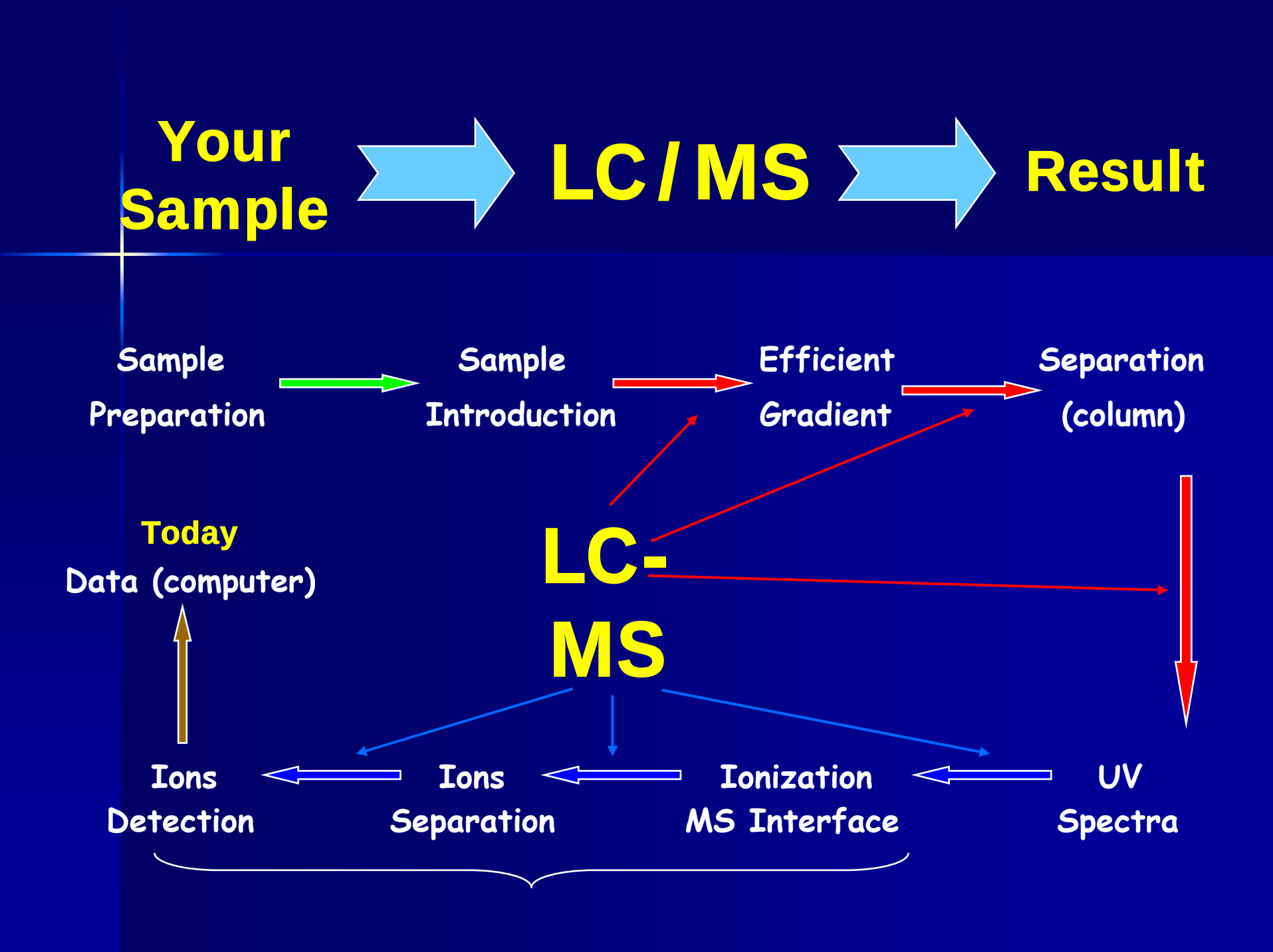
High Resolution LC-MS Data Output and Analysis


Your Sample
LC / MS
Result
Sample Preparation
Sample Introduction
Efficient Gradient
Separation (column)
Today
Data (computer)
LC-MS
Ions Detection
Ions Separation
Ionization MS Interface
UV Spectra

Mass Spectrometer

1. Breaks up constituents into molecular ions and other fragments

2. The ions are separated according to their mass-to-charge ratio (m/z)

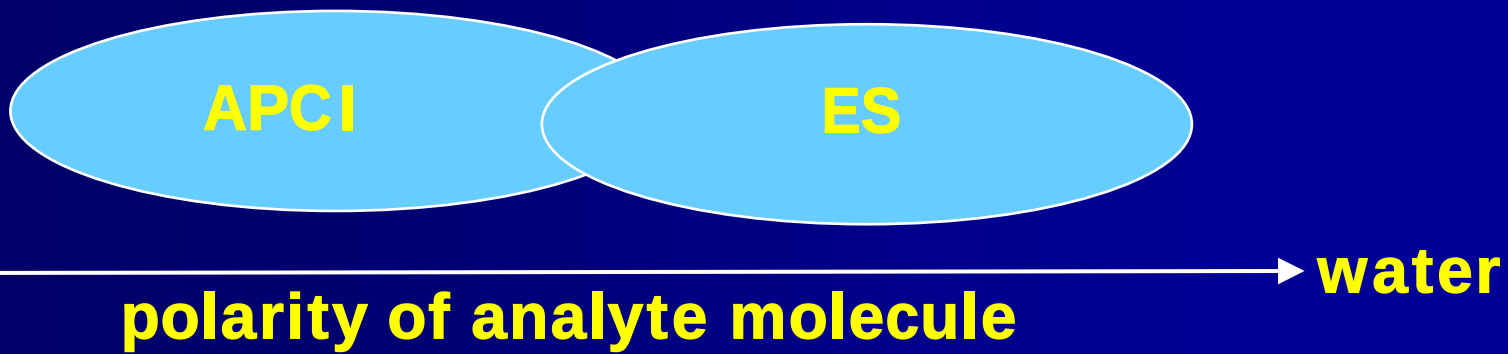
3. Measures masses

Mass Spectrometer

4. Structural information on metabolite
    * fragmentation pattern
    * accurate mass

Alternative Ionization Modes

- Atmospheric Pressure Ionization (API), in LC-MS
  - Electrospray Ionisation (ESI): polar and semi-polar
  - Atmospheric Pressure Chemical Ionization (APCI): less polar



Positive or Negative Modes?

The formation of positive or negative ions depends on the sign of the applied electrical field

ES+: $(M+H)^+$

Good ionization of basic compounds (get proton)
E.g. amino, amide, ester, aldehyde/keto functional groups (formic acid in sample solution to help ionize)

ES-: $(M-H)^-$

Acidic Compounds (give proton) E.g. organic acids, containing OH
(ammonium buffer in sample solution to help ionize)

Mass Analyzers

- *Ion Cyclotron (FT-ICR-MS)*
- *Time of Flight (TOF)*
- *Magnetic Sector*
- *Quadrupole Ion Trap*
- *Quadrupole*

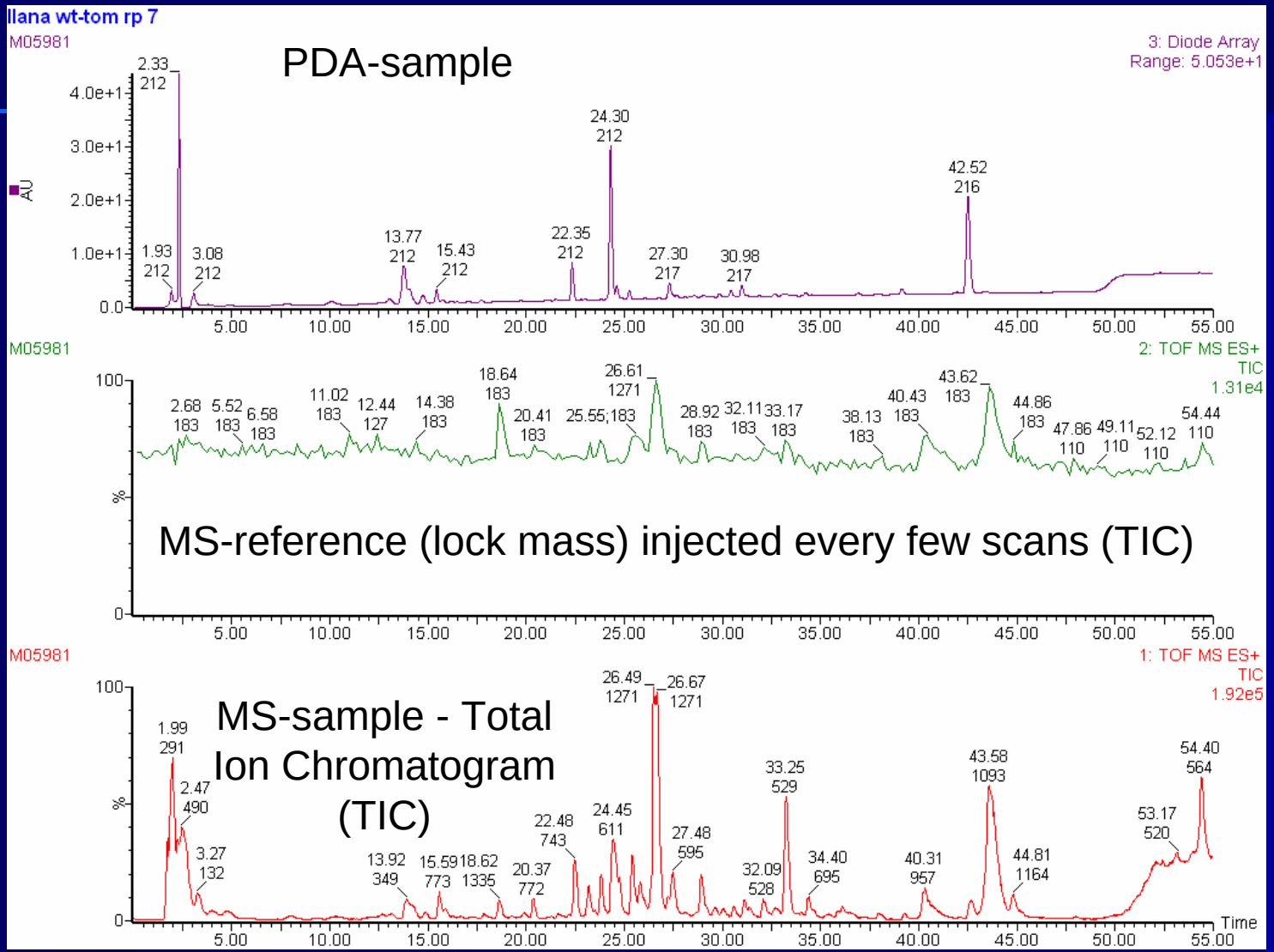
"High Resolution" Instruments

"Low Resolution" Instruments

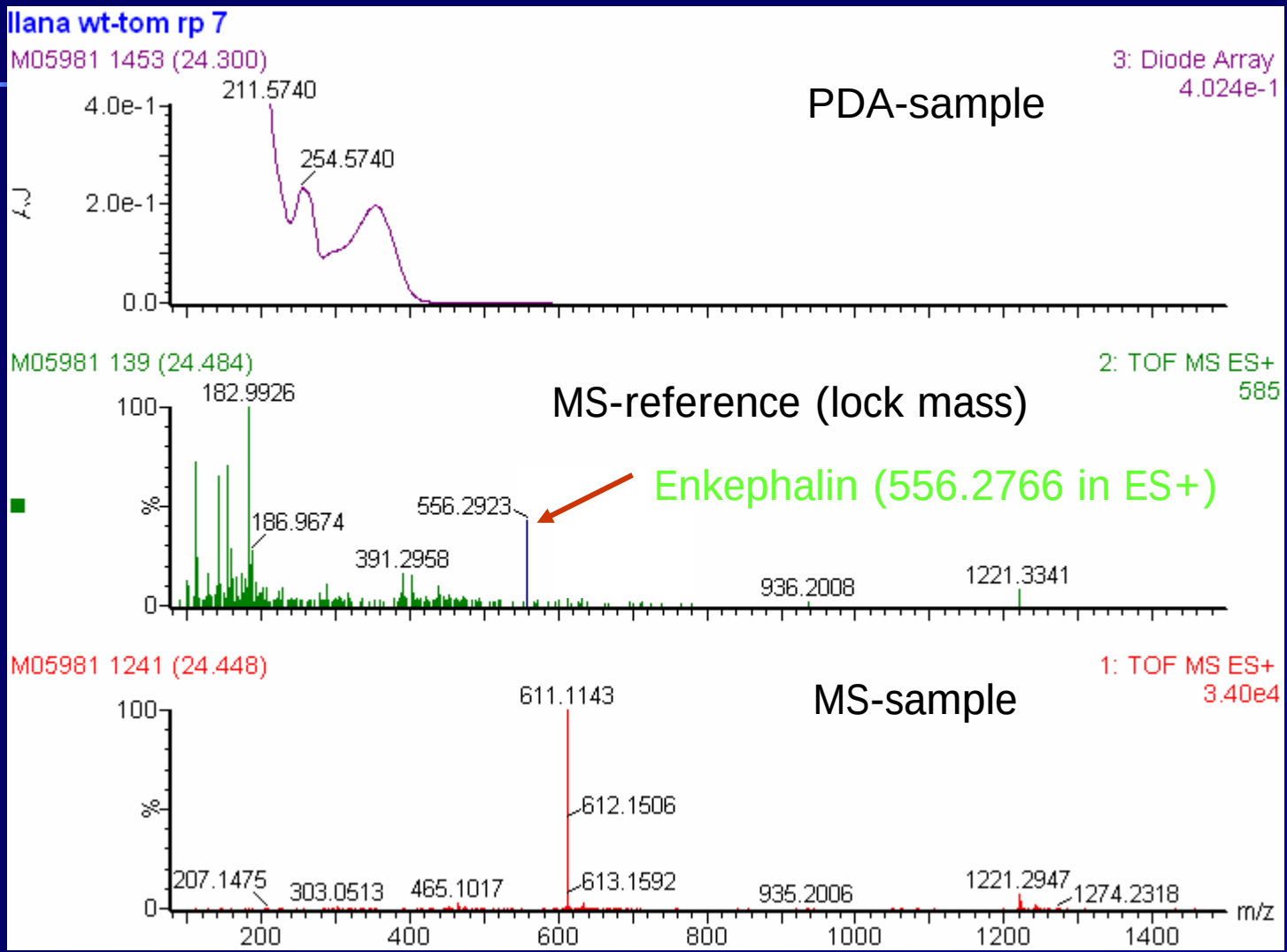
Mass Analyzers

	Mass Accuracy
• Ion Cyclotron (FT-ICR-MS)	<1ppm
• Time of Flight (TOF)	3-10ppm
• Magnetic Sector	2-5ppm
• Quadrupole Ion Trap	n/a
• Quadrupole	n/a

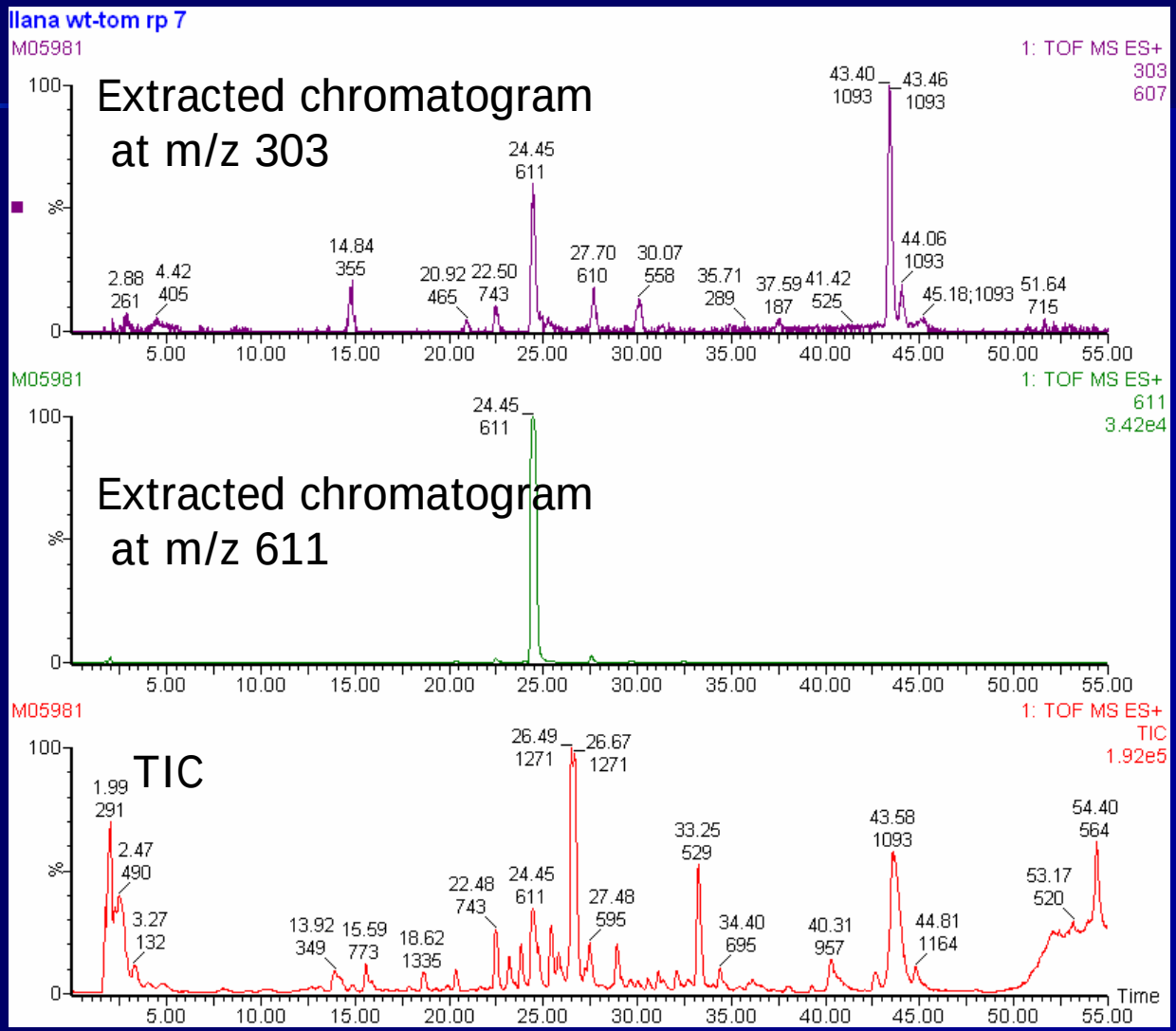
LC-PDA-QTOF-MS of a Tomato Sample



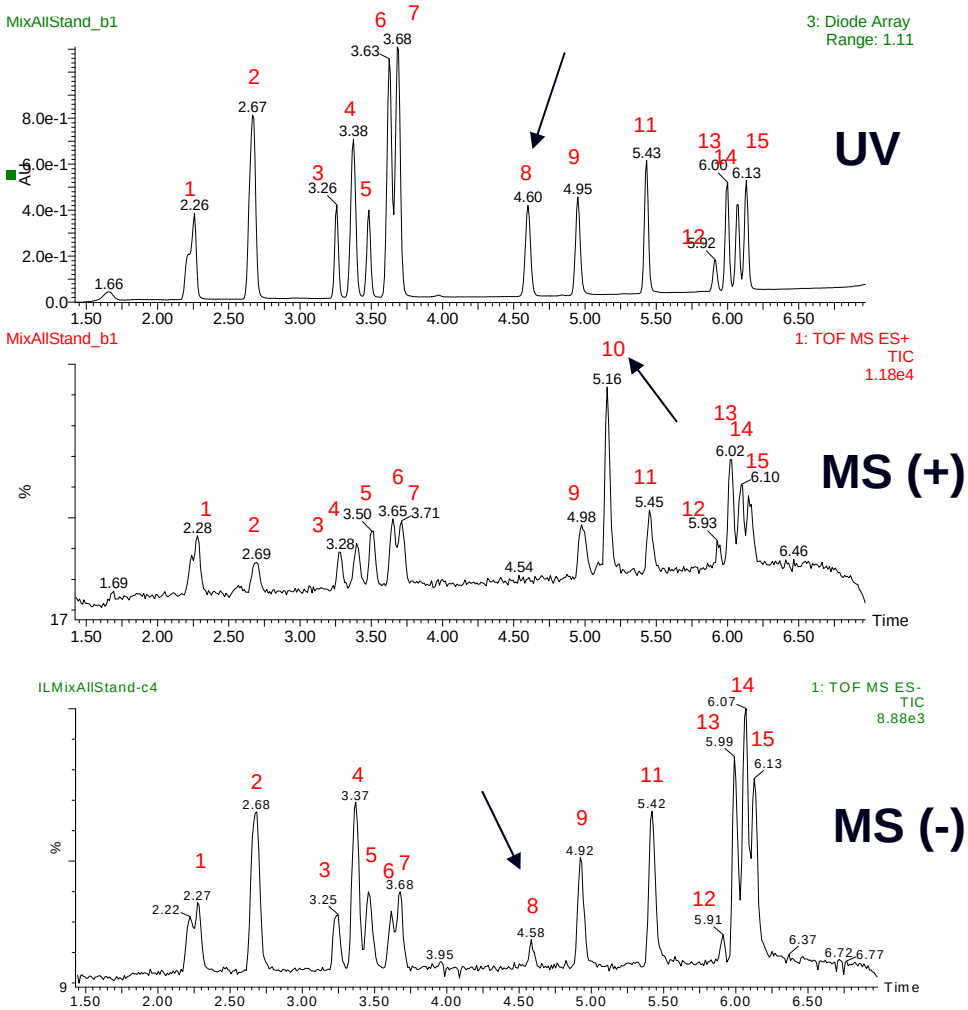
Spectra Obtained at Each Scan



Extracted chromatogram of a Specific Ion



LC/UV/MS Chromatograms of Standard's Mixture (UV at 240nm, ES+ and ES-)

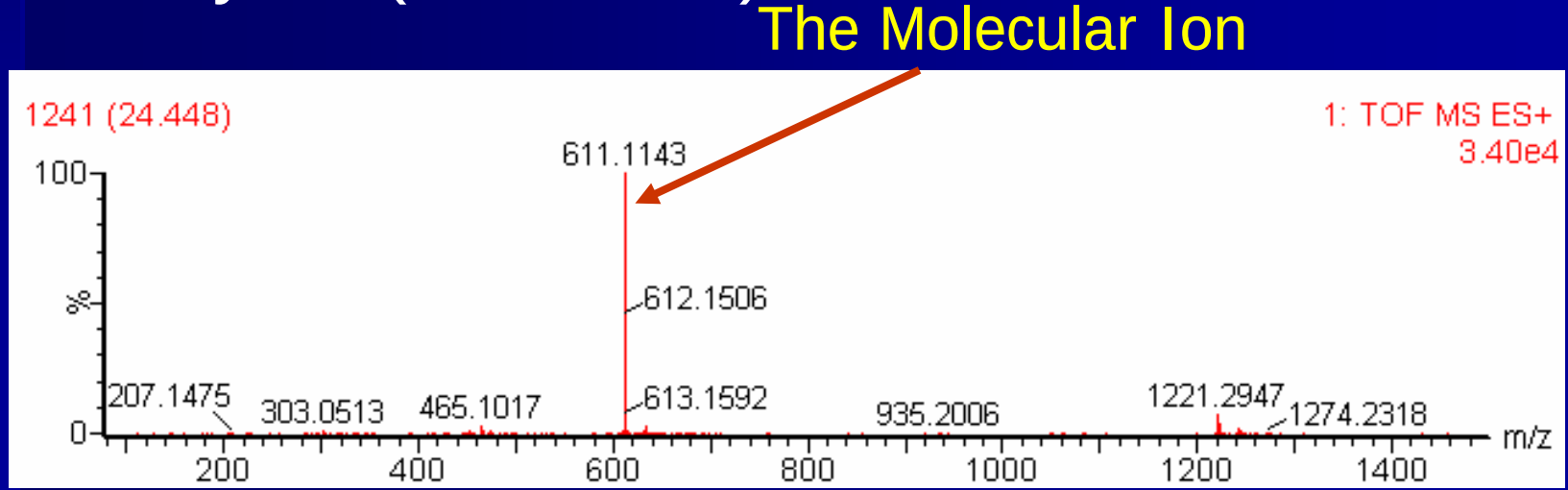


1 – Chlorogenic acid
2 – Caffeic acid
3 – Rutin
4 – p-Coumaric
5 – Quercetin-3-glucoside
6 – Ferulic acid
7 – Sinapic acid
8 – Benzoic acid
9 – Morin (Internal Standard)
10 – Tomatin
11 – Quercetin
12 – Cinnamic acid
13 – Naringenin Chalcone
14 – Naringenin
15 – Kaempferol

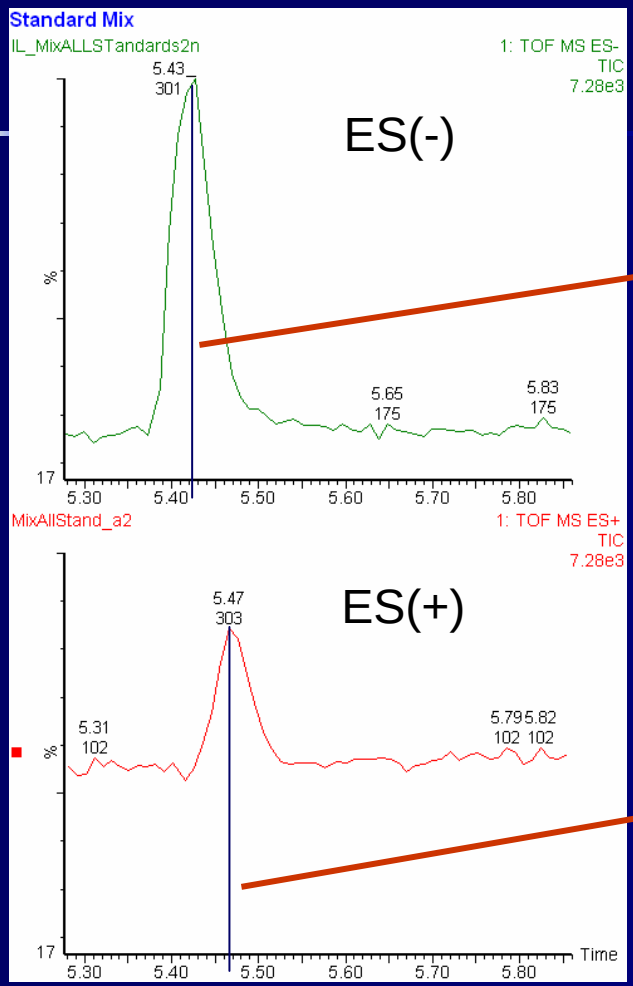
In the Spectra: Molecular Ion

- An **unfragmented** (parent) ion formed by loss or gain of an proton from a molecule

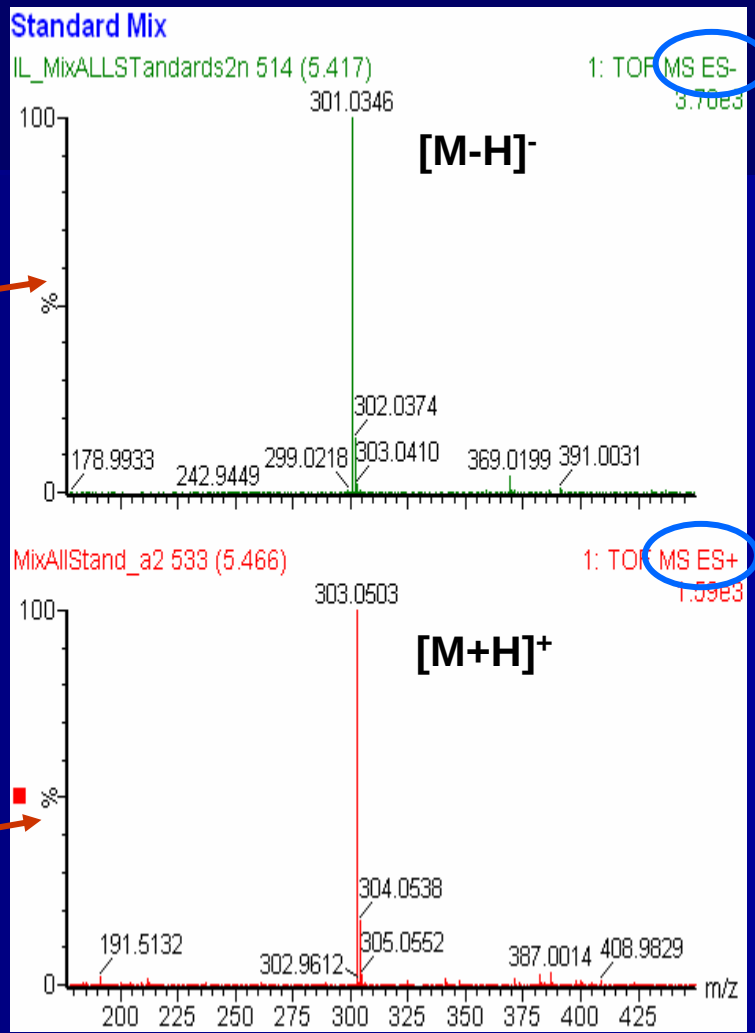
- Has the same mass as the metabolite being analyzed (+ H or - H)



Chromatogram and Spectrum of Morin

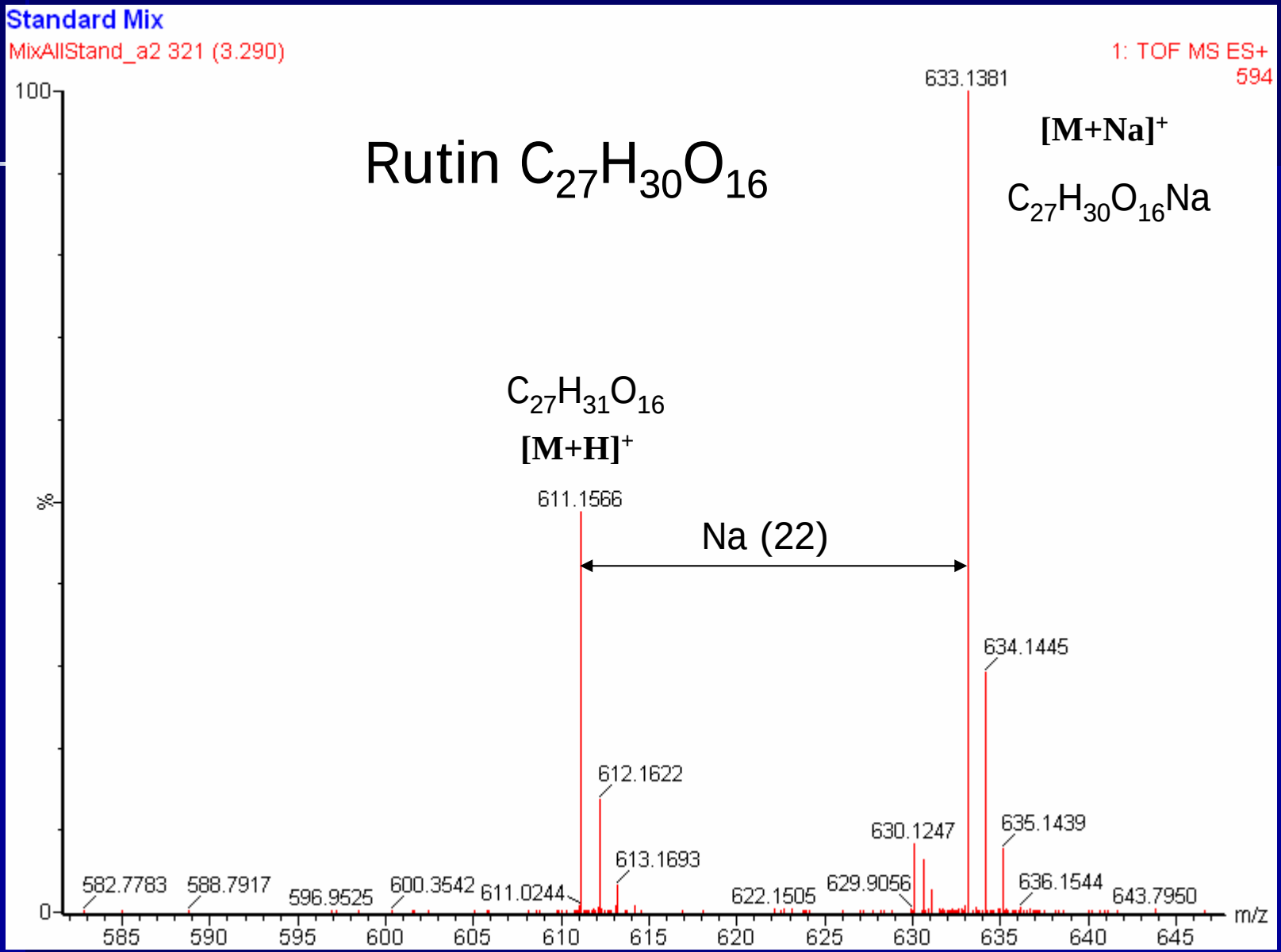


Chromatograms in ES(+) & ES(-) modes



Spectra's in ES(+) & ES(-) modes

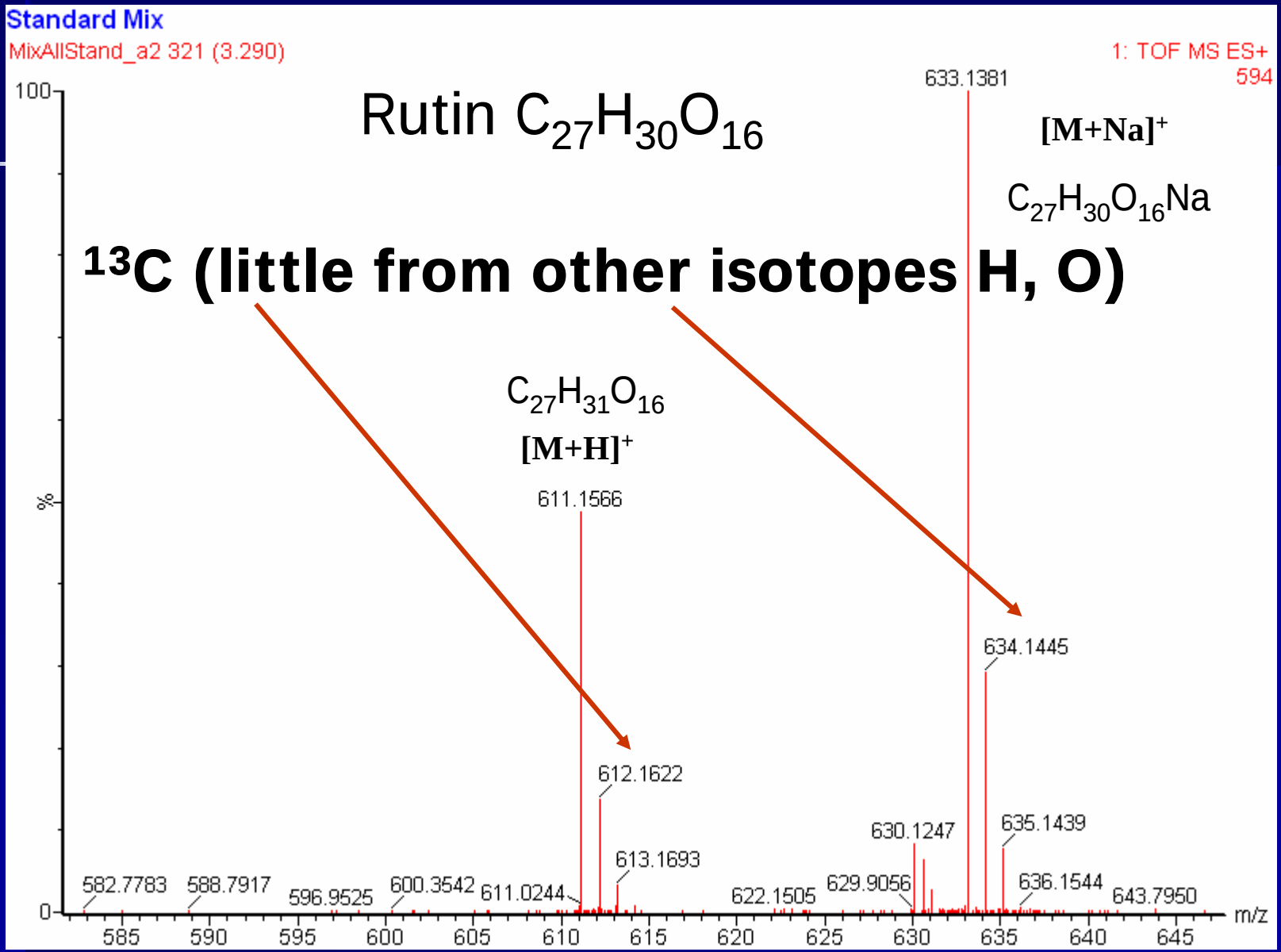
Adduct Formation in Spectra (Na)



Typical Adducts formed in LCMS

Adducts Ion	Source of Adduct	Mass of Adduct Ion
$[M+CH_3COO]^-$	Acetic acid	M+59
$[M+Cl]^-$	Chlorinated solvent	M+35
$[M+NH_4]^+$	Ammonia	M+18
$[M+Na]^+$	Sodium Salt	M+23
$[M+K]^+$	Potassium Salt	M+39
$[M+CH_3CNH]^+$	Acetonitrile	M+42
$[M+CH_3OHH]^+$	Methanol	M+33
$[M+H_2O]^+$	Water	M+19

Isotopes in Spectra (^{13}C)

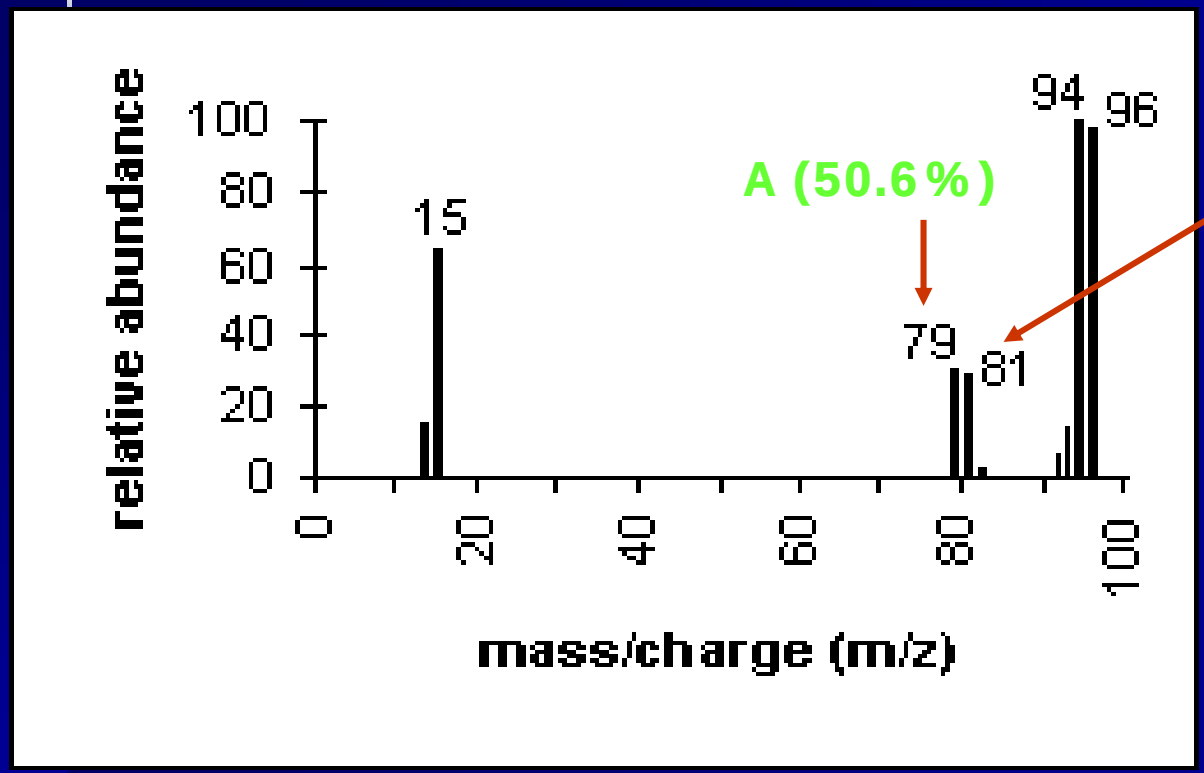


Abundance of Isotopes (in Spectra)

Atomic Masses and Abundances for a Subset of Naturally Occurring Biologically Relevant Isotopes

Iso	A	%	Iso	A+1	%	Iso	A+2	%	Iso	A+3	%	Iso	A+4	%
^{12}C	12	98.93(8)	^{13}C	13.0033548378 (10)	1.07(8)	^{14}C	14.003241988(4)	-	-	-	-	-	-	-
^{1}H	1.0078250321(4)	99.9885(70)	^{2}H	2.0141017780(4)	0.0115 (70)	^{3}H	3.0160492675(11)	-	-	-	-	-	-	-
^{14}N	14.0030740052(9)	99.632(7)	^{15}N	15.0001088984(9)	0.368(7)	-	-	-	-	-	-	-	-	-
^{16}O	15.9949146221(15)	99.757(16)	^{17}O	16.99913150(22)	0.038(1)	^{18}O	17.9991604(9)	0.205(14)	-	-	-	-	-	-
^{32}S	31.97207069(12)	94.93(31)	^{33}S	32.97145850(12)	0.76(2)	^{34}S	33.96786683(11)	4.29(28)	-	-	-	^{36}S	35.96708088(25)	0.02(1)
^{19}F	18.99840320(7)	100	-	-	-	-	-	-	-	-	-	-	-	-
^{23}Na	22.98976967(23)	100	-	-	-	-	-	-	-	-	-	-	-	-
^{39}K	38.9637069(3)	93.2581(44)	^{40}K	39.96399867(29)	0.0117(1)	^{41}K	40.96182597(28)	6.7302(44)	-	-	-	-	-	-
^{31}P	30.97376151(20)	100	-	-	-	-	-	-	-	-	-	-	-	-
^{35}Cl	34.96885271(4)	75.781(4)	-	-	-	^{37}Cl	36.96590260(5)	24.22(4)	-	-	-	-	-	-
^{55}Mn	54.9380496(14)	100	-	-	-	-	-	-	-	-	-	-	-	-
^{54}Fe	53.9396148(14)	5.845(35)	-	-	-	^{56}Fe	55.9349421(15)	91.754(36)	^{57}Fe	56.9353987(15)	2.119 (10)	^{58}Fe	57.9332805(15)	0.282(4)
^{63}Cu	62.9296011(15)	69.17(3)	-	-	-	^{65}Cu	64.9277937(19)	30.83(3)	-	-	-	-	-	-
^{79}Br	78.9183376(20)	50.69(7)	-	-	-	^{81}Br	80.916291(3)	49.31(7)	-	-	-	-	-	-
^{127}I	126.904468(4)	100	-	-	-	-	-	-	-	-	-	-	-	-

An example of how isotopes can aid in peak identification



$Br{-}CH_3$

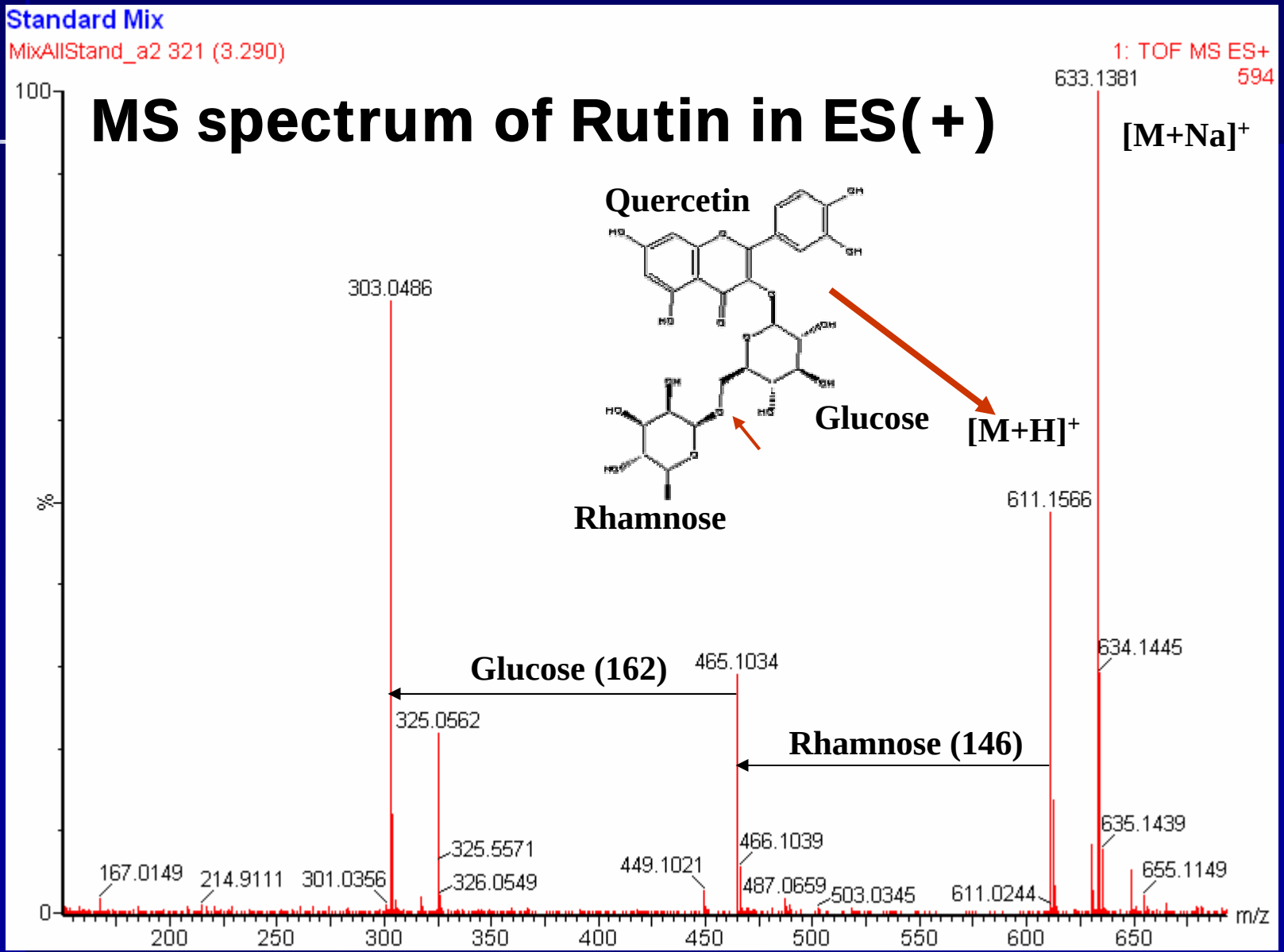
m/z = 15

(79)BrCH3 m/z = 94

(81)BrCH3 m/z = 96

The ratio of peaks containing ^{79}Br and its isotope ^{81}Br (100/98) confirms the presence of bromine in the compound.

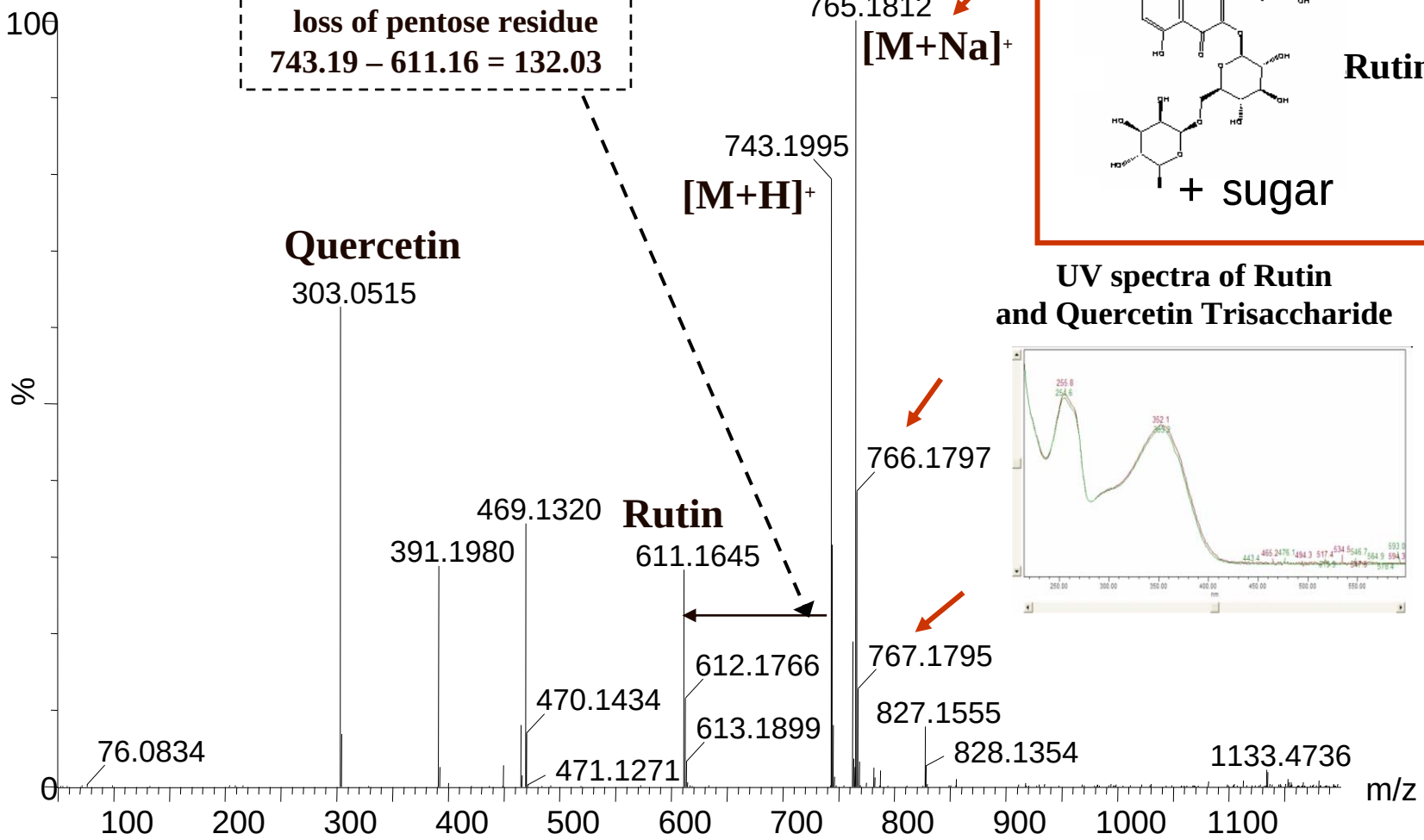
Fragments of a Metabolite in Spectra



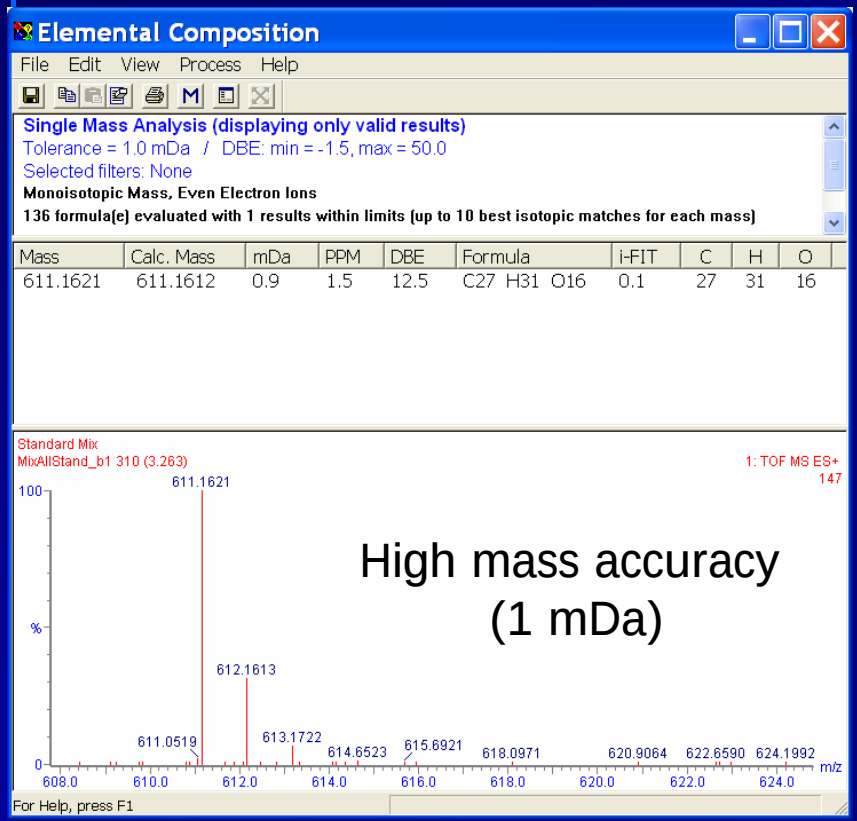
Spectra Components

- Molecular Ion
- Fragments of Metabolite
- Adducts (from solvent or di-tri-mers)
- Isotopes

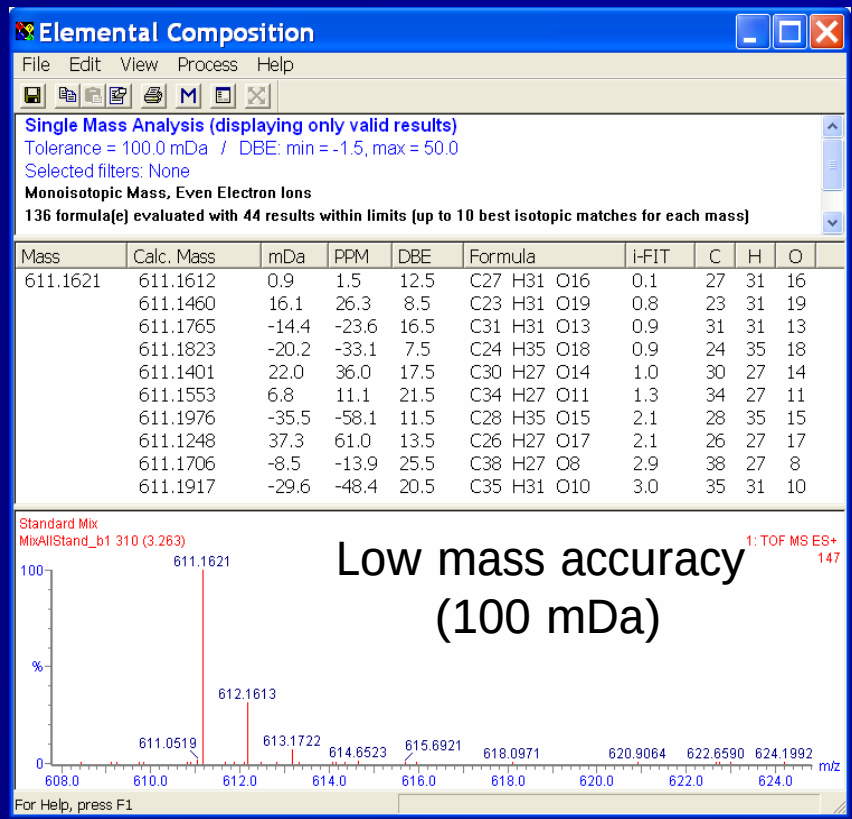
Spectrum of Quercetin Trisaccharide (a Rutin Derivative)



Mass Accuracy and Structure Elucidation



High mass accuracy
(1 mDa)



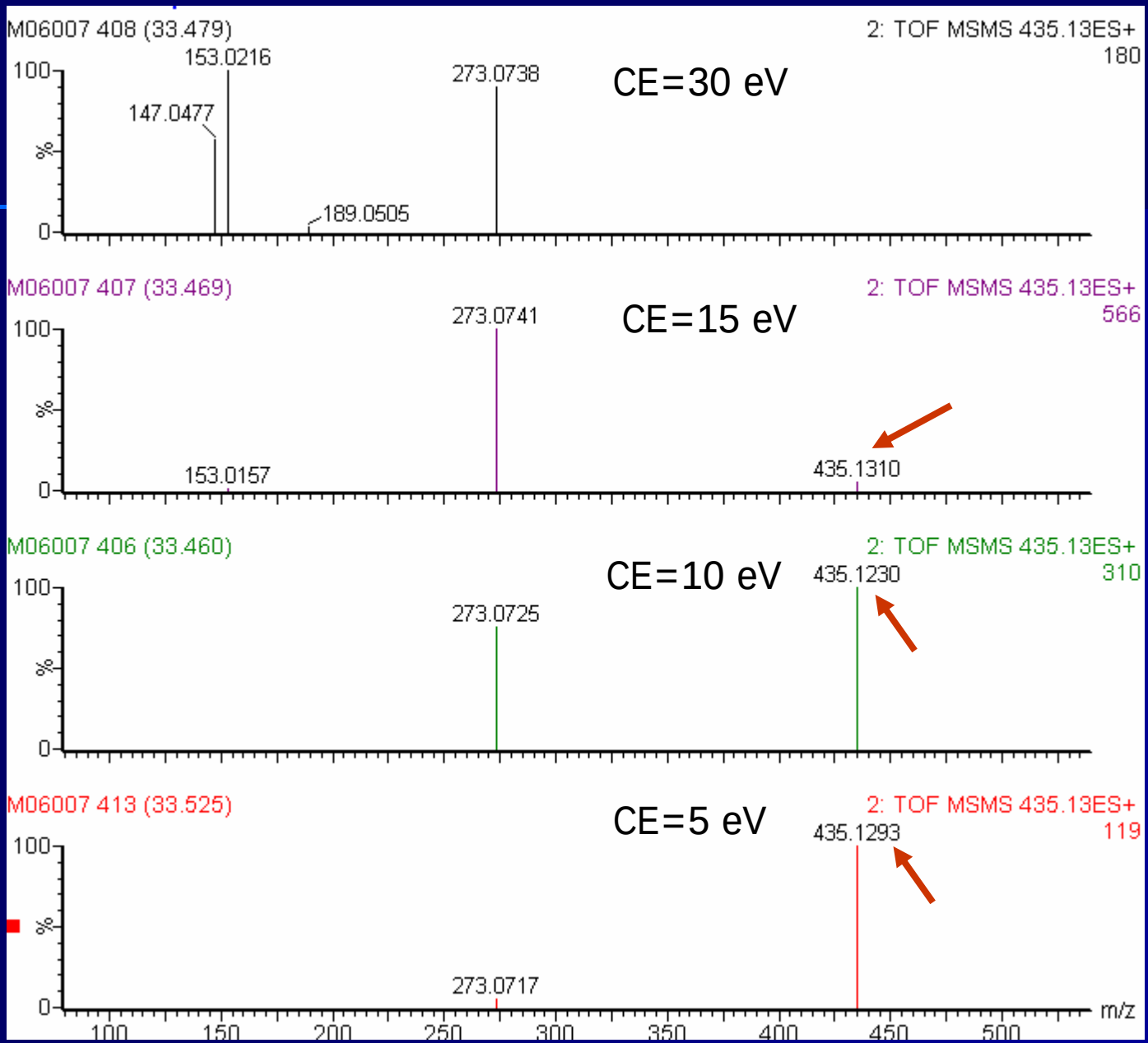
Low mass accuracy
(100 mDa)

Mass Accuracy and Structure Elucidation

C	18	H	19	NO	4	Sinoacutin	(-)-form,	N-De-Me
C	18	H	19	NO	4	Salutaridin	(+)-form,	N-De-Me
C	18	H	19	NO	4	Tembamid	(±)-form,	Ac
C	18	H	19	NO	4	1,2,3,9-Tet	(R)-form,	2,3-Di-Me ether
C	18	H	19	NO	4	Corytuberir	(S)-form,	N-De-Me
C	18	H	19	NO	4	Cularidine;	(S)-form,	N-De-Me
C	18	H	19	NO	4	Isocorytube	(S)-form,	N-De-Me
C	18	H	19	NO	4	Liriotulipife	(S)-form,	N-De-Me
C	18	H	19	NO	4	1,2,3,9-Tet	(S)-form,	1,2-Di-Me ether
C	18	H	19	NO	4	Tyrosine;	(S)-form,	N-Benzoyl, Et ester
C	18	H	19	NO	4	Erythrinine	11-Epimer	
C	18	H	19	NO	4	Erysodine;	11-Oxo	
C	18	H	19	NO	4	Erysovine;	11-Oxo	
C	18	H	19	NO	4	Erythratine	2-Ketone	
C	18	H	19	NO	4	14-Hydroxy	6-Ketone	
C	18	H	19	NO	4	4,6,7-Trihy	7-(3-Methy	4,6-di-Me ether
C	18	H	19	NO	4	Caranine;	Ac	
C	18	H	19	NO	4	4-(2-Aminc	N-(4-Hydroxy-3-methoxycinnamoyl)	
C	18	H	19	NO	4	Pallidine;	N-De-Me	
C	18	H	19	NO	4	Pachyconfi	N-Oxide	
C	18	H	19	NO	4	Haplopine;	O-(3-Methyl-2-butenyl)	
C	18	H	19	NO	4	Isohaplopir	O-(3-Methyl-2-butenyl)	
C	18	H	19	NO	4	Artavenustine		
C	18	H	19	NO	4	Celtisine		
C	18	H	19	NO	4	Cephalotaxinone		
C	18	H	19	NO	4	Claviculine		
C	18	H	19	NO	4	Culacorine		
C	18	H	19	NO	4	Erysodienone		
C	18	H	19	NO	4	Erythrinine		
C	18	H	19	NO	4	N-Feruloyltyramine		
C	18	H	19	NO	4	Flavinine		
C	18	H	19	NO	4	Glaufine		
C	18	H	19	NO	4	Hernovine		
C	18	H	19	NO	4	Isocephalotaxinone		
C	18	H	19	NO	4	Krepowine		
C	18	H	19	NO	4	Laurelliptine		

Selected natural compounds with formula $C_{18}H_{19}NO$ (monoisotopic mass 313.1314 Da)

Performing MS-MS: MS-MS of m/z 435 at different collision energies

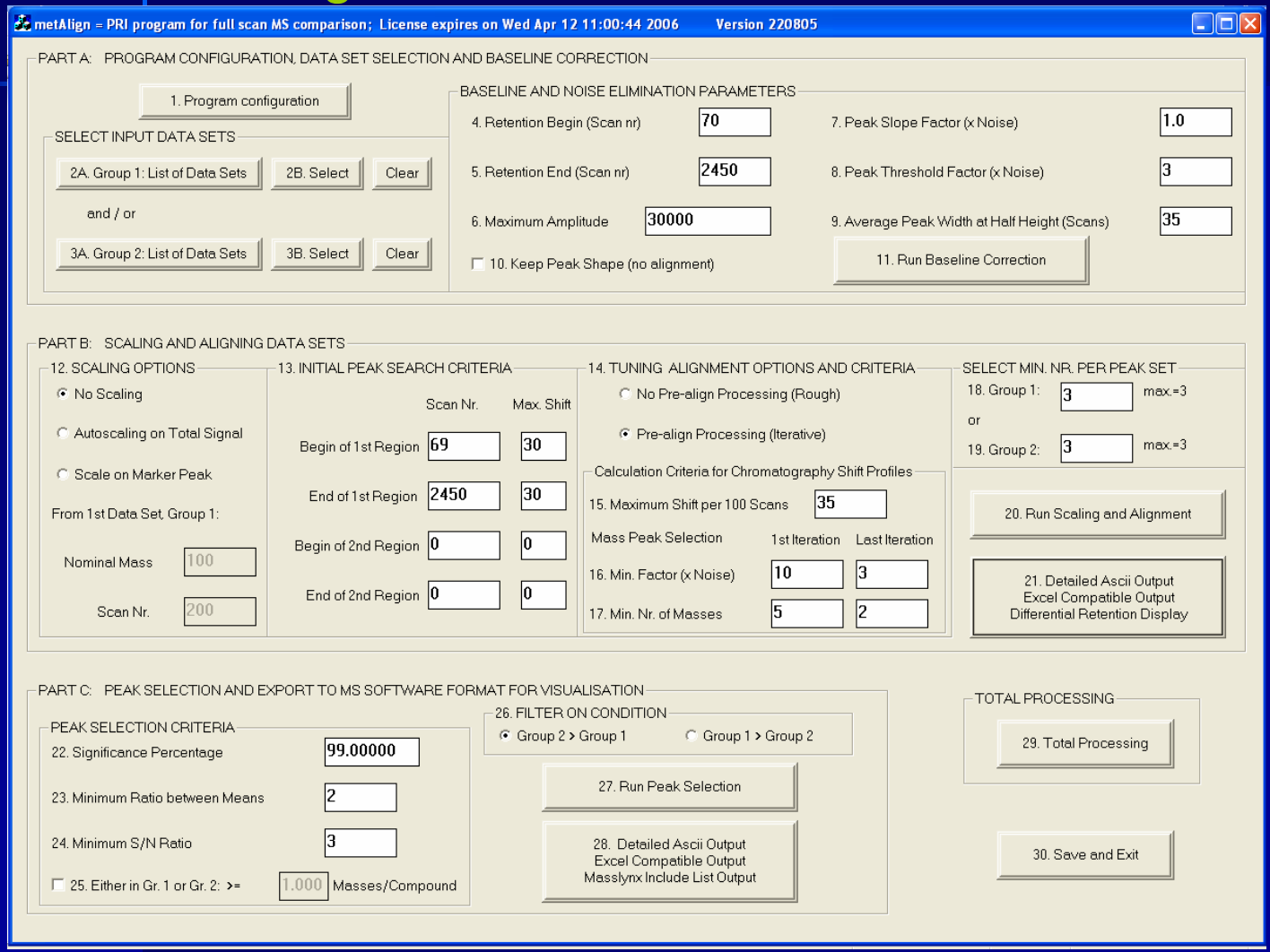


Output Data For High Res. LC-MS Based Metabolomics

scan	rt (umin)	Nominal mass	area	Accurate mass	Accumass database	calc mass	ppm
112	2166433	147	1277	147.0767	glutamine	147.0764	2.2
115	2218350	138	652	138.0544	TRIGONELLINE	138.0550	-4.2
121	2339467	133	117	133.0627	ASPARAGINE	133.0608	14.7
123	2374533	284	100	284.0990	guanosine	284.0989	0.2
124	2391750	182	113	182.0839	TYROSINE	182.0812	14.9
124	2391750	132	64	132.1047	LEUCINE	132.1019	21.5
125	2409000	148	2730	148.0590	GLUTAMIC-ACID	148.0604	-9.7
125	2409000	150	85	150.0631	METHIONINE	150.0583	31.8
128	2477900	343	75	343.1250	SUCROSE	343.1235	4.3
128	2477900	134	434	134.0460	ASPARTIC-ACID	134.0448	8.9
133	2564183	304	22	304.1520	NICOTIANAMINE	304.1503	5.5
170	3273917	193	2774	193.0352	CITRIC-ACID	193.0343	5.0
170	3273917	245	575	245.0794	uridine	245.0768	10.7
171	3291367	308	595	308.0946	glutathione	308.0916	9.8
172	3326083	268	1583	268.1056	ADENOSINE	268.1040	5.8
172	3326083	182	272	182.0827	TYROSINE	182.0812	8.4
174	3360967	132	1111	132.1022	LEUCINE	132.1019	2.0
243	4672433	166	145	166.0883	PHENYLALANINE	166.0863	12.1
244	4706900	118	155	118.0663	PHENYLACETONITRILE	118.0651	9.7
404	7771017	146	417	146.0615	1H-Indole-3-carboxaldehyde, 9CI	146.0600	10.0
634	12210317	265	119	265.1470	ABSCISIC-ACID	265.1434	13.4
707	13614350	147	97	147.0448	coumarin	147.0441	4.8
1107	21309401	465	1031	465.1039	quercetin-glucoside (quercitrin)	465.1028	2.4
1107	21309401	743	502	743.2054	q-trisaccharide	743.2029	3.3
1107	21309401	303	1151	303.0510	Quercetin	303.0499	3.4
1107	21309401	611	568	611.1642	Quercetin 3-O-rutinoside (rutin)	611.1607	5.8
1212	23333666	303	3323	303.0482	Quercetin	303.0499	-5.9
1212	23333666	611	806	611.1646	Quercetin 3-O-rutinoside (rutin)	611.1607	6.5
1224	23557133	449	199	449.1092	QUERCETIN-3-O-RHAMNOSIDE	449.1078	3.1
1225	23591383	595	111	595.1677	Kaempferol-3-O-rutinoside	595.1657	3.3

Software for comparing full-scan datasets

MetAlign method



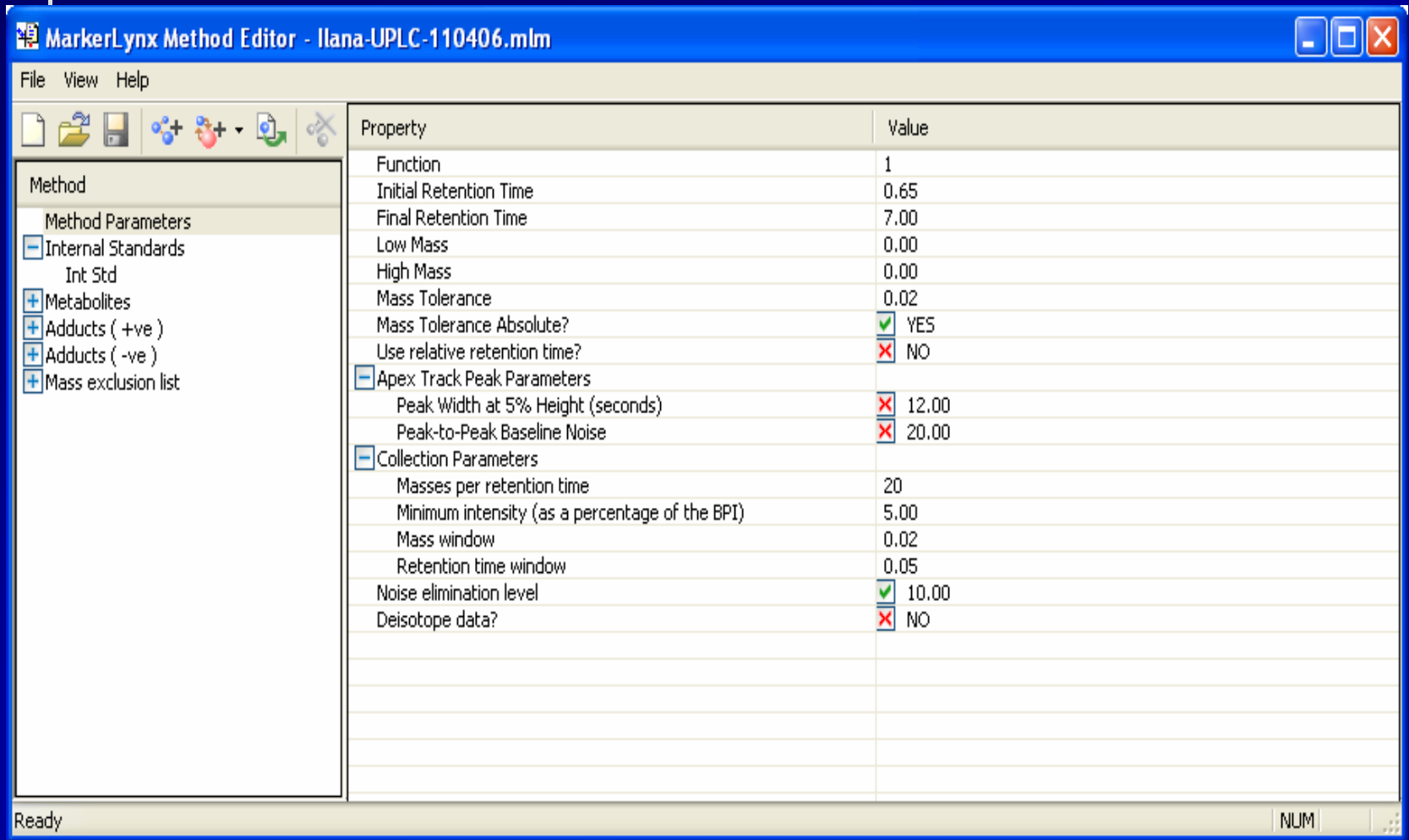
Peak picking

Alignment

Statistics

Software for comparing full-scan datasets

MarkerLynx method



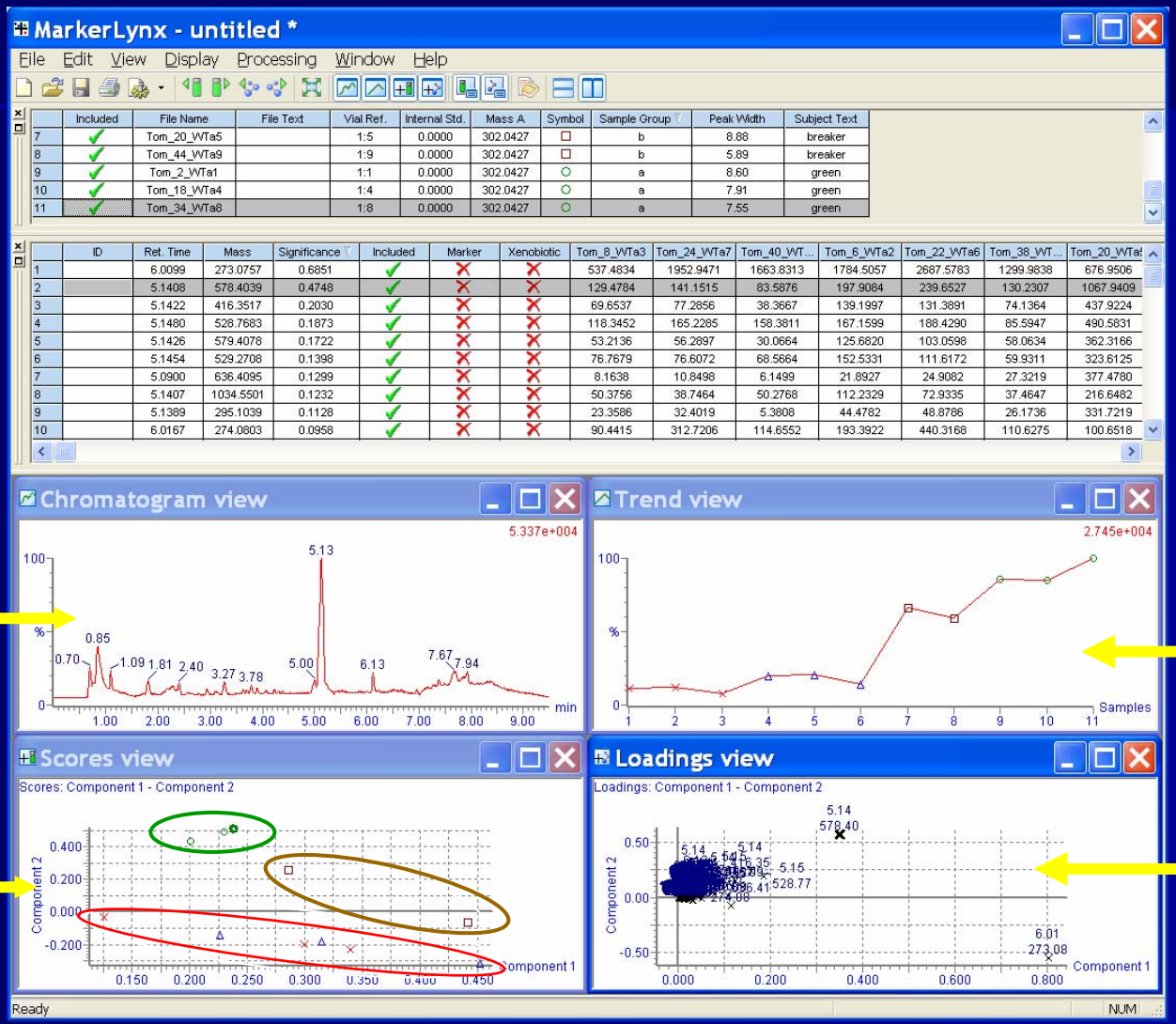
Out-put from the MarkerLynx (Waters) Program : Analyses of Wild-Type Tomato Peel (Different Stages of Development; ES+)

Samples Processed

Markers Detected

TIC of currently selected sample

Principle Components Analysis



Response of a marker across all samples

Markers responsible for clustering (by PCA)

A Case Study in Tomato

Small Green | Middle Green | Big Green | Breaker | Orange | Red

Sample Preparation for Tomato Fruit Metabolome Analysis

Harvesting tomato fruit at different developmental stages

Separation of peel and flesh tissues

Immediate freezing in liquid nitrogen

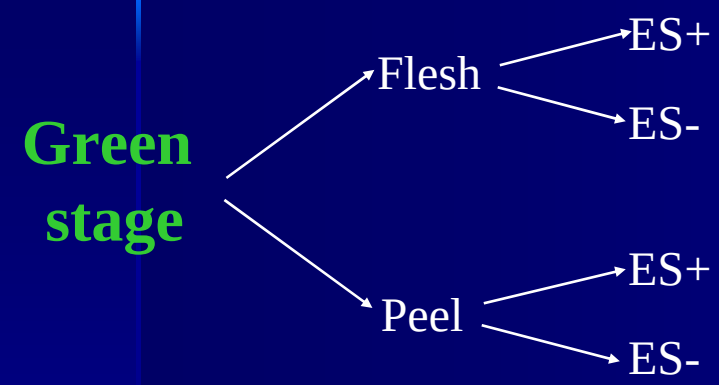
Grinding to fine powder

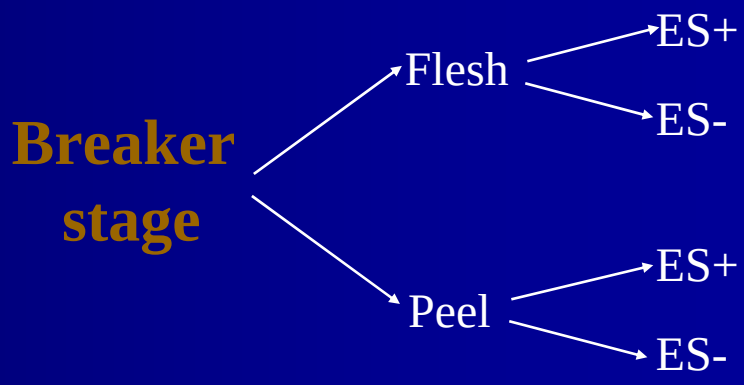
Weighing frozen samples

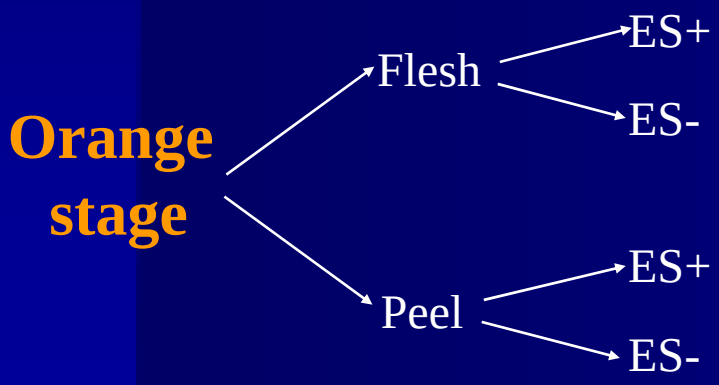
Extraction in 100% MeOH by vortexing and sonication

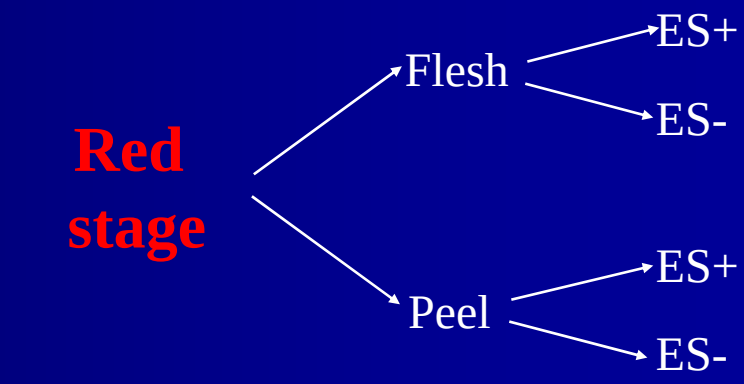
Analyzing with UPLC-MS [ES (+) and ES (-)]

Experimental Set-Up for LC-MS Analysis: PEEL and FLESH during tomato fruit development

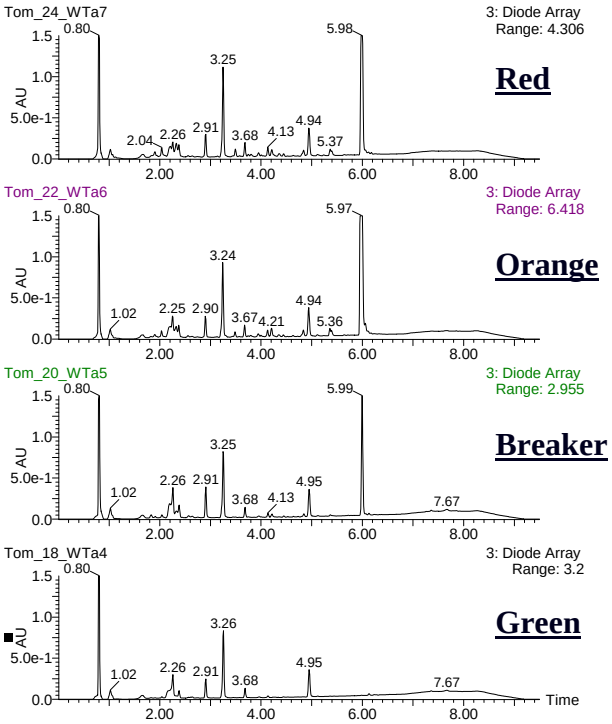




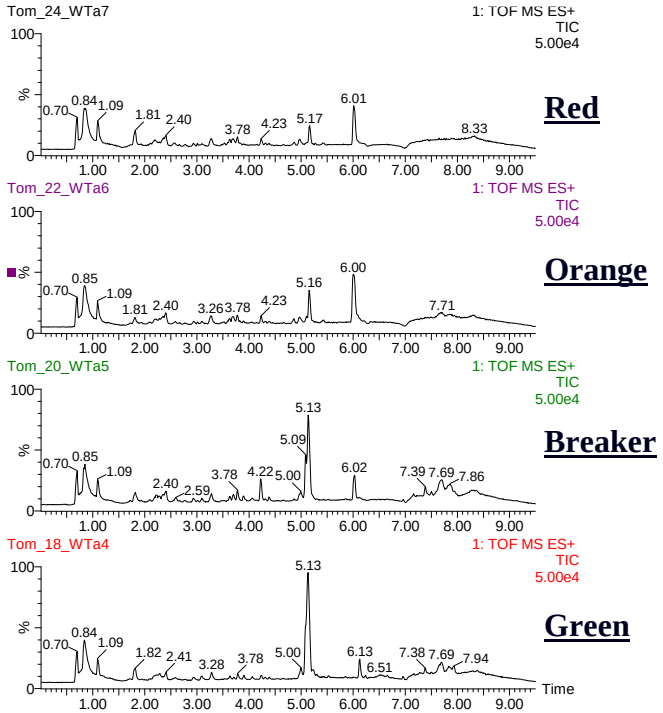




UPLC-Q-Tof-MS Analysis of Four Stages of Tomato Fruit Development

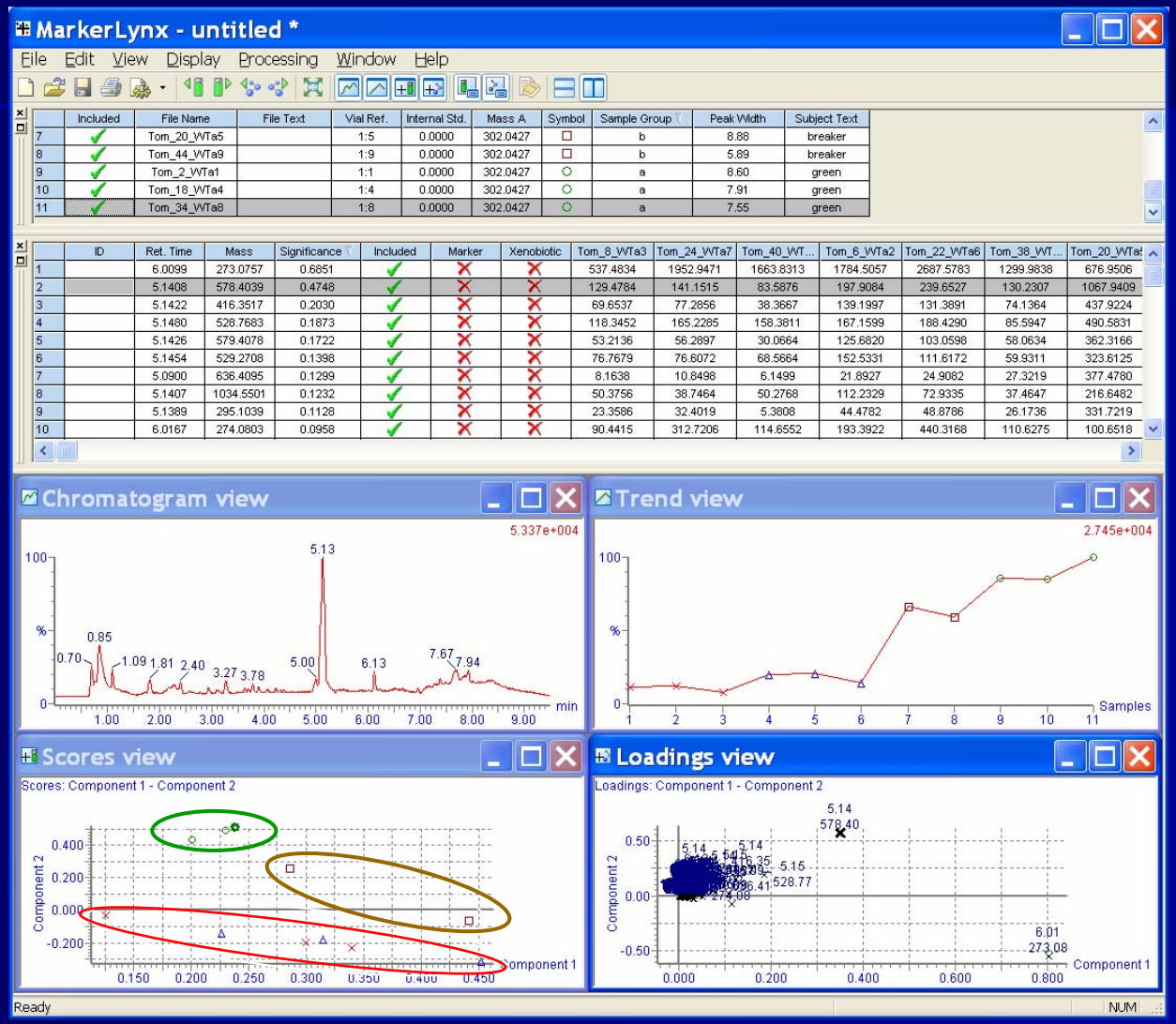


UV (240 nm)

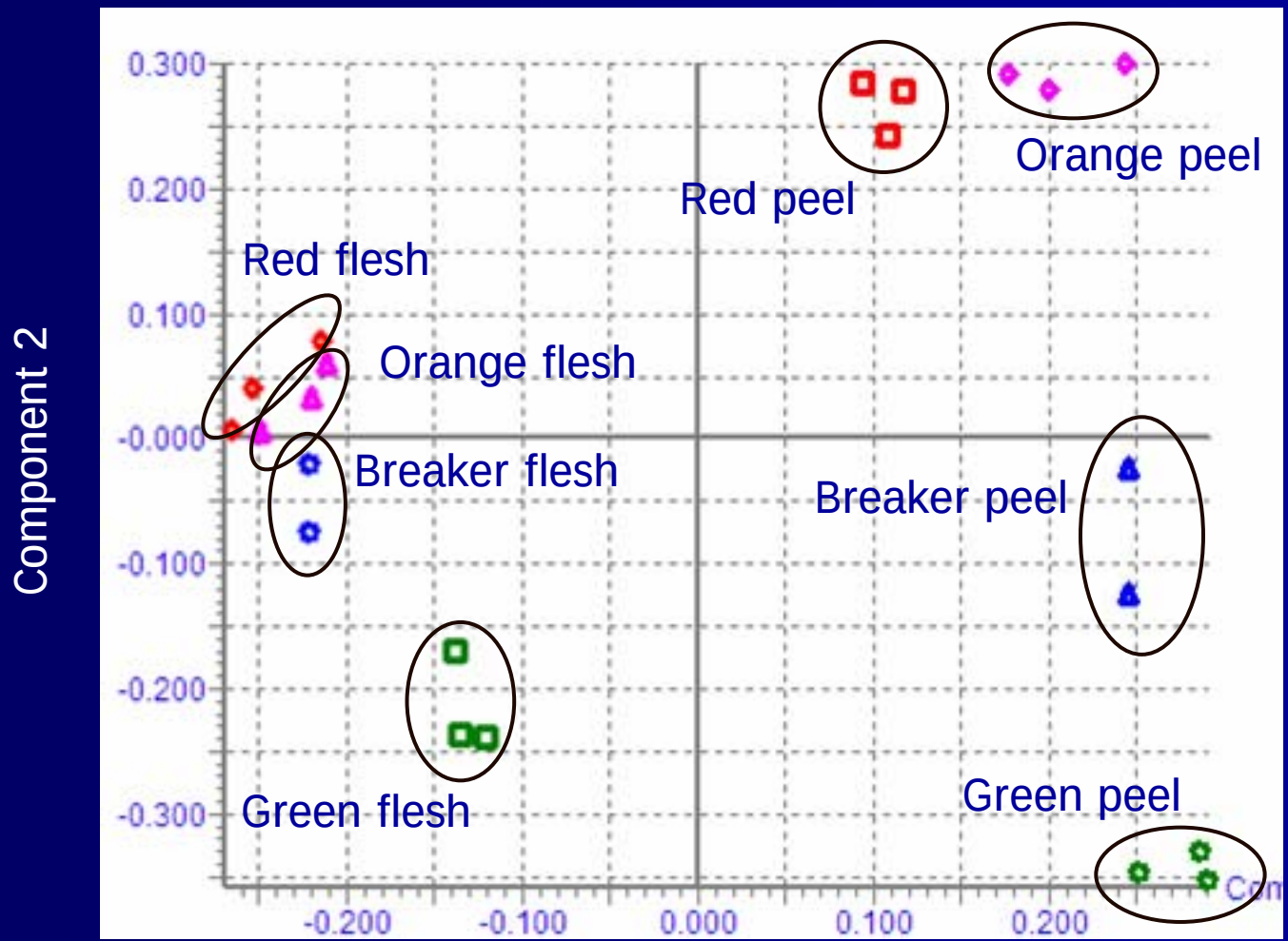


MS (ES(+),TIC)

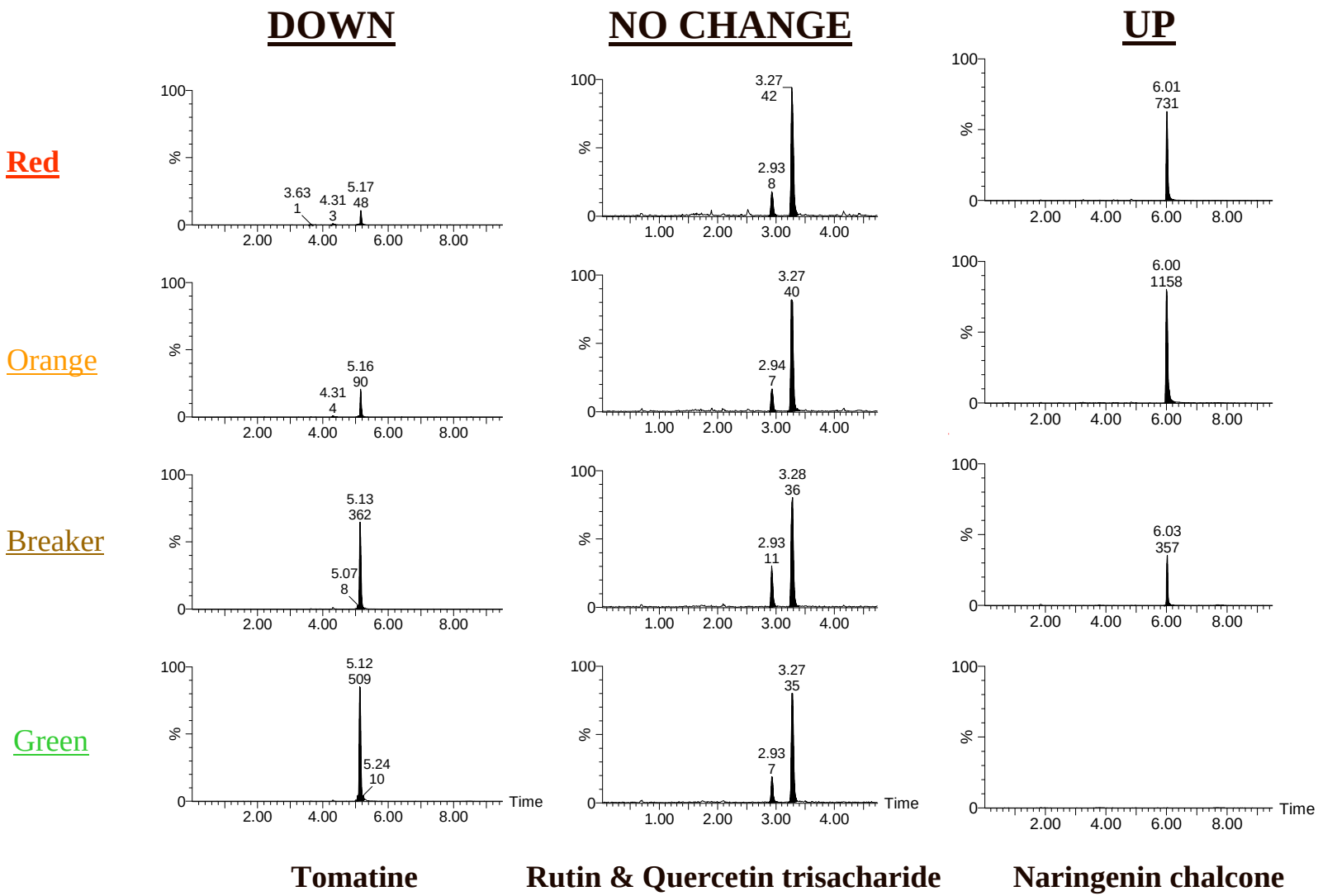
Out-put from the MarkerLynx (Waters) Program : Analyses of Wild-Type Tomato Peel (Different Stages of Development; ES+)



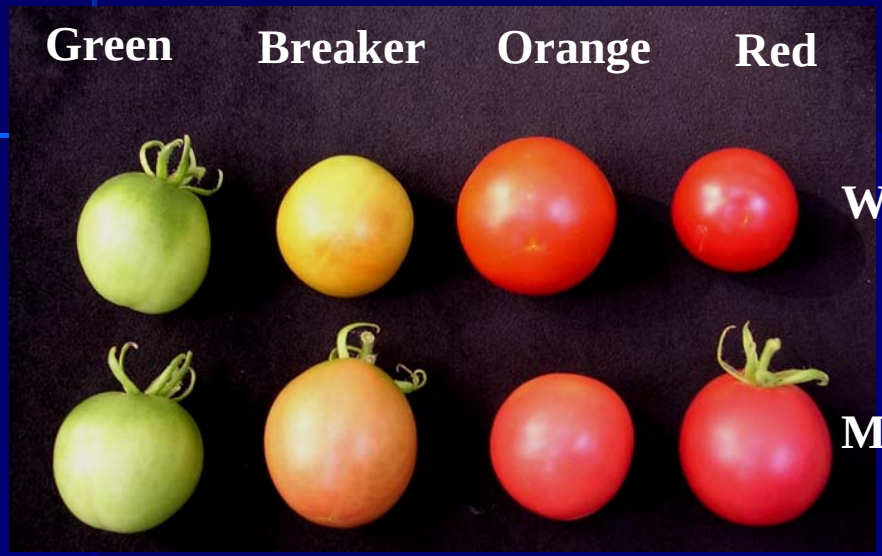
Peel-Flesh Profiles During Fruit Development



Wild-type Tomato Peel, Different Developmental Stages

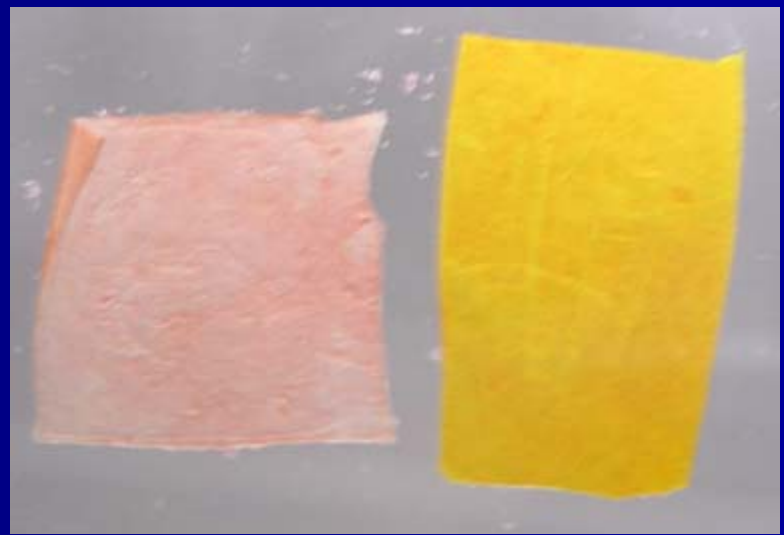


Tomato (var. Ailsa Craig) and the Mutant “Y”, at different ripening stages

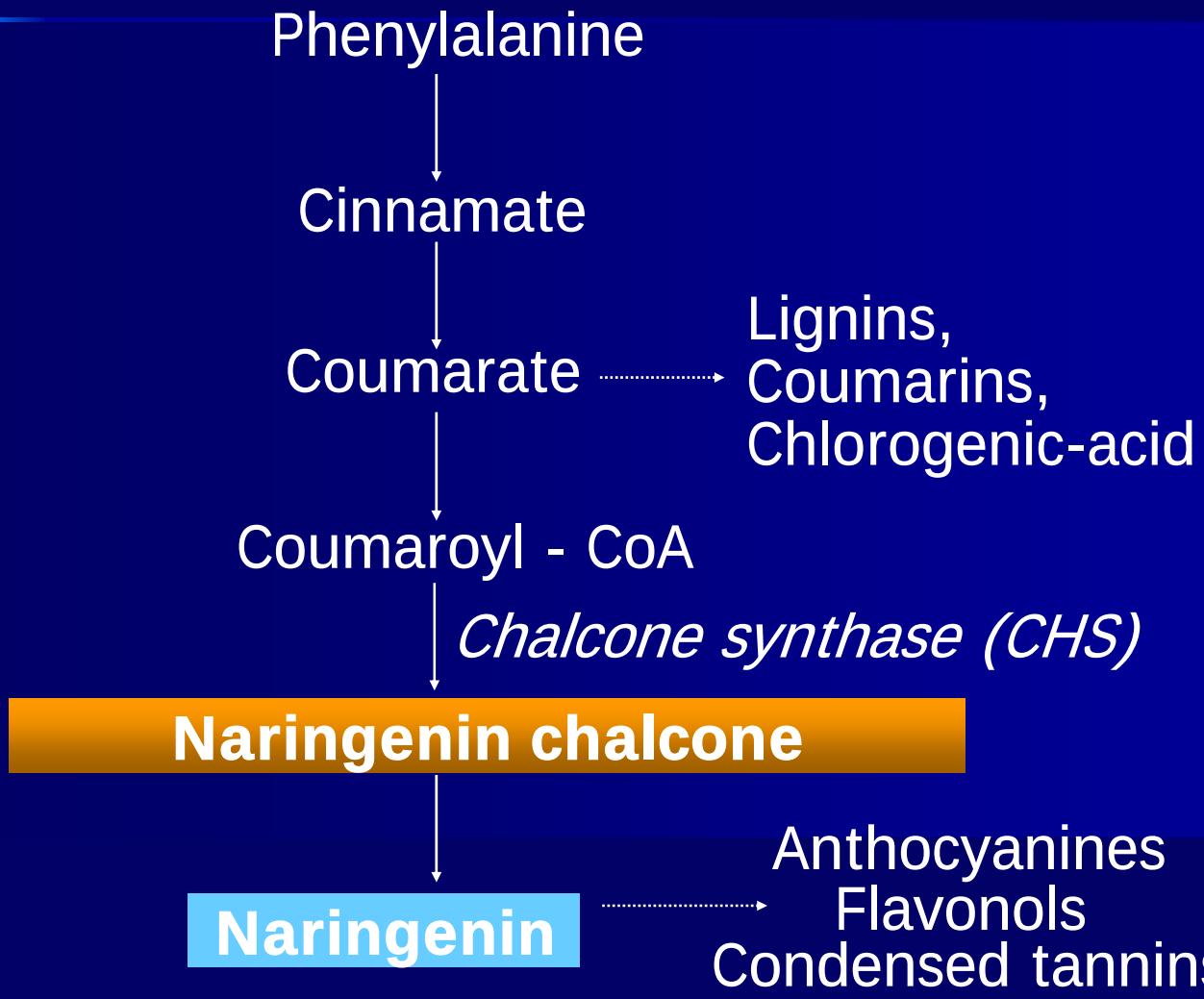


Tomato peel

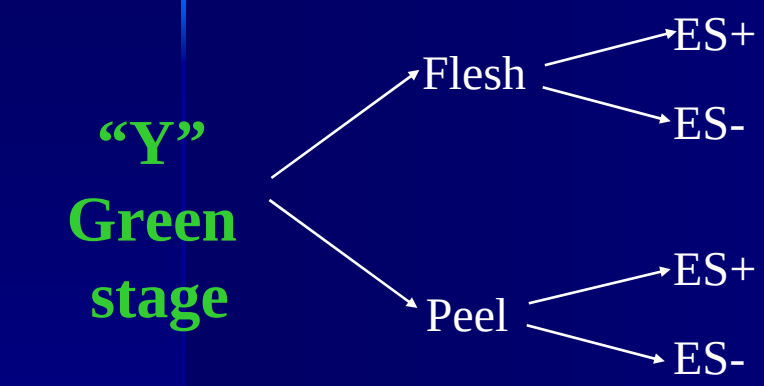
of Mutant “Y” and Wild-type

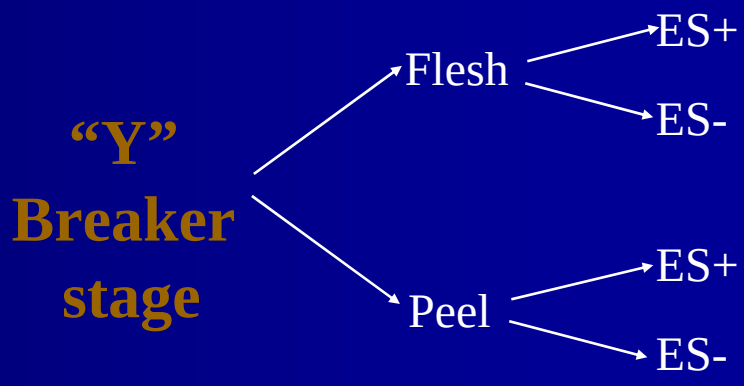


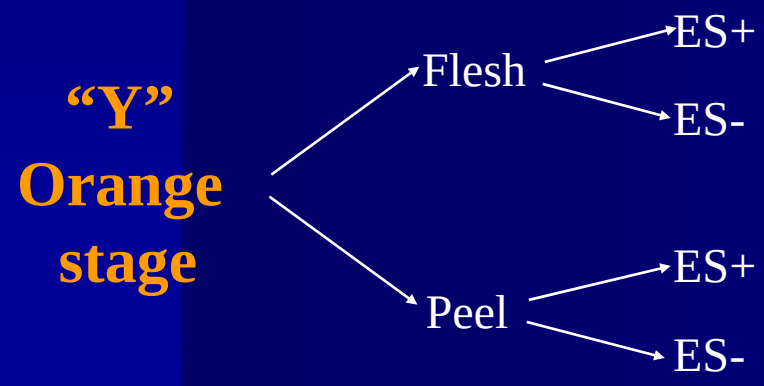
Biosynthesis of Naringenin Chalcone in the Flavonoid Pathway

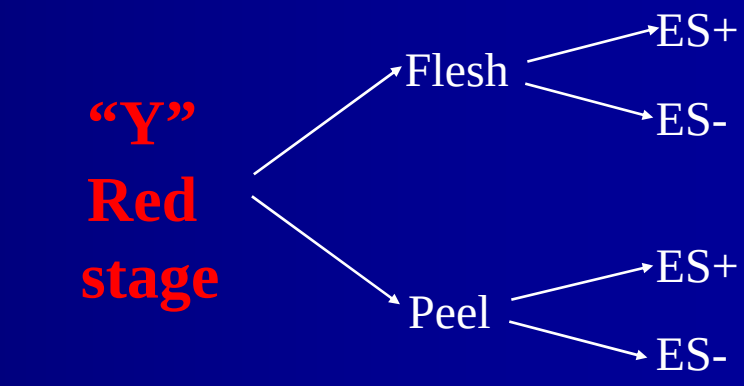


Experimental Set-Up for LC-MS Analysis: PEEL and FLESH during "Y" fruit development

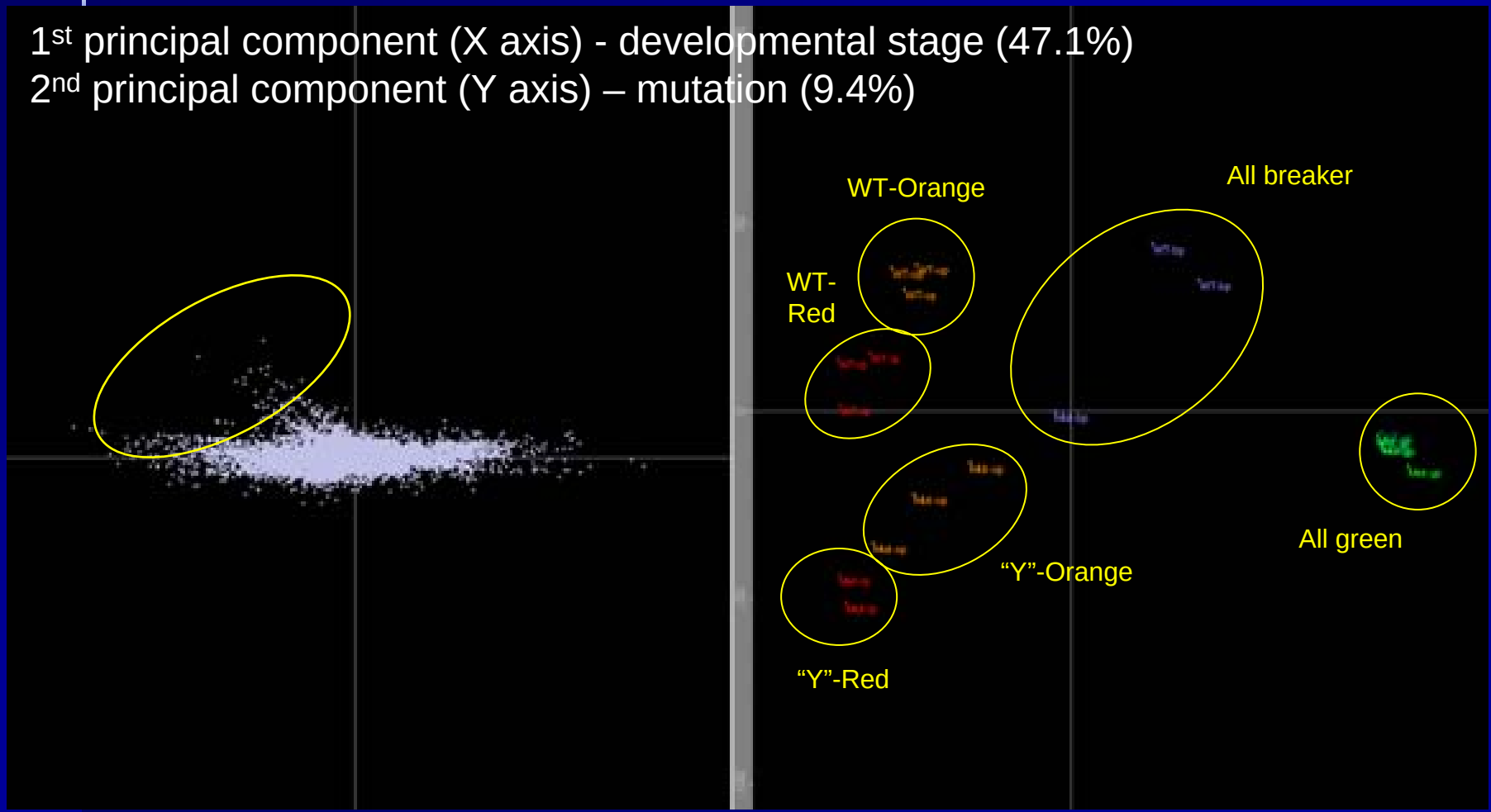




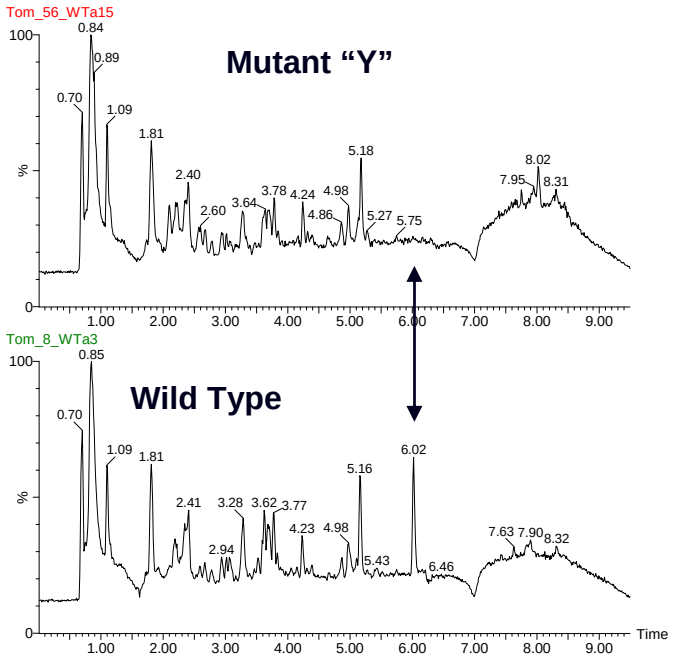




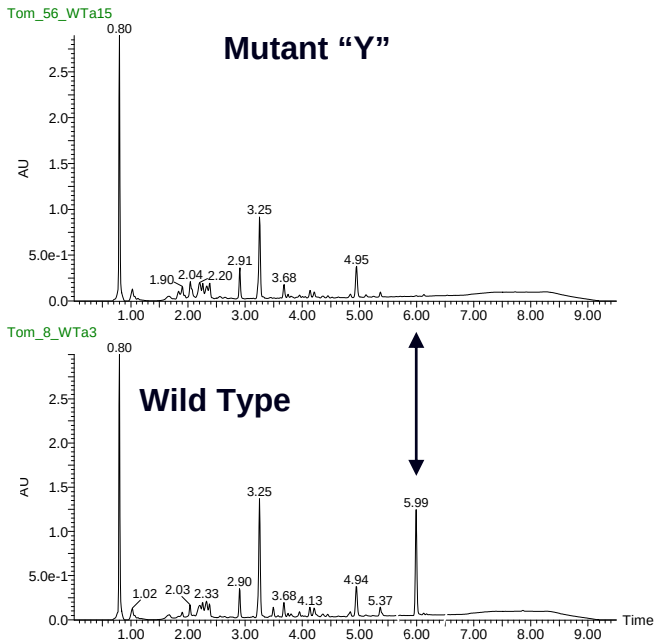
HPLC-QTOF-MS (Ultima-PRI) - Wild Type and “Y” Peels



UV and MS Chromatograms of Red Tomato Peel (Wild Type and Mutant “Y”)

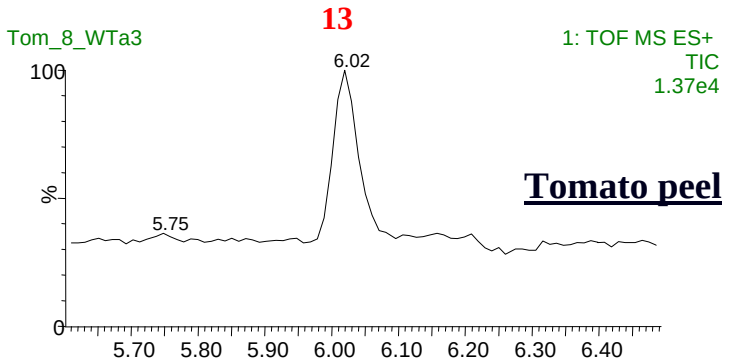


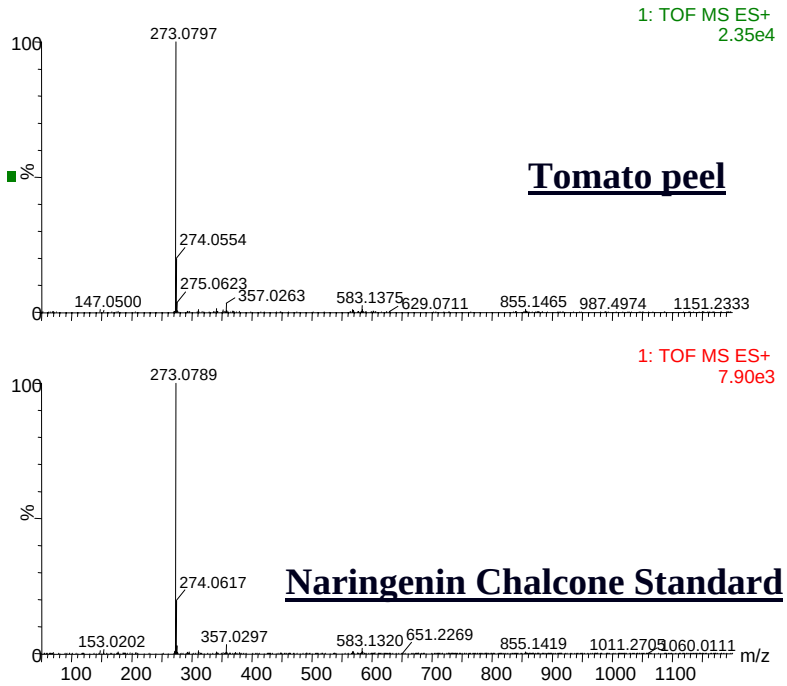
UV (240 nm)



MS (ES(+),TIC)

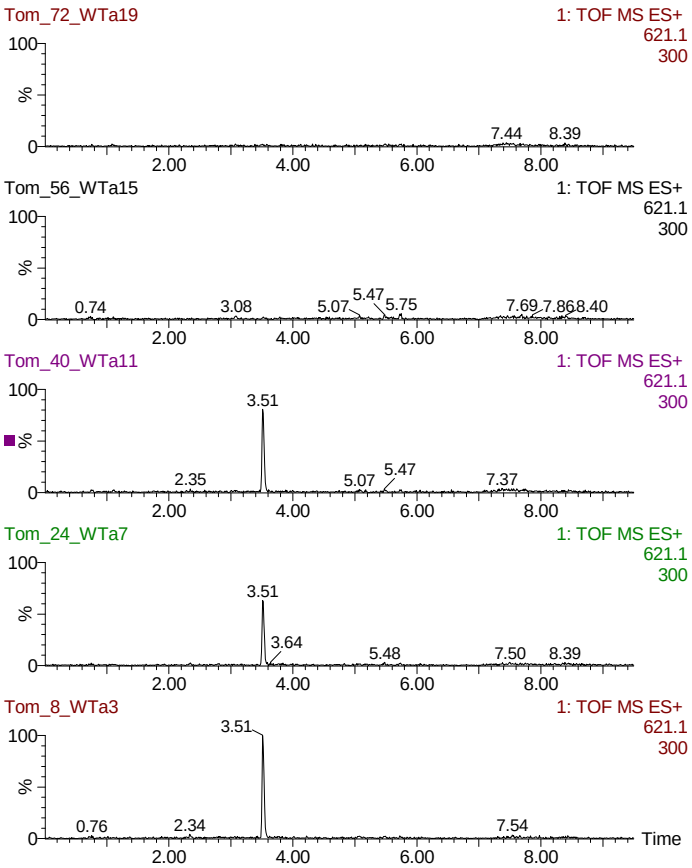
The Yellow Pigment Absent in the “Y” Mutant Fruit Peel is Naringenin Chalcone





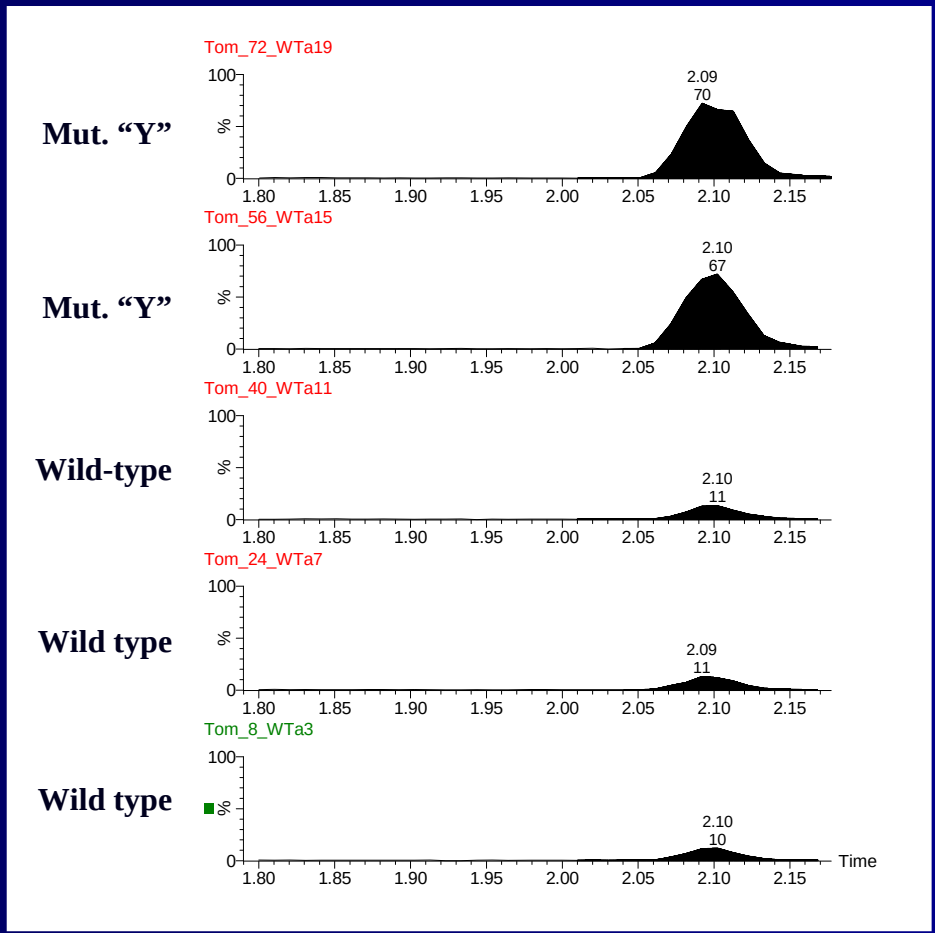
12 – Cinnamic acid
13 – Naringenin Chalcone
14 – Naringenin
15 – Kaempferol

Unknown Mass peak (RT=3.51 min) Detected Only in Wild Type



Extracted mass chromatogram at m/z 621.1 Da

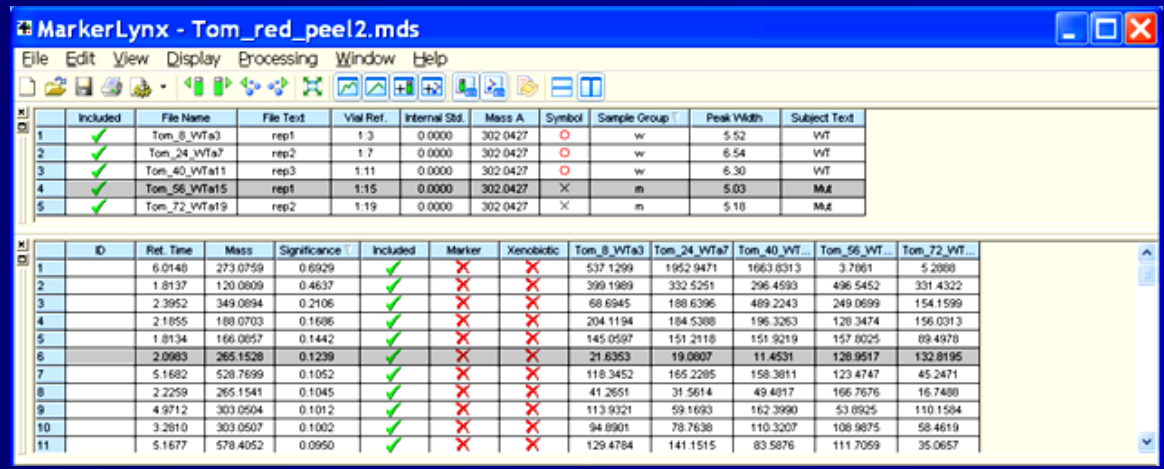
Unknown Mass peak Detected in Higher Levels in “Y”



Extracted mass chromatogram at m/z 265.1 Da

In-depth Analysis of Red Tomato Peel (ES+) Wild-type vs. the “Y” Mutant

MarkerLynx
Differential Markers



	Included	File Name	File Text	Vial Ref.	Internal Std.	Mass A	Symbol	Sample Group	Peak Width	Subject Text
1	✓	Tom_8_WTa3	rep1	1:3	0.0000	302.0427	○	w	5.52	WT
2	✓	Tom_24_WTa7	rep2	1:7	0.0000	302.0427	○	w	6.54	WT
3	✓	Tom_40_WTa11	rep3	1:11	0.0000	302.0427	○	w	6.30	WT
4	✓	Tom_56_WTa15	rep1	1:15	0.0000	302.0427	×	m	5.03	Mut
5	✓	Tom_72_WTa19	rep2	1:19	0.0000	302.0427	×	m	5.18	Mut

	ID	Ret. Time	Mass	Significance	Included	Marker	Xenobiotic	Tom_8_WTa3	Tom_24_WTa7	Tom_40_WT...	Tom_56_WT...	Tom_72_WT...
1		6.0148	273.0759	0.6929	✓	×	×	537.1299	1952.9471	1663.0313	3.7861	5.2888
2		1.8137	120.0809	0.4637	✓	×	×	399.1989	332.5251	296.4593	496.5452	331.4322
3		2.3952	349.0894	0.2106	✓	×	×	68.6945	188.6396	489.2243	249.0899	154.1599
4		2.1855	188.0703	0.1686	✓	×	×	204.1194	184.5388	196.3263	128.3474	156.0313
5		1.8134	166.0857	0.1442	✓	×	×	145.0597	151.2118	151.9219	157.8025	69.4978
6		2.0983	265.1528	0.1239	✓	×	×	21.6353	19.0807	11.4531	128.9517	132.8195
7		5.1682	528.7699	0.1052	✓	×	×	118.3452	165.2285	158.3811	123.4747	45.2471
8		2.2259	265.1541	0.1045	✓	×	×	41.2651	31.5614	49.4817	166.7676	16.7488
9		4.9712	303.0504	0.1012	✓	×	×	113.9321	59.1693	162.3990	53.8925	110.1584
10		3.2610	303.0507	0.1002	✓	×	×	94.8901	78.7638	110.3207	108.9875	58.4619
11		5.1677	578.4052	0.0950	✓	×	×	129.4784	141.1515	83.5876	111.7059	35.0657

- Filtering out non-replicated data
- Identifying molecular ions, isotopes, adducts, fragments and passing from Markers to Metabolites
- Analysis of differential metabolites and identification

What are the Differentially Expressed Metabolites between WT and “Y” ?

Peak assignment by:

- Accurate mass and elemental composition (MassLynx and natural products database)
- MS-MS fragmentation
- UV spectrum
- Literature
- UV & MS spectra databases

(e.g. for tomato in PRI)

Differential Metabolites between the "Y" and Wild Type Peel UPLC-QTOF-MS in ES- Mode

	Differential metabolites (ES-)	
	WT > "Y"	"Y" > WT
R only	10	11
O only	12	16
Both R & O	26	4
Total	48	31

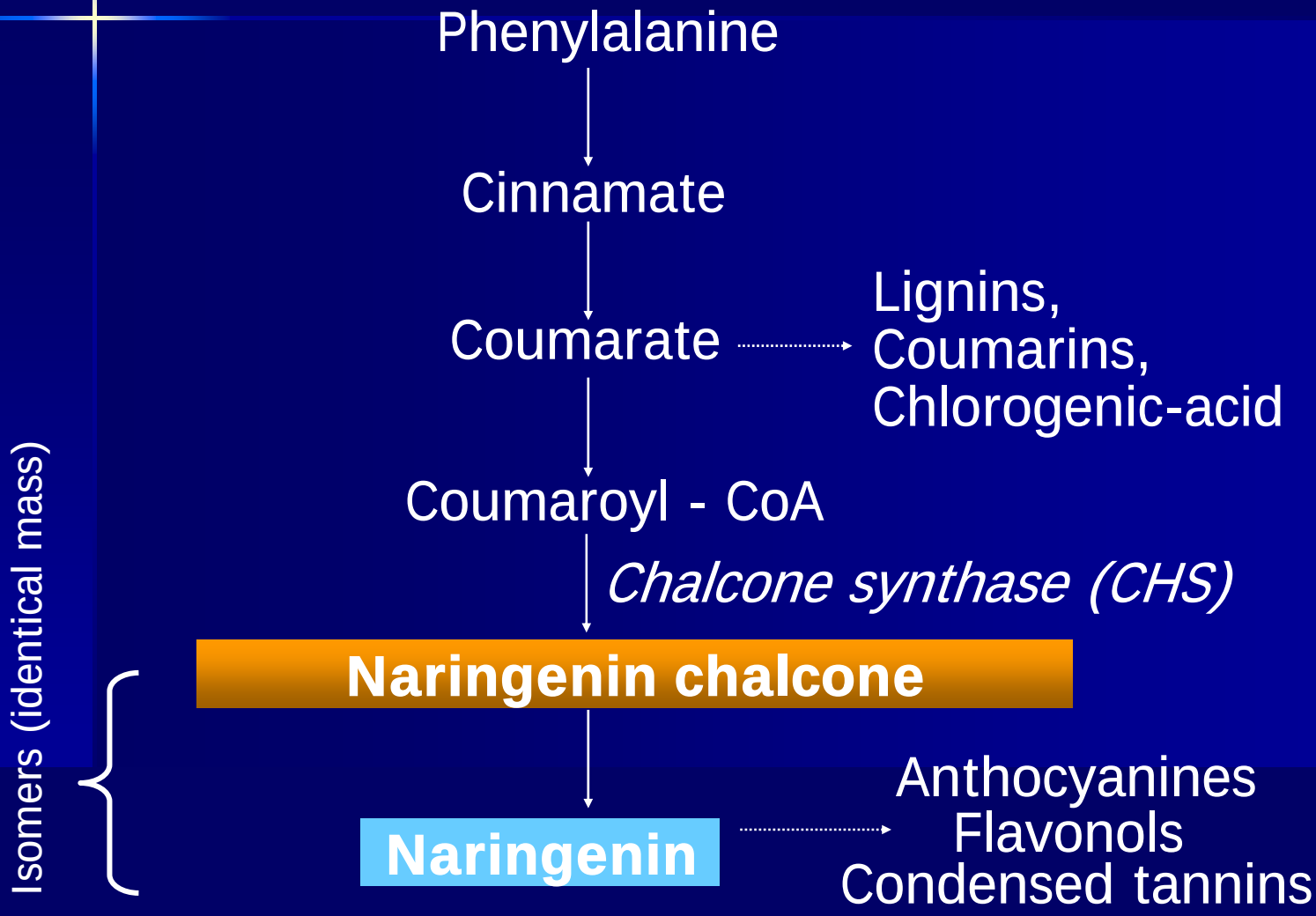
Differential Metabolites between the "Y" and Wild Type Peel UPLC-QTOF-MS in ES+ Mode

	Differential metabolites (ES+)	
	WT >"Y"	"Y" > WT
R only	3	2
O only	0	2
Both R & O	16	8
Total	19	12

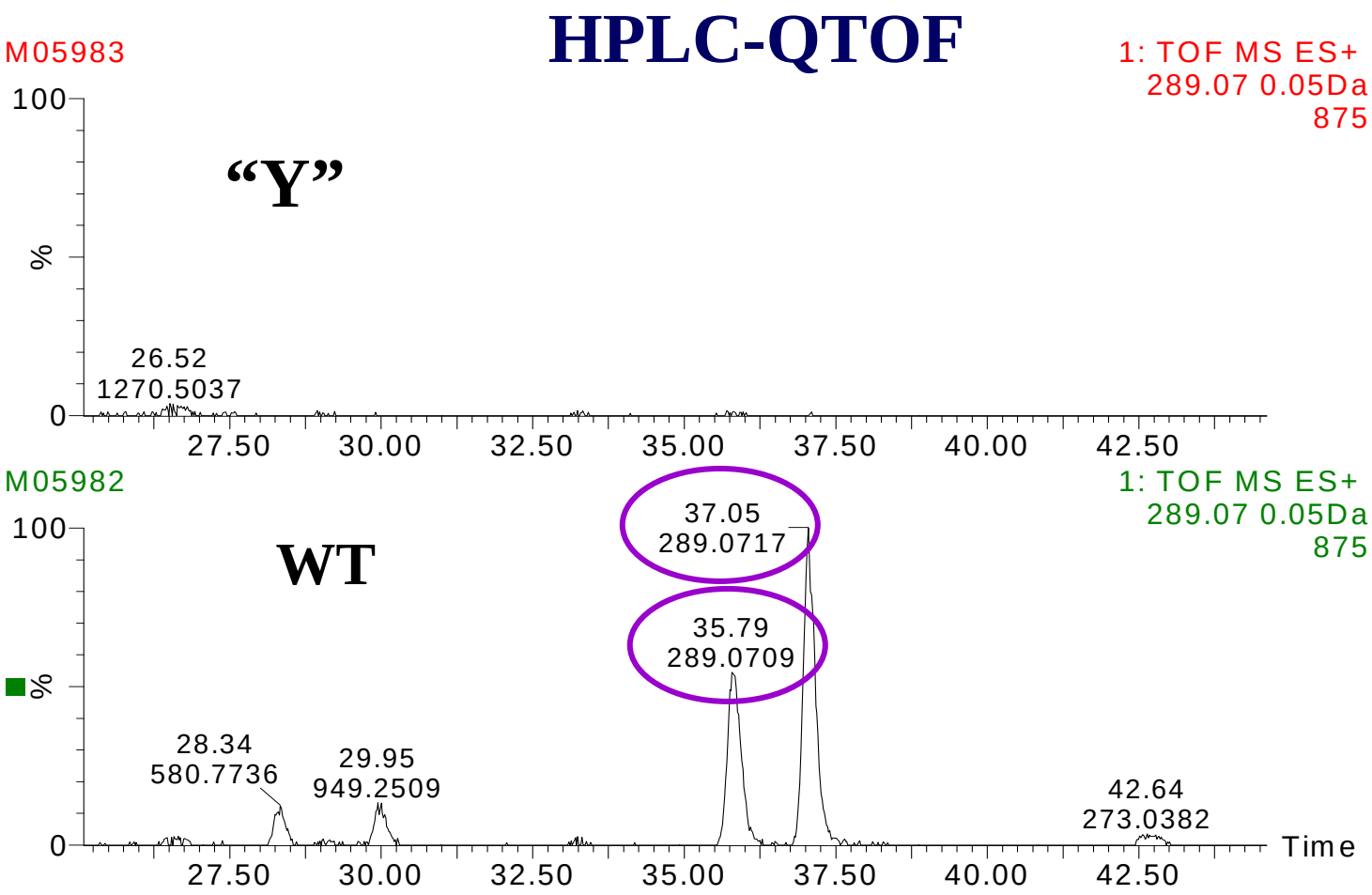
What are the Differentially Expressed Metabolites between WT and “Y” ?

Most mass peaks showing higher levels in WT than in the “Y” mutant are derivatives of Naringenin Chalcone or Naringenin.

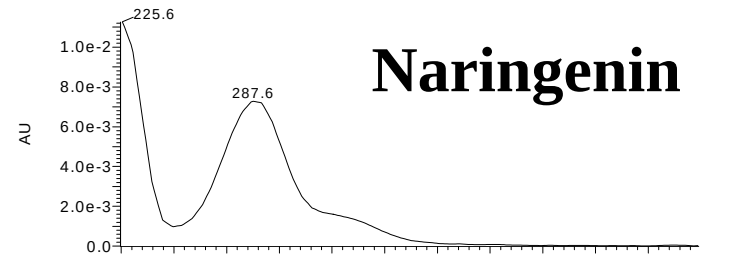
Biosynthesis of Naringenin Chalcone in the Flavonoid Pathway



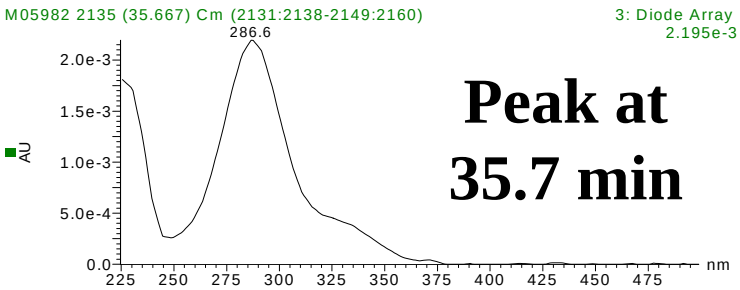
Hydroxylated Naringenin & Naringenin- Chalcone



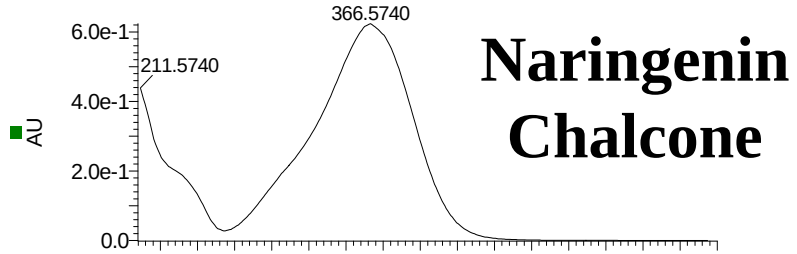
UV Spectra of Differential Peaks (35.7 min. and 37.05 min.)



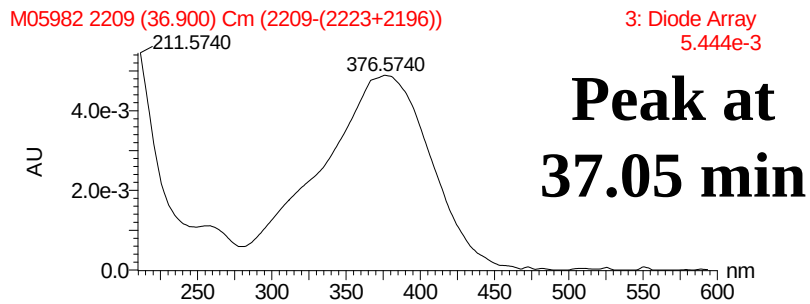
Naringenin



Peak at 35.7 min

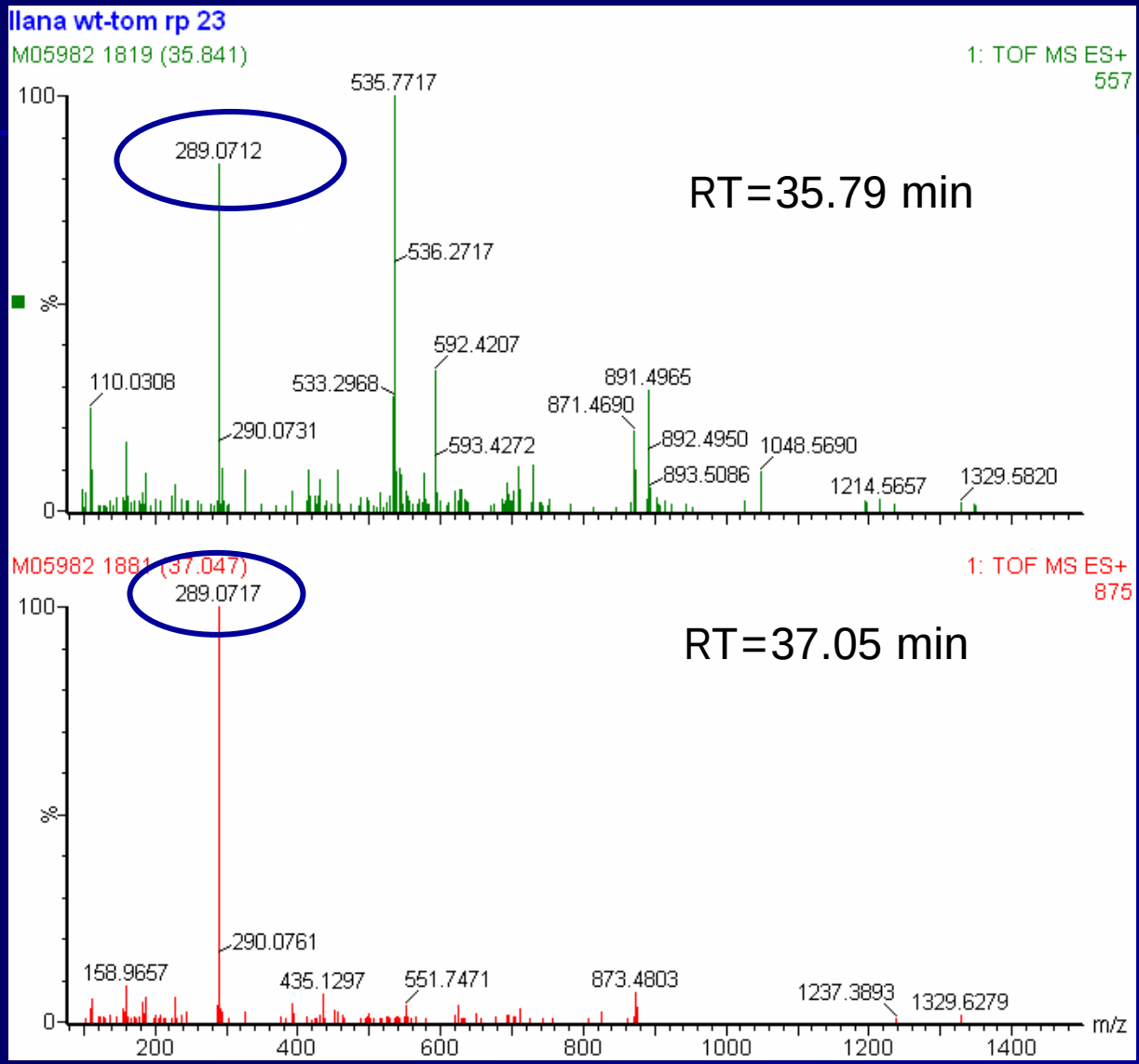


Naringenin Chalcone

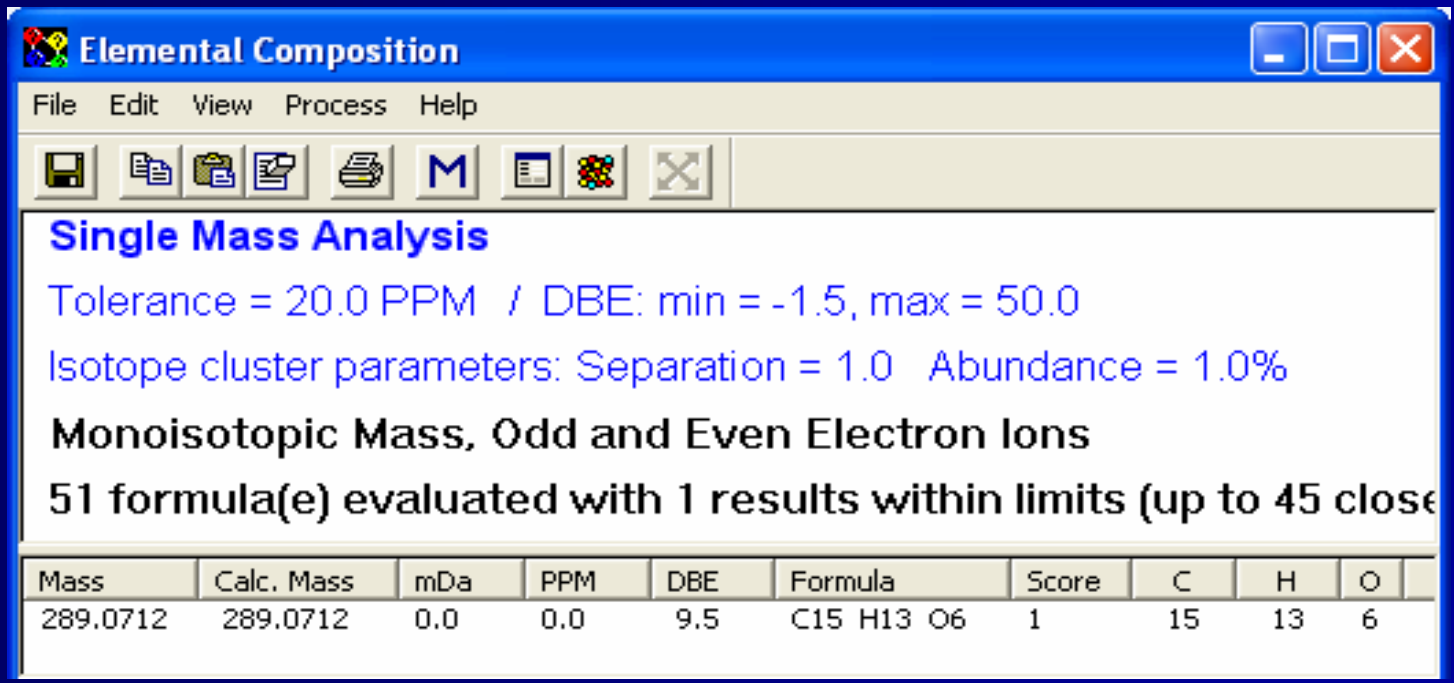


Peak at 37.05 min

Spectra of differential compounds at RT=35.79 min and 37.05 min



Elemental Composition (289.0712)

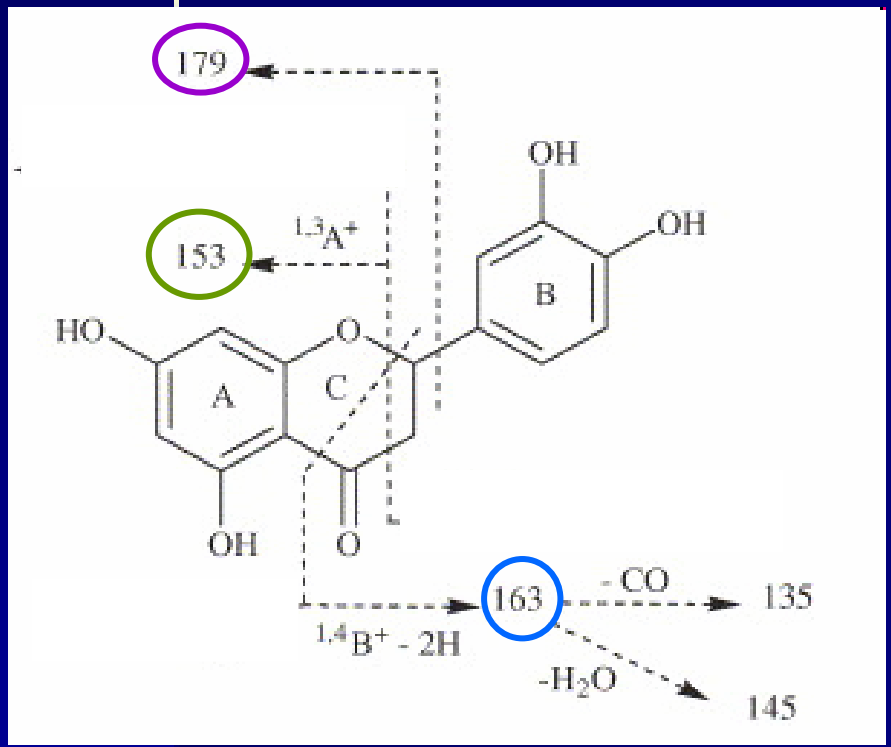


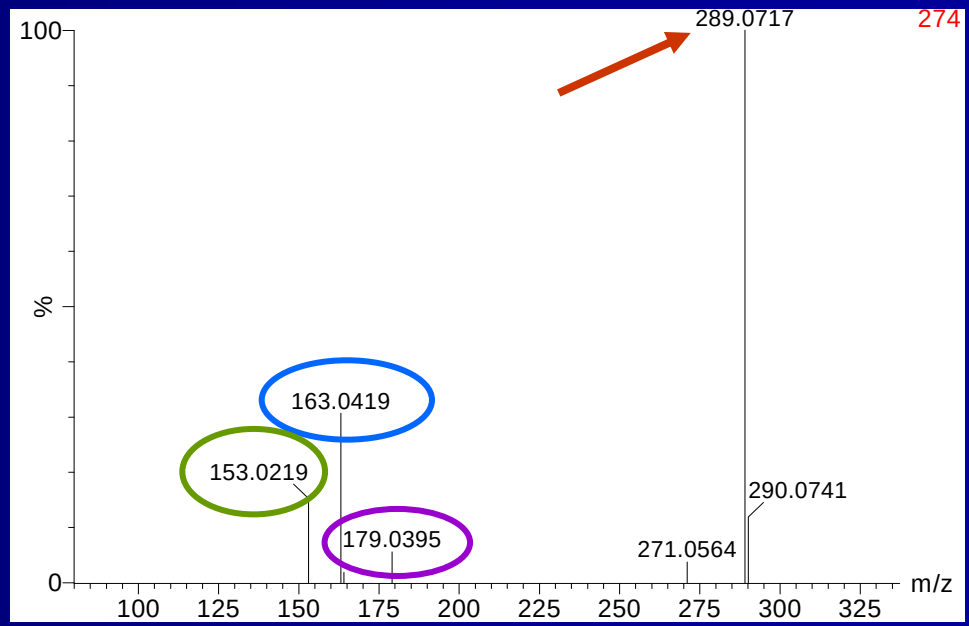
Mass	Calc. Mass	mDa	PPM	DBE	Formula	Score	C	H	O
289.0712	289.0712	0.0	0.0	9.5	C15 H13 O6	1	15	13	6

Elemental composition: $C_{15}H_{12}O_6$ for neutral compound
Elemental composition of Naringenin: $C_{15}H_{12}O_5$
Difference: OH group instead of H

Suggested Metabolite

MS-MS Fragmentation of m/z 289.07



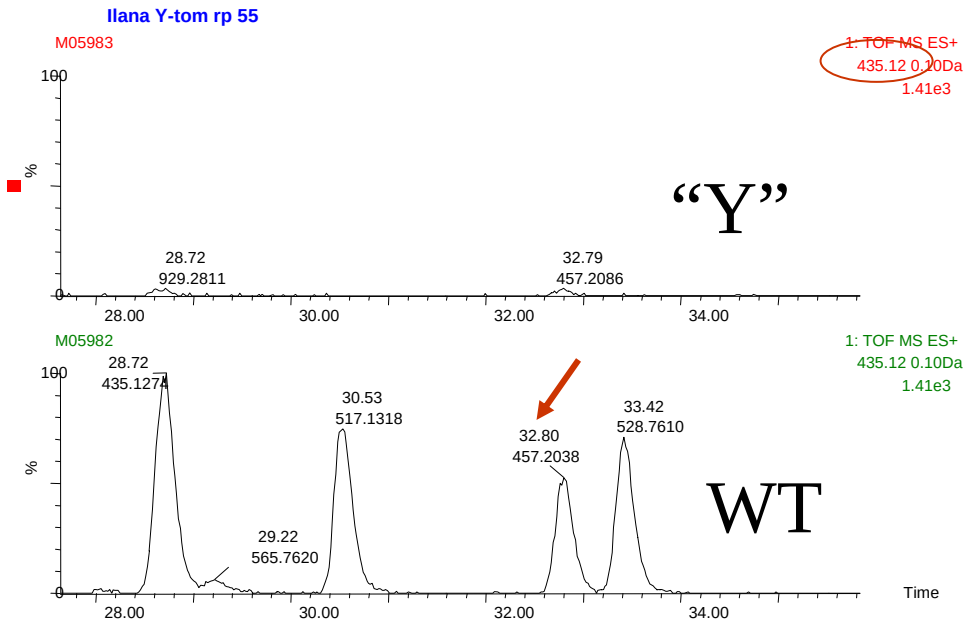


MS-MS fragmentation of m/z 289.0717

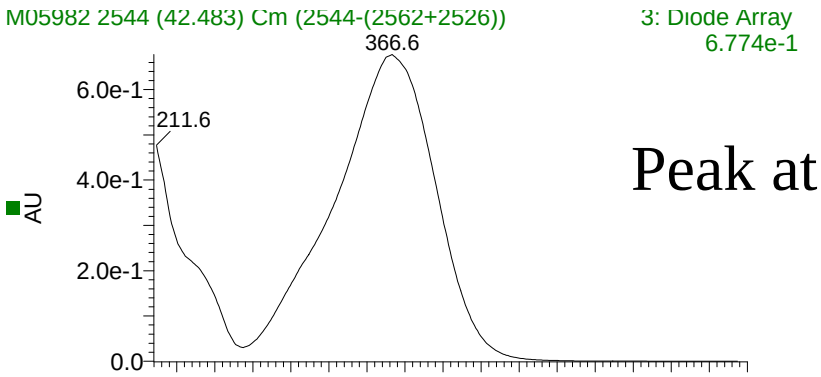
Eriodictyol ($C_{15}H_{12}O_6$)
Main fragmentations in reference:
I. J. M. S. 247 (2005) 93–100

Naringenin and Naringenin Chalcone Glycosides

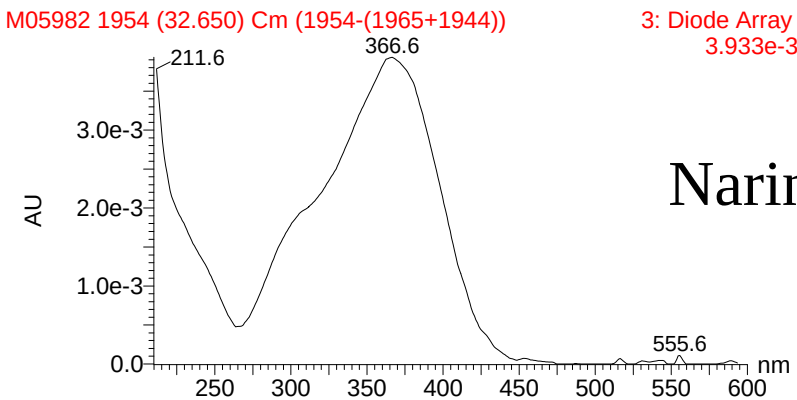
HPLC-QTOF



UV Spectra of Peak at 32.8 min. and Naringenin Chalcone



Peak at 32.8 min

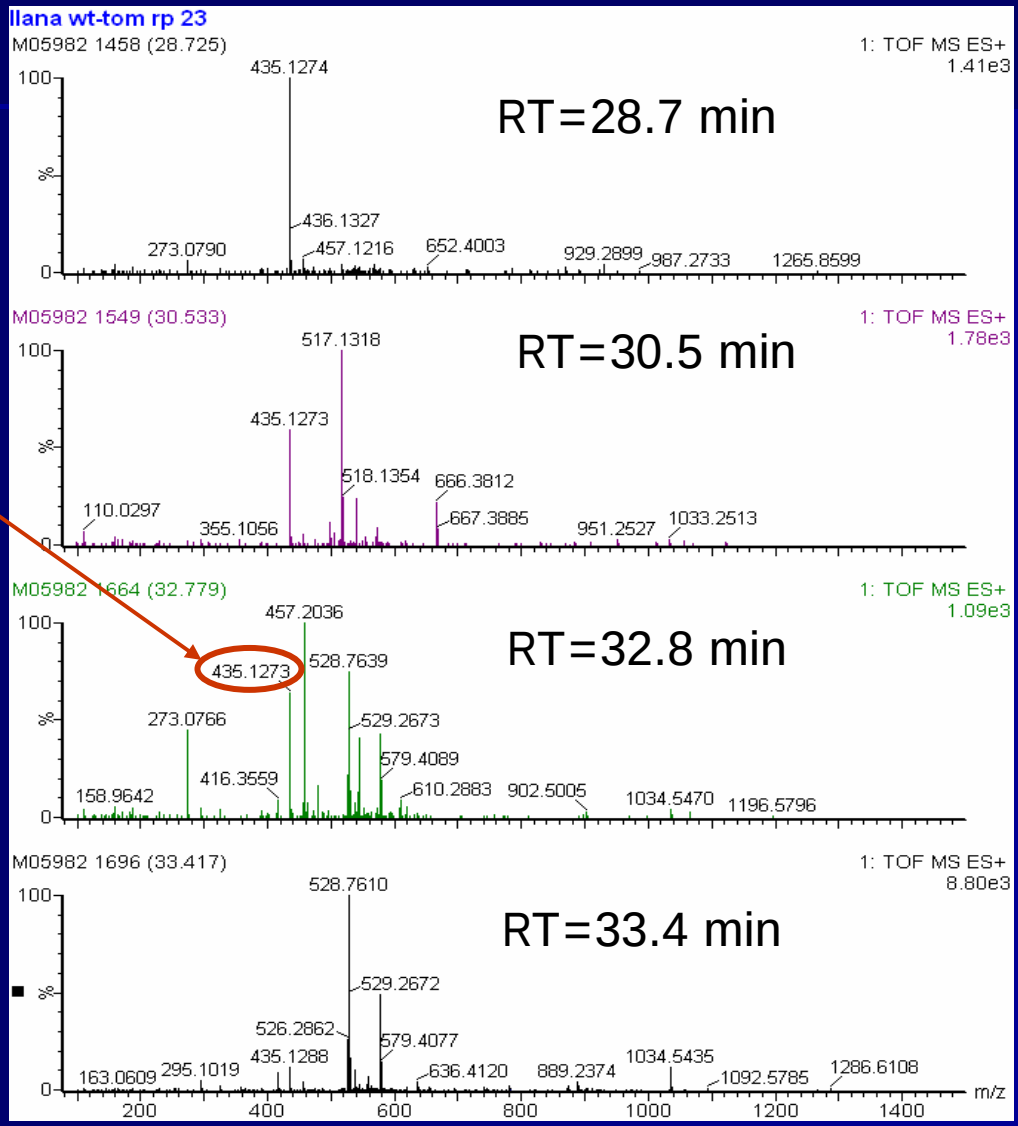


Naringenin Chalcone

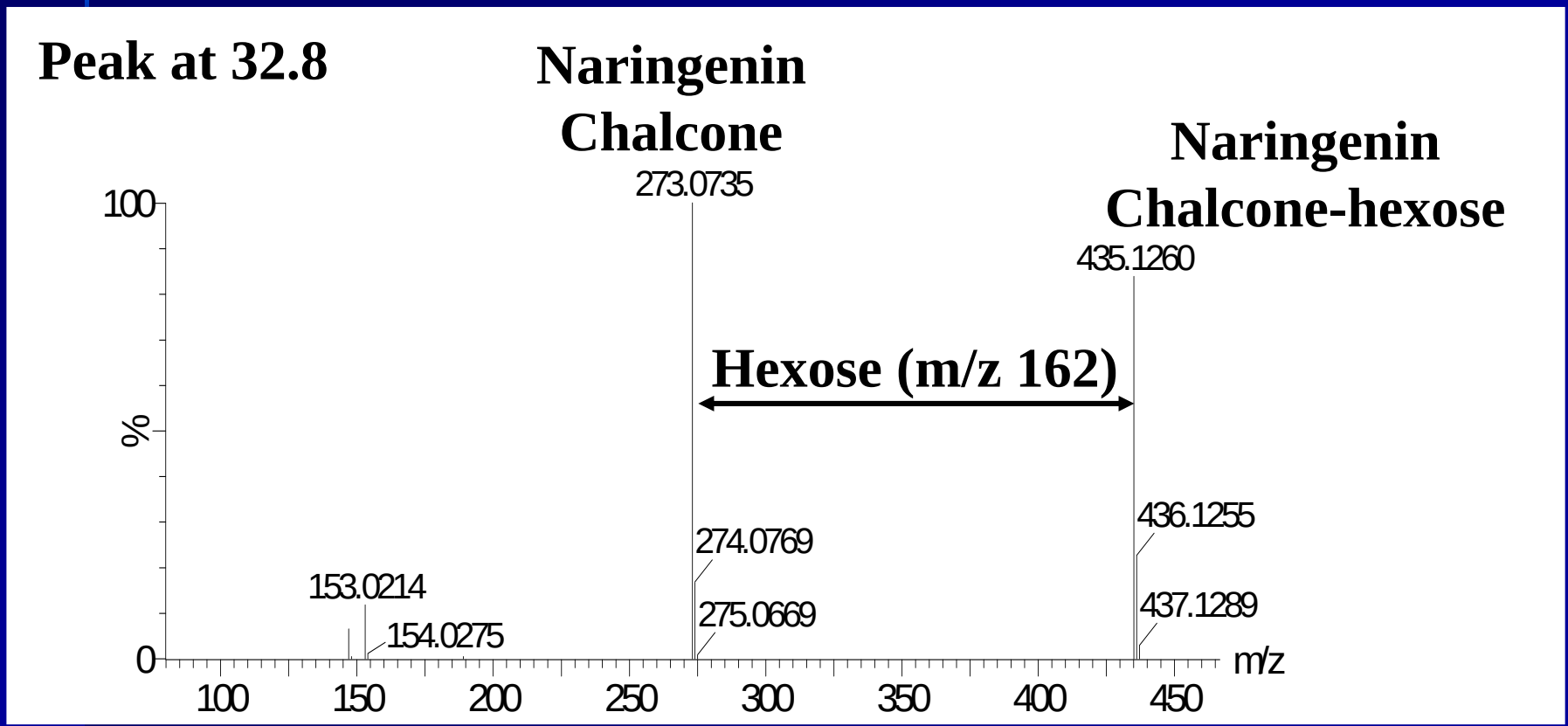
MS spectra at RT=28, 30 32 and 33 min

For MS-MS

Exact mass of 435.1273 shows elemental composition of $C_{21}H_{22}O_{10}$



MS-MS Fragmentation of m/z 435.12 for Peak at RT=32.8

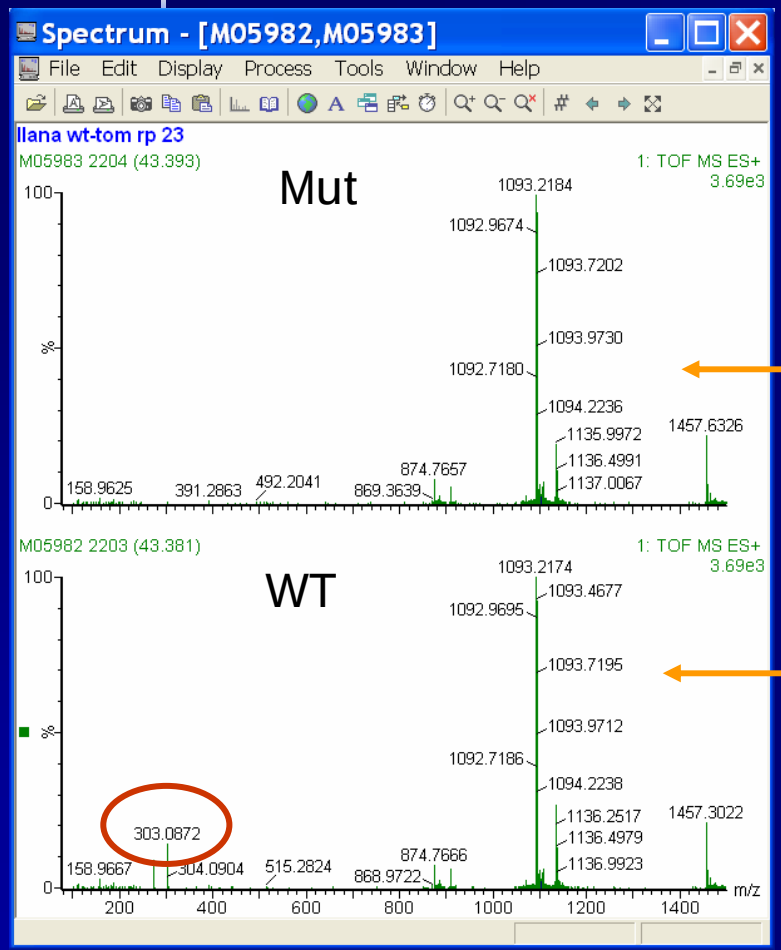


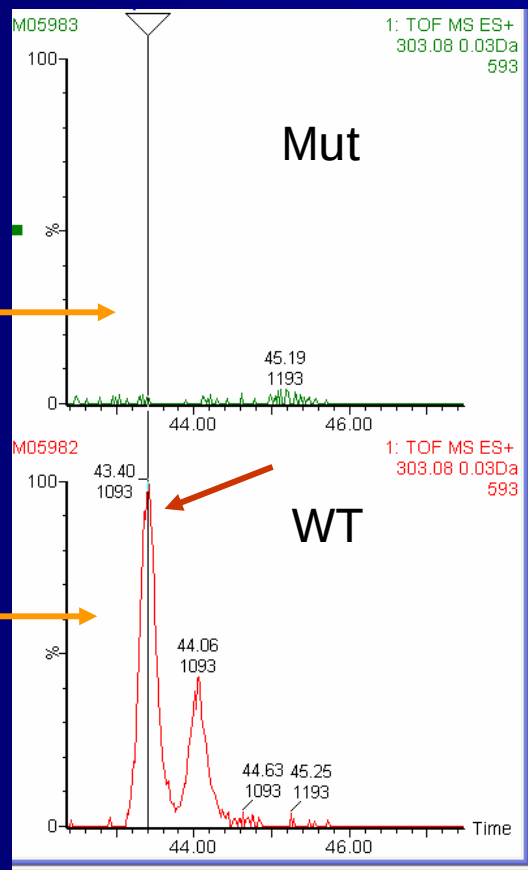
Mass Peak at RT=43.40min (>WT)

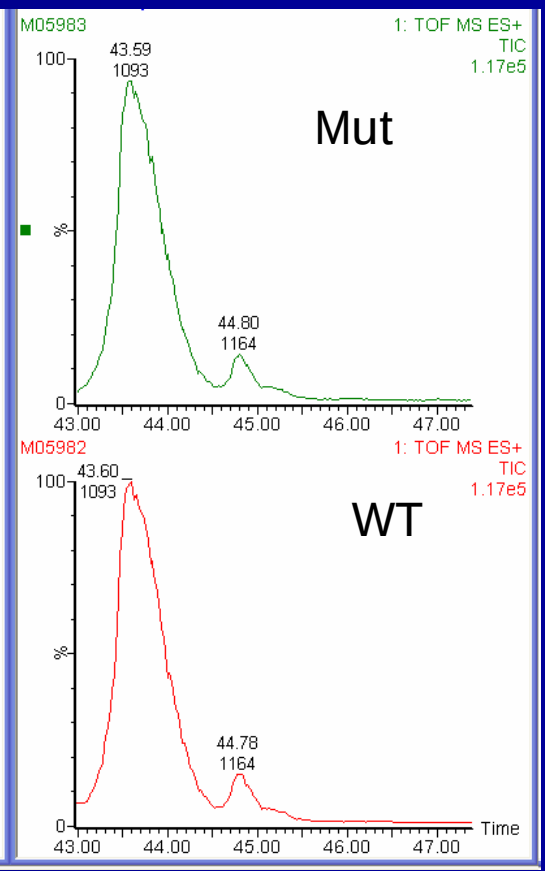
MS spectra at RT=43.40min

Selected Mass Chromatogram m/z 303.08 Da

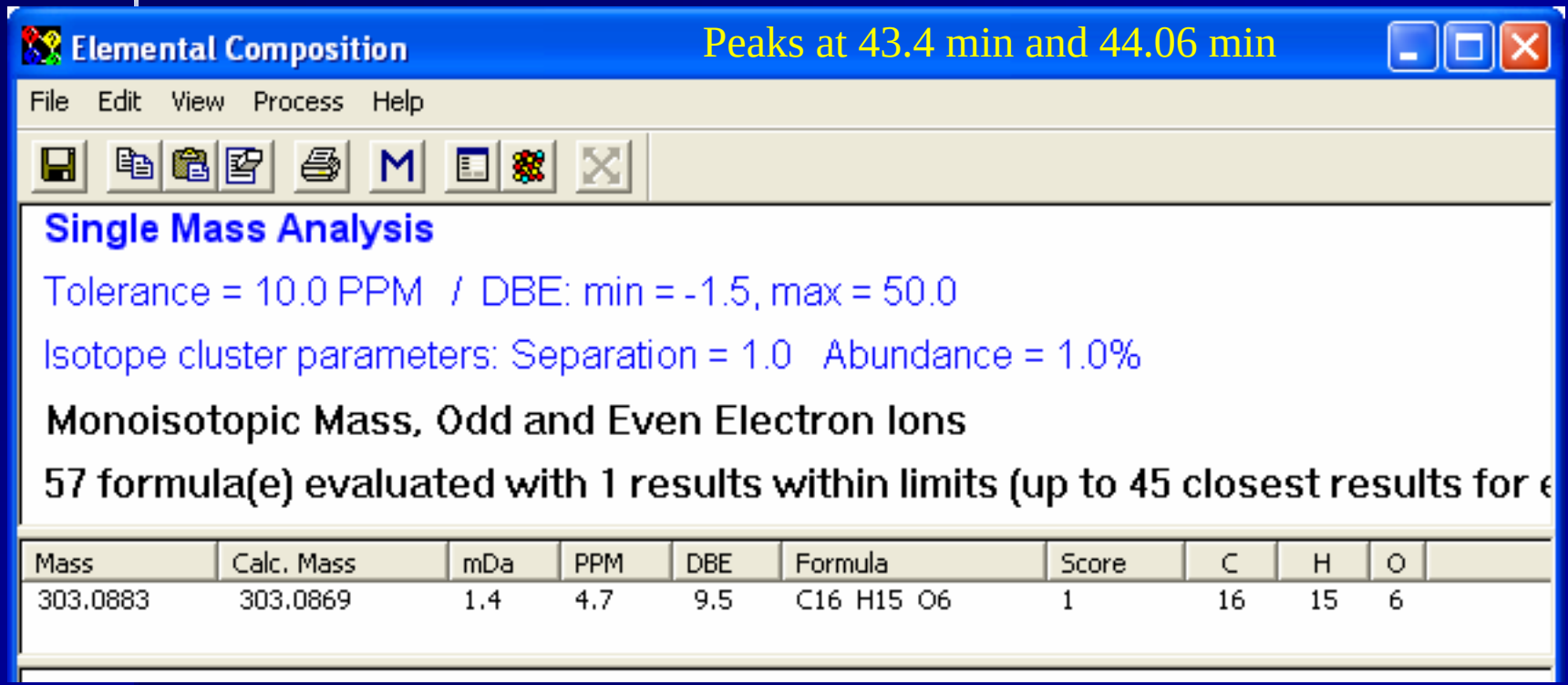
TIC chromatogram





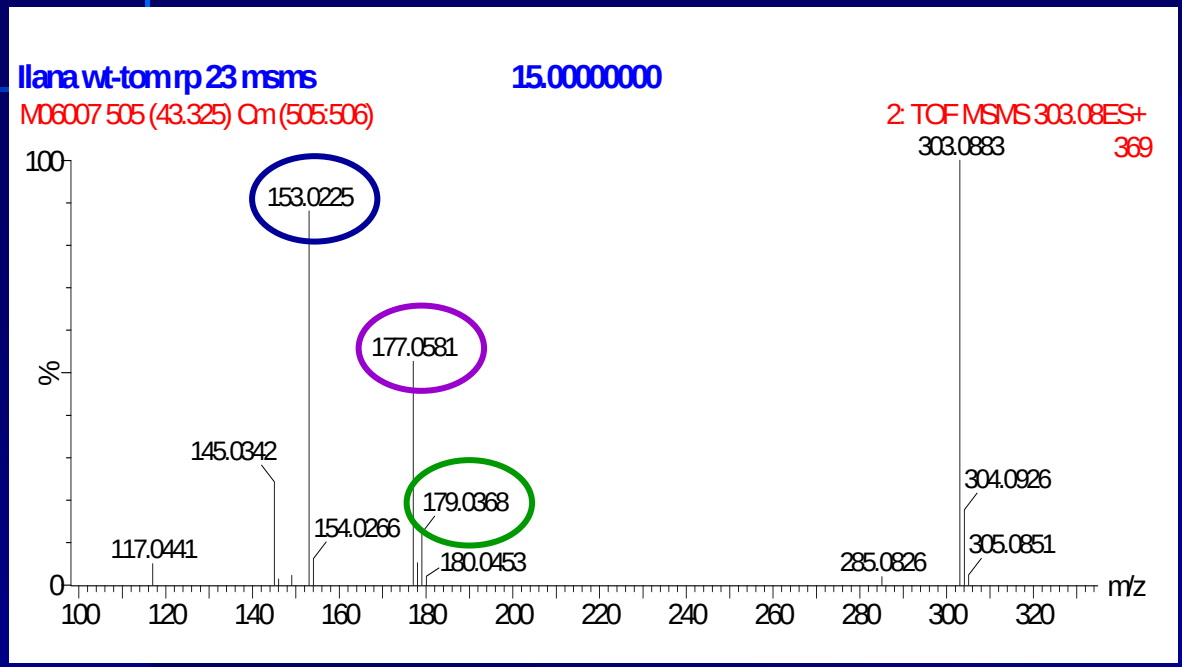


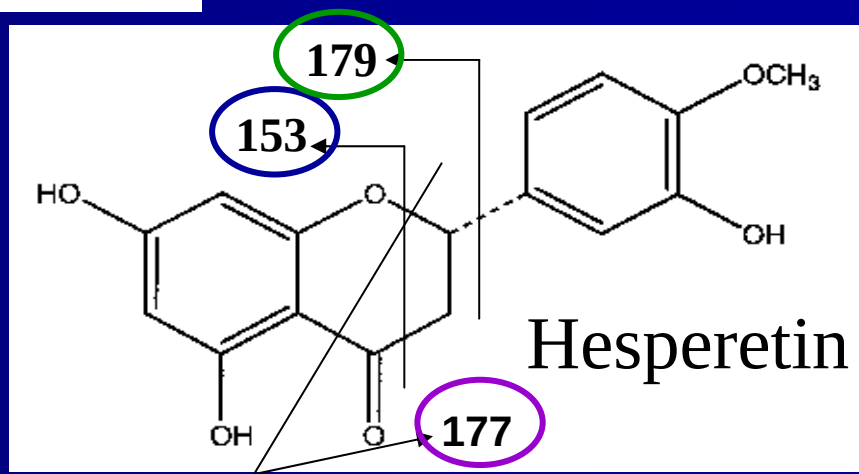
Methyl ether of Hydroxylated Naringenin and Naringenin Chalcone



- Calculated formula is $C_{16}H_{14}O_6$ based on exact mass
- Proposed structure for the peak at 43.4 min: Hesperetin or a B-ring positional isomer

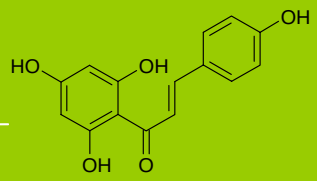
MS-MS Fragmentation of m/z 303.08 (43.4 min.)



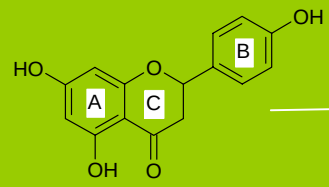


Proposed Scheme of Naringenin & Naringenin Chalcone Hydroxylation, Methylation and Glycosylation

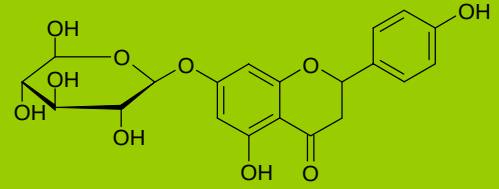
Glycosylation



Naringenin Chalcone

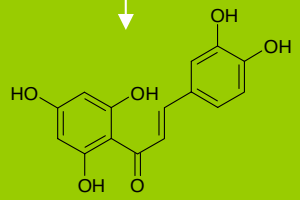


Naringenin

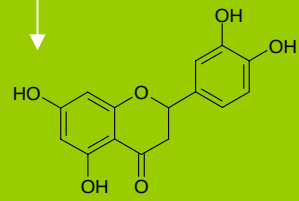


Glycosylation
e.g. Naringenin glucoside

Glycosylation



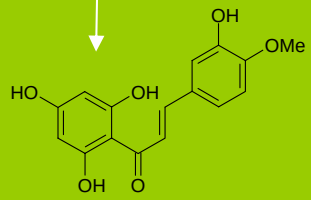
Hydroxylated Naringenin Chalcone



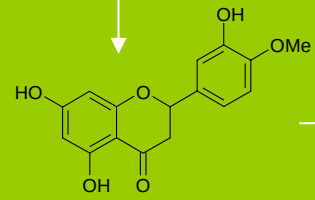
Glycosylation

Hydroxylated Naringenin
e.g. Eriodyctyol or a B-ring isomer

Glycosylation



Methyl ether of Hydroxylated Naringenin Chalcone



Glycosylation

Methyl ether of Hydroxylated Naringenin
e.g.Hesperetin or a B-ring isomer

Biosynthesis of Naringenin Chalcone in the Flavonoid Pathway