Novel MS approaches for understanding flavonoid bioavailability and metabolism

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V&E and FST Symposium:
Vision for the future

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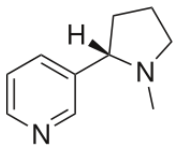
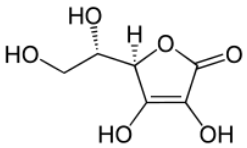
Fruit, Vegetables & the American Diet

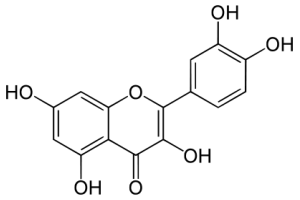
- Epidemiological studies indicate people who consume diets rich in fruits and vegetables have a reduced risk of chronic diseases
  - stroke, type II diabetes, some cancers and heart disease
- Accordingly, AHA, AICR, NIH, CDC and USDA began promoting F&V consumption more than a decade ago
  - Today USDA guidelines call for 2 cups fruit and 2.5 cups vegetables for adults eating 2000 calorie per day
- The US population is still not eating enough F&V to gain these benefits from them
- To improve human health new approaches, based upon an analytical understanding of the food-human interface, are needed to provide better information to guide dietary guidelines

Why Focus on Fruit & Vegetables

- Primary dietary source of vitamins, minerals, fiber and a wide array of *non-essential* nutrient phytochemicals
  - Polyphenolic antioxidants (e.g. flavonoids), carotenoids (e.g. lycopenes, β-carotene), alkaloids, glucosinolates, etc.
- The health benefits associated with F&V consumption are largely thought to be due to the *synergistic* activities of these bioactive compounds
- However, human studies are often contradictory and hard to interpret…
  - Why?



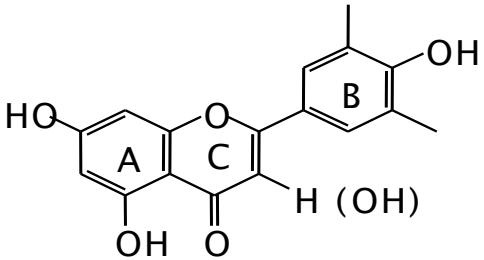




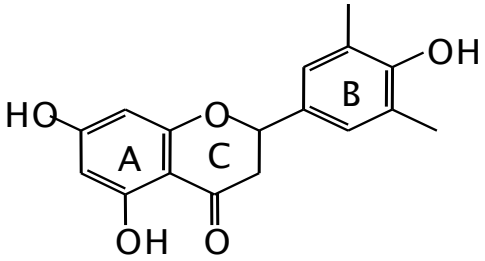
Food is More Complex Than a Drug

- Non-essential nutrient bioactives represent a wide range of chemical structures with tremendous variability in foods
- Little understanding of synergistic reactions between dietary components in foods
- Limited understanding of chemical composition of foods
  - Cultivar variability, agronomic inputs, growing region, etc.,
- Limited understanding on how processing, storage, formulation, and packaging influence bioactive chemistry
  - Oxidation, polymerization, Maillard reaction, etc.
  - Food-matrix chemistry
- Limited information on how humans absorb and metabolize bioactives in foods

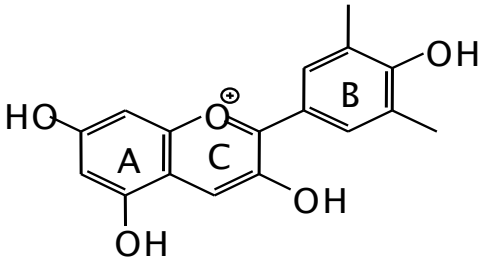
The Flavonoids



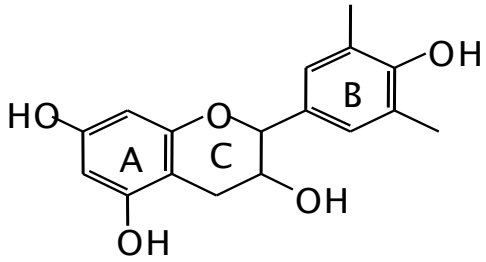
Flavone (Flavonol)



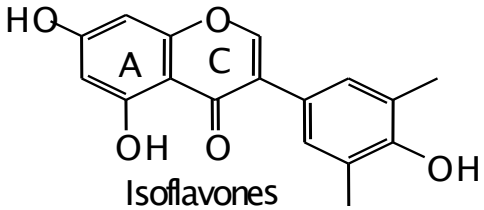
Flavanones



Anthocyanidins



Favan-3-ols

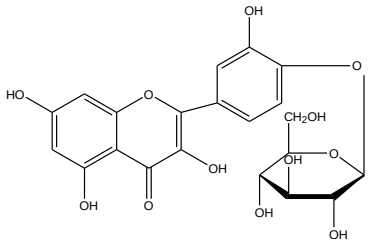


Isoflavones

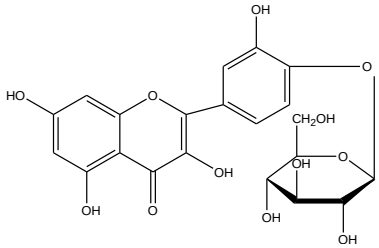
Flavonoids are diphenylpropanes based upon a C_6-C_3-C_6 carbon backbone
Roughly 2% of all hydrocarbons fixated in photosynthesis are converted into flavonoids
Flavonoids have diverse biological properties with human health implications

Quercetin: A Model Flavonol

- Major flavonoid in the US diet
  - Antioxidant, anti-inflammatory, improves vascular activity, etc.
  - Exists with different sugars attached (glycosides)
  - Sources include:
    - Onions 210 mg/100 g fresh wt
    - Apple peel 30 mg/100 g fresh wt
- Biological activity depends upon bioavailability
  - Bioavailability is highly dependent upon the glycoside
  - The dominant type of glycoside varies between foods and cultivars



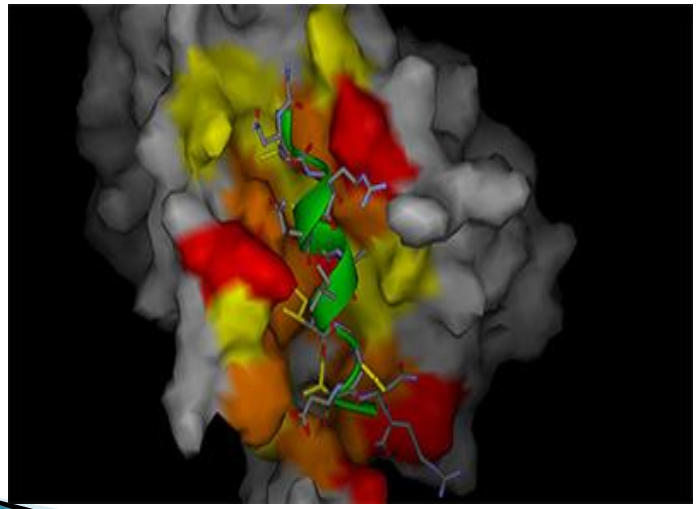
Q 4'-*O*- glucoside
Primary form found in onions

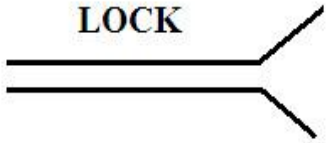


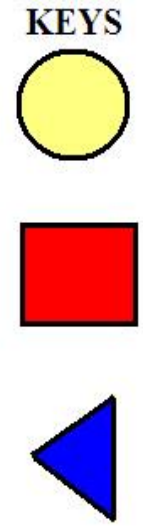
Q 3-*O*-galactoside
Primary form found in apples

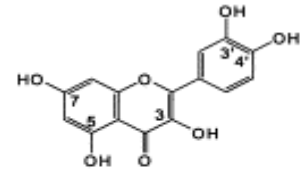
Why the Glycoside is Important

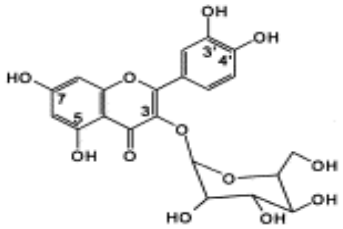
- Receptors recognize molecules largely by shape
  - Protein interactions are specific, intermolecular distances are critical for interactions between amino acids and active sites on molecules
- Receptors in the lumen of the gut have differing affinities for the gylcosides of quercetin
- Will this influence absorption ?

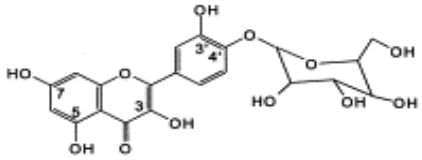






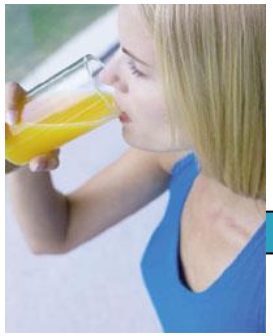

Quercetin aglycone (I)

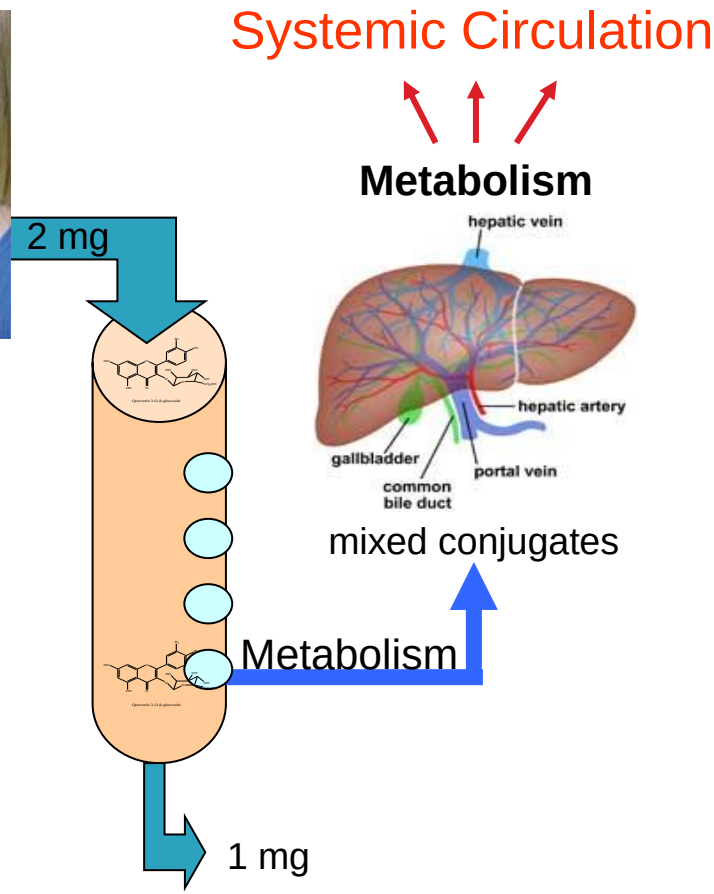

Quercetin-3-*O*-ß-D-galactoside (III)


Quercetin-4'-*O*-ß-D-glucoside (II)

Bioavailability

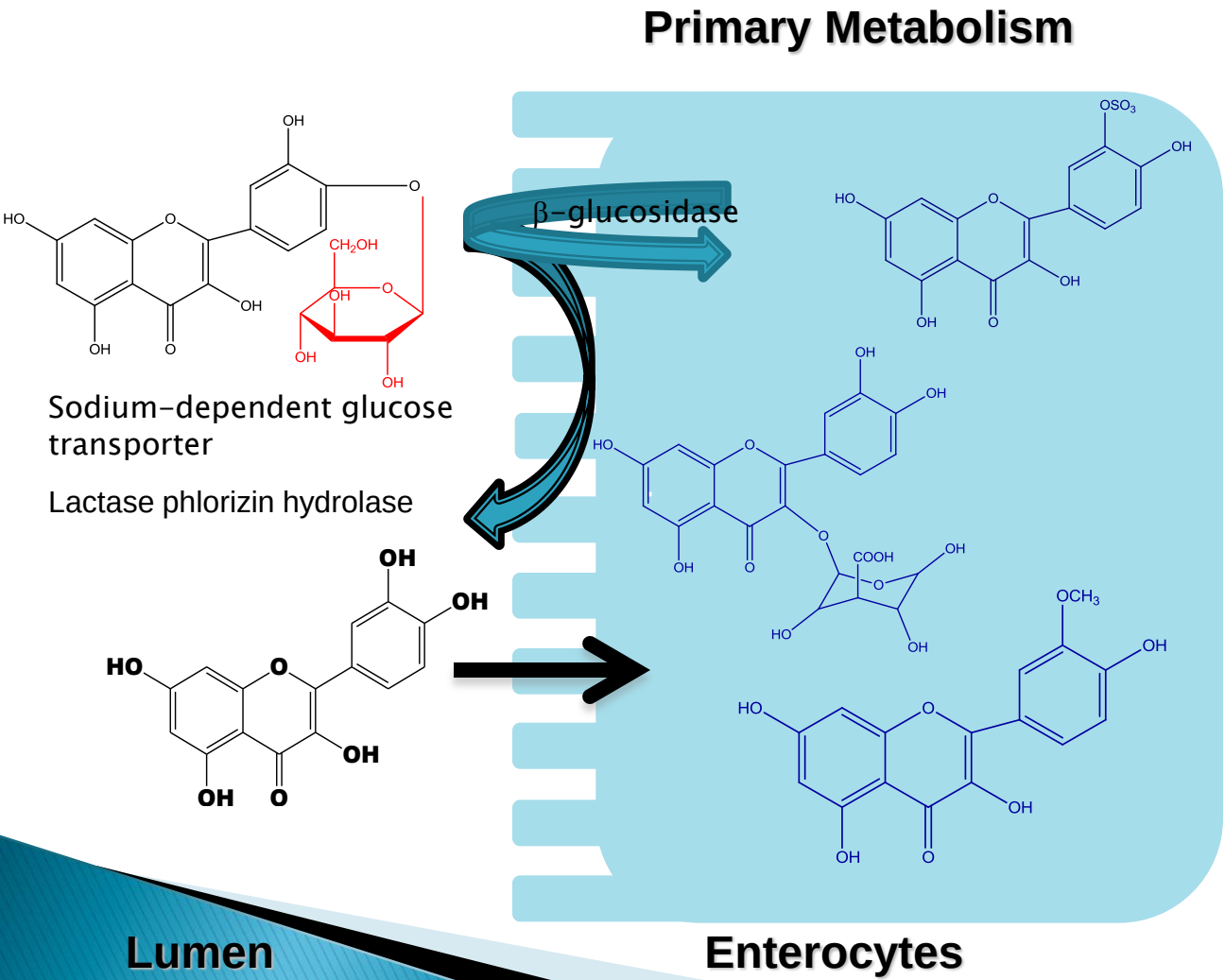
- The fraction of an administered dose of *unchanged* compound that is available to the body for use in normal physiological functions, metabolism or for storage (i.e. reaches systemic circulation)



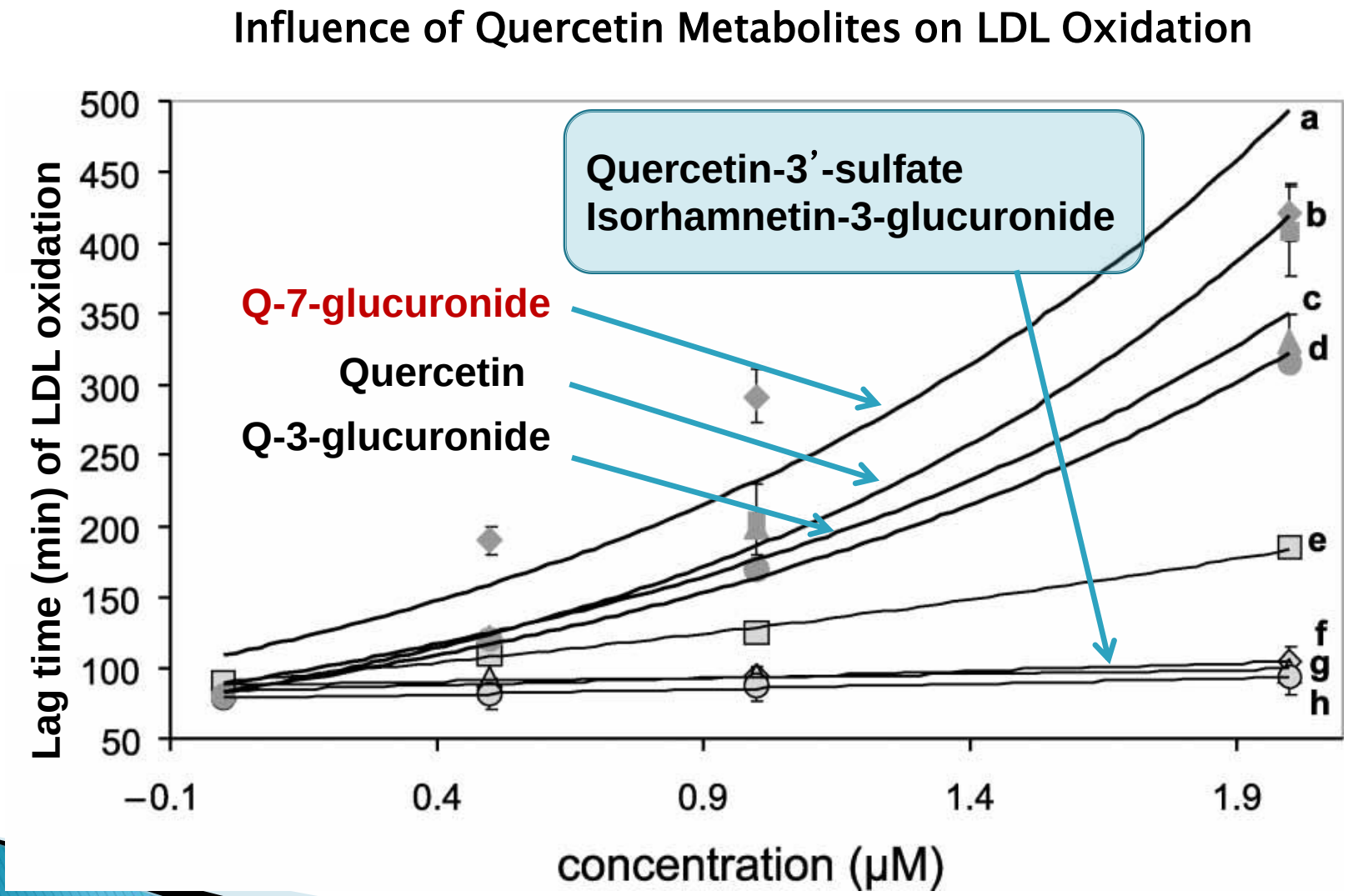


- Absorption is a receptor-mediated event
- Uptake is dependent upon molecular recognition
- *why the glycoside matters*

Quercetin Uptake & Metabolism

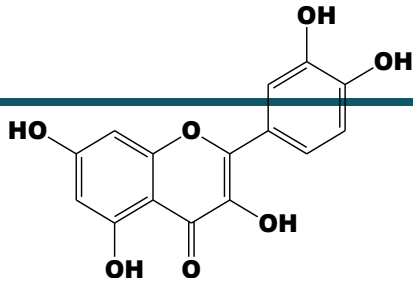


Metabolites and Biological Activity



(Janisch et al., 2004, Free Radic. Res. 38(8): 877-

Influence of Glycoside on Metabolite Profiles

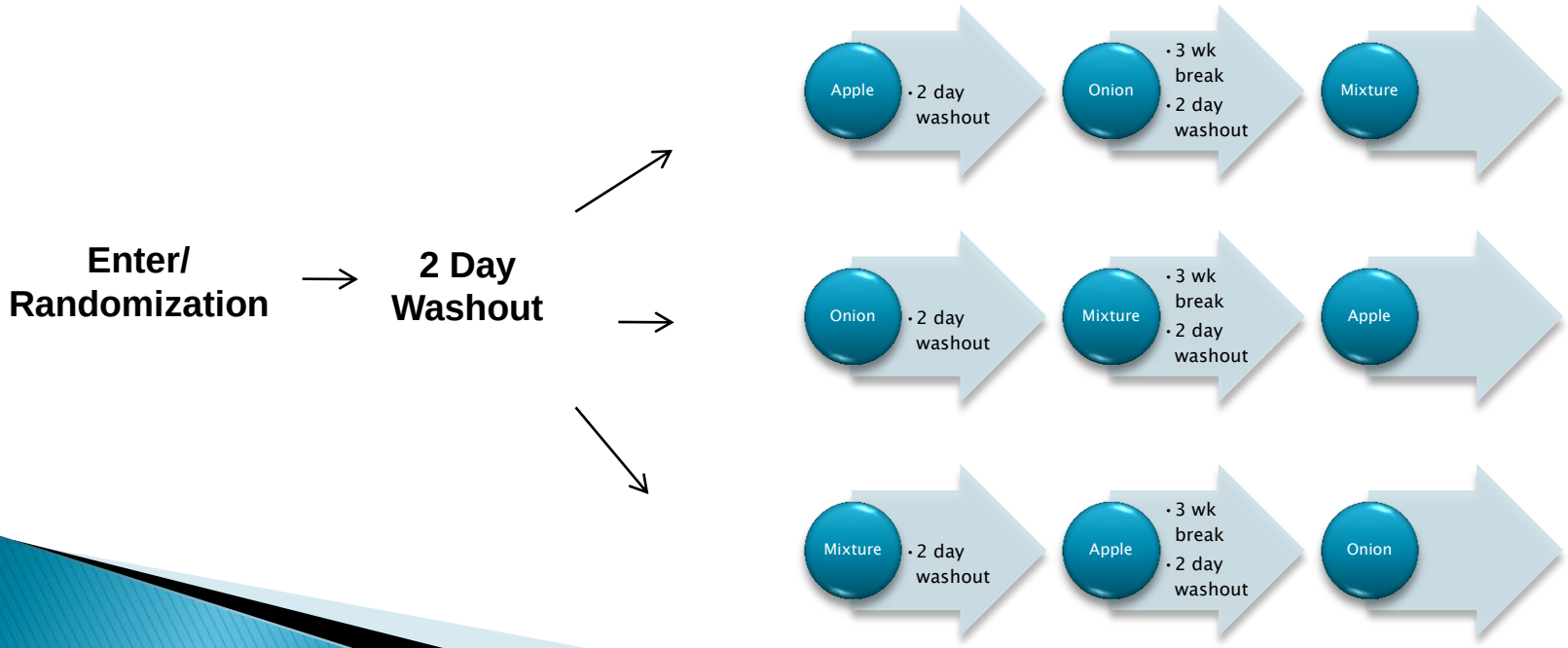


- Study Rational:
- To investigate the complement of quercetin metabolites in plasma in response to two different foods with the same level of quercetin aglycone equivalents yet composed of differing quercetin glycosides

- Interest is in how the glycoside influences basic pharmacokinetic parameters (absorption and metabolism) and potential bioactivity

Clinical Study Design

- Feeding Trial – randomized crossover design
- 16 subjects (8 male/ 8 female)
- Plasma taken at 0, 0.5, 1, 2, 4, 6, 8, and 24 hr post consumption of applesauce fortified with micronized apple peel or micronized onion powder (100 mg quercetin eqivalents)

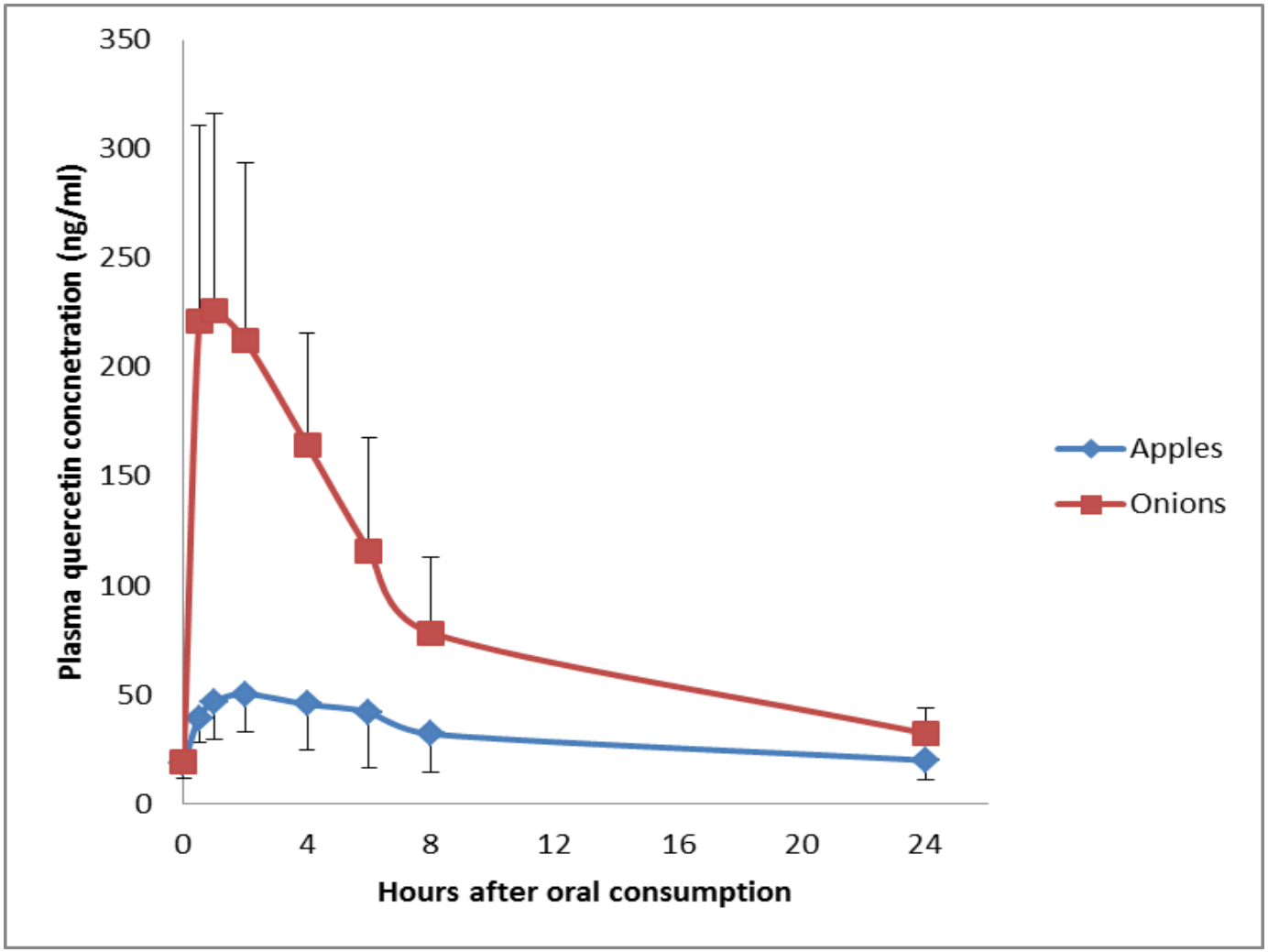


Quercetin Profile of Fortified Applesauce (mg)

quercetin glycosides	100 g applesauce fortified with	
	apple peel powder	onion powder
quercetin 3,4′-O-diglucoside	0	44.9
quercetin 3-O–rutinoside (rutin)	6.5	0
quercetin 3-O-galactoside	16.8	0
quercetin 3-O-glucoside	16.1	4.9
quercetin -O-xyloside	9.3	0
quercetin -O-arabinofuranose	3.0	0
quercetin -O-arabinopyranose	17.6	0
quercetin 3-O-rhamnoside	30.6	0
quercetin 4′-O-glucoside	0	48.7
total	99.9 ± 2.3	98.5 ± 2.1

(JH Lee and AE Mitchell, 2012, J. Agric. Food Chem., 60: 3874-3881)

Bioavailability of Total Plasma Quercetin



JH Lee and AE Mitchell, 2012, J. Agric. Food Chem., 60: 3874-3881

Accurate Mass Q –TOF MS Analysis of Quercetin Metabolites

- The next step was to identify the range of quercetin metabolites in these samples
  - Instrument: Agilent 1290 UHPLC interfaced with a 6530 Accurate Mass Q-TOF MS
  - Separation: Poroshell C_{18} (2.1 x 100, 2.7 µm) column
  - Mobile phase: a linear gradient of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile
  - The limit of detection was 6 ng/mL in plasma and the linear dynamic range spanned three orders of magnitude
  - The extraction recovery was 87-108% when the absolute recovery was measured

Accurate Mass and Library Assisted Identification of Quercetin Metabolites

1. Build a Library of All Possible Quercetin Metabolites

- The exact masses of all *potential* quercetin metabolites were compiled into a Personal Compound Database Library (PCDL) and imported into MassHunter Qualitative Analysis Software (Agilent Tech)

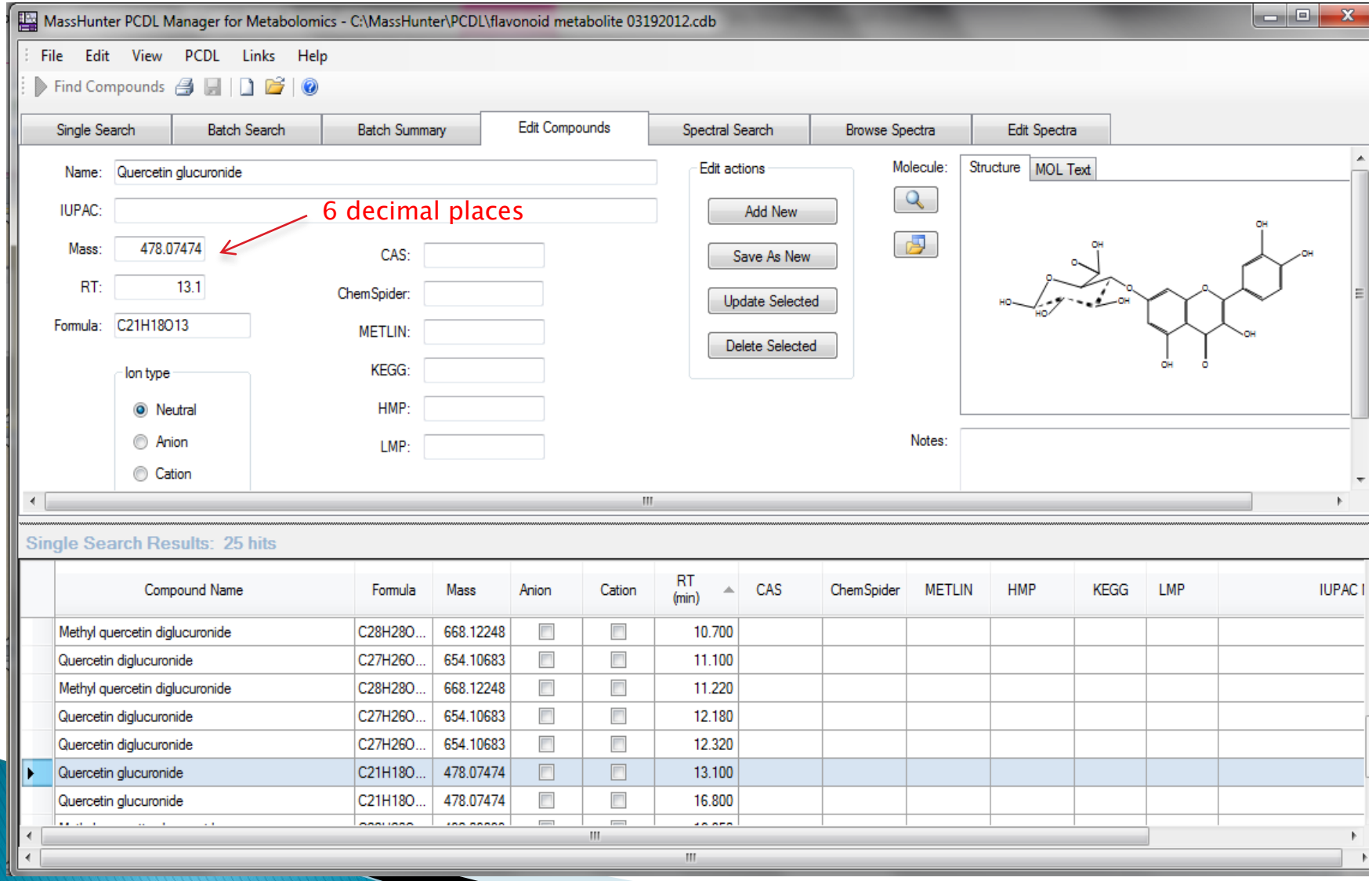
2. Q-TOF: MS1 Scan – Targeted Unknowns

- MS1 scan was search against the PCDL library and quercetin metabolites in plasma were identified based on accurate mass, isotope abundance and isotope spacing

3. Q-TOF: MS2 Scan – Targeted MS/MS

- Tandem MS was used for building structural confidence of quercetin metabolites identified by accurate match formula matching

Building an Accurate Mass Library of Quercetin Metabolites

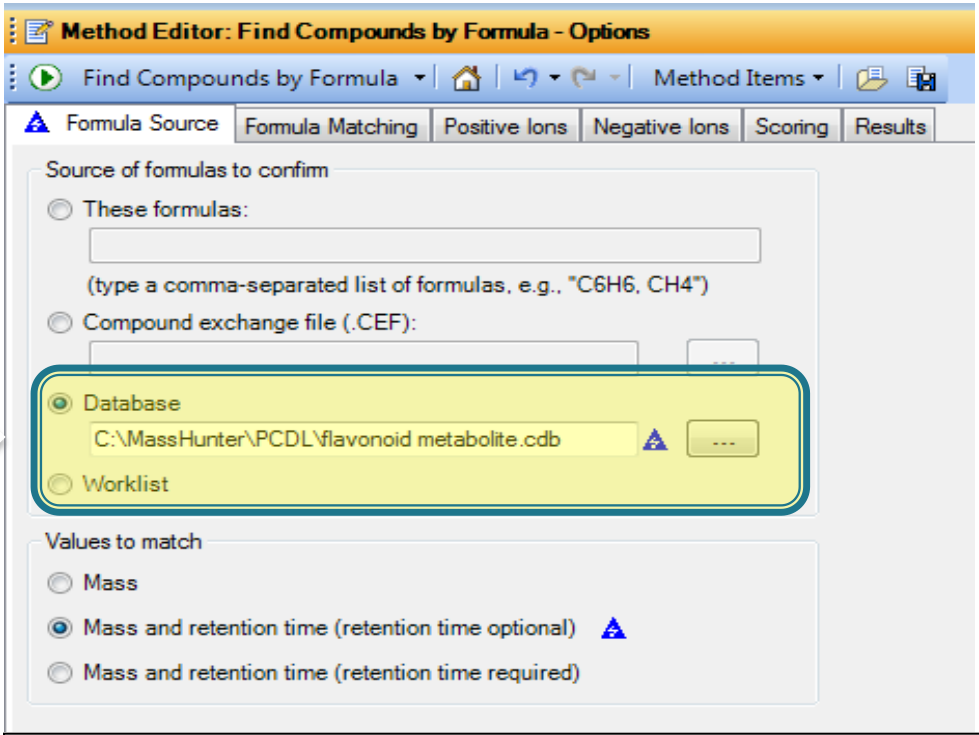


Compound Name	Formula	Mass	Anion	Cation	RT (min)	CAS	ChemSpider	METLIN	HMP	KEGG	LMP	IUPAC I
Methyl quercetin diglucuronide	C28H28O...	668.12248			10.700							
Quercetin diglucuronide	C27H26O...	654.10683			11.100							
Methyl quercetin diglucuronide	C28H28O...	668.12248			11.220							
Quercetin diglucuronide	C27H26O...	654.10683			12.180							
Quercetin diglucuronide	C27H26O...	654.10683			12.320							
Quercetin glucuronide	C21H18O...	478.07474			13.100							
Quercetin glucuronide	C21H18O...	478.07474			16.800							

Q TOF MS1 Scan: Targeted Unknown Identification

MS1 Scan Accurate Mass

Database (PCDL)

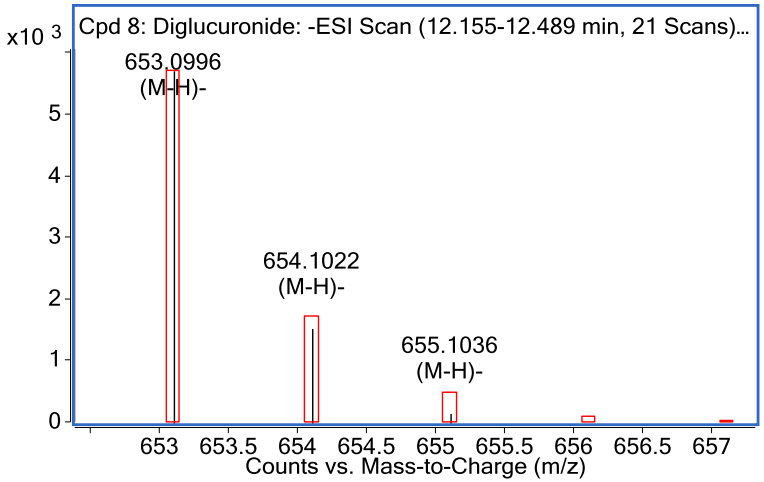


Search QTOF accurate mass data for compounds in the PCDL database

Generate list of possible compounds

Identification Based Upon Accurate Mass and Isotope Fidelity

peak	RT (min)	possible compounds	type of molecular ion	predicted m/z	observed m/z	error (ppm)
1	8.86	Methyl Q diglucuronide	$[M-H]^-$	667.1152	667.1146	0.9
2	10.07	Q diglucuronide	$[M-H]^-$	653.0996	653.0987	1.4
3	10.70	Methyl Q diglucuronide	$[M-H]^-$	667.1152	667.1136	2.4
4	11.10	Q diglucuronide	$[M-H]^-$	653.0996	653.0986	1.5
5	11.22	Methyl Q diglucuronide	$[M-H]^-$	667.1152	667.1147	0.6
6	12.18	Q diglucuronide	$[M-H]^-$	653.0996	653.0989	1.1
7	12.32	Q diglucuronide	$[M-H]^-$	653.0996	653.0989	1.1
8	13.38	Q glucuronide sulfate	$[M-H]^-$	557.0243	557.0240	0.5
9	13.10	Q-3-glucuronide	$[M-H]^-$	477.0675	477.0671	0.8
10	16.80	Q-3′-glucuronide	$[M-H]^-$	477.0675	477.0673	0.4
11	16.85	Methyl Q 3 glucuronide	$[M-H]^-$	491.0831	491.0832	-0.2
12	16.90	Q glutathione	$[M+HCOO]^-$	651.1012	651.0998	2.2
13	17.07	Q glucuronide	$[M-H]^-$	477.0675	477.0672	0.6
14	17.10	Methyl Q glucuronide	$[M-H]^-$	491.0831	491.0831	0.0
15	17.45	Q-3-sulfate	$[M-H]^-$	380.9922	380.9925	-0.8



MS1 scan → Identifies list of possibilities

↓

Evaluated based on accurate mass and isotope fidelity

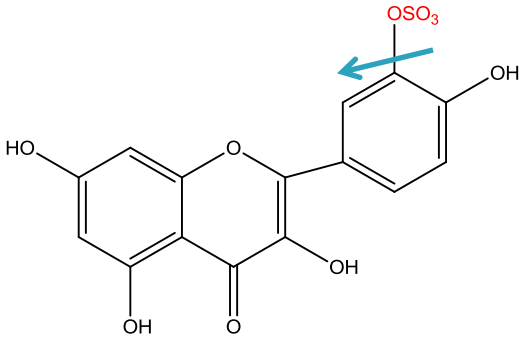
↓

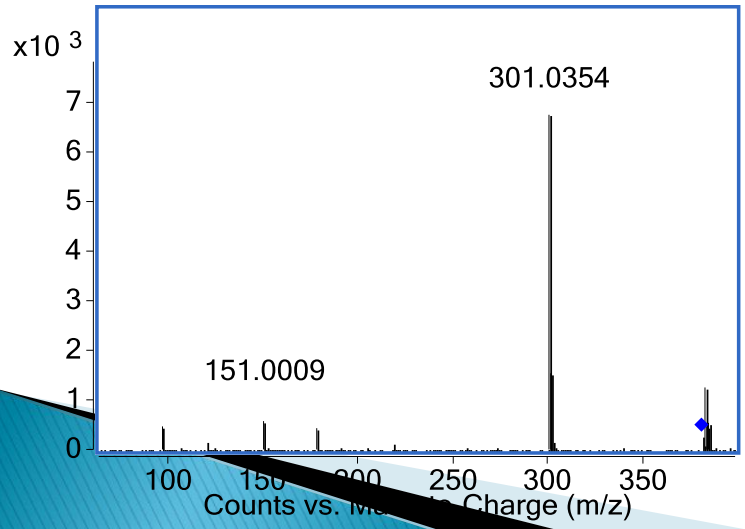
MS2 scan: Fragments

Targeted MS/MS of Library-Assisted Identified Metabolites: Building Confidence

Quercetin sulfate

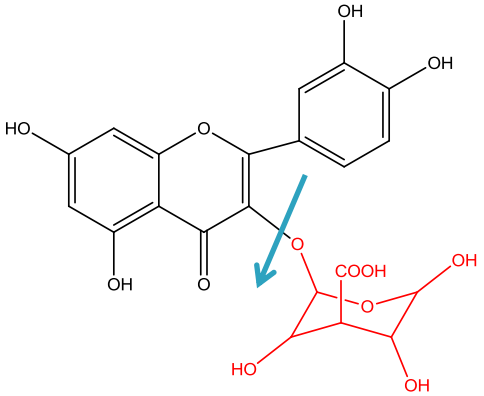
Peak 8

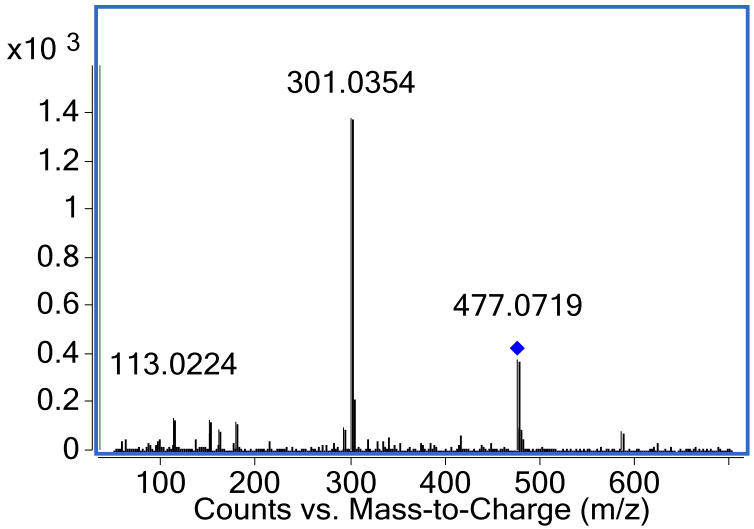




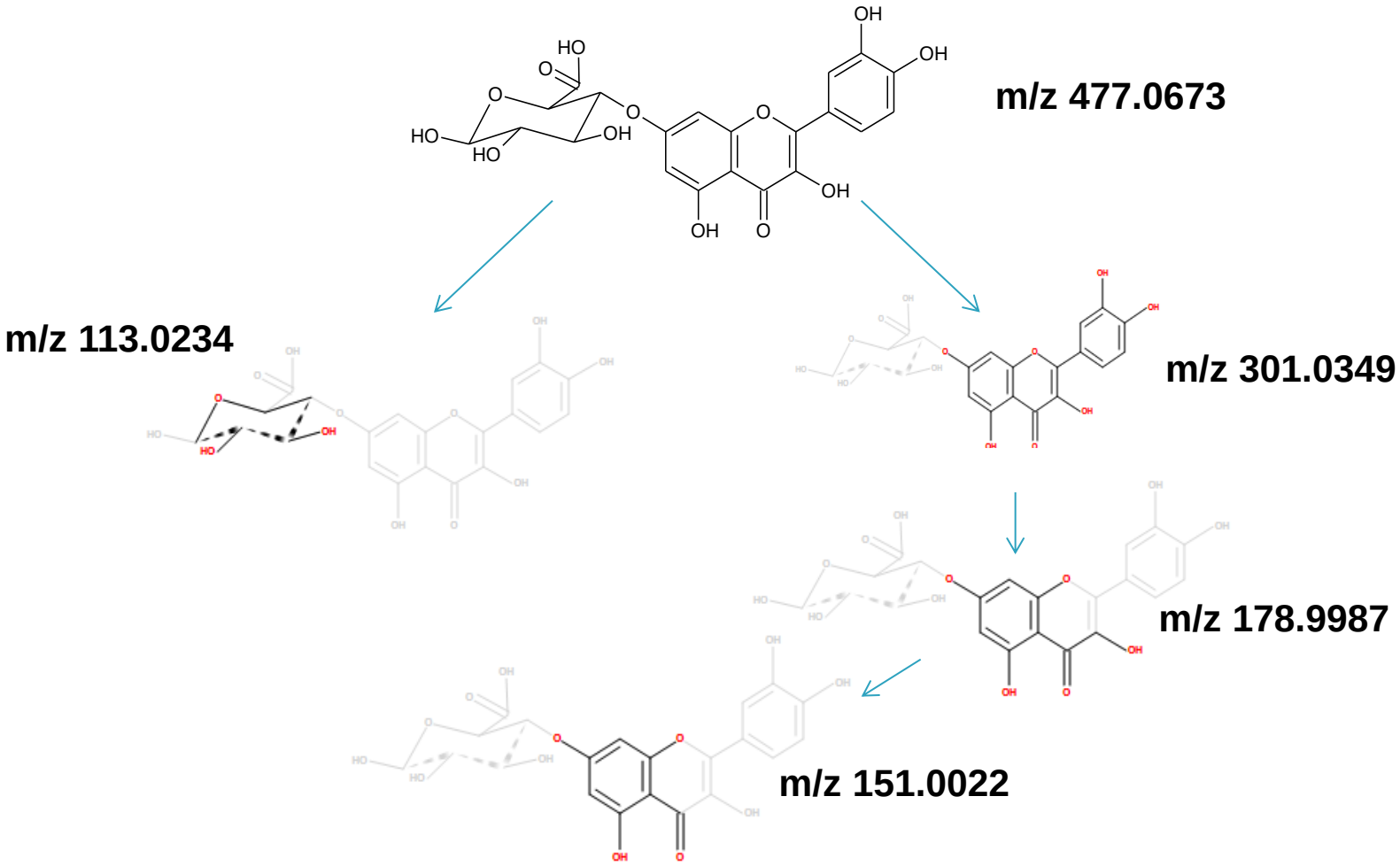
Quercetin glucuronide

Peaks 9, 10, 13





Fragmentation of Quercetin Glucuronide



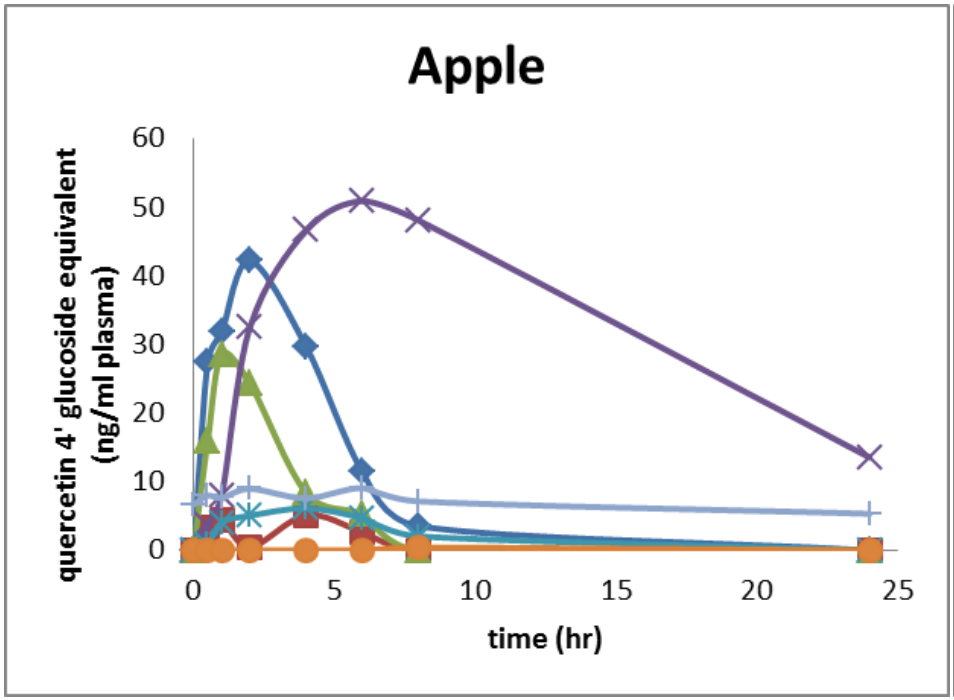
Fragmentation patterns (and R_t) can be used to help distinguish isomers

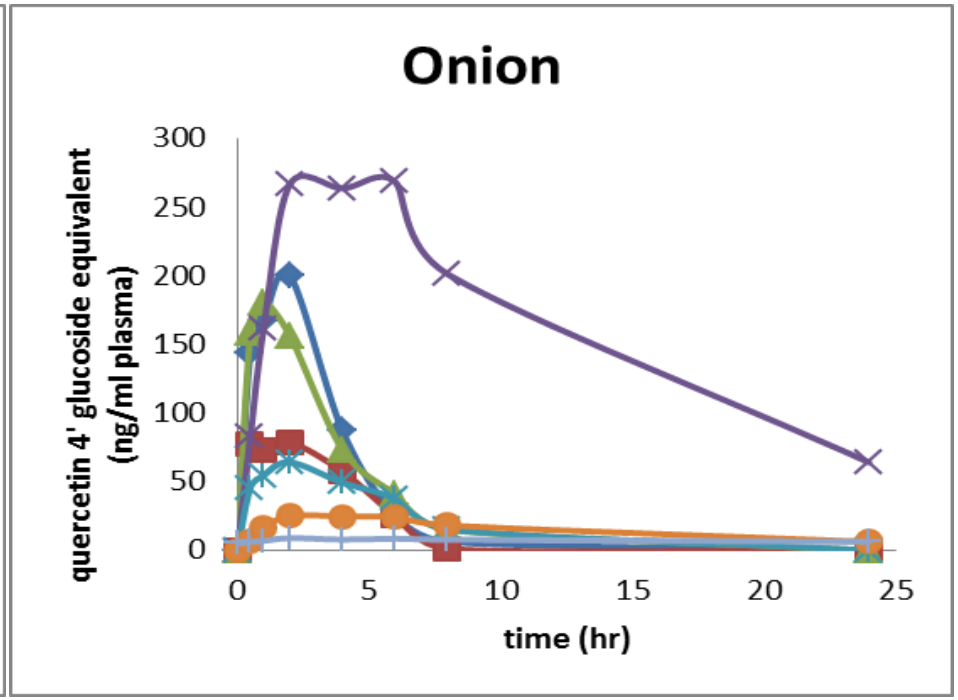
Simplifying Metabolite Profiling: *Grouping Metabolites*

peak	RT (min)	possible compounds	observed m/z
1	8.86	Methyl Q diglucuronide	667.1146
2	10.07	Q diglucuronide	653.0987
3	10.70	Methyl Q diglucuronide	667.1136
4	11.10	Q diglucuronide	653.0986
5	11.22	Methyl Q diglucuronide	667.1147
6	12.18	Q diglucuronide	653.0989
7	12.32	Q diglucuronide	653.0989
8	13.38	Q glucuronide sulphate	557.0240
9	13.10	Q-3-glucuronide	477.0671
10	16.80	Q-3′-glucuronide	477.0673
11	16.85	Methyl Q 3 glucuronide	491.0832
12	16.90	Q glutathione	651.0998
13	17.07	Q glucuronide	477.0672
14	17.10	Methyl Q glucuronide	491.0831
15	17.45	Q-3-sulphate	380.9925

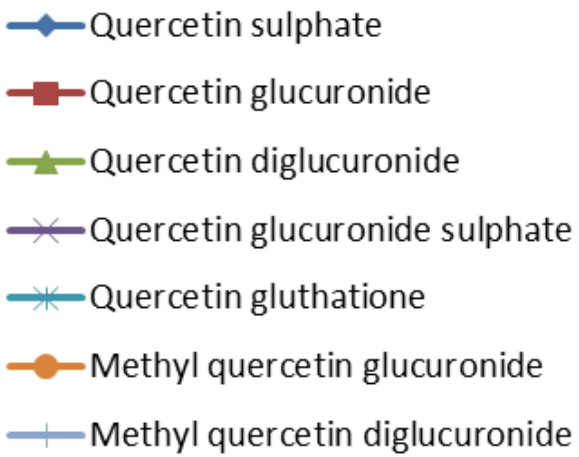
group	Q metabolite groups
1	Q sulphate
2	Q glucuronide
3	Q diglucuronide
4	Q glucuronide sulphate
5	Q glutathione
6	Methyl Q glucuronide
7	Methyl Q diglucuronide

Plasma Quercetin Metabolites (ESI-)

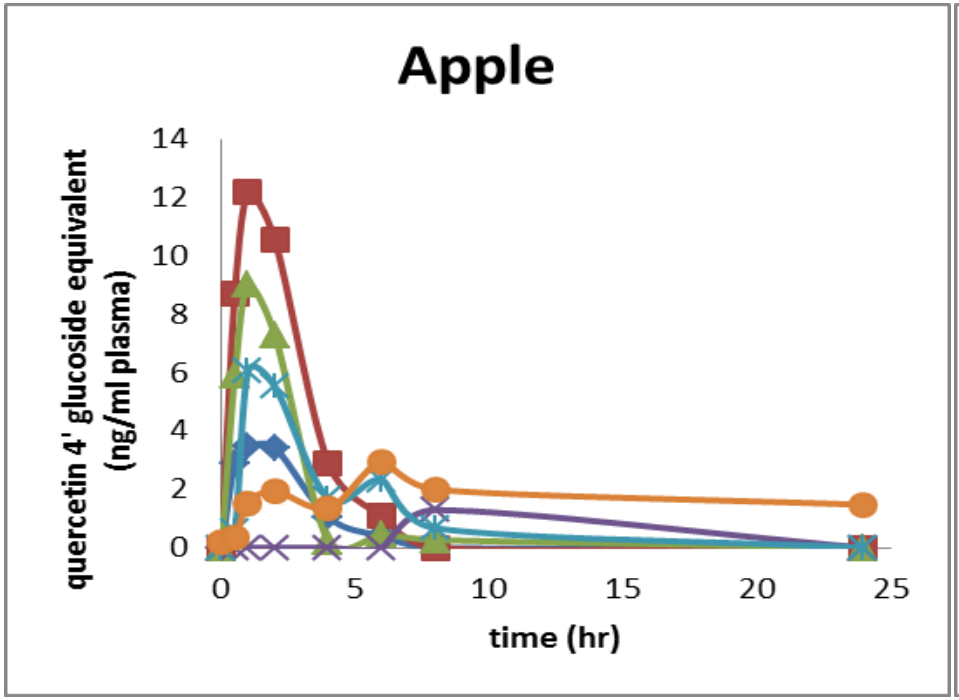


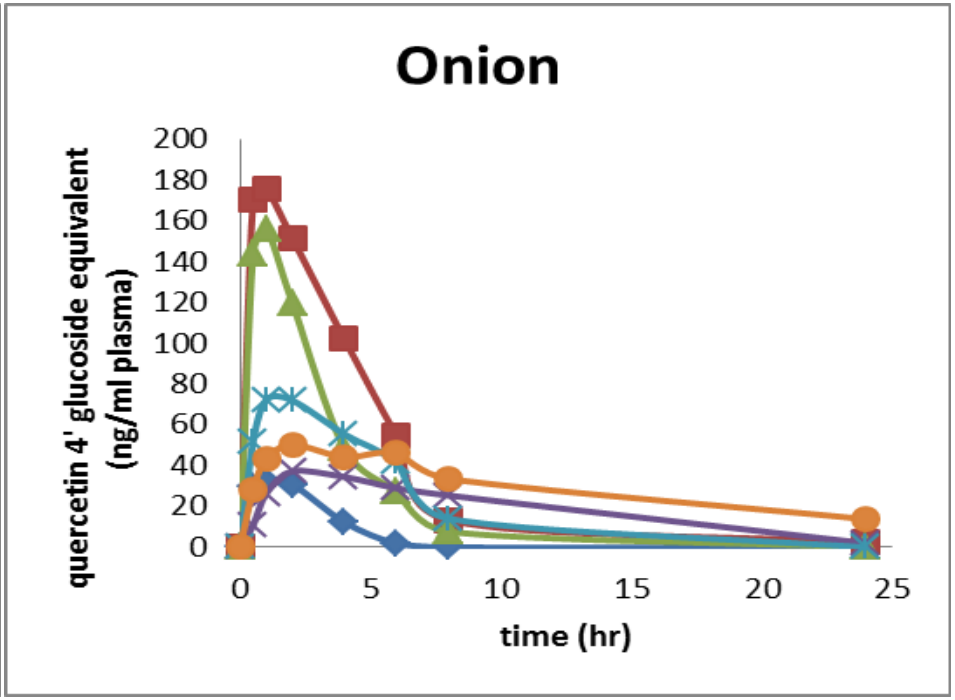


- Q metabolites profiles were similar for apples and onions
- Total bioavailability differed significantly
- Gluc-sulphate, sulphate, di-glucuronide
- GSH-conj

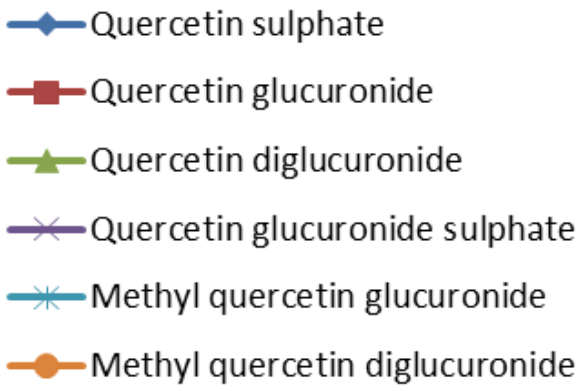


Plasma Quercetin Metabolites (ESI+)





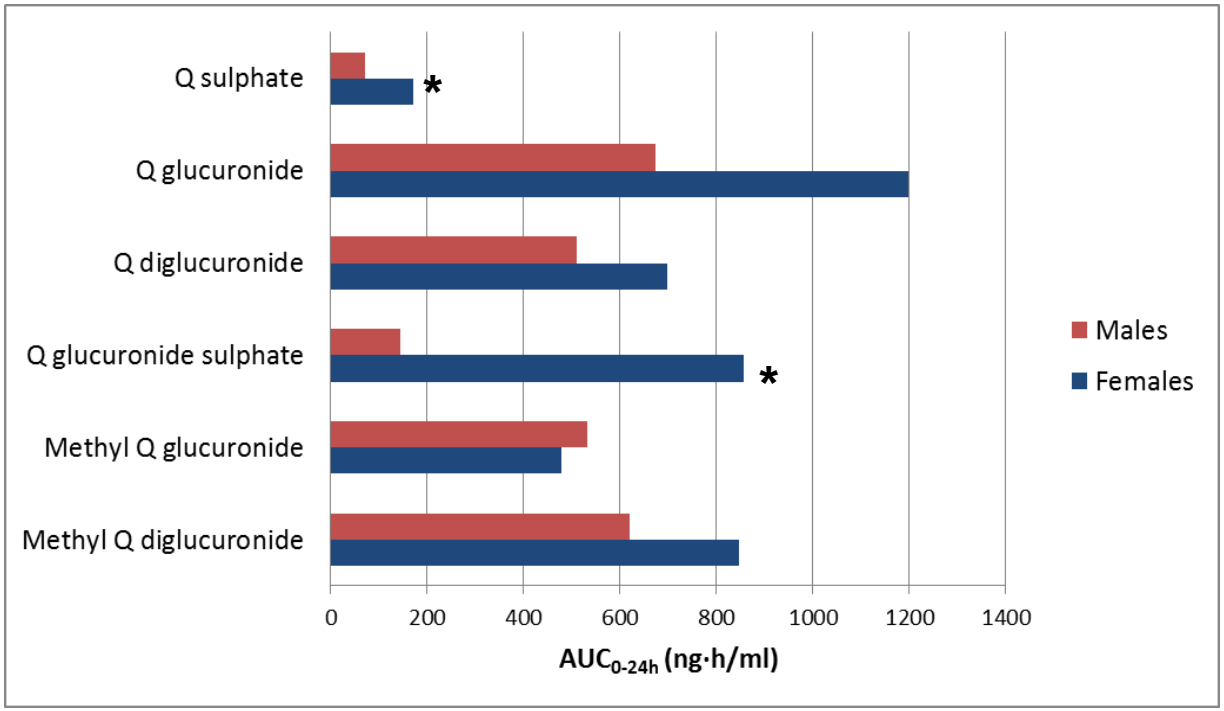
- The Q metabolite profiles were similar for apples and onions
- Total bioavailability differed significantly
- Glucuronide, di-glucuronide, methyl-glucuronide
- Polarity of ESI impacts ionization efficiency of quercetin metabolites



Pharmacokinetic Parameters (ESI+)

	C_{max} (ng/ml)	$AUC_{0\text{-}24h}$ (ng·h/ml)	T_{max} (h)
Apple peel fortified			
Q sulphate	4.6 ± 6.3	*12.0 ± 17.3*	1.7 ± 1.2
Q glucuronide	15.5 ± 13.3	37.3 ± 36.9	2.2 ± 1.6
Q diglucuronide	9.3 ± 15.2	24.4 ± 40.6	2.0 ± 1.6
Q glucuronide sulphate	1.3 ± 4.8	11.6 ± 43.6	*8.0 ± 0.0*
Methyl Q glucuronide	7.5 ± 8.7	*27.2 ± 41.8*	2.4 ± 1.8
Methyl Q diglucuronide	3.6 ± 5.4	42.6 ± 85.8	*5.6 ± 2.2*
Onion powder fortified			
Q sulphate	51.9 ± 67.7	113.9 ± 86.7	1.3 ± 0.7
Q glucuronide	284.7 ± 329.9	897.9 ± 656.3	2.4 ± 1.9
Q diglucuronide	267.7 ± 330.8	591.0 ± 293.5	1.8 ± 1.6
Q glucuronide sulphate	86.7 ± 182.9	453.6 ± 668.4	*4.0 ± 1.4*
Methyl Q glucuronide	158.2 ± 285.1	509.6 ± 204.3	2.6 ± 2.0
Methyl Q diglucuronide	87.3 ± 94.2	717.2 ± 284.0	*3.5 ± 2.2*

Gender-Related Differences



- AUCs of Q sulphate and Q glucuronide sulphate were greater in females than in males ($p<0.05$)

Conclusions

- UHPLC(ESI) Q-TOF MS/MS methods facilitated identification of quercetin metabolites in plasma
  - Improved sensitivity allowed for kinetic monitoring of individual quercetin metabolites in plasma over 24 hours
  - Polarity of ESI impacts ionization efficiency of quercetin metabolites
- The major quercetin metabolites included quercetin glucuronide, quercetin sulphate and quercetin diglucuronide regardless of dietary source
- T_{max} of methyl quercetin diglucuronide and quercetin glucuronide sulphate differ between treatment groups
- AUCs of Q sulphate and Q glucuronide sulphate were greater in females than in males ($p < 0.05$)

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