2013 Missouri Life Sciences Week Workshop

Practical application of UPLC-MS/MS for targeted metabolite analysis

Abraham J.K. Koo

What is Metabolomics?

"Metabolomics is the scientific **study of chemical processes involving metabolites**. " -wikipedia.org

"Metabolomics is a newly emerging field of "omics" research concerned with the **comprehensive characterization of the small molecule metabolites** in biological systems."

-Metabolomics Society

How many metabolites are there?

KEGG (Kyoto Encyclopedia of Genes and Genomes): ~15,000 compounds (from animals, plants and bacteria)

E. coli and yeast: **600** ~ **800** (Forster et al., 2003: Keseler et al., 2005)

Human: **2,900** (Human Metabolome Project)

Plant kingdom: **200,000** ~ **1,000,000** (Dixon and Strack, 2003)

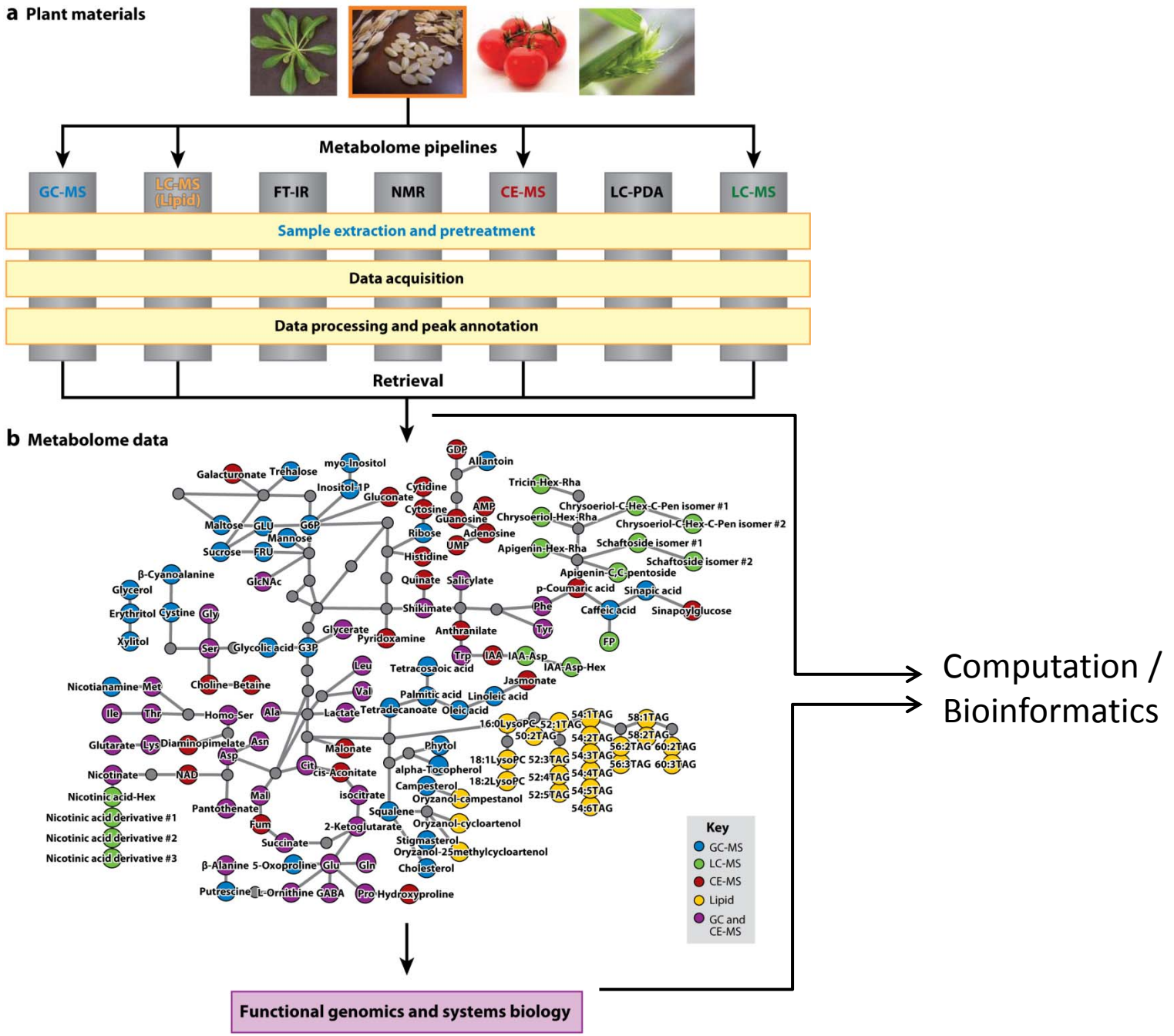
Arabidopsis thaliana: ~ **5,000**

Structural Diversity (chemical properties)

Wide Concentration Range (10^6)

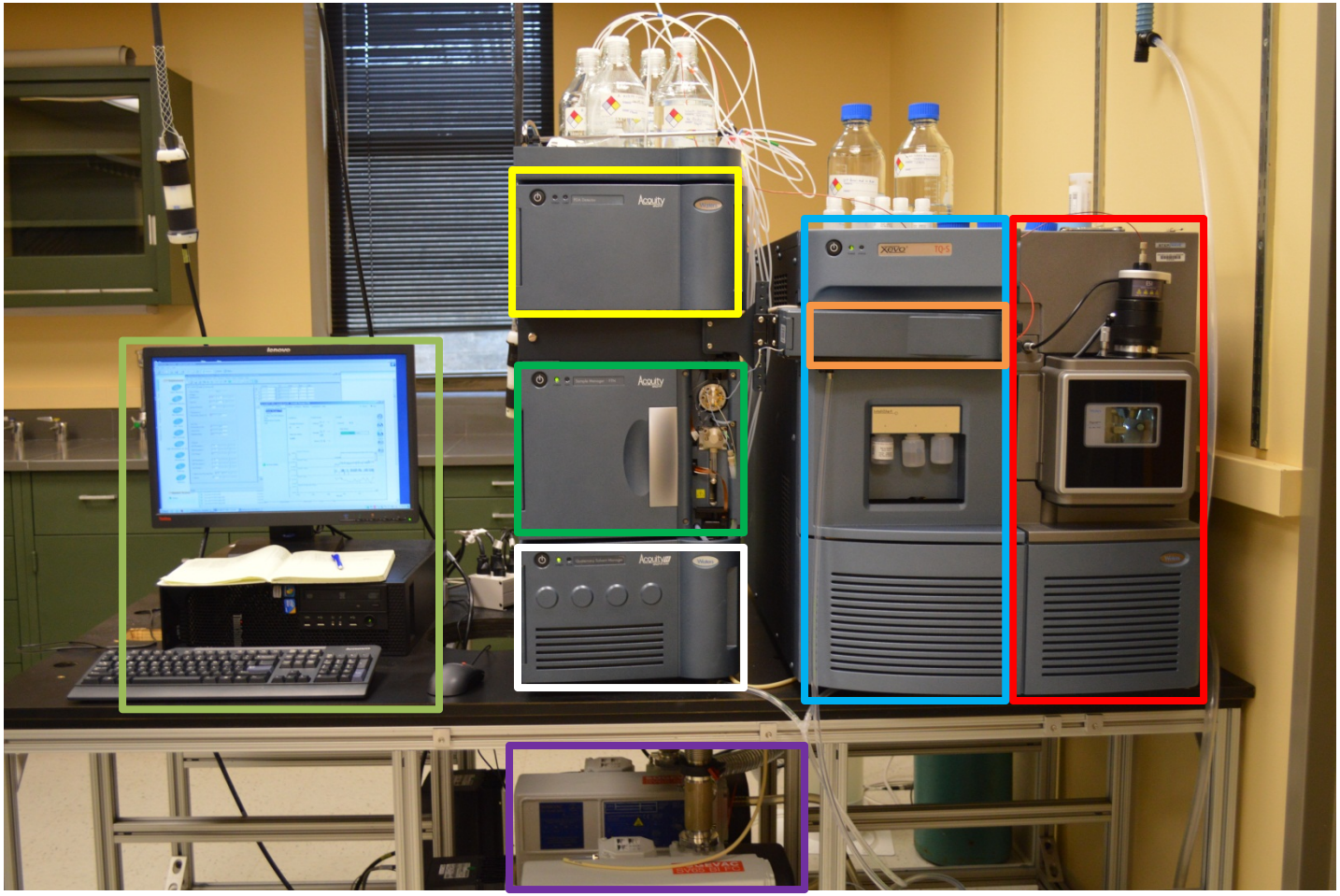
PlantMetabolomics mics.org) :
detect approximate emically defined.

Human Metabolom ca/): experimental MS/MS data for **800** compounds, experimental 1H and 13C NMR data for **790** compounds and GC/MS spectral and retention index data for **260** compounds.



Saito K, Matsuda F. 2010.
Annu. Rev. Plant. Biol. 61:463–89

UPLC–MS/MS system at MU (19 Schweitzer Hall)



HPLC unit (ACQUITY UPLC)

Chromatographic Separation of Complex Sample Mixture

Mass Spectrometer (Xevo TQ-S)

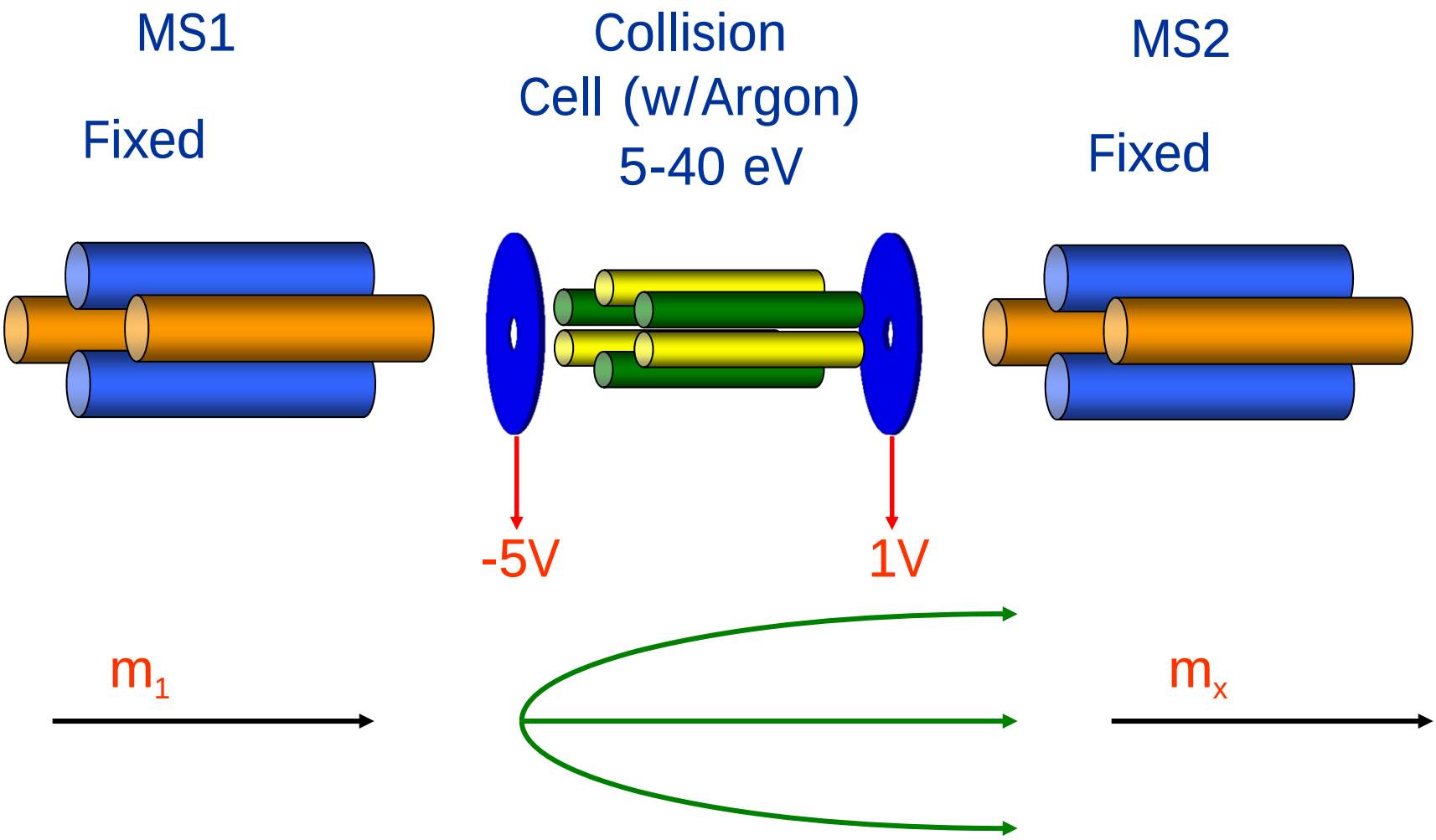
Ionization and detection of ions' **mass-to-charge ratio (m/z)**

Triple quadrupole: MS/MS Tandem Mass Spectroscopy (MRM)

PDA (ACQUITY UPLC PDA detector)

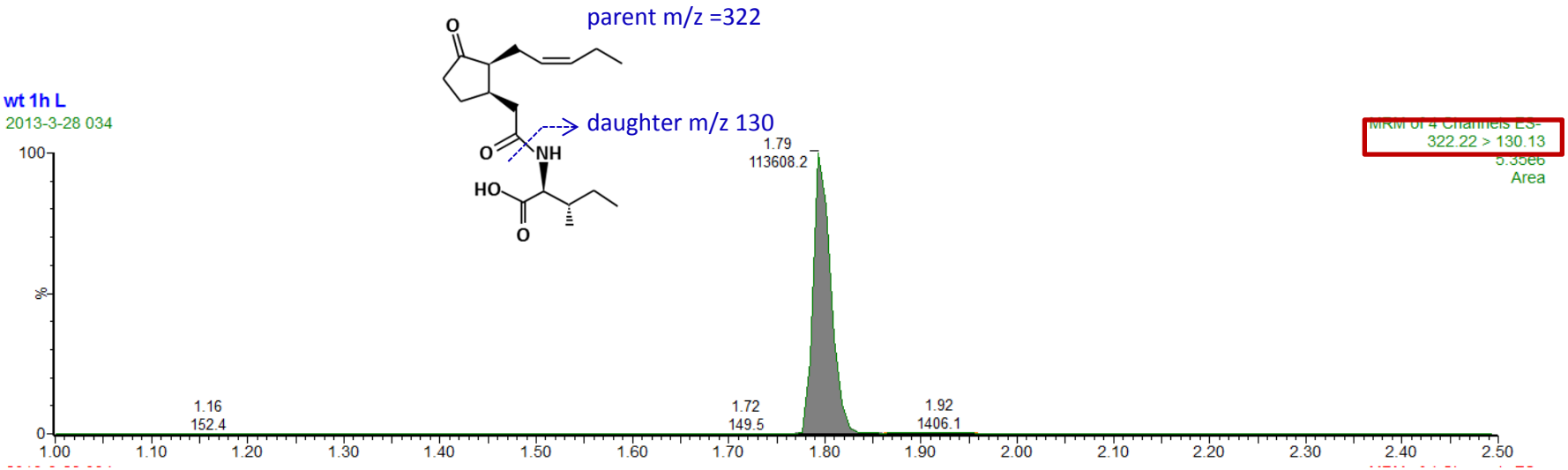
Spectrophotometric detection of **absorbance** at wavelength range 190 nm – 500 nm with 1.2 nm resolution

MRM (Multiple Reaction Monitoring)



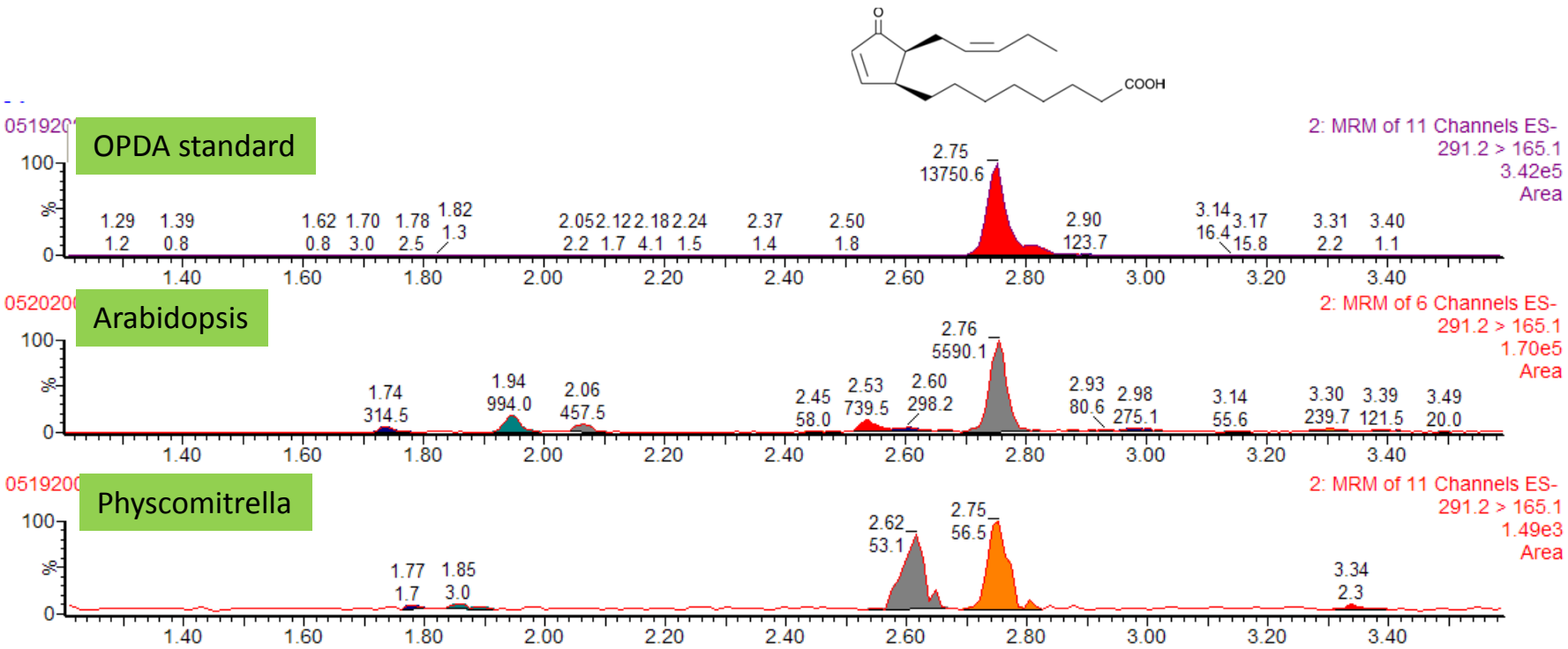
MRM's are used to monitor selected analyte(s) via their daughter ions

Selective detection of a target compound in complex sample matrix

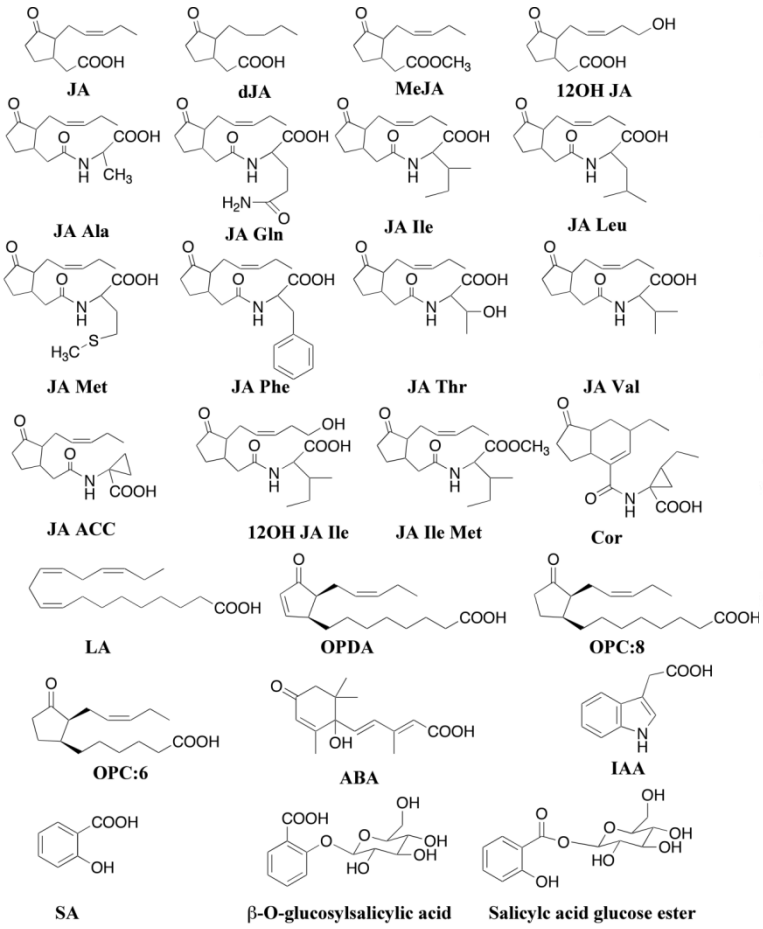


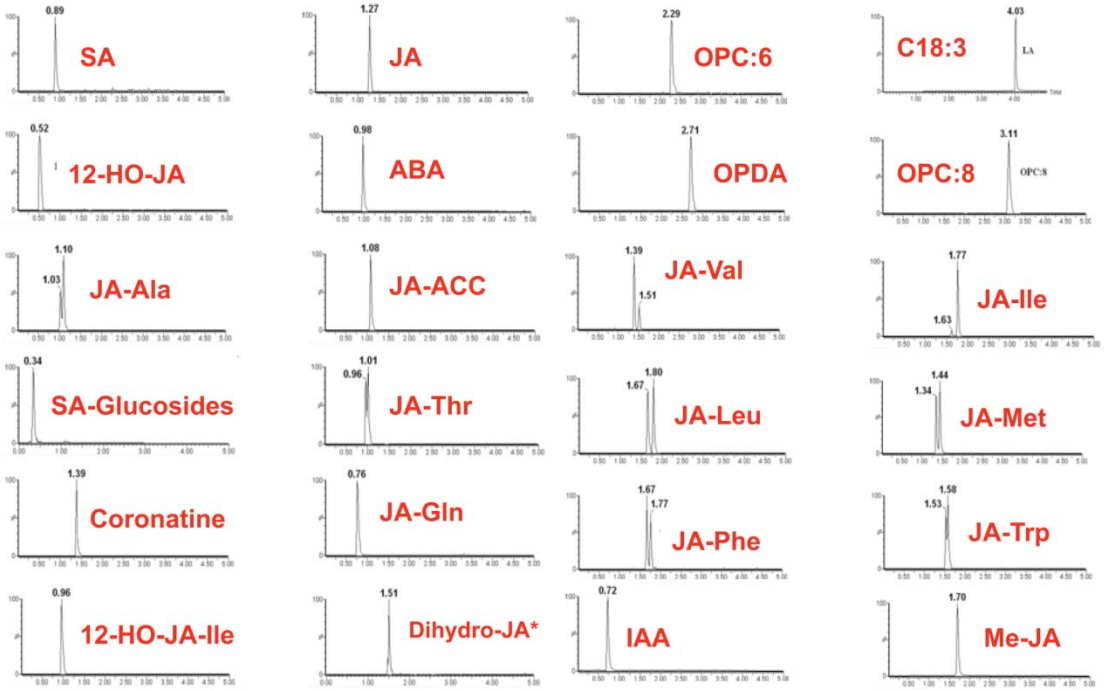
Methanol extract from Arabidopsis leaf

Chromatography improves specificity



MRM allows Simultaneous Detection of Multiple Ions





Metabolite analysis using MRM requires pre-knowledge about the compound (molecular mass) or to have standards

Public metabolomics database (KEGG >15,000 compounds)

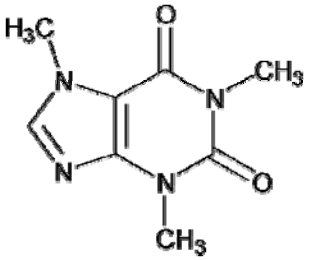
> 212,000 compounds available from Sigma-Aldrich

What kind of molecules can be analyzed?

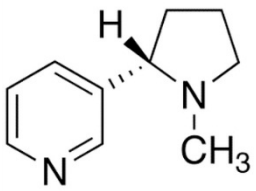
In the fields of pharmacology and biochemistry, **a small molecule is a low molecular weight (<800 Daltons) organic compound** that may serve as an enzyme substrate or regulator of biological processes. The upper molecular weight limit for a small molecule is approximately 800 Daltons which allows for the possibility to rapidly diffuse across cell membranes so that they can reach intracellular sites of action.

Mass range: 2 to 2048 m/z

Caffeine

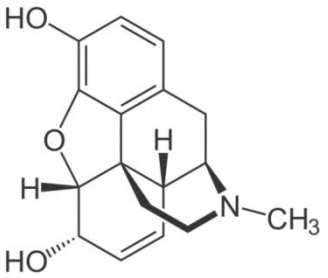


Nicotine

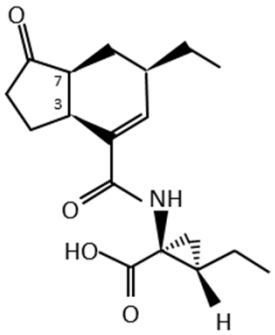


m/z = 137

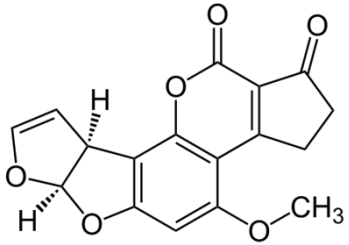
Morphine



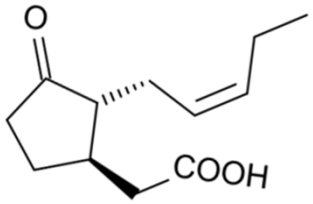
Coronatine



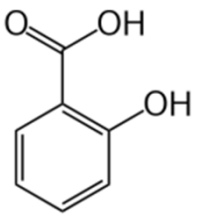
Aflatoxin B1



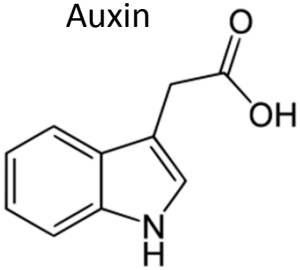
Jasmonic Acid



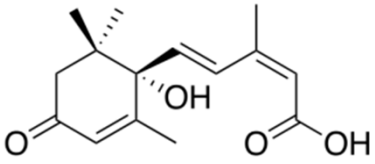
Salicylic Acid



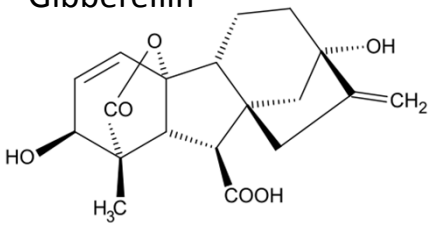
Auxin



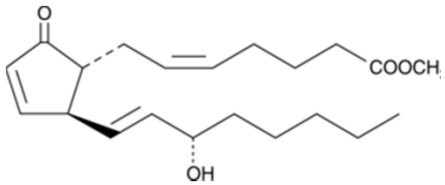
Abscisic Acid



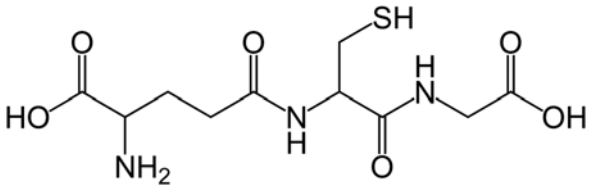
Gibberellin



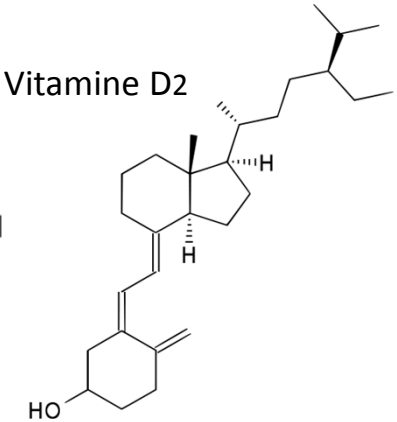
Prostaglandin A2



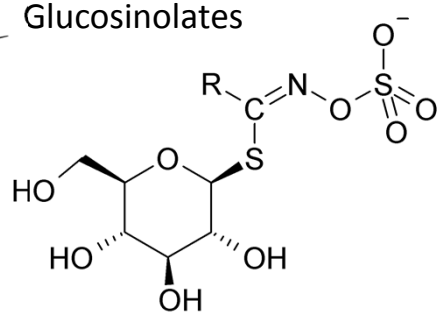
Glutathione (GSH)



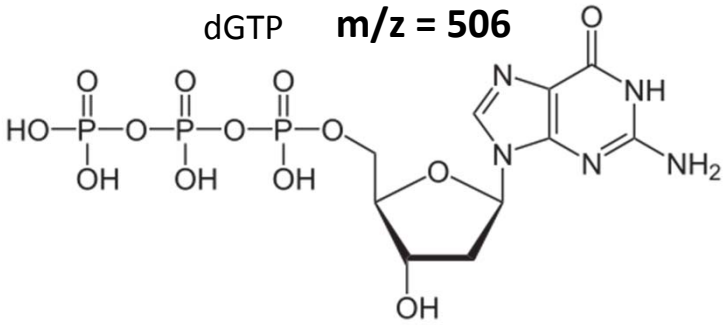
Vitamine D2



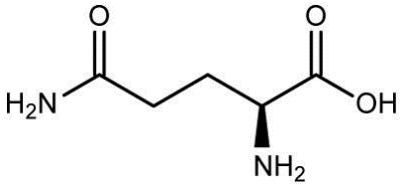
Glucosinolates



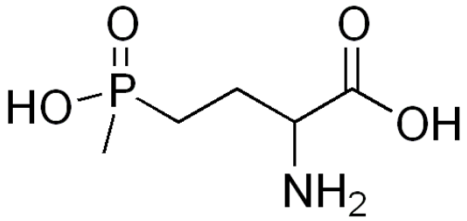
dGTP **m/z = 506**



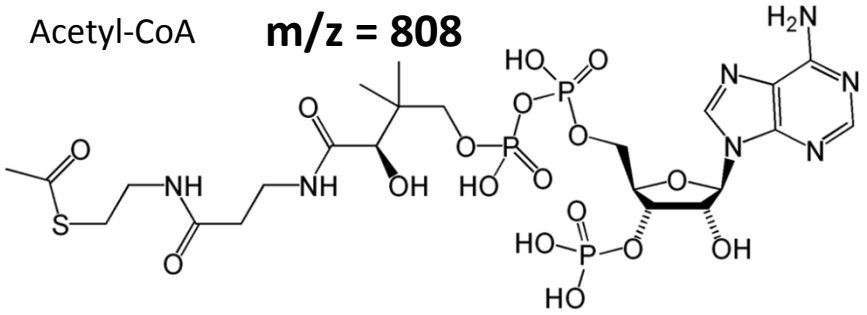
Glutamine



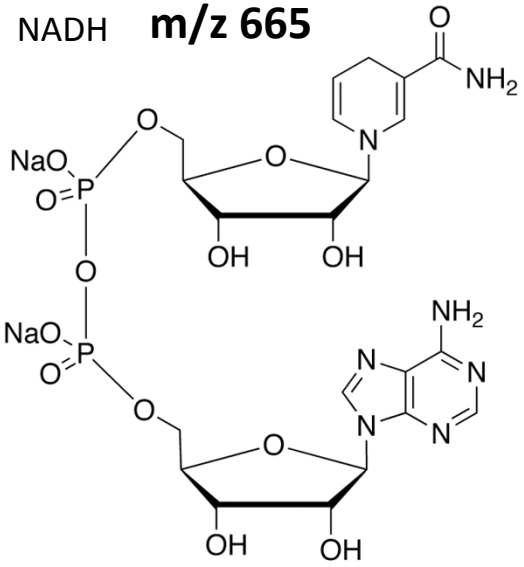
Glycophosphate



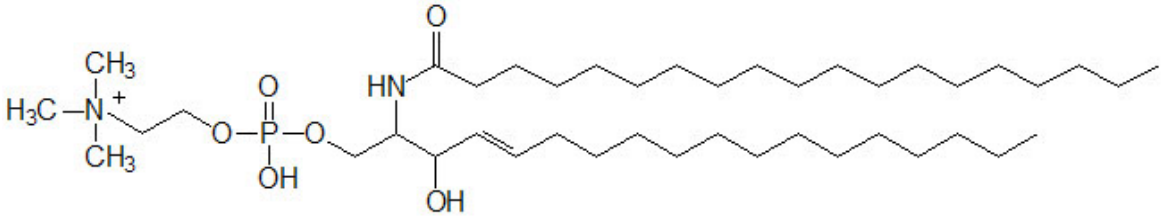
Acetyl-CoA **m/z = 808**



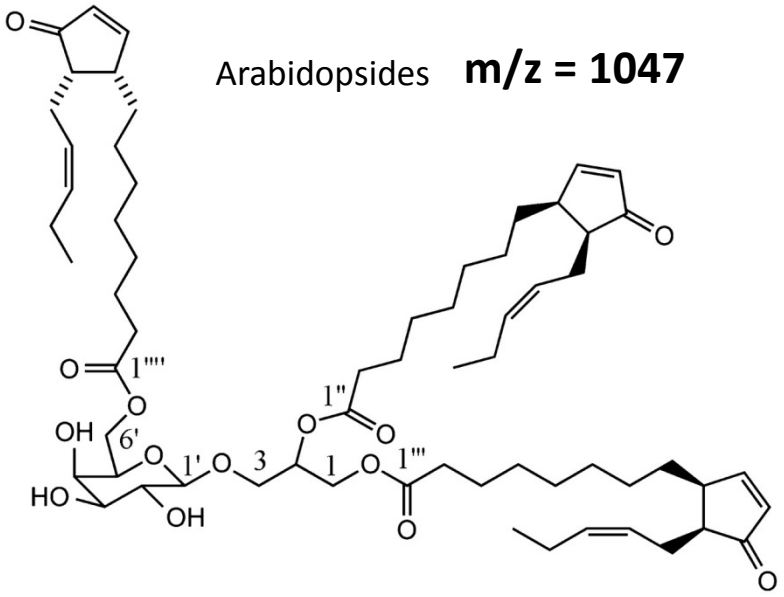
NADH **m/z 665**



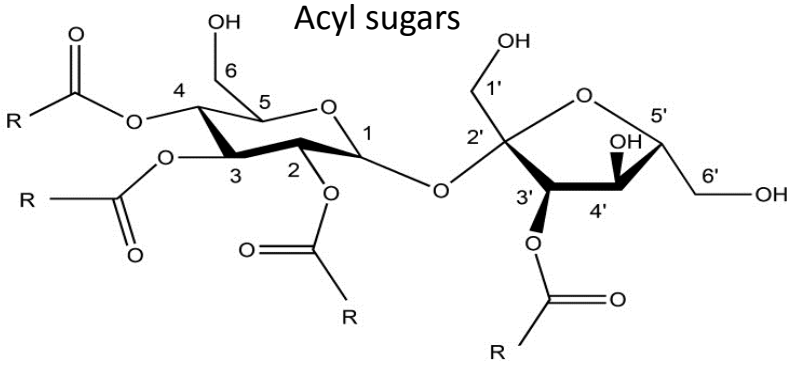
Sphingomyelin



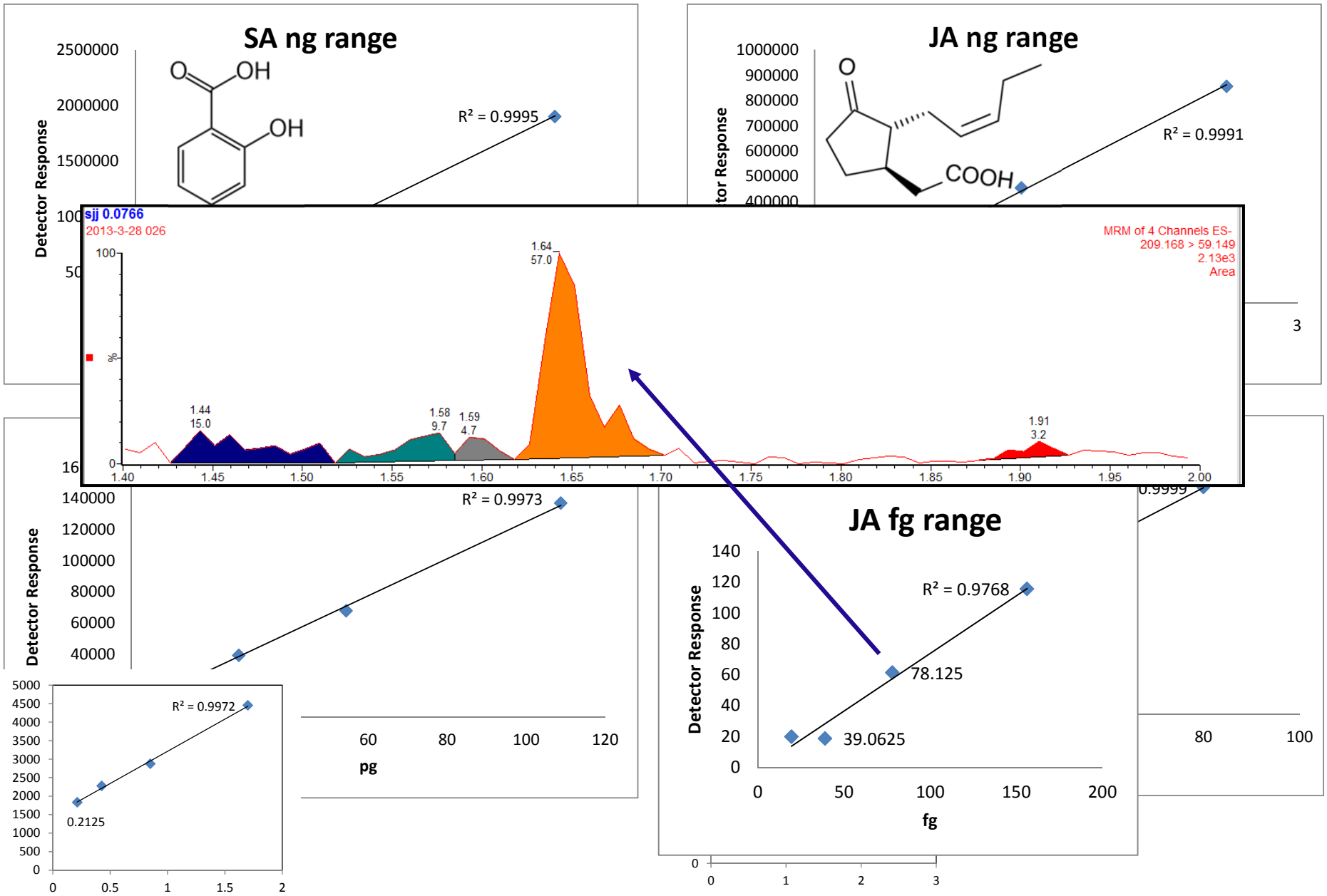
Arabidopsides **m/z = 1047**

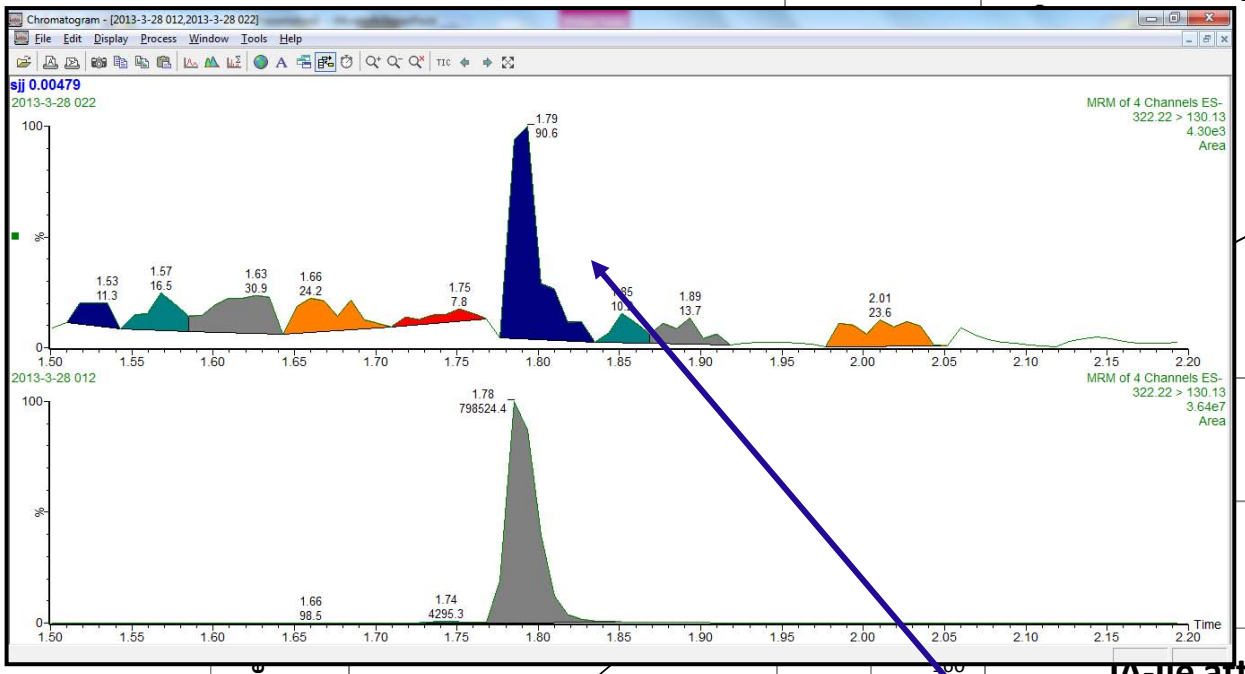


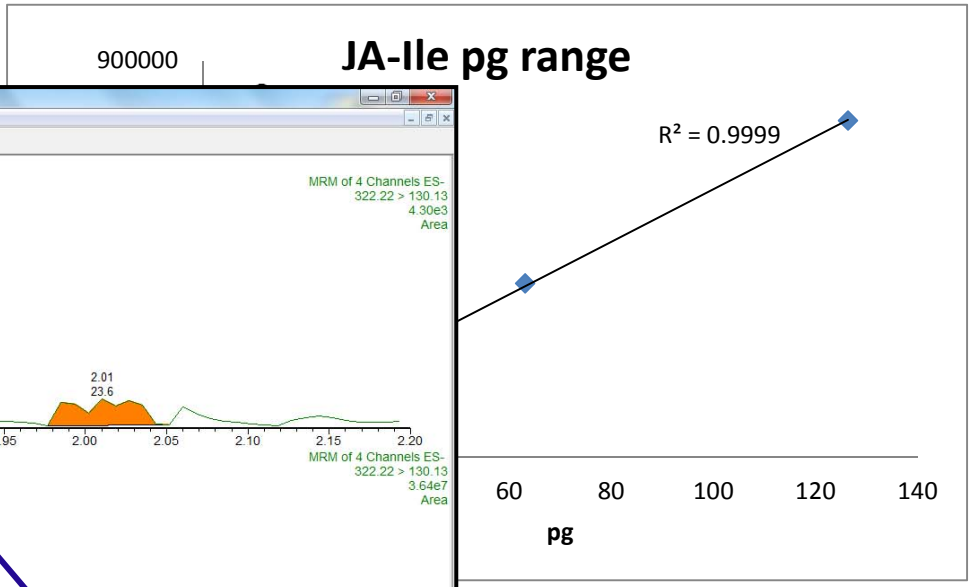
Acyl sugars

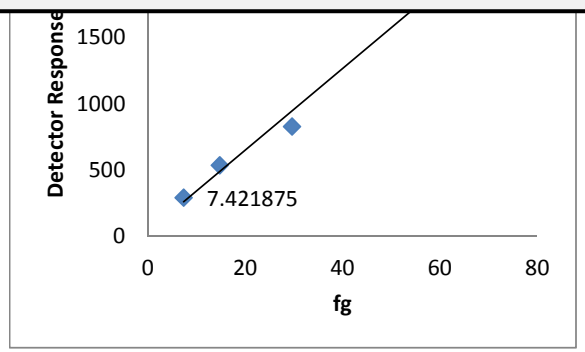


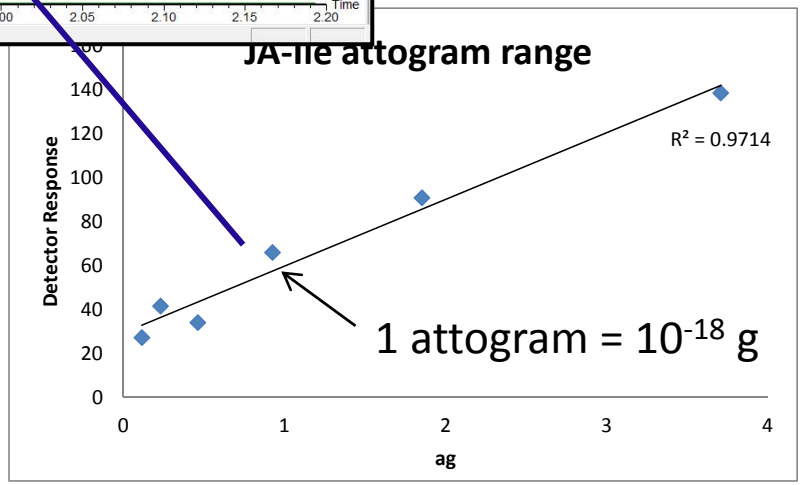
How Sensitive is it? (detection limit)




Chromatogram - [2013-3-28 012,2013-3-28 022]
File Edit Display Process Window Tools Help
sjj 0.00479
2013-3-28 022
MRM of 4 Channels ES-
322.22 > 130.13
4.30e3
Area
1.79
90.6
1.53
11.3
1.57
16.5
1.63
30.9
1.66
24.2
1.75
7.8
1.89
13.7
2.01
23.6
2013-3-28 012
MRM of 4 Channels ES-
322.22 > 130.13
3.64e7
Area
1.78
798524.4
1.66
98.5
1.74
4295.3
%
Time


JA-Ile pg range
900000
R² = 0.9999
60
80
100
120
140
pg


Detector Response
1500
1000
500
0
7.421875
0
20
40
60
80
fg


JA-Ile attogram range
Detector Response
140
120
100
80
60
40
20
0
R² = 0.9714
1 attogram = 10-18 g
0
1
2
3
4
ag

What does this mean in real tissue?

Mechanical wounding 1h

↓

Bulk leaf tissue (~400 mg)

↓

Frozen liquid N2

↓

Grind into powder

↙ ↙ ↓ ↓ ↓ ↘

Divide into smaller weights (mg)

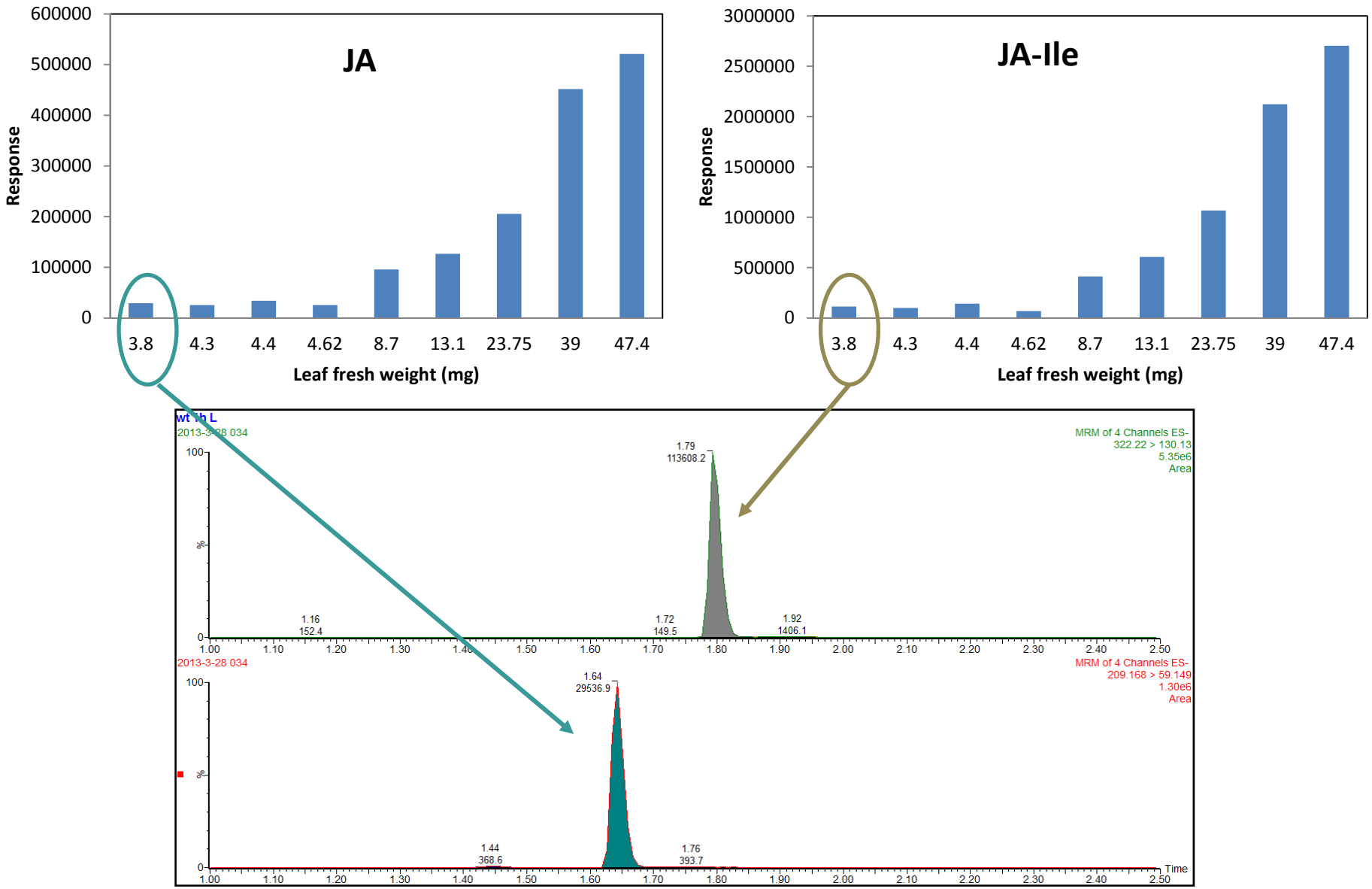
3.8, 4.3, 4.4, 4.62, 8.7, 13.1, 23.75, 39, 47.4, 88.2

↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓

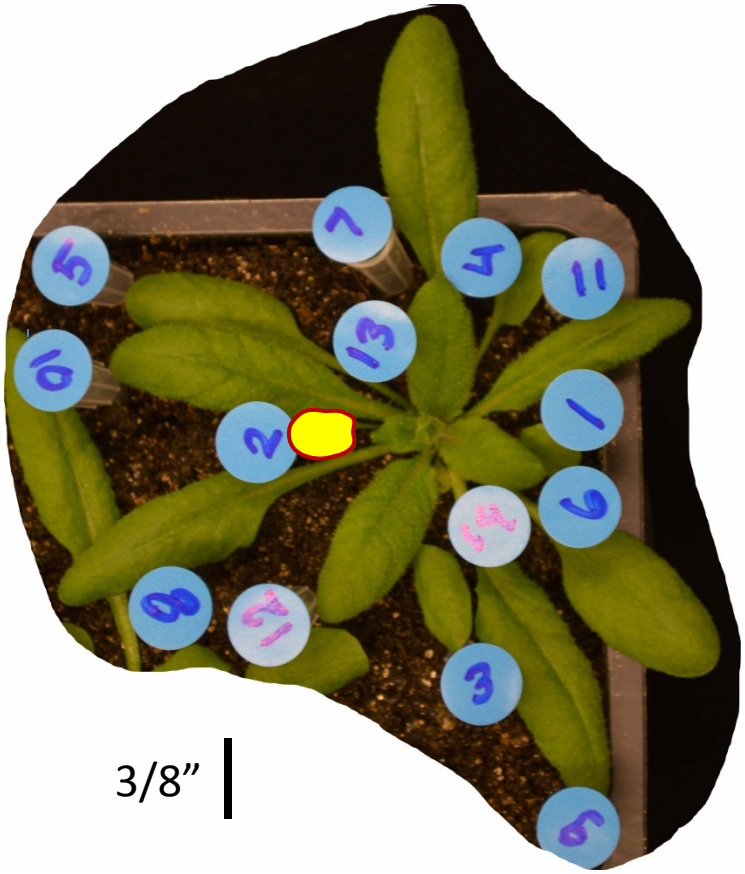
Hormone extraction (100 μL)

↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓

UPLC MS/MS (5 μL)


JA
Response
600000
500000
400000
300000
200000
100000
0
3.8
4.3
4.4
4.62
8.7
13.1
23.75
39
47.4
Leaf fresh weight (mg)
JA-Ile
Response
3000000
2500000
2000000
1500000
1000000
500000
0
3.8
4.3
4.4
4.62
8.7
13.1
23.75
39
47.4
Leaf fresh weight (mg)
2013-3-28 034
MRM of 4 Channels ES-
322.22 > 130.13
5.35e6
Area
1.79
113608.2
1.16
152.4
1.72
149.5
1.92
1406.1
2013-3-28 034
MRM of 4 Channels ES-
209.168 > 59.149
1.30e6
Area
1.64
29536.9
1.44
368.6
1.76
393.7
Time

How small is 3.8 mg?



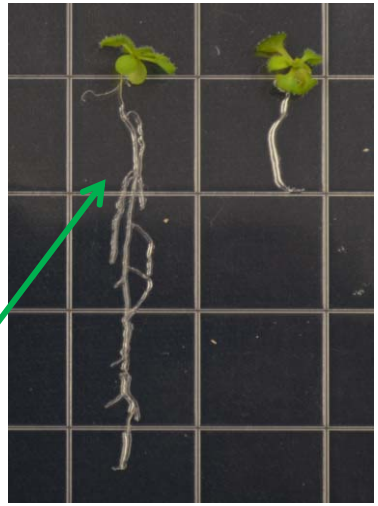
leaf no.	mg
1	6.89
2	7.3
3	22.9
4	20.7
5	39.7
6	50.85
7	83.6
8	65.05
9	81.33
10	67.61
11	45.35
12	41.25
13	32.32
14	16.71

30d old Col-0

Arati Nepal

Hormone analysis from individual leaves possible
-phenotyping of mutants and transgenics

Hormone analysis from individual seedlings



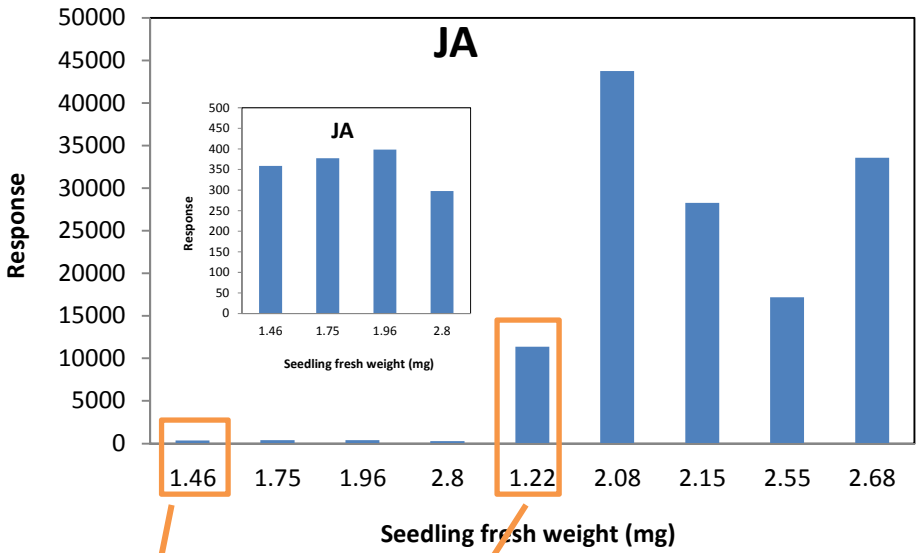
3 %

10 d old plate grown

anbhag

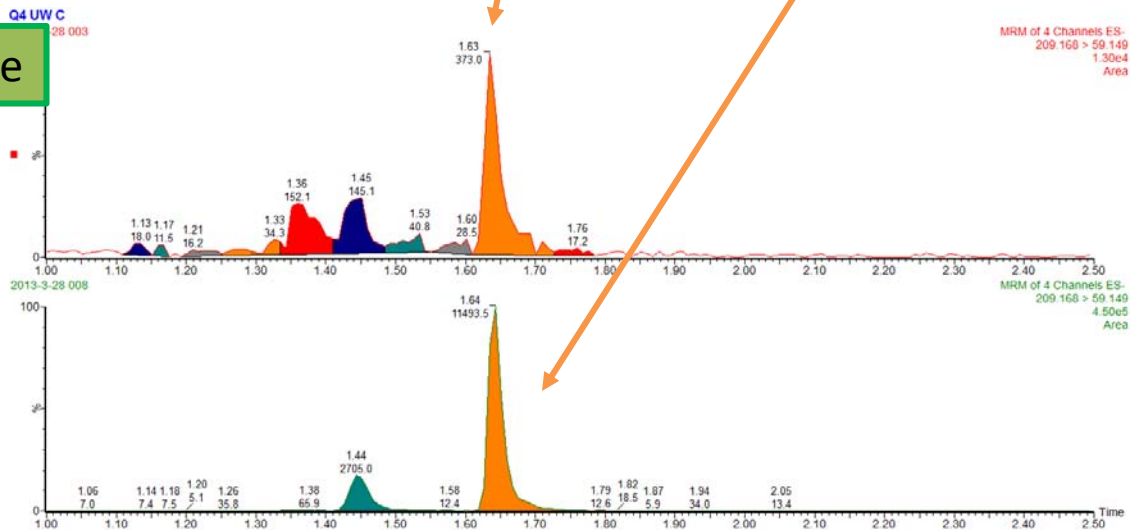
JA-Ile ~40 fold JA

0.08 % ~ 1 µg tissue

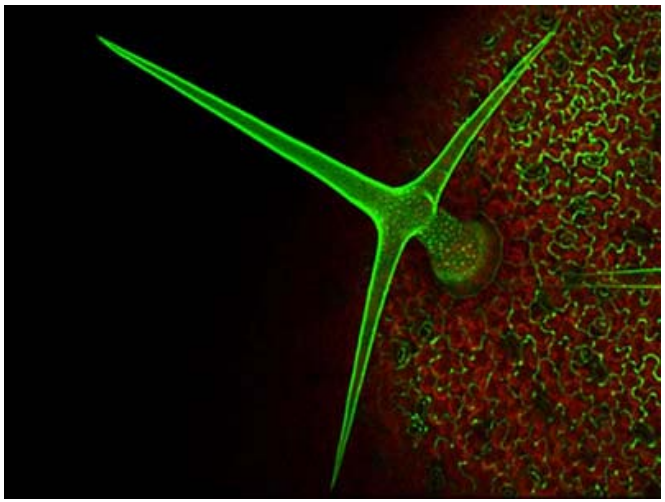


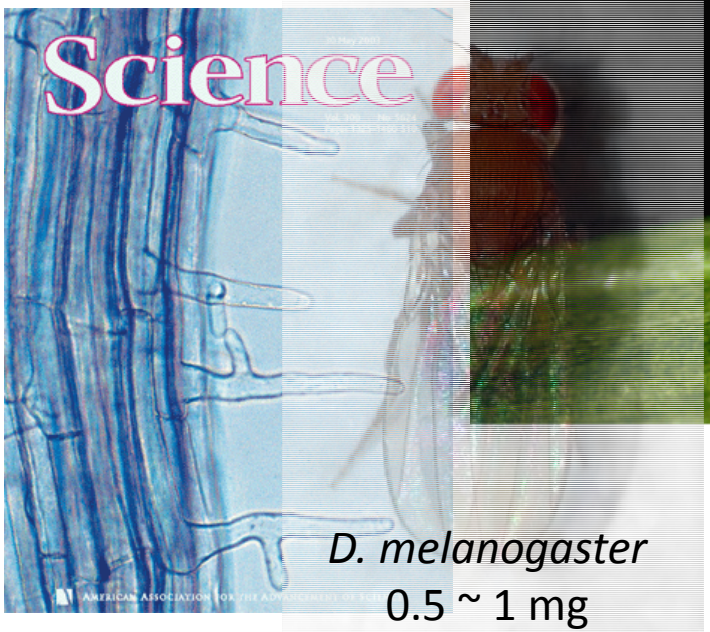
unwounded

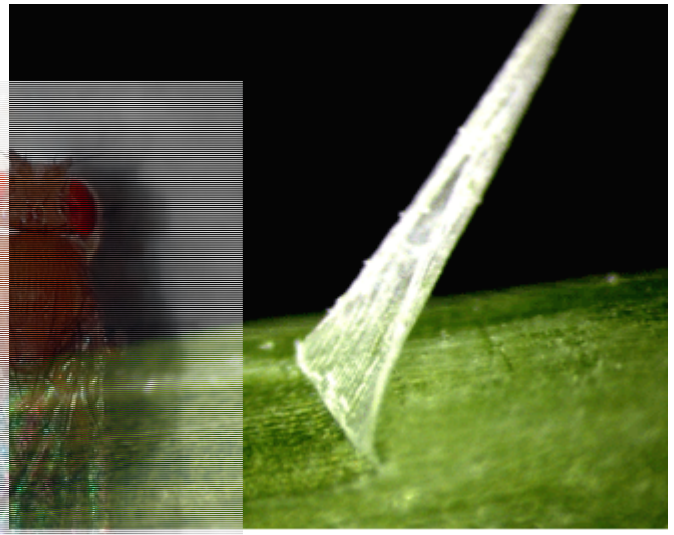
Wounded



Organ specific metabolite analysis



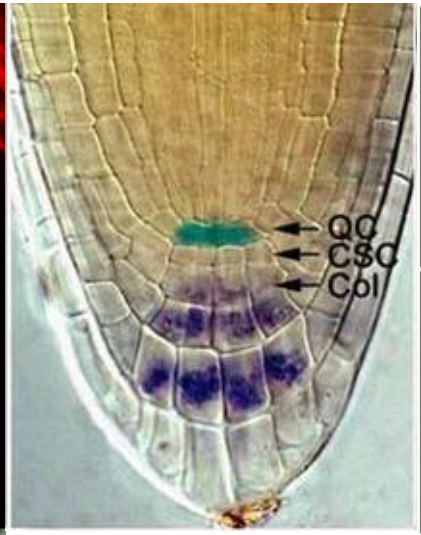


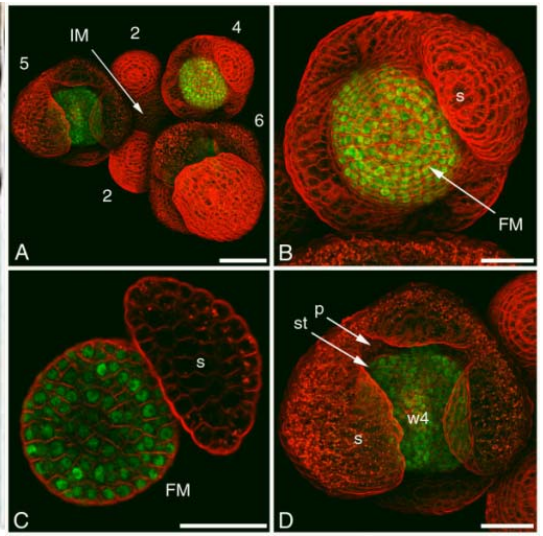


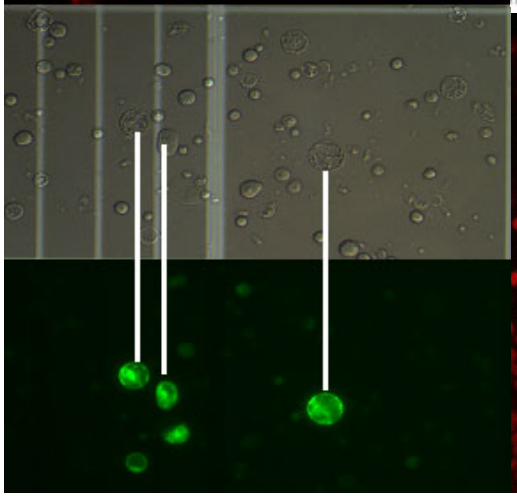
D. melanogaster
0.5 ~ 1 mg

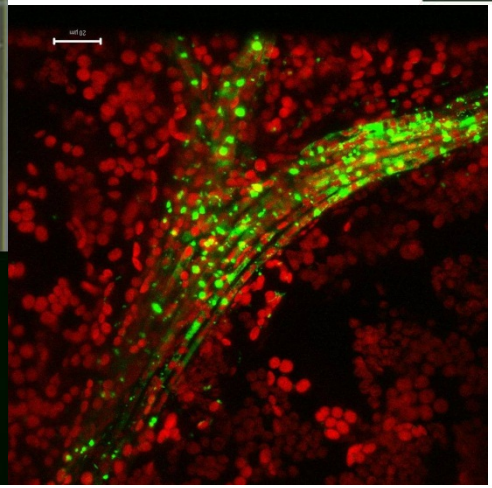
Canola
Soybean
Corn

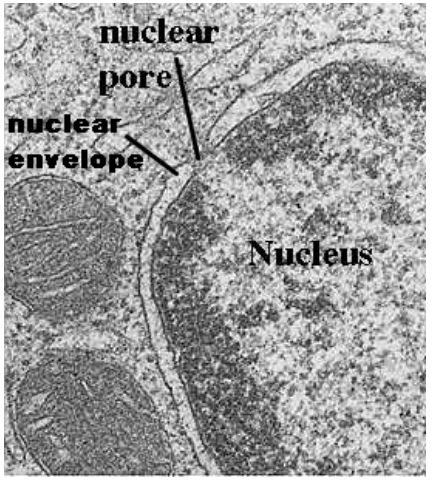
Cell type specific metabolite analysis (abundant metabolites)







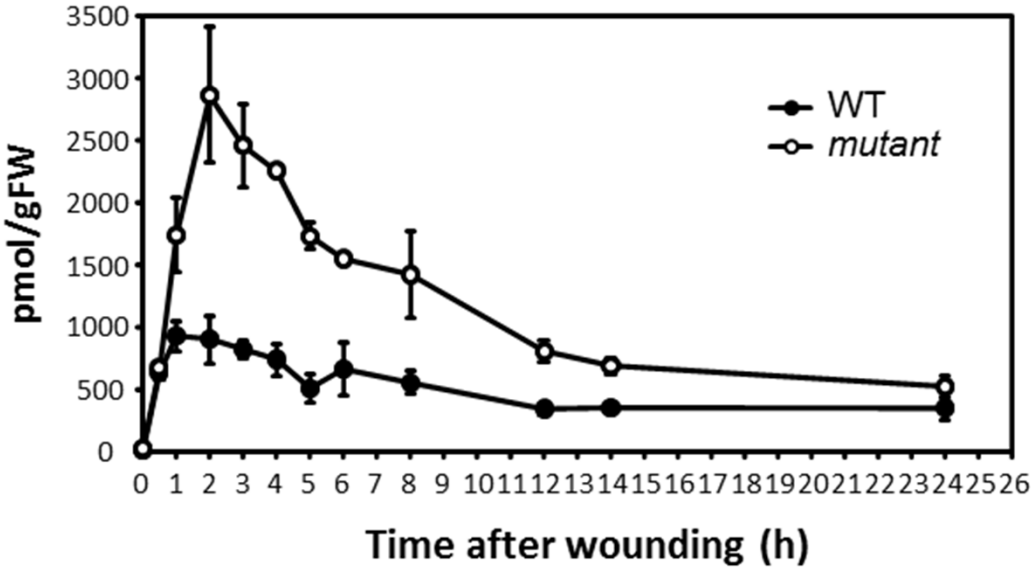




Organelle specific metabolite analysis

Higher Sample Throughput (Shorter Run Time) using UPLC

Conventional chromatography often takes long time!



12 time points X 4 replicates X 2 genotypes = 96 samples

45 minute method X 96 samples = 4320 minutes = 72 hours **$$$**

Higher Sample Throughput (Shorter Run Time) by UPLC-MS/MS

3 min method X 96 samples = 288 minutes = **4.8** hours

Inlet parameters

Column: ACQUITY UPLC® BEH C18 **1.7 µm**, 2.1 x 50 mm
Mobile phase A: Water, 0.1 % formic acid
Mobile phase B: Methanol
Column Temperature: 40 °C
Flow rate: **0.4 mL/min**
Injection volume: 5 µL

Time (min)	Flow Rate (mL/min)	%A	%B
0.00	0.4	70	30
1.50	0.4	0	100
2.50	0.4	0	100
2.51	0.4	70	30
3.00	0.4	70	30

Higher Sample Throughput (Shorter Run Time) by UPLC-MS/MS

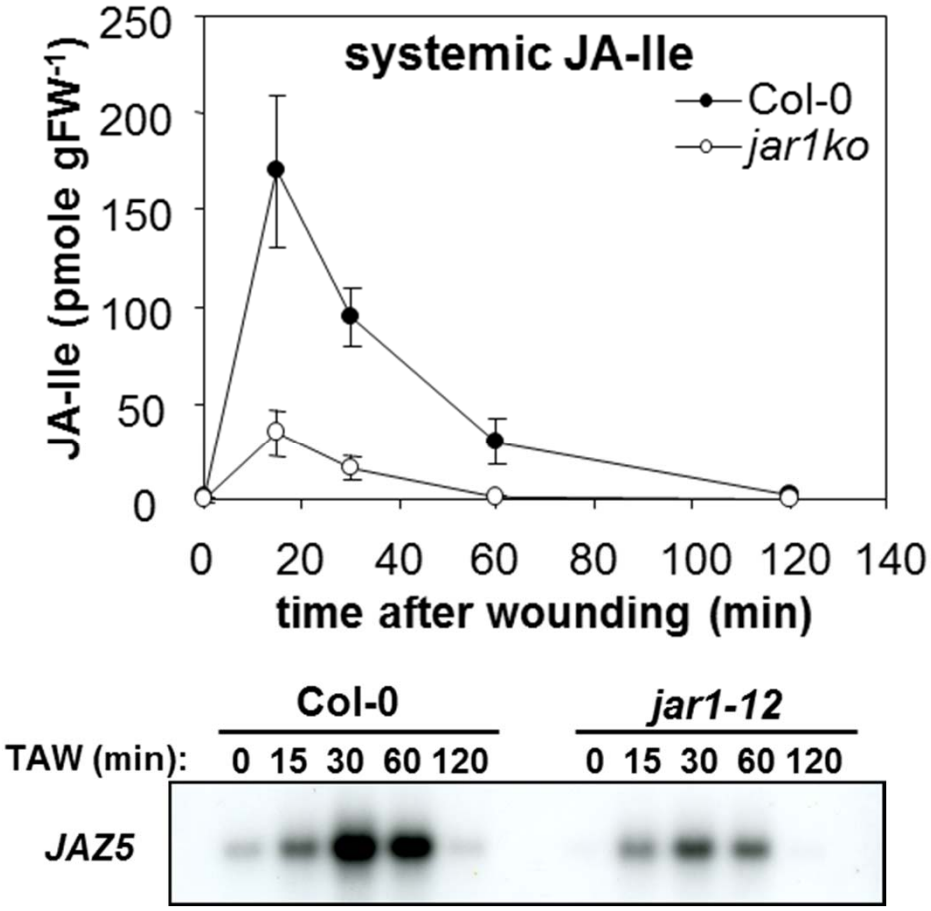
Higher operating pressure (back pressure) → ACQUITY UPLC H-Class (**15,000 psi** or 1,034 bar)

- Narrower peak width require **faster detector sampling rate**
- Low system and dwell volume (minimal dispersion in the detector cell)
- Injection valves should prevent extreme pressure fluctuations on column
- Fast injection cycle time
- Low volume injections with minimal carryover

Applications that need running many samples

Not just yes/no but How Much?

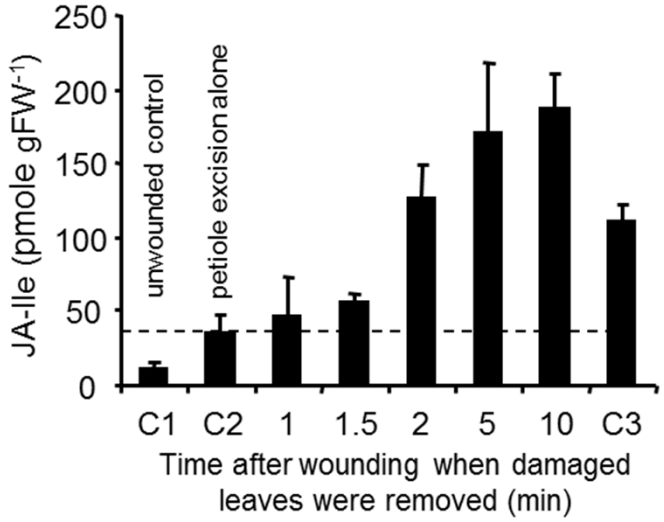
Triple Quadrupole good for Quantitative Analysis



Not just yes/no but How Much?

Triple Quadrupole good for Quantitative Analysis

systemic 15 min post wounding



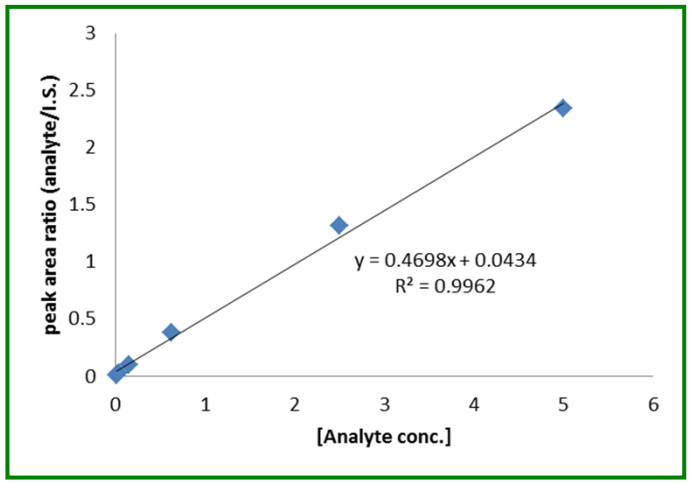
Not just yes/no but How Much?

Triple Quadrupole good for Quantitative Analysis

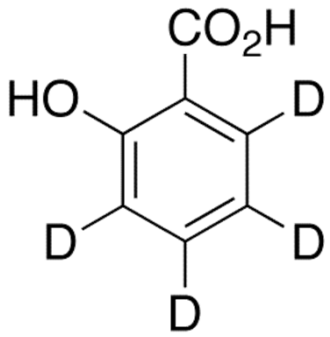
Internal standards (to account for Experimental Drift)

- very similar but not identical to the analyte
- known amount added before extraction or LC injection

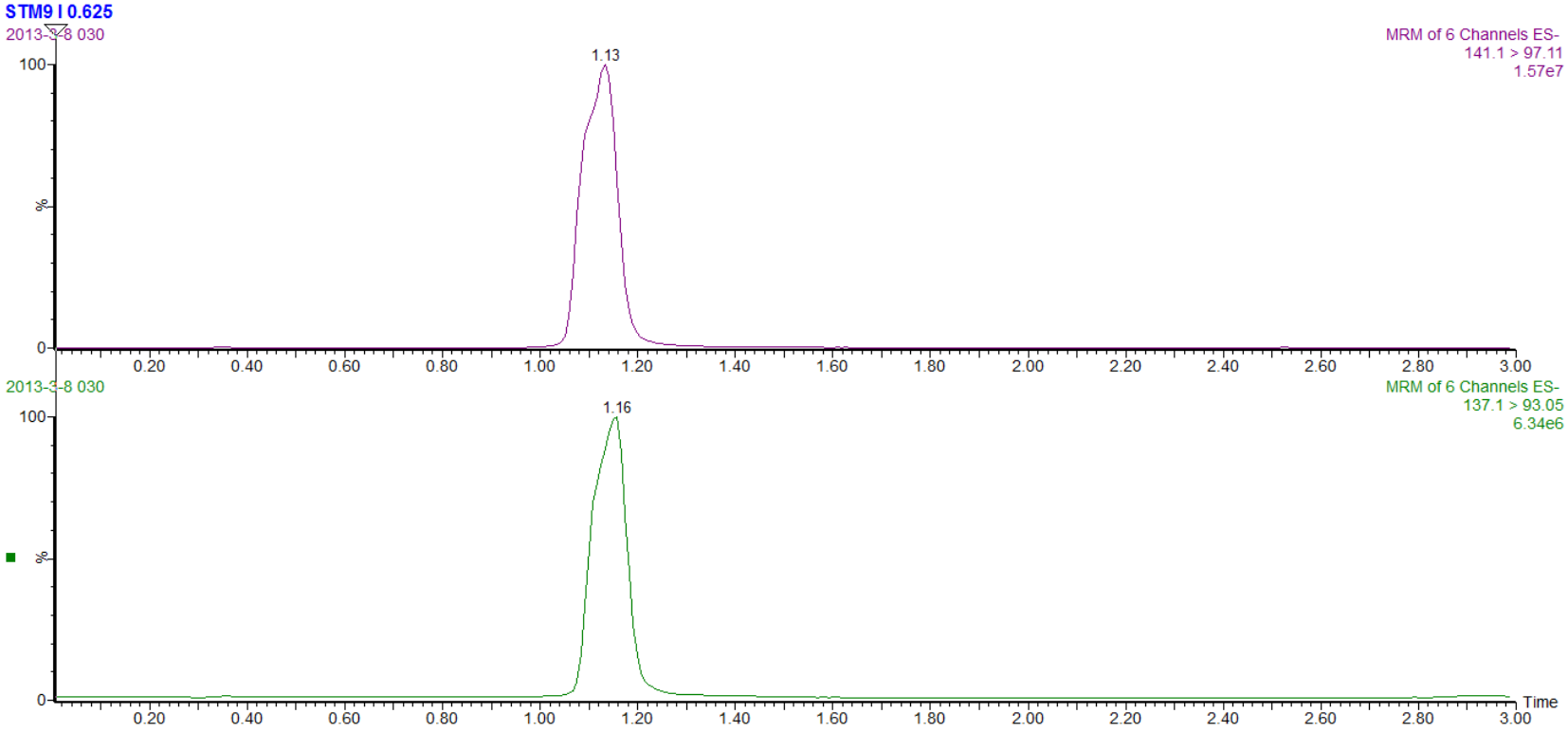
Calibration curves



Example of an Internal Standard



	MRM transition $[M-H]^-$ (m/z)
d4-SA	141 > 97
SA	137 > 93



Simple and Quick Sample Preparations

- No derivatization necessary

Things to consider

How much tissue sample is needed? (final volume can be adjusted)

Record tissue weight (or other **normalization** factors)

Solubility in extraction solvent / buffer

Extractability (tissue homogenization)

Rapid Quenching (stop chemical reactions, hot isopropanol)

Stability (temp, pH, purge w/ inert gas, anti-oxidants, fresh extract, storage)

Remember to include known amount of **Internal Standard**

Remove insoluble debris (column life, filter / precipitate / centrifuge hard)

Sample Prep Example:
Jasmonate Extraction Protocol

Tissue collect

↓

Weigh

F ep with beads)

T

A 0.5% acetic acid)

M

M 00 RPM 30 min

Tr

Larger Scale (0.1- 1g)

Tissue collect

↓

Weigh

↓

Freeze in liquid N2 (until use)

↓

Grind

↓

Add I.S. and Ethyl Acetate and Mix

↓

Spin down (10,000 x g)

↓

Transfer organic phase to glass tubes

↓

Adjust pH and re-extract from pellet

↓

Dry down under N2 gas / heating

↓

Reconstitute dried residue in 70% methanol

↓

Keep in -20 °C

↓

Micro-centrifuge 4 °C 13,000 RPM 30 min

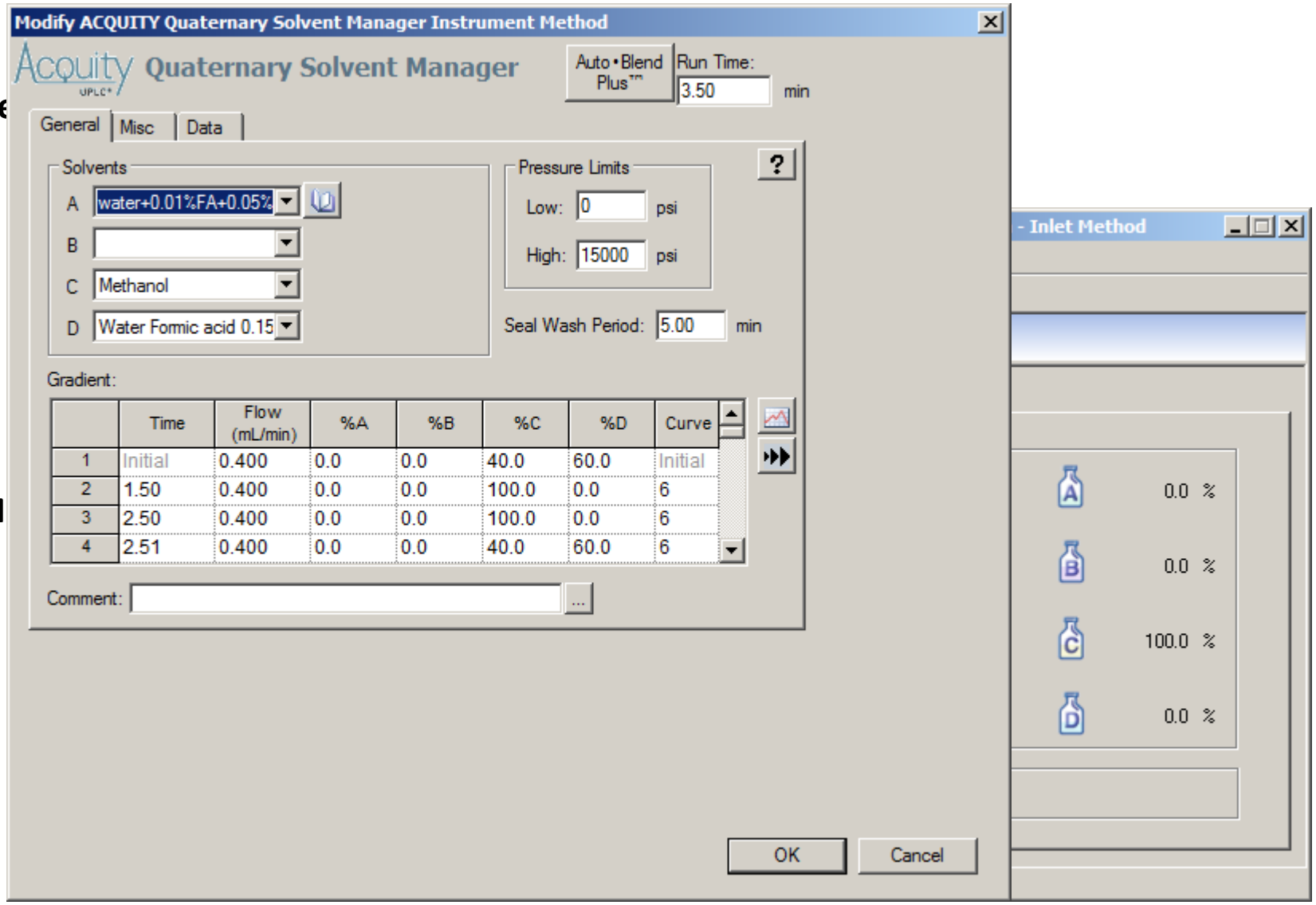
↓

Transfer to LC vials

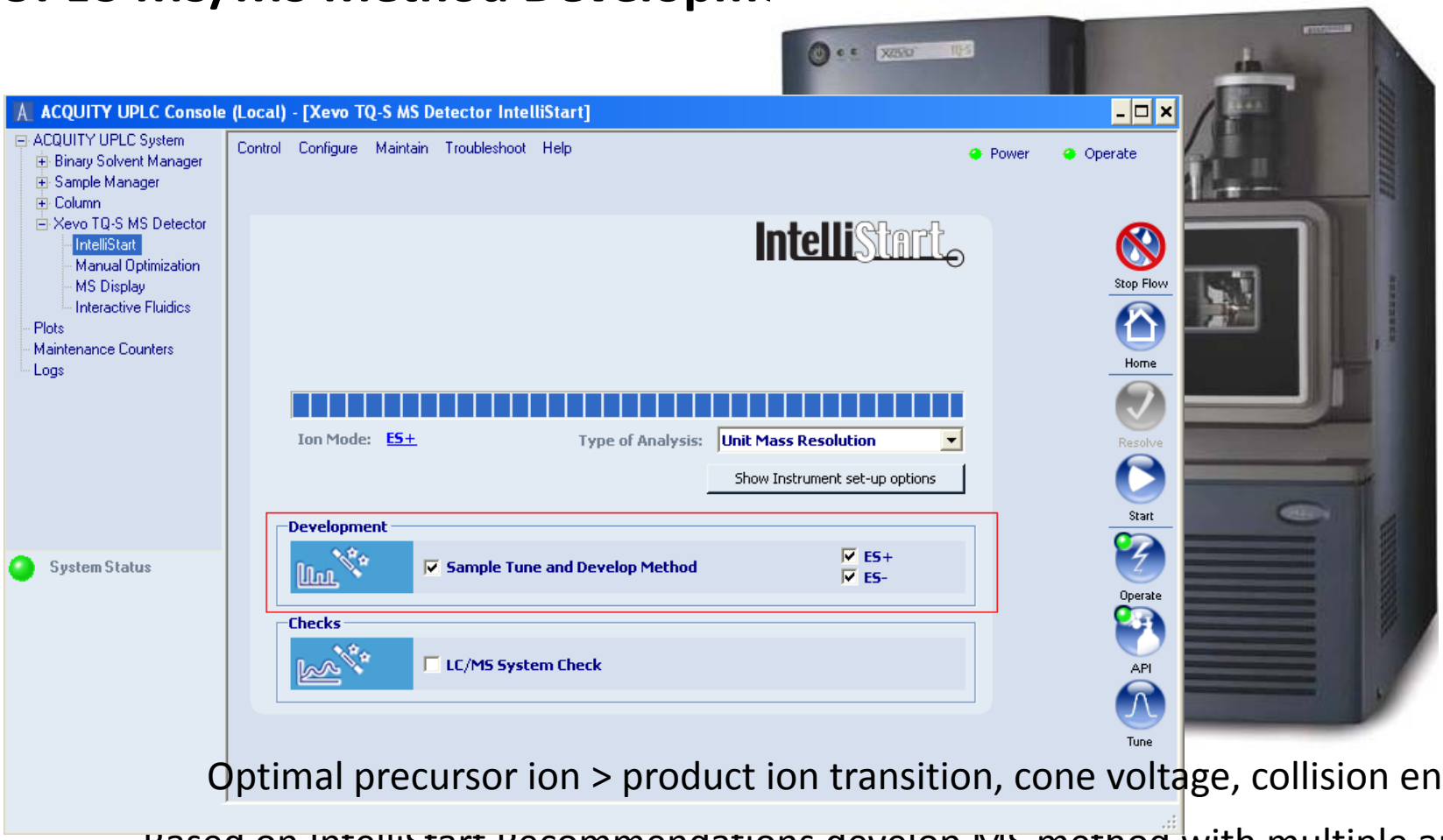
UPLC-MS/MS Method Development

LC Me

MS M



UPLC-MS/MS Method Development

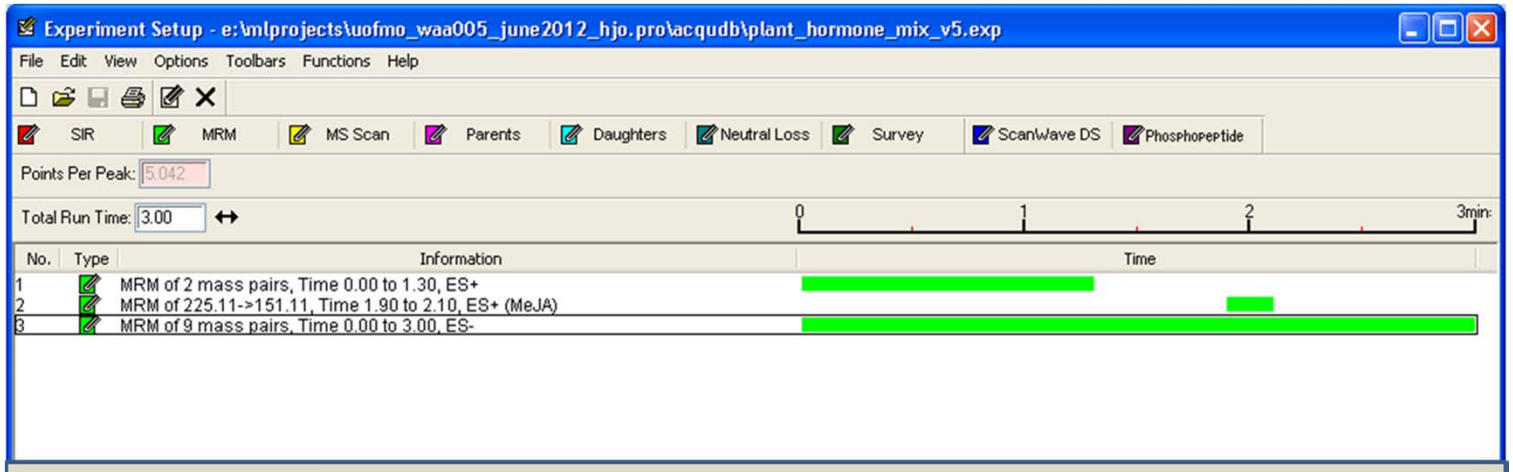


Optimal precursor ion > product ion transition, cone voltage, collision energy

- Based on IntelliStart Recommendations develop MS method with multiple analytes

UPLC-MS/MS Method Development

MS method editor



F1 (ES+)
(0-1.3 min)

	Compound Na	Parent (m/z)	Daughter (m/z)	Aut	Dwell (s)	Cone (V)	Collision (V)
1	MeSA	153.0200	135.0000	☐	0.025	35	8
2	IAA	176.0314	130.0604	☐	0.025	16	16

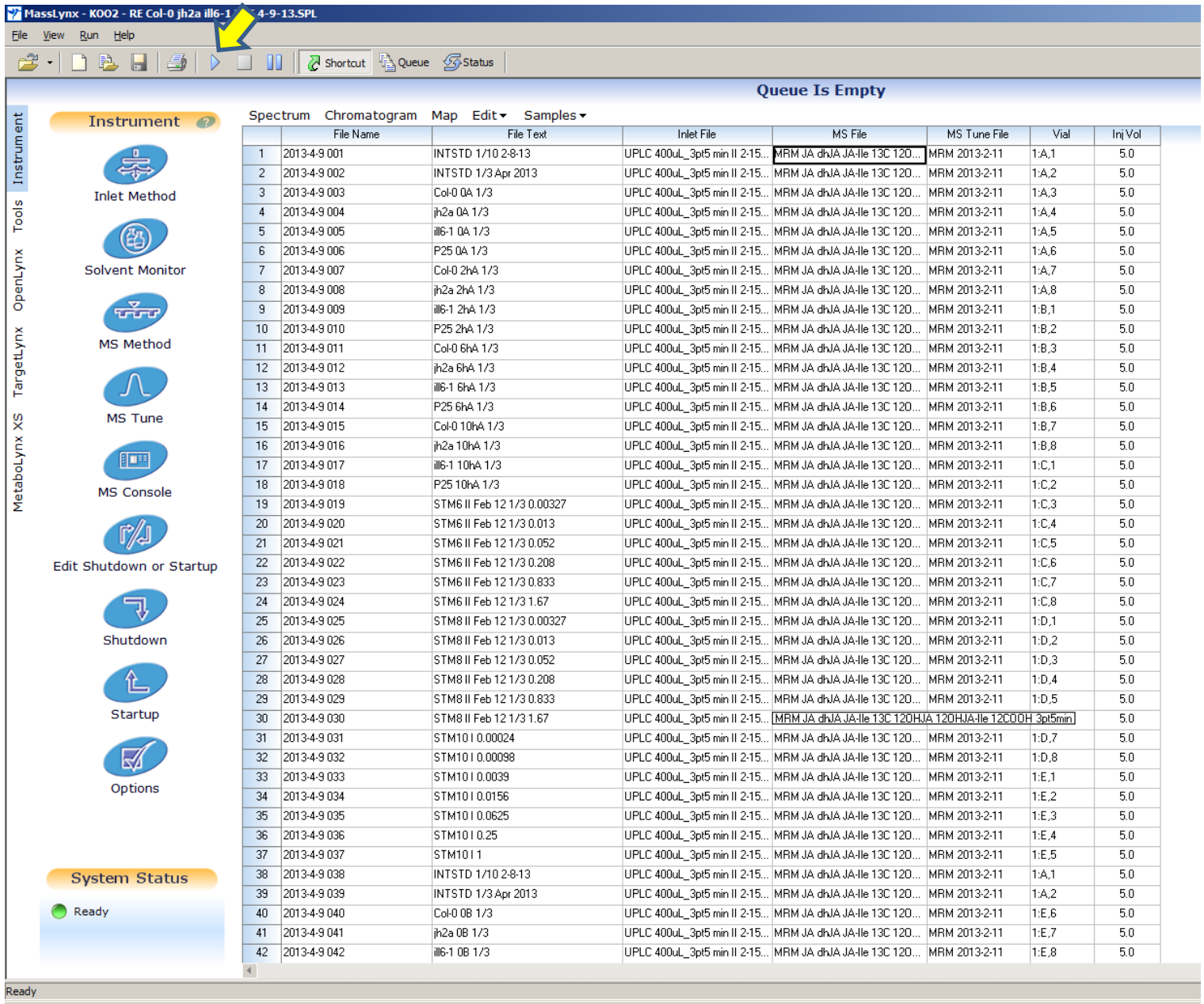
F2 (ES+)
(1.9-2.1 min)

	Compound Na	Parent (m/z)	Daughter (m/z)	Auto	Dwell (s)	Cone (V)	Collision (V)
1	MeJA	225.1093	151.1060	☐	0.025	2	14

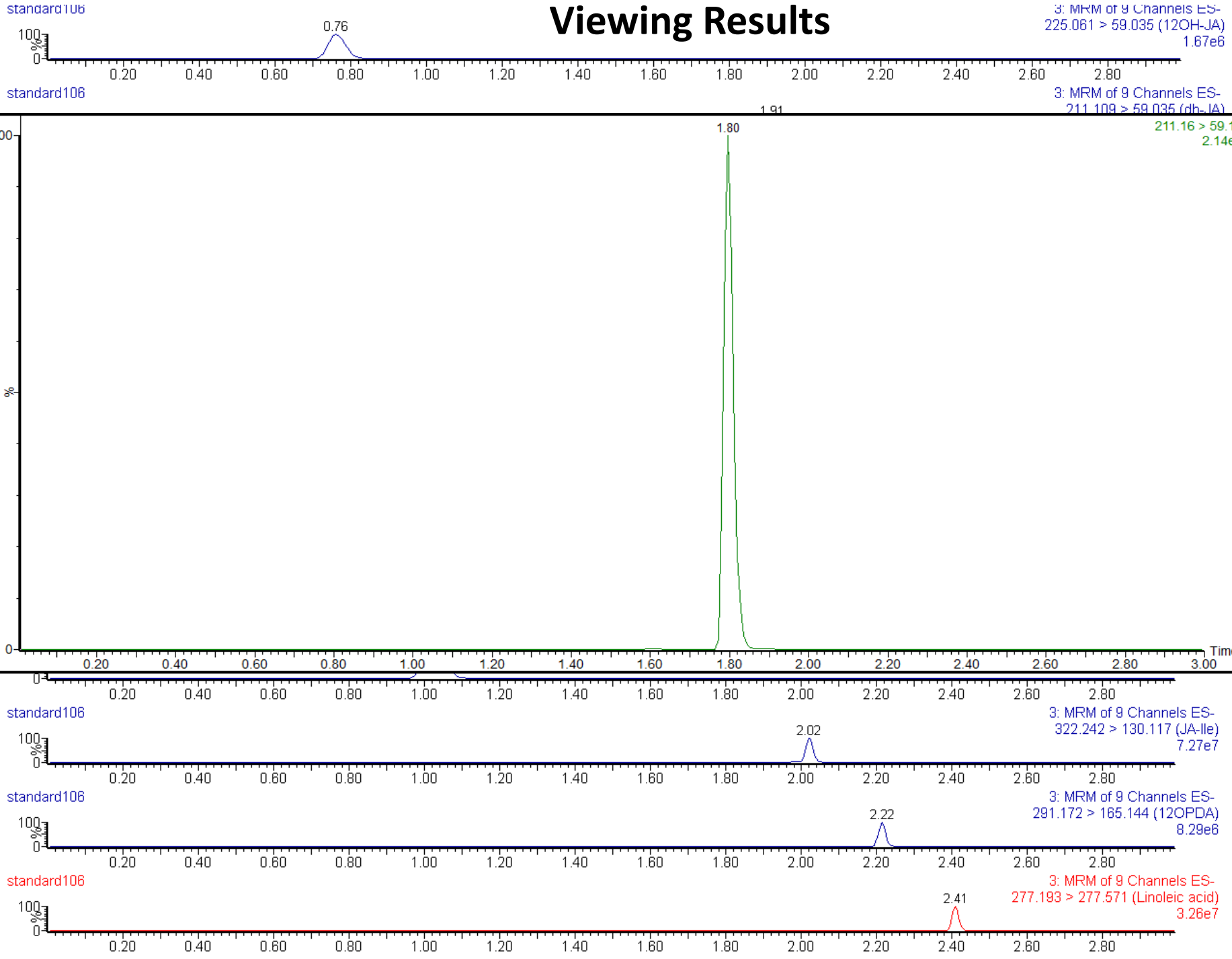
F3 (ES-)
(0-3 min)

	Compound Na	Parent (m/z)	Daughter (m/z)	Auto	Dwell (s)	Cone (V)	Collision (V)
1	SA	136.9998	92.9775	☐	0.025	32	14
2	JA	209.0937	59.0384	☐	0.025	32	12
3	dh-JA	211.1093	59.0350	☐	0.025	6	14
4	12OH-JA	225.0612	59.0352	☐	0.025	28	14
5	Linoleic acid	277.1927	277.5712	☐	0.025	44	18
6	12OPDA	291.1719	165.1438	☐	0.025	2	20
7	JA-Ile	322.2416	130.1169	☐	0.025	34	20
8	GA3	345.1735	239.1464	☐	0.025	36	14
9	Rutin	609.1215	300.1908	☐	0.025	60	36

Running the Sample



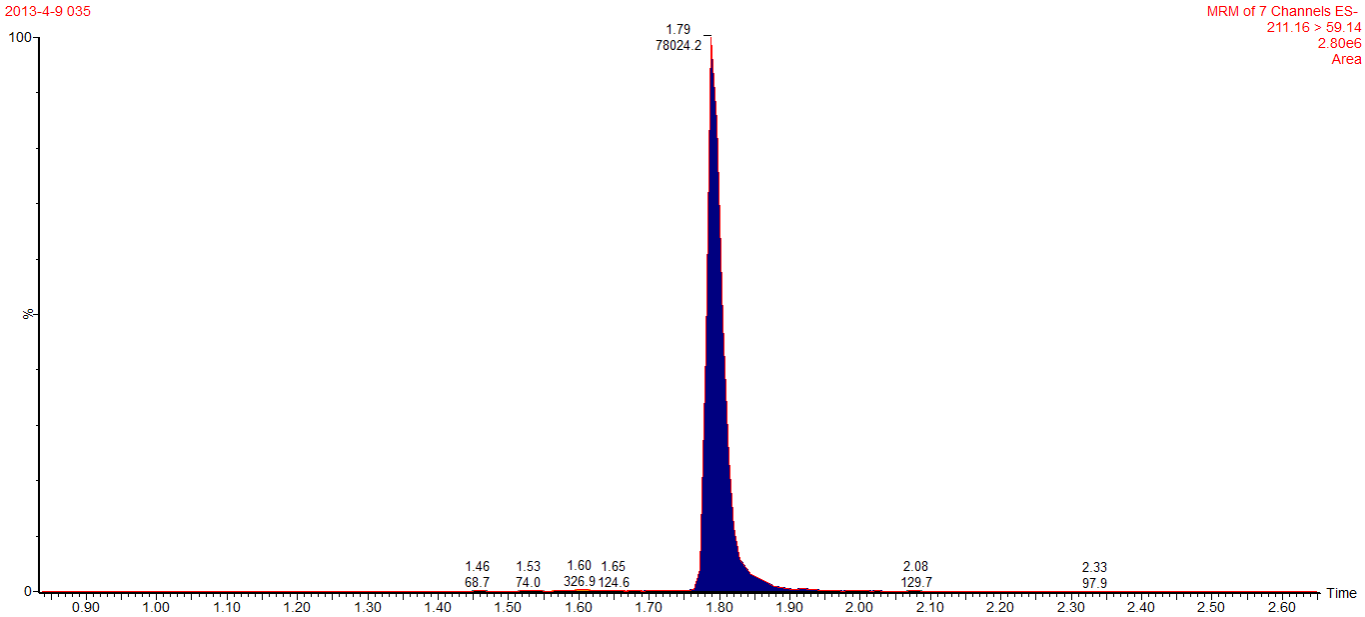
Viewing Results



Data Analysis

-manual

-TargetLynx



#	Name	Sample Text	Type	Std. Conc	RT	IS Area	Area	Response	Conc.
1	standard083	Blank, 5 ul inj.	Blank						
2	standard084	Mix, 0.4166 nM, 5 ul inj.	Standard	0.417	1.91		59	58.501	0.3396
3	standard085	Mix, 4.166 nM, 5 ul inj.	Standard	4.166	1.91		593	593.480	4.0583
4	standard086	Mix, 41.66 nM, 5 ul inj.	Standard	41.660	1.91		6506	6505.699	45.1543
5	standard087	Mix, 416.6 nM, 5 ul inj.	Standard	416.600	1.91		62892	62892.266	437.0986
6	standard088	Mix, 833.3 nM, 5 ul inj.	Standard	833.300	1.91		113679	113678.523	790.1151
7	standard092	Blank, 5 ul inj.	Blank						
8	standard093	Mix, 0.4166 nM, 5 ul inj.	Standard	0.417	1.91		67	66.805	0.3974
9	standard094	Mix, 4.166 nM, 5 ul inj.	Standard	4.166	1.91		608	607.897	4.1585
10	standard095	Mix, 41.66 nM, 5 ul inj.	Standard	41.660	1.91		6702	6701.528	46.5155
11	standard096	Mix, 416.6 nM, 5 ul inj.	Standard	416.600	1.91		63870	63869.531	443.8916
12	standard097	Mix, 833.3 nM, 5 ul inj.	Standard	833.300	1.91		114581	114580.672	796.3860
13	standard101	Blank, 5 ul inj.	Blank						
14	standard102	Mix, 0.4166 nM, 5 ul inj.	Standard	0.417	1.91		58	58.281	0.3381
15	standard103	Mix, 4.166 nM, 5 ul inj.	Standard	4.166	1.91		613	612.683	4.1918
16	standard104	Mix, 41.66 nM, 5 ul inj.	Standard	41.660	1.91		6950	6949.885	48.2418
17	standard105	Mix, 416.6 nM, 5 ul inj.	Standard	416.600	1.91		65053	65052.539	452.1147
18	standard106	Mix, 833.3 nM, 5 ul inj.	Standard	833.300	1.91		117320	117319.914	815.4265

Summary

- UPLC-MS/MS for targeted analysis of known metabolites
- Highly sensitive detections
- Short running time, increased throughput
- Quantitative analysis
- Quick and simple sample preparations
- Overview of UPLC-MS/MS steps
- User friendly software interface
- Robust system (low maintenance / large sample number)

What is your compound of interest?

ENVIRONMENTAL Science & Technology

ARTICLE

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Quantification of a Male Sea Lamprey Pheromone in Tributaries of Laurentian Great Lakes by Liquid Chromatography−Tandem Mass Spectrometry

Xiaodan Xi,[†] Nicholas S. Johnson,[†,∥] Cory O. Brant,[†] Sang-Seon Yun,[†,⊥] Keali L. Chambers,[†] A. Daniel Jones,[‡,§] and Weiming Li*[,†]