Novel Approaches for Food Verification & Authentication

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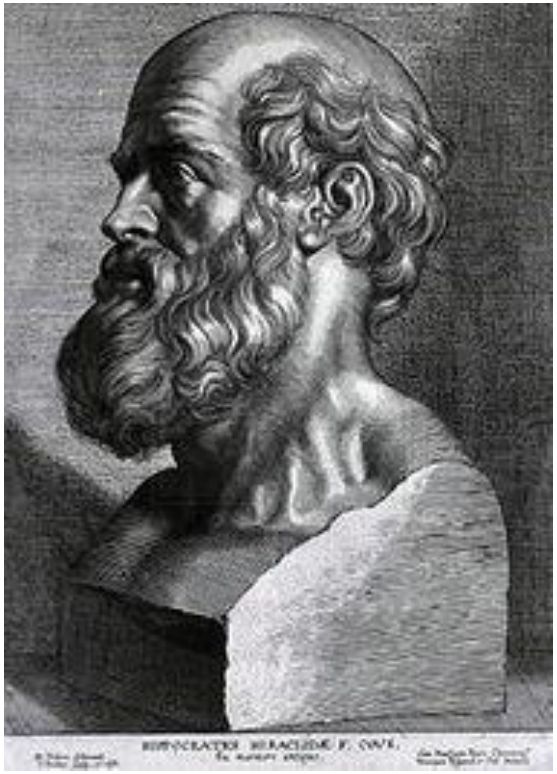
Food is Complex

- "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)

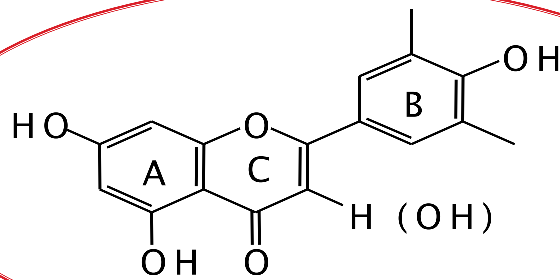
Agronomic and Environmental Pressures
Secondary Plant Metabolites

Post-Harvest Handling, Transport, Processing & Storage

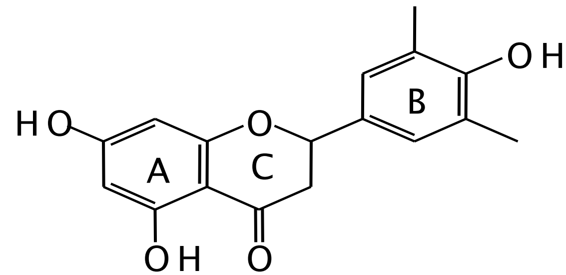
Consumer

Human Interface: Absorption, Bioavailability and Biological Activity

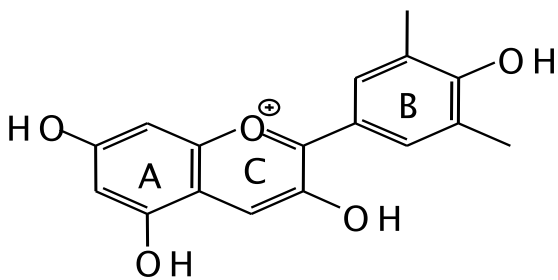
The Flavonoids



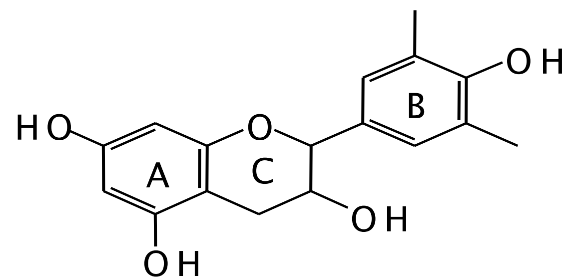
Flavone (Flavonol)



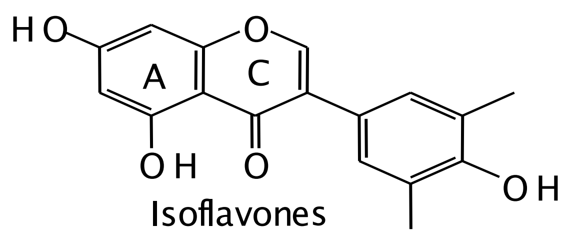
Flavanones



Anthocyanidins



Favan-3-ols

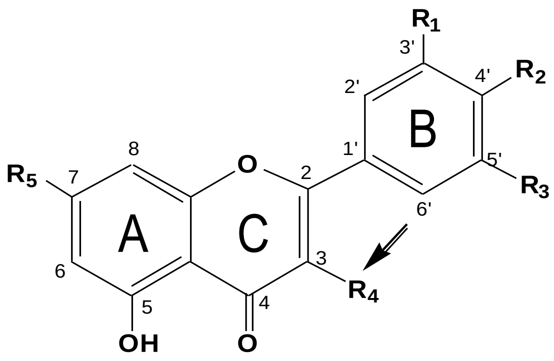


Isoflavones

Roughly 2% of all hydrocarbons fixated in photosynthesis are converted into flavonoids and their derivatives

A Model Flavonol: Quercetin

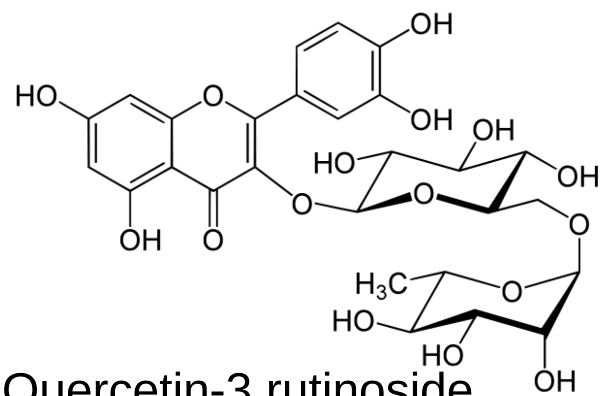
- Quercetin [3,3’,4’,5,7-pentahydroxyflavone]
- Hydroxylation, methylation and glycosylation pattern influences chemical diversity (5 forms)



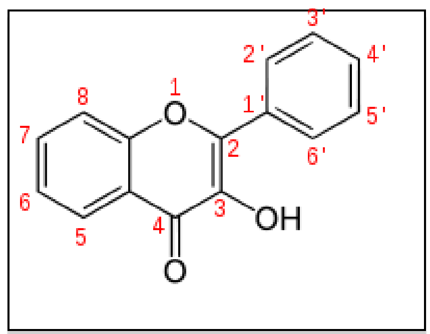
Compound	R_1	R_2	R_3	R_4	R_5
Quercetin	OH	OH	H	OH	OH
Kaempferol	H	OH	H	OH	OH
Myricetin	OH	OH	OH	OH	OH
Morin	OH	H	OH	H	OH
Isorhamnetin	OCH_3	OH	H	OH	OH

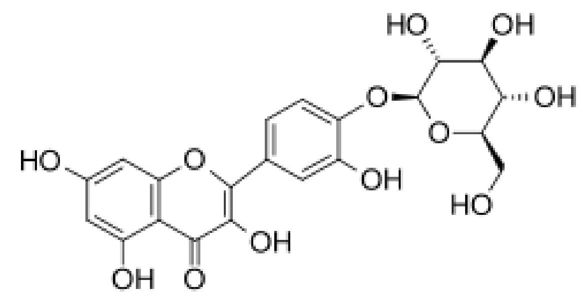
It Gets Even More Complex

- 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

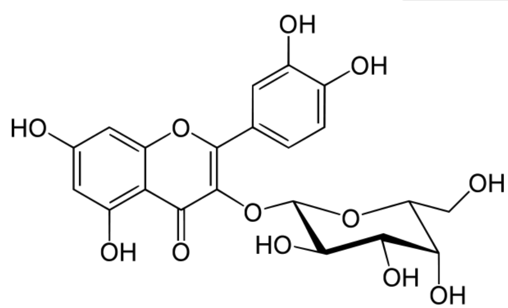


Quercetin-3 rutinoside

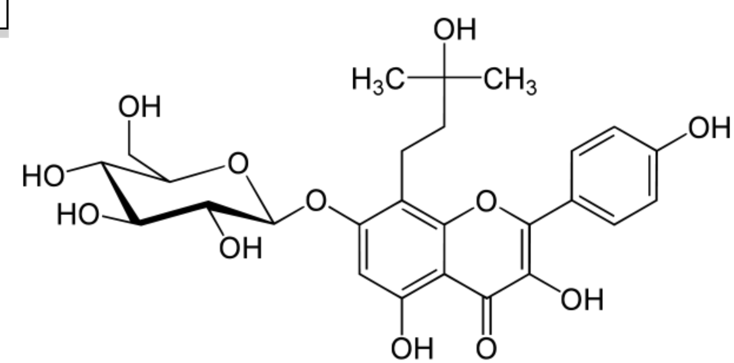




Quercetin-4'-O-glucoside



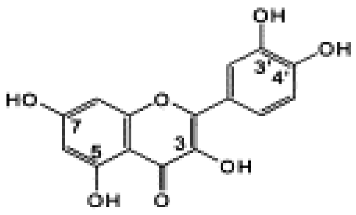
Hyperoside -3-O-galactoside



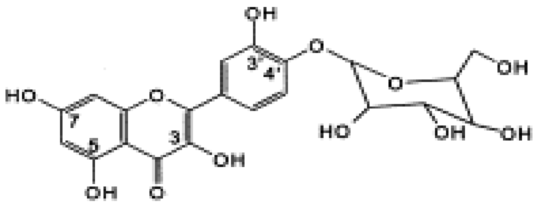
Amurensin tert-amyl alcohol derivative of kaempferol 7-O-glucoside

So Why Is this Important? The Source Matters

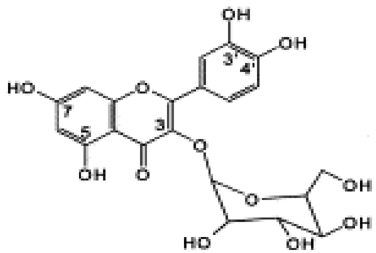
- The compliment of flavonoid conjugates differs in various F&Vs
- For example: The major US dietary sources of quercetin are
  - Onions: 210 mg/kg fresh weight
  - Apples: 30 mg/kg fresh weight
  - *Most human and cell culture data is derived on forms not even found in foods*



Quercetin aglycone (I)



Quercetin-4'-*O*-ß-D-glucoside (II)



Quercetin-3-*O*-ß-D-galactoside (III)

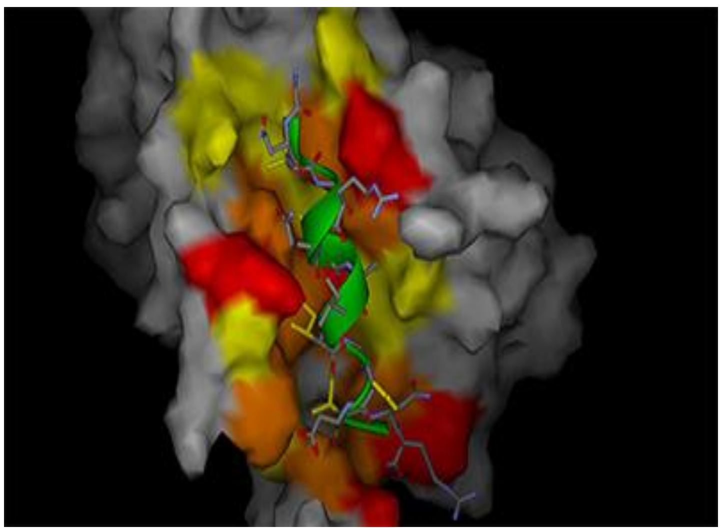
Used in cell culture studies, clinical trials, sold as an ingredient
Poor Bioavailability

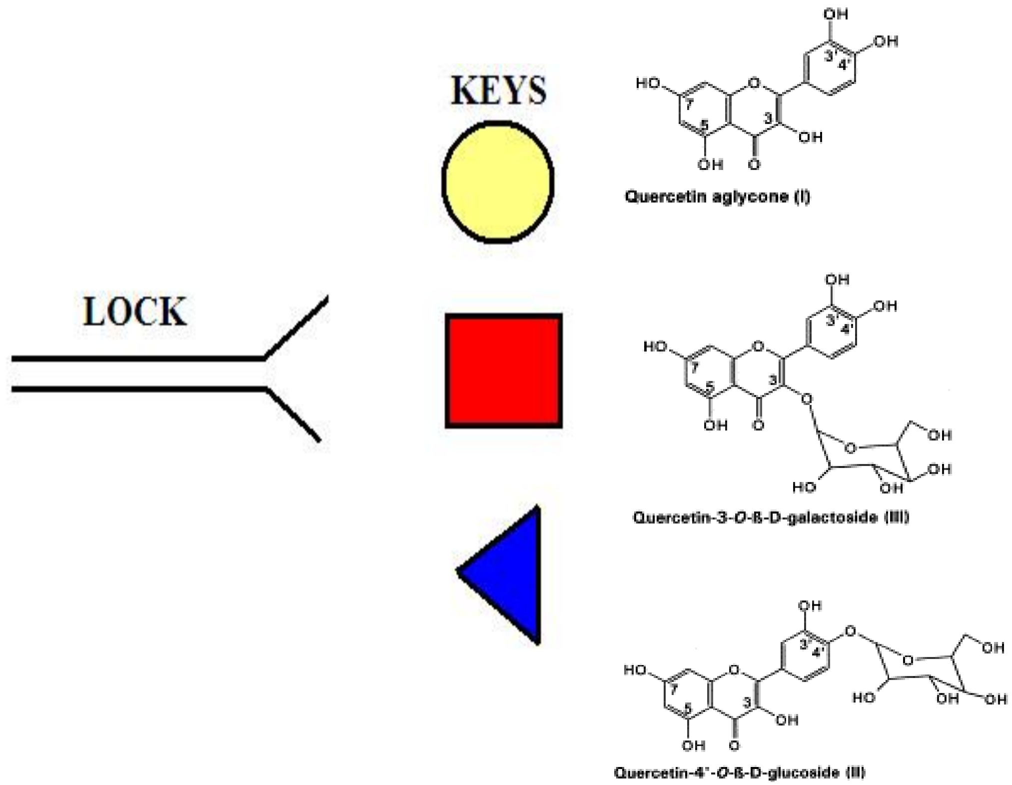
Primary form found in onions
Highly Bioavailable

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





What Form is in These Products?

Characterizing Quercetin in Onions

Variety Matters

variety	quercetin 3,4'-diglucoside	quercetin 3-glucoside	quercetin 4'-glucoside	isorhamnetin 4'-glucoside	quercetin aglycone	sum
Cougar	**541.3 ± 3.9**	42.0 ± 0.8	**480.0 ± 4.2**	79.2 ± 0.4	**256 ± 2**	1398.4 ± 3.3
Don Victor	9.6 ± 0.4	Below LOQ	56.1 ± 1.2	7.2 ± 0.2	20.1 ± 0.1	93.1 ± 1.7
Gobi	94.6 ± 2.8	10.4 ± 0.2	102.5 ± 1.6	20.5 ± 0.6	29.8 ± 0.1	257.8 ± 4.3
Milestone	**49.3 ± 0.7**	53.8 ± 0.7	**536.1 ± 3.3**	16.7 ± 0.3	**1047.0± 29.1**	1703.0 ± 29.9
Natasha	35.8 ± 2.8	4.3 ± 0.0	88.7 ± 2.2	11.6 ± 0.1	26.3 ± 0.4	166.7 ± 4.8
Warrior	242.4 ± 1.2	9.9 ± 0.0	229.9 ± 2.2	34.1 ± 0.2	256.0 ± 2.4	739.1 ± 3.4

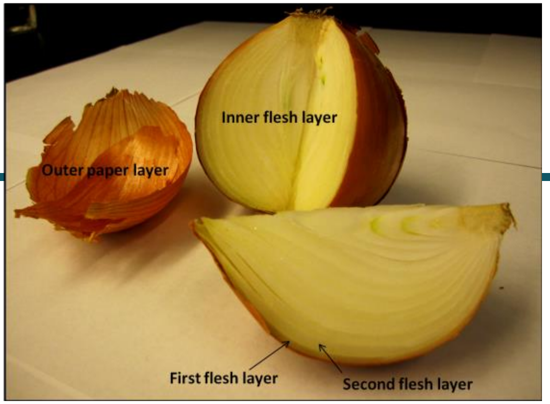
*Five primary forms found in onions LC-(ESI)MS/MS

Lee and Mitchell, 2011 J Agric Food Chem. 59(3):857-63

Q-TOF LC/MS Identification of Target Unkowns 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

Sample Extraction



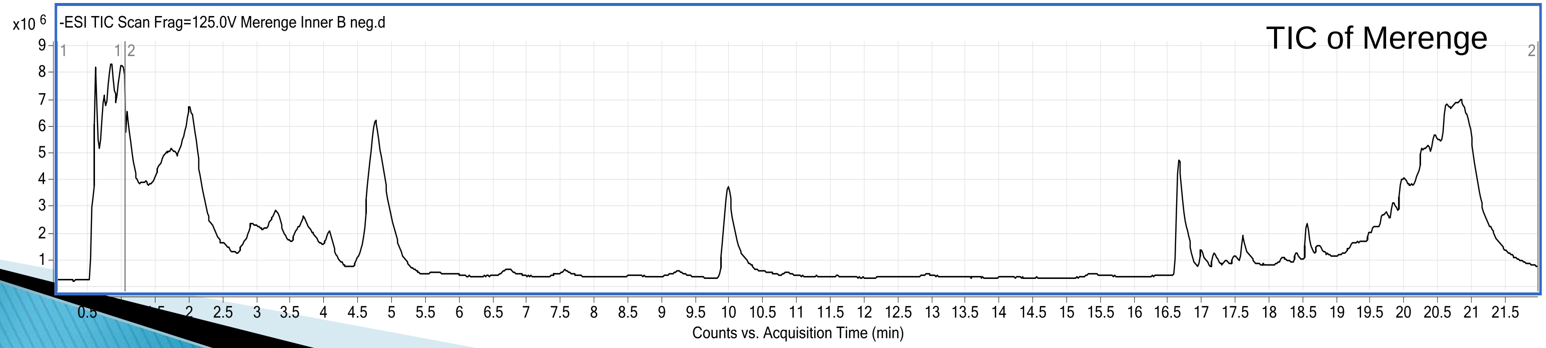
- Seven varieties of onions were evaluated
  - Yellow: Cowboy, Chief, Vaquero, Sommerset
  - Red: Red Rock, Salsa, Merenge
- Inner layer of the onion were separated from the outer layers and used in this study
  - Inner layers have limited anthocyanidins (red color)
  - Onions were lyophilized and extracted with 80% MEOH for 20 minutes
    - Anthocyanins will not be extracted under these conditions
    - All samples run in triplicate

Q-TOF LC/MS Analysis

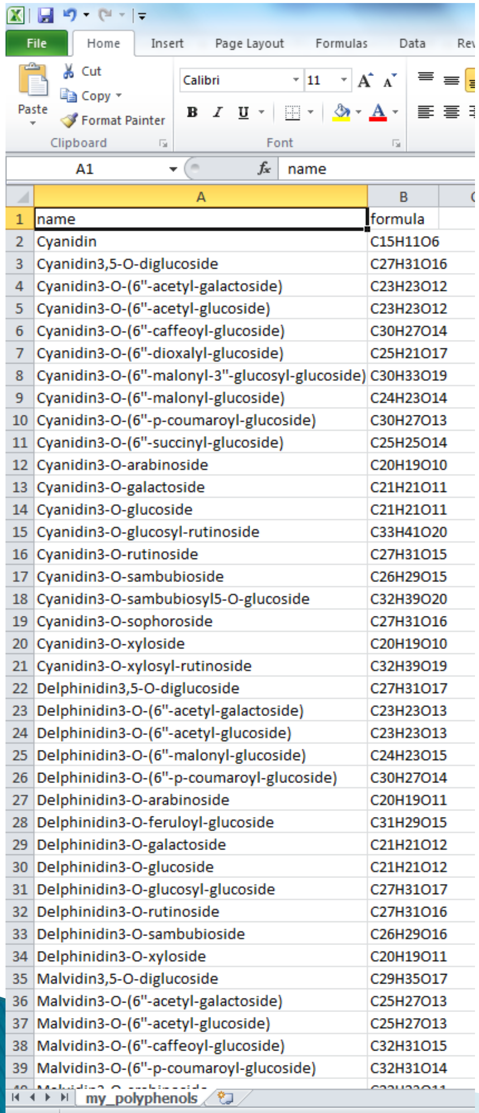
- Methanolic extracts (80:20 MeOH:H_20 v/v) were separated on a Poroshell 120 EC-C_{18} (2.1 x 100 mm, 2.7 mm) column using a 1200 RRLC (Agilent Technologies)
- Mobile Phase:
  - A: 0.1% formic acid in H2O
  - B: 0.1% formic acid in ACN
    - 22 min gradient to 90% B at 0.4 mL/min
- Spectra were collected 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 posible 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



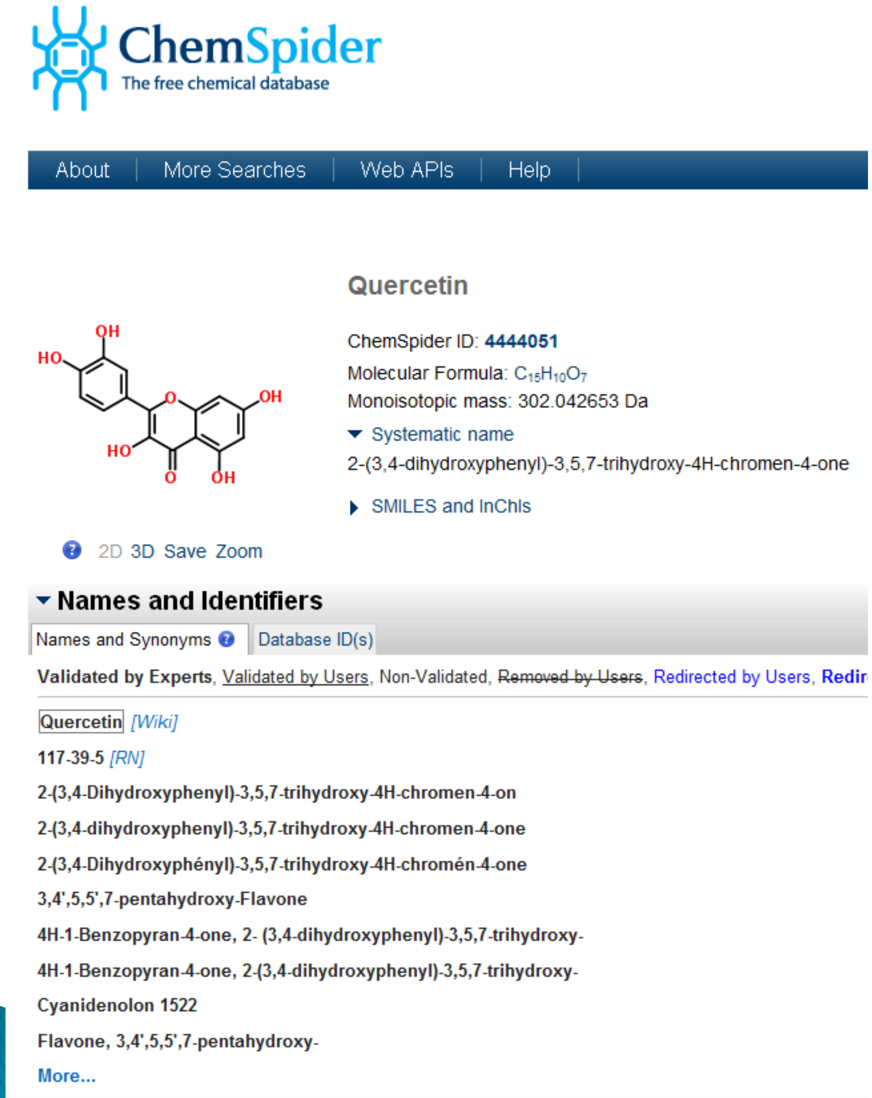
Phenol Explorer Library
Polyphenol content of foods



	A	B
1	name	formula
2	Cyanidin	$C_{15}H_{11}O_{6}$
3	Cyanidin3,5-O-diglucoside	$C_{27}H_{31}O_{16}$
4	Cyanidin3-O-(6''-acetyl-galactoside)	$C_{23}H_{23}O_{12}$
5	Cyanidin3-O-(6''-acetyl-glucoside)	$C_{23}H_{23}O_{12}$
6	Cyanidin3-O-(6''-caffeoyl-glucoside)	$C_{30}H_{27}O_{14}$
7	Cyanidin3-O-(6''-dioxalyl-glucoside)	$C_{25}H_{21}O_{17}$
8	Cyanidin3-O-(6''-malonyl-3''-glucosyl-glucoside)	$C_{30}H_{33}O_{19}$
9	Cyanidin3-O-(6''-malonyl-glucoside)	$C_{24}H_{23}O_{14}$
10	Cyanidin3-O-(6''-p-coumaroyl-glucoside)	$C_{30}H_{27}O_{13}$
11	Cyanidin3-O-(6''-succinyl-glucoside)	$C_{25}H_{25}O_{14}$
12	Cyanidin3-O-arabinoside	$C_{20}H_{19}O_{10}$
13	Cyanidin3-O-galactoside	$C_{21}H_{21}O_{11}$
14	Cyanidin3-O-glucoside	$C_{21}H_{21}O_{11}$
15	Cyanidin3-O-glucosyl-rutinoside	$C_{33}H_{41}O_{20}$
16	Cyanidin3-O-rutinoside	$C_{27}H_{31}O_{15}$
17	Cyanidin3-O-sambubioside	$C_{26}H_{29}O_{15}$
18	Cyanidin3-O-sambubiosyl5-O-glucoside	$C_{32}H_{39}O_{20}$
19	Cyanidin3-O-sophoroside	$C_{27}H_{31}O_{16}$
20	Cyanidin3-O-xyloside	$C_{20}H_{19}O_{10}$
21	Cyanidin3-O-xylosyl-rutinoside	$C_{32}H_{39}O_{19}$
22	Delphinidin3,5-O-diglucoside	$C_{27}H_{31}O_{17}$
23	Delphinidin3-O-(6''-acetyl-galactoside)	$C_{23}H_{23}O_{13}$
24	Delphinidin3-O-(6''-acetyl-glucoside)	$C_{23}H_{23}O_{13}$
25	Delphinidin3-O-(6''-malonyl-glucoside)	$C_{24}H_{23}O_{15}$
26	Delphinidin3-O-(6''-p-coumaroyl-glucoside)	$C_{30}H_{27}O_{14}$
27	Delphinidin3-O-arabinoside	$C_{20}H_{19}O_{11}$
28	Delphinidin3-O-feruloyl-glucoside	$C_{31}H_{29}O_{15}$
29	Delphinidin3-O-galactoside	$C_{21}H_{21}O_{12}$
30	Delphinidin3-O-glucoside	$C_{21}H_{21}O_{12}$
31	Delphinidin3-O-glucosyl-glucoside	$C_{27}H_{31}O_{17}$
32	Delphinidin3-O-rutinoside	$C_{27}H_{31}O_{16}$
33	Delphinidin3-O-sambubioside	$C_{26}H_{29}O_{16}$
34	Delphinidin3-O-xyloside	$C_{20}H_{19}O_{11}$
35	Malvidin3,5-O-diglucoside	$C_{29}H_{35}O_{17}$
36	Malvidin3-O-(6''-acetyl-galactoside)	$C_{25}H_{27}O_{13}$
37	Malvidin3-O-(6''-acetyl-glucoside)	$C_{25}H_{27}O_{13}$
38	Malvidin3-O-(6''-caffeoyl-glucoside)	$C_{32}H_{31}O_{15}$
39	Malvidin3-O-(6''-p-coumaroyl-glucoside)	$C_{32}H_{31}O_{14}$

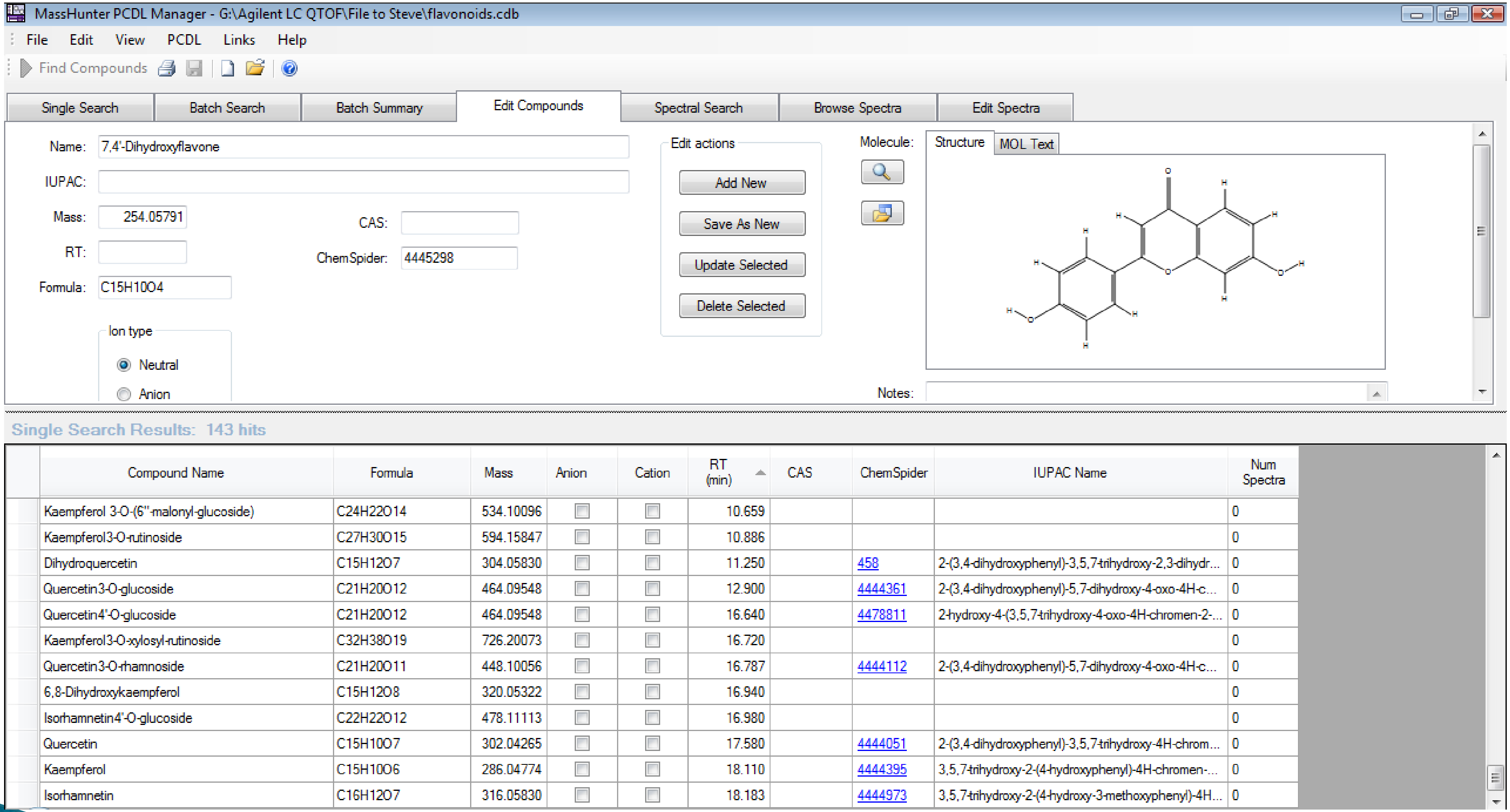
- Phenol Explorer Library (INRA)
  - ~500 different polyphenols in over 400 foods
- http://www.phenol-explorer.eu
  - Provides molecular formulas of flavonoids
    - Numerous flavonoids have the same molecular weight due to variation in glycosylation patterns
  - Onions
    - Isorhamnetin, Isorhamnetin 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



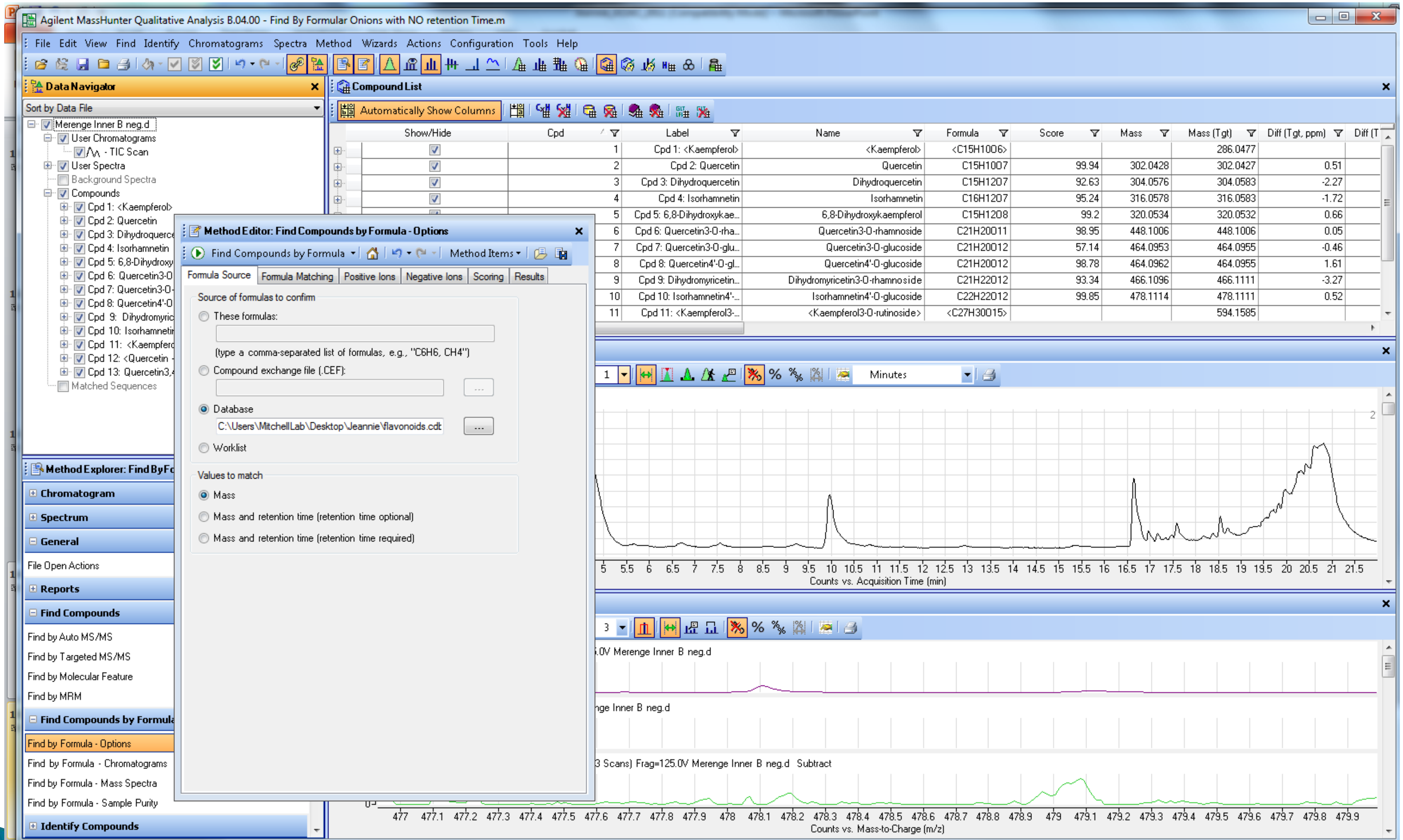
- ChemSpider provides molecular formula, monoisotopic mass, systematic names and structure for a wide range of compounds
- All of these values can be downloaded into MassHunter PCDL for library development

MassHunter PCDL Manager

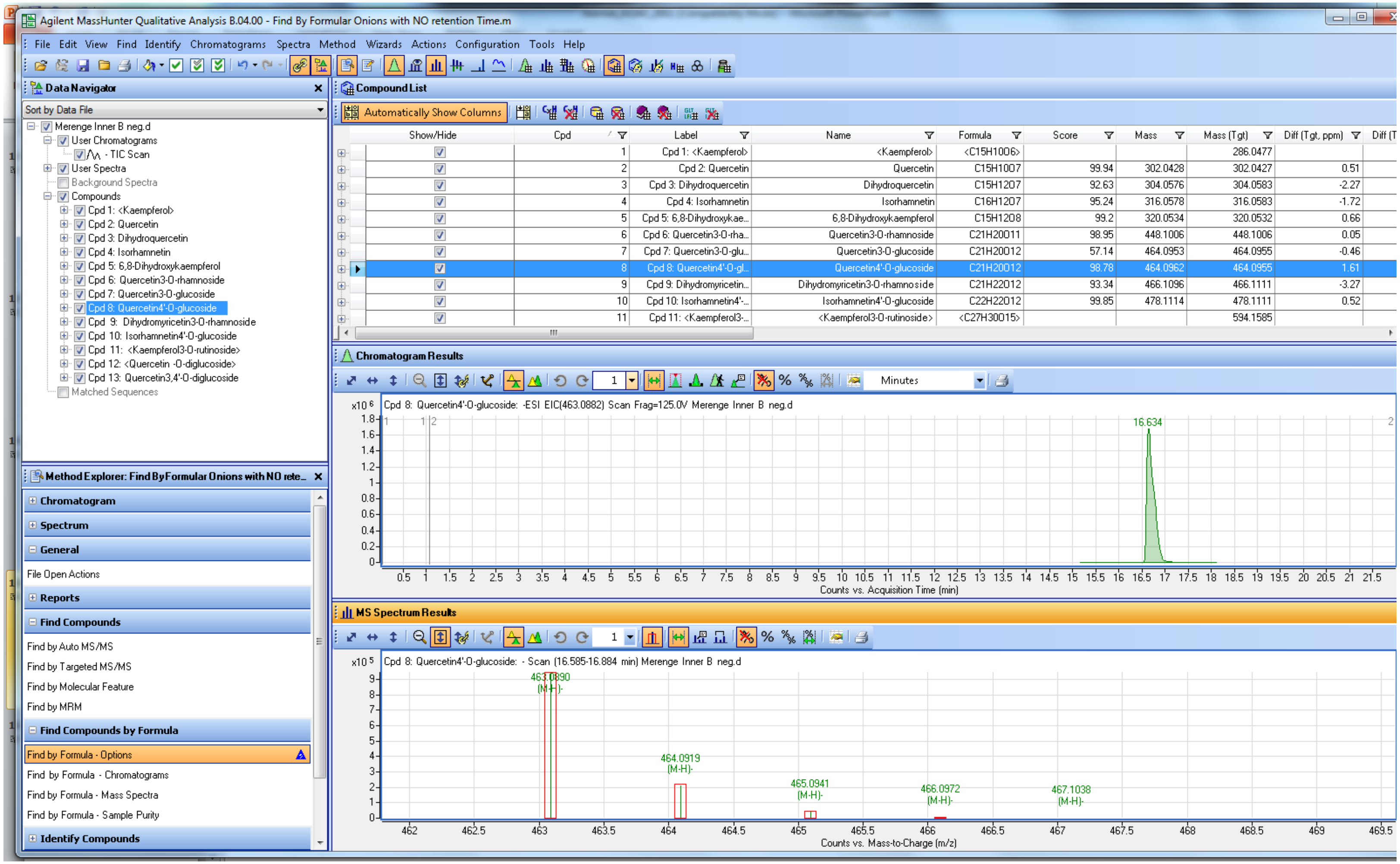


Compound Name	Formula	Mass	Anion	Cation	RT (min)	CAS	ChemSpider	IUPAC Name	Num Spectra
Kaempferol 3-O-(6''-malonyl-glucoside)	C24H22O14	534.10096			10.659				0
Kaempferol3-O-rutinoside	C27H30O15	594.15847			10.886				0
Dihydroquercetin	C15H12O7	304.05830			11.250		458	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-2,3-dihydr...	0
Quercetin3-O-glucoside	C21H20O12	464.09548			12.900		4444361	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-c...	0
Quercetin4'-O-glucoside	C21H20O12	464.09548			16.640		4478811	2-hydroxy-4-(3,5,7-trihydroxy-4-oxo-4H-chromen-2-...	0
Kaempferol3-O-xylosyl-rutinoside	C32H38O19	726.20073			16.720				0
Quercetin3-O-rhamnoside	C21H20O11	448.10056			16.787		4444112	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-c...	0
6,8-Dihydroxykaempferol	C15H12O8	320.05322			16.940				0
Isorhamnetin4'-O-glucoside	C22H22O12	478.11113			16.980				0
Quercetin	C15H10O7	302.04265			17.580		4444051	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chrom...	0
Kaempferol	C15H10O6	286.04774			18.110		4444395	3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-...	0
Isorhamnetin	C16H12O7	316.05830			18.183		4444973	3,5,7-trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4H...	0

MassHunter Qualitative Analysis



Evaluating the Data



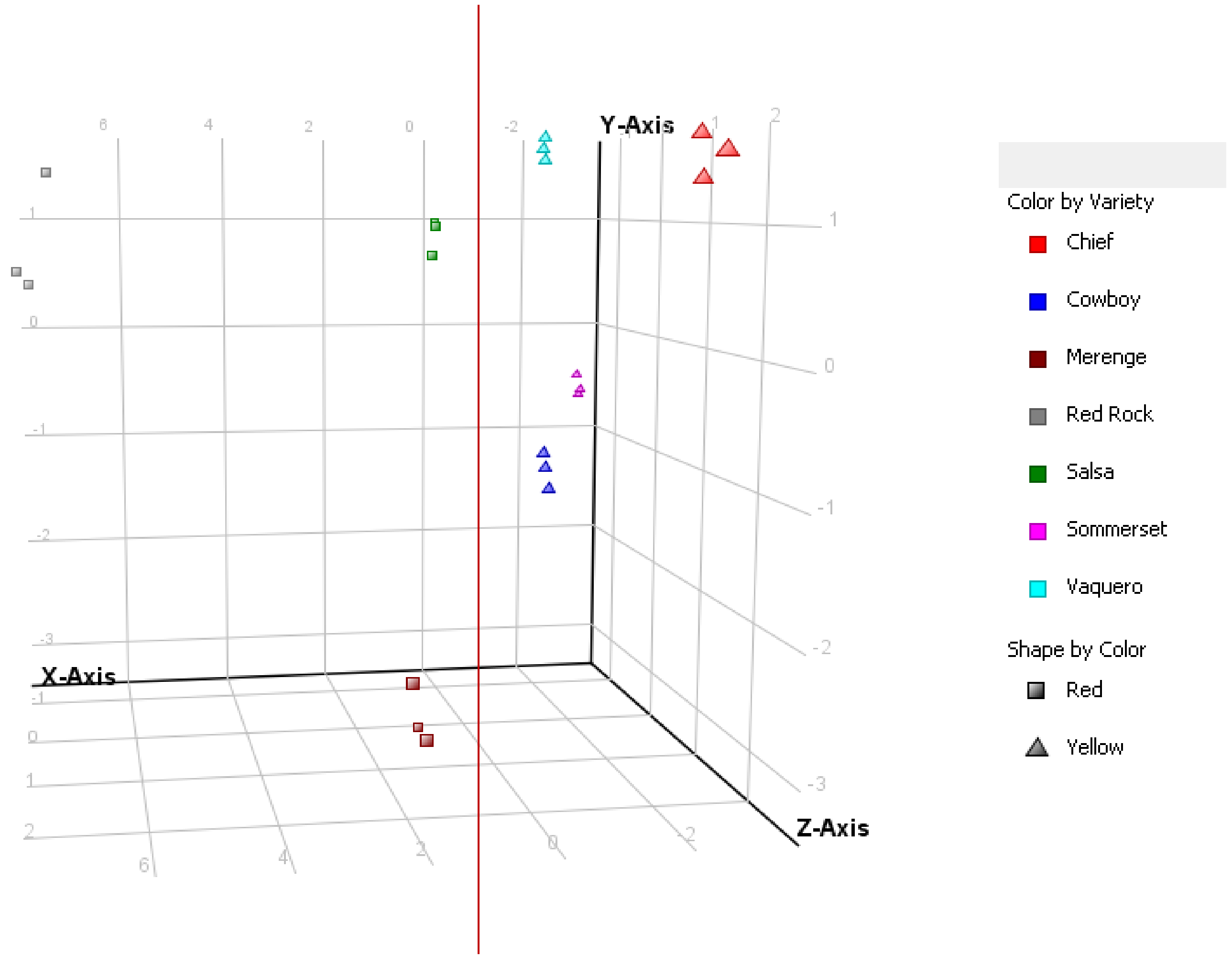
- Compounds identified are evaluated based on exact mass, t_R and isotope spacing

Data Mining Results

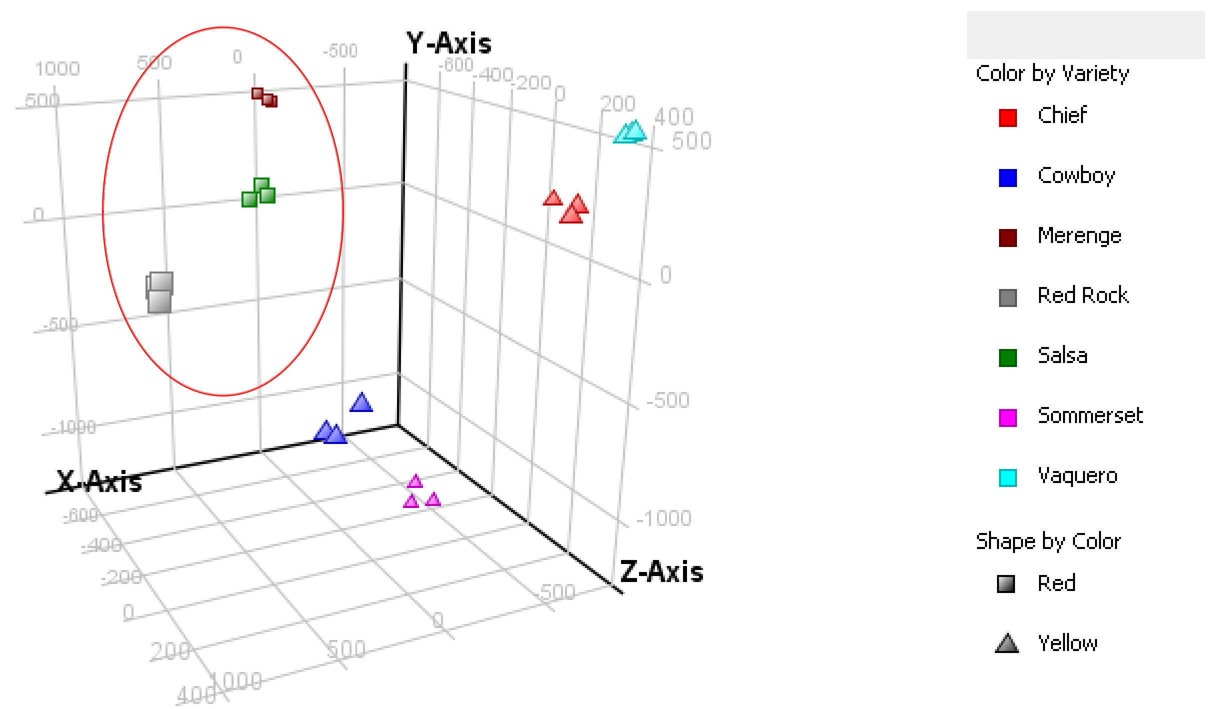
- A compound list of 19 flavonoids were identified in the 7 varieties of onions:

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)

Compound	Component 1 (80.91%)	[Red](raw)	[Yellow](raw)	Mass	Retention Time	Imperical Formula	Possible compounds (ChemSpider link)						Memo
401.2771@17.854668	2.448897	1	148,110	401.2771	17.854668	C17H35N7O4	8128513	10137334	16569011	16572992	16575753	16575939	
366.0947@12.518	2.449304	1	101,734	366.0947	12.518	C22H14N4S	1730076	3422011	4424188	9489554	9958189	24762963	(These are not all. There are 3 possible compounds more for this formula)
565.3451@18.173601	2.449187	1	66,407	565.3451	18.173601	C27H51NO11	19738984						
471.1688@10.042999	2.44908	1	98,651	471.1688	10.042999	C28H25NO6	2149487	2148324	2193205	2950892	4246898	3128669	(These are not all. There are 94 possible compounds more for this formula)
678.2377@16.946	2.449251	1	72,799	678.2377	16.946	C30H38N4O14	9149629	9160477					
318.0735@16.160666	-2.4492757	137,619	1	318.0735	16.160666	C16H14O7	89997	2107568	808939	2854063	423348	2793250	(These are not all. There are 113 possible compounds more for this formula)
322.1623@15.643333	-2.4491868	159,363	1	322.1623	15.643333	C14H26O8	81088	469143	469180	472191	487171	487181	(These are not all. There are 31 possible compounds more for this formula)
304.0578@11.303	-2.4492521	279,442	1	304.0578	11.303	C15H12O7	458	388626	19214	621332	46250	5140861	(These are not all. There are 81 possible compounds more for this formula)
696.1538@11.1189995	-2.4489334	300,917	1	696.1538	11.1189995	C30H32O19	4478818	8732006	10188633	24843605	24844550	24844875	
552.1116@10.594334	-2.4492724	646,870	1	552.1116	10.594334	C24H24O15	10476794	13611569					

Compounds Tentatively Identified Responsible for Colored Differentiation

- 6,8-Dihydroxykaempferolkaempferol
  Dihydroquercetin
  Kaempferol 3-O-(6"-malonyl-glucoside)
  Kaempferol di glucoside
  Kaempferol di glucoside (isomer, different T_R)
  Kaempferol3-O-acetyl-glucoside
  Quercetin
  Quercetin3,4'-O-diglucoside
  Quercetin diglucoside (isomer, different T_R)
  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

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