Novel Approaches for Food Verification & Authentication

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“Let thy food be thy medicine.”
- Hippocrates (ca. 460 BC – ca. 370 BC)

But which foods?
- Are all foods created equal?
- Are all apples created equal?
- Which one will provide me with what benefits?
Fruit & Vegetables

- Primary dietary source of vitamins, minerals, fiber and a wide array of *non-essential* nutrient phytochemicals in the American diet
  - polyphenolic antioxidants (e.g. flavonoids), carotenoids (e.g. lycopenes, β-carotene), alkaloids, glucosinolates, etc.
  - Epidemiological studies indicate people who consume diets rich in F&Vs have a reduced risk of chronic diseases
    - stroke, type II diabetes, some cancers and heart disease
- These benefits are largely thought to be due to the synergistic activities of these bioactive phytochemicals
- Natural plant components in *traditional* medicine
- However, human studies on F&Vs are often contradictory and hard to interpret…
  - Why?
The Pharmacology Model Doesn’t Apply:

- **Non-essential** nutrient bioactives represent a wide range of chemical structures with **tremendous variability** in foods
  - Little understanding of synergistic reactions between dietary components
  - Often comparing in vitro data with animal and human data
  - Types of intervention studies differ [acute, subacute, chronic]
  - Different endpoints and biomarkers are measured
  - Differences in dose and composition (foods)

- **Limited understanding of chemical composition of foods**
  - Cultivar variability, season, growing region, etc.,
  - Processing, storage, formulation, and packaging

- This lack of knowledge makes the medicinal or personalized nutritional use of foods difficult

- Manufacturing challenge
  - Ingredient sourcing and consistency in product composition
What Influences Non-Essential Nutrient Bioactives in Foods?

Genotype Selection
(e.g. Cultivar or Variety)

Agronomic and Environmental Pressures
*Secondary Plant Metabolites*

Post-Harvest Handling, Transport, Processing & Storage

*Human Interface: Absorption, Bioavailability and Biological Activity*
The Flavonoids

Roughly 2% of all hydrocarbons fixated in photosynthesis are converted into flavonoids and their derivatives.
A Model Flavonol: Quercetin

- Quercetin [3,3',4',5,7-pentahydroxyflavone]
- Hydroxylation, methylation and glycosylation pattern influences chemical diversity (5 forms)

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<th>R₃</th>
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Flavonoids can also be acylated, malonylated and sulfated as well as methylated and glycosylated.

Glycosides can be mono-, di-, and tri-saccharide substituted.

- The 5 forms just became 20+ or more forms.

Quercetin-3 rutinoside

Quercetin-4'-O-glucoside

Hyperoside-3-O-galactoside

Amurensin tert-amyf alcohol derivative of kaempferol 7-O-glucoside
So Why Is this Important?  
The Source Matters

- The compliment of flavonoid conjugates differs in various F&Vs
- For example: The major US dietary sources of quercetin are
  - Onions: 210 mg/kg fresh weight
  - Apples: 30 mg/kg fresh weight
  - Most human and cell culture data is derived on forms not even found in foods

Used in cell culture studies, clinical trials, sold as an ingredient
Poor Bioavailability

Primary form found in onions
Highly Bioavailable

Primary form found in apples
Some Bioavailability
Why Conjugation is Important

- Receptors recognize molecules largely by shape
- Protein interactions are specific, intermolecular distances are critical for interactions between amino acids and active sites on molecules

![Diagram showing molecular interactions](image)

**KEYS**
- Quercetin aglycone (I)
- Quercetin-3-O-β-D-galactoside (III)
- Quercetin-4'-O-β-D-glucoside (II)
What Form is in These Products?

Quercetin in Skincare
**Characterizing Quercetin in Onions**  
**Variety Matters**

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<tr>
<th>Variety</th>
<th>Quercetin 3,4’-diglucoside</th>
<th>Quercetin 3’-glucoside</th>
<th>Quercetin 4’-glucoside</th>
<th>Isorhamnetin 4’-glucoside</th>
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<td>Below LOQ</td>
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*Five primary forms found in onions LC–(ESI)MS/MS*  

Q-TOF LC/MS Identification of Target
Unknowns for Variety Verification

- The predominant factor influencing the compliment of flavonoids in food is genetics
  - Species and cultivar specific
  - This has important impact when sourcing ingredients that will have specific characteristics (e.g. bioactives, flavors, etc.)

- Could Q-TOF LC/MS be used to identify varietal differences based upon a target unknown analysis of flavonoids?

- How would this data compare with non-target analysis of the same sample?

- Onions as a Model
  - Gills Onions, Oxnard CA
  - Flavonoid profiles in onions relatively simple
Seven varieties of onions were evaluated

- Yellow: Cowboy, Chief, Vaquero, Sommerset
- Red: Red Rock, Salsa, Merenge

Inner layer of the onion were separated from the outer layers and used in this study

- Inner layers have limited anthocyanidins (red color)
- Onions were lyophilized and extracted with 80% MEOH for 20 minutes
  - Anthocyanins will not be extracted under these conditions
  - All samples run in triplicate
Methanolic extracts (80:20 MeOH:H₂O v/v) were separated on a Poroshell 120 EC-C₁₈ (2.1 x 100 mm, 2.7 mm) column using a 1200 RRLC (Agilent Technologies)

Mobile Phase:
- A: 0.1% formic acid in H₂O
- B: 0.1% formic acid in ACN
  - 22 min gradient to 90% B at 0.4 mL/min

Spectra were collected using a 6530 Accurate-Mass Q-TOF LC/MS (Agilent Technologies)

Data processed using Mass Profiler Plus (Agilent Technologies)
Building a Library

- A targeted unknown library was developed by importing the empirical formula of flavonoids from various databases into MassHunter PCDL Manager
  - Phenol-Explorer and ChemSpider
- We found 250 possible flavanoids to import
  - caution there are mistakes in these databases
- Narrowed down list into a library of 150 possible flavonoids in onions
- Searched against the TIC for these compounds
Phenol Explorer Library
Polyphenol content of foods

- Phenol Explorer Library (INRA)
  - ~500 different polyphenols in over 400 foods
- [http://www.phenol-explorer.eu](http://www.phenol-explorer.eu)
  - Provides molecular formulas of flavonoids
    - Numerous flavonoids have the same molecular weight due to variation in glycosylation patterns
  - Onions
    - Isorhamnetin, Isorhamnetin 4’-glucoside, quercetin, quercetin 3,4’-diglucoside, quercetin 3-glucoside, quercetin 3-rutinoside, quercetin 4’-glucoside
      - Delphinidin 3-O-glucosyl-glucoside; Cyanidin 3-O-(6''-malonyl-glucoside); Cyanidin 3-O-(6''-malonyl-3''-glucosyl-glucoside)
ChemSpider provides molecular formula, monoisotopic mass, systematic names and structure for a wide range of compounds.

All of these values can be downloaded into MassHunter PCDL for library development.
MassHunter PCDL Manager

![Chemical structure and data analysis interface](image)

**Single Search Results:** 143 hits

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<tr>
<th>Compound Name</th>
<th>Formula</th>
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<th>RT (min)</th>
<th>CAS</th>
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### MassHunter Qualitative Analysis

#### Chromatograms

- **Method Editor:** Find Compounds by Formula - Options
- **Formula Source:** Formula matching, Positive ions, Negative ions, Scoring, Results
- **Values to match:** Mass
- **Compound exchange file:** (CEF)
- **Library:** Q/View/MethodLib: Expected/Jeannie/Phenolacova.cdf
- **Peaks:** Mass and retention time (retention time constrained)
- **Mass:** Mass and retention time (retention time required)

#### Data Navigator

- **Set by Data File:** Automatically Show Columns
- **Compounds:**
  - **Column:**
    - Show/Hide
    - Cod
    - Label
    - Name
    - Formula
    - Score
    - Mass
    - Mass [ppm]
    - Diff [ppm]
    - Diff

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</table>
Compounds identified are evaluated based on exact mass, $t_R$ and isotope spacing.
A compound list of 19 flavonoids were identified in the 7 varieties of onions:

- Delphinidin3-O-(6''-malonyl-glucoside)
- Dihydromyricetin3-O-rhamnoside
- Dihydroquercetin
- Isorhamnetin
- Isorhamnetin4'-O-glucoside
- Kaempferol
- Kaempferol 3-O-(6''-malonyl-glucoside)
- Kaempferol3,7-O-diglucoside
- Kaempferol3-O-acetyl-glucoside
- Kaempferol3-O-rutinoside
- Kaempferol3-O-xylosyl-rutinoside
- Quercetin
- Quercetin 3,7,4'-triglucoside
- Quercetin -O-diglucoside
- Quercetin3,4'-O-diglucoside
- Quercetin3-O-glucoside
- Quercetin3-O-rhamnoside
- Quercetin4'-O-glucoside
Principle Component Analysis on 19 Targeted Flavonoids for Varietal Difference
Principle Component Analysis On Non-Target Compounds Varietal & Color Difference

PCA analysis performed on data obtained using MFE
### Non-pigment Compounds Responsible for Color Differentiation

- **Chief (yellow) vs. Merenge (red)**

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<th>Compound</th>
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<th><a href="caw">Yellow</a></th>
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<th>Retention Time</th>
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<th>Possible compounds (ChemSpider link)</th>
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Compounds Tentatively Identified Responsible for Colored Differentiation

- 6,8-Dihydroxykaempferol
  - Kaempferol
- Kaempferol 3-O-(6'-malonyl-glucoside)
- Kaempferol di glucoside
- Kaempferol di glucoside (isomer, different T<sub>R</sub>)
- Kaempferol3-O-acetyl-glucoside
- Quercetin
- Quercetin3,4'-O-diglucoside
- Quercetin diglucoside (isomer, different T<sub>R</sub>)
- Quercetin 3-rhamnoside
Conclusions

- A PCDL was established for 250 flavonoids
  - A PCDL was extracted from this specific for onion flavonoids and included 150 entries
- 19 flavonoids were identified in methanolic extracts of onions
  - Principle component analysis of these 19 target compounds demonstrate clear separation in varietal difference and color difference
- Non-target analysis resulted in similar results
  - Tentative identification of the top 10 flavonoids correlating strongly with color and variety differences
- More sampling of varieties grown under different conditions over time are needed to establish clear correlations
Acknowledgements

- UC Davis
  - JiHyun Lee
  - Susan Ebeler, PhD
  - Carolyn Doyle, PhD
  - Tom Collins

- Agilent Technologies
  - Jerry Zweigenbaum, PhD
  - Steven Fischer, PhD

- Gills Onions