

H$_2$O/ACN +0.1%FA

High Resolution Mass Spectrometry

MALDI-ICR

ESI-QQQ-ICR

Q-TOF w/HPLC
FTICR Technology

- 3 Modes of Motion
  - Magnetonic
  - Cyclotronic
  - Translational

- Fluctuation of RF Field changes Gyration radius

\[ \frac{m}{z} \propto \frac{B}{\omega} \]

- Multiple ions in one cell

Fourier Transform Deconvolution

Lebrilla, C.B. 2013
## Sample of Current ICR Conditions

<table>
<thead>
<tr>
<th>Compound</th>
<th>Exact Mass</th>
<th>Experimental Mass</th>
<th>Mass Error (ppm)</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malvidin-3-(6-acetyl)-monoglucoside</td>
<td>535.1452</td>
<td>535.1450</td>
<td>0.3</td>
<td>54000</td>
</tr>
<tr>
<td>Catechin-malvidin-3-glucoside (T-A Dimer)</td>
<td>781.1980</td>
<td>781.1975</td>
<td>0.6</td>
<td>44000</td>
</tr>
<tr>
<td>Malvidin-3-glucoside-catechin (A-T Dimer)</td>
<td>782.2058</td>
<td>782.2079</td>
<td>2.6</td>
<td>42000</td>
</tr>
<tr>
<td>Malvidin-3-glucose-4-vinyl-catechin</td>
<td>805.1980</td>
<td>805.1967</td>
<td>1.6</td>
<td>39000</td>
</tr>
<tr>
<td>Malvidin-3-(acetyl)glucose-4-vinyl-catechin</td>
<td>847.2086</td>
<td>847.2070</td>
<td>1.8</td>
<td>38000</td>
</tr>
<tr>
<td>Malvidin-3-(p-coumaroyl)glucose-4-vinyl-catechin</td>
<td>951.2348</td>
<td>951.2333</td>
<td>1.5</td>
<td>34000</td>
</tr>
<tr>
<td>Malvidin-3-glucoside-dicatechin (A-T Trimer)</td>
<td>1070.2692</td>
<td>1070.2679</td>
<td>1.2</td>
<td>29000</td>
</tr>
<tr>
<td>Malvidin-3-glucose-4-vinyl-dicatechin</td>
<td>1093.2614</td>
<td>1093.2575</td>
<td>3.5</td>
<td>29000</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>1.7</strong></td>
<td></td>
<td></td>
<td><strong>38000</strong></td>
</tr>
</tbody>
</table>
1990 Cabernet Sauvignon
FTICR Spectra

1990 Cabernet Sauvignon
ICR Values
7 Pinotin Variations
  ➢ Grape Derived
  ➢ 1 Vanillin bridged Petunidin Cat Dimer
  ➢ Oak Derived
  ➢ 1.7ppm Mass Error

Flavan-3-ol-vanillyl-anthocyanin (mediated by vanillin)*

*He et al. (3)
QTOF Technology

Why Nano-HPLC?

- Nano-ESI
- Enrichment column
  - Concentration
  - Cleanup on Loading
  - Narrows injection band

Our Chip

- Custom Diol Chip
- Normal Phase Gradient
- Separation by length


http://www.kjemi.com/artikkel/1947
Nano-HPLC QTOF Method

- Modification of Kelm et al.
- Expansion of elution gradient
- Modification of Kelm Fluorescence to MS
- FA 3.75 vs. Acetic 4.76
  - Order of magnitude greater proton strength
- Proton accessibility of solvent increased
- ACN:HOAc (98:2) → ACN:H₂O:FA (95:3:2)
- MeOH:H₂O:HOAc (95:3:2) → MeOH:H₂O:FA (95:3:2)
QTOF of Tannin

QTOF fragmentation spectrum of ion 1443.3, Catechin Pentamer M+H⁺, from 2010 Caymus Cabernet Sauvignon.
Current Status

- >4,000 Compounds of Interest
- Isomeric Complications
- ~450 Distinct Signals
- ~150 signals matched to database
- Remainder could be new, fragments

- Boost QTOF Sensitivity
- Other Wine Varieties and Ages
- Fragmentation Analysis – By Hand
Impact on Wine Production

- Insight into desired control features
  - Mechanisms of pigmented tannin development
- Which pigments are important
- Refined aging conditions for desired traits
  - Oak, Micro-ox, etc.
- Enhancement of precursors
- Implications for oxygen management
- Basis for innovation in production techniques
- Greater Stylistic Control
With these methods in place we proceed on the following objectives.

35 Year Vertical  (Observe pigmented tannin evolution throughout aging)

- Employ our method of complimentary mass spectrometric techniques for comprehensive identification of wine matrix compounds.
- Observe the changes in relative abundance, depletion and accumulation in pigmented tannin composition.
- Postulate wine pigment precursors for examination of mechanistic pathways.
- Employ standards to quantitate the classes of polymeric pigments in wine.
Thank You

• American Vineyard Foundation
• Evan Parker
• Andres Guerrero
• Carlito Lebrilla
• James A. Kennedy
• Waterhouse Lab
References


