

# MS/MS interpretation in identification of unknowns

Jeevan Prasain, PhD

[jprasain@uab.edu](mailto:jprasain@uab.edu)

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## Outline

- Introduction
- How to interpret LC-MS and MS/MS data.
- Identification of some conjugated metabolites.
- Sensitivity enhancement through derivatization
- Dereplication of natural products, sub-structure analysis of unknowns

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Triglycerides

Genistein  
(a plant secondary metabolite)

Taxol

## Small molecules are important!!

- 89% of all known drugs and 50% of all drugs are derived from pre-existing metabolites.
- Small molecules are cofactors and signalling molecules to 1000's of proteins.
- 100,000 (lipidome)

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## Knowing the unknowns

NMR  
(non-destructive,  
lack of sensitivity for detecting  
minor compounds)

LC-MS/MS  
(selective, highly sensitives)

High  
resolution)

MS/MS

public/commercial  
mass spec chemical database

An unknown compound is a small molecule that can reproducibly be detected and quantified in a metabolomics experiment, but whose chemical identification has not been elucidated yet (Krumsiek et al., 2012).

Unknown unknown- not previously cited (Little et al., 2011)

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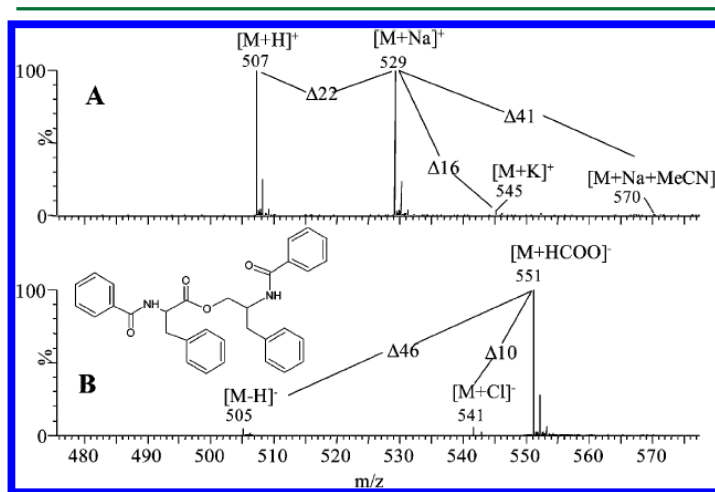
**Keys to identifying unknown structures (putative/definitive) by mass spectrometry**

- Knowing the precursor ion
- Retention time of metabolites in LC
- Accurate mass
- Isotope distribution
- Nitrogen rule
- Fragmentation pattern of a precursor ion
- Product/precursor ion intensity ratio
- Comparison with authentic standards (definitive)

Moco et al. Trends in Analytical Chemistry, 2007

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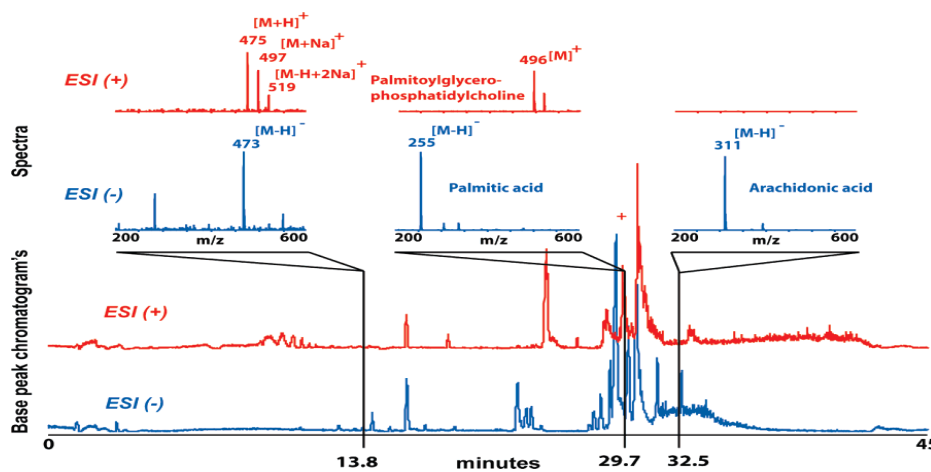
**Adduct formation in +/-ve ion modes**



Nielsen et al., J Nat Prod. 2011

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## Increasing metabolite coverage using +ve and -ve ion mode



Representative Q1 scans of a methanolic extract of human blood serum

Source: Nordstrom et al. Analytical Chemistry, 2007

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## Isotopic distribution and MS

1H = 99.9%, 2H = 0.015%

12C = 98.9%, 13C = 1.1%

35Cl = 75.7%, 37Cl = 24.2%

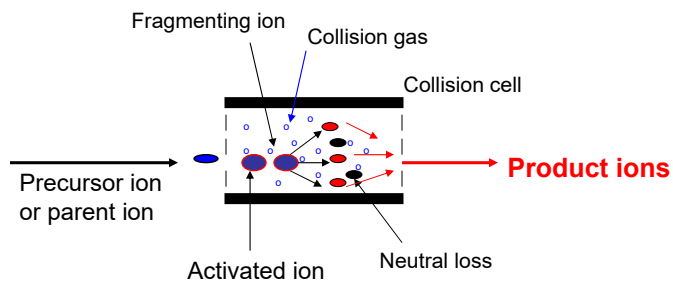
**Monoisotopic mass** - the mass of the most abundant isotope

**Average mass** - the abundance weighted mass of all isotopic components.

<https://www2.chemistry.msu.edu/faculty/reusch/OrgPage/mass.htm>

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## What is Collision Induced Dissociation (CID) or Collisionally Activated Dissociation (CAD) ?



Schematic of CID fragmentation

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## Applications of MS/MS

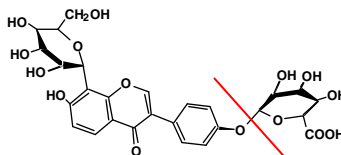
- **Pharmaceuticals-** Identification and quantification of drug metabolites, PK/PD
- **Academic/biotechnology-** analysis of protein/peptides, authentication and profiling of chemical components in a crude mixture, substructure analysis of unknown components
- **Clinical-** eg. neonatal screening, steroids in athletes etc.
- **Environment-** eg. dioxins in fish..
- **Geological-** eg. oil compositions...

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## Interpreting MS/MS spectra

- Likely sites of protonation or deprotonation.
- Likely leaving group after cleavage (C-C vs C-O bond)
- Relative abundance of fragment ions
- Neutral molecules are lost

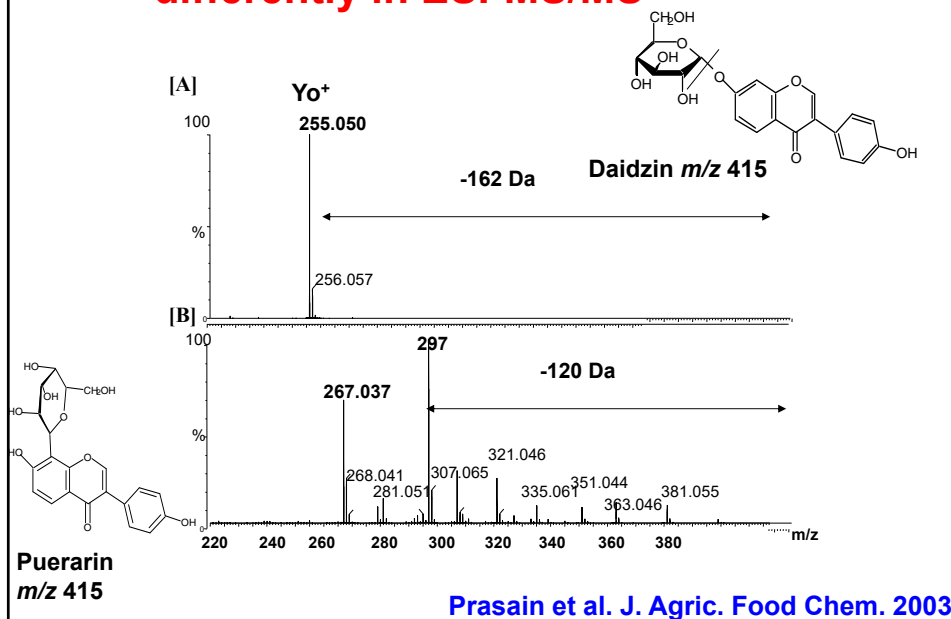
Where are the sites of deprotonation/protonation?  
What is the most likely leaving group in this molecule?



Fragmentation always follows the basic rules of chemistry- resonance stabilization

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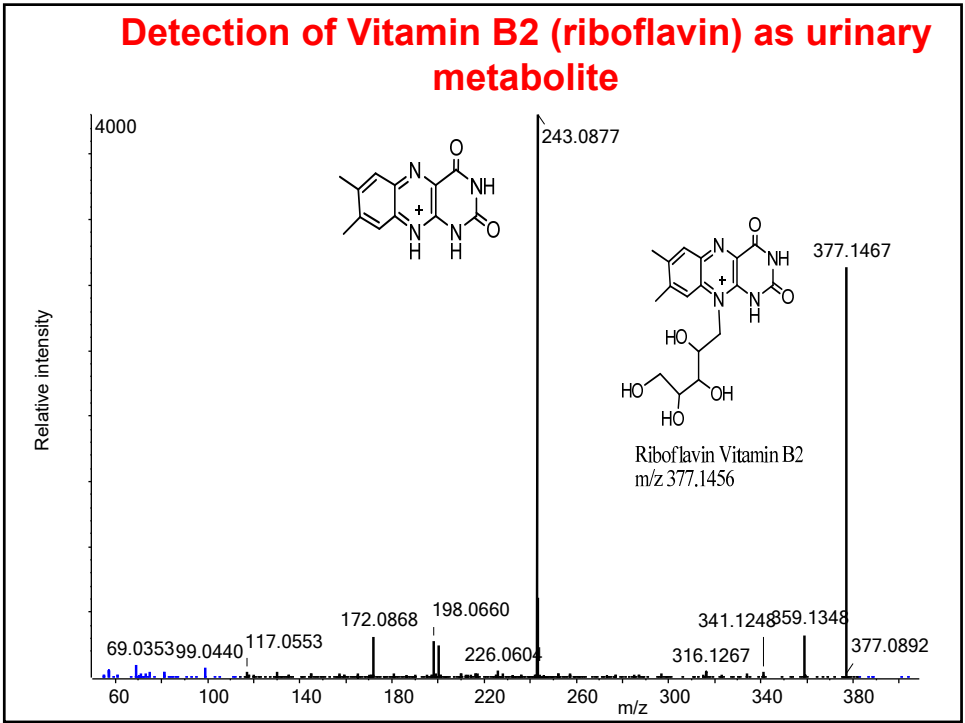
## O- and C-glucosides fragment differently in ESI-MS/MS



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- XCMSonline screen shot showing list of potential
- metabolites, with p-values and fold changes

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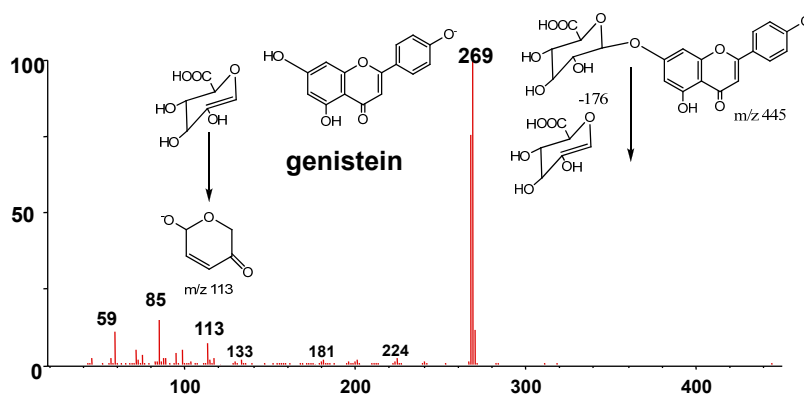
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## Ion fragmentation for identification of phase II drug metabolites (glucuronide/sulfate conjugates)

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### What fragment ions are characteristic for glucuronide conjugates?

#### Product ion spectrum of genistein glucuronide in ESI-MS/MS

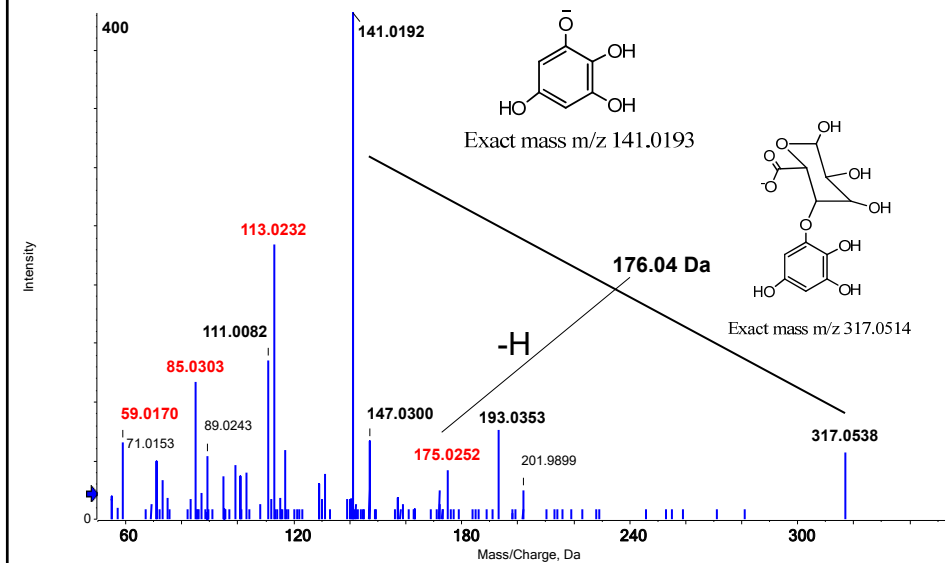


Glucosides/glucuronides conjugates are easily cleaved off by higher potential at orifice

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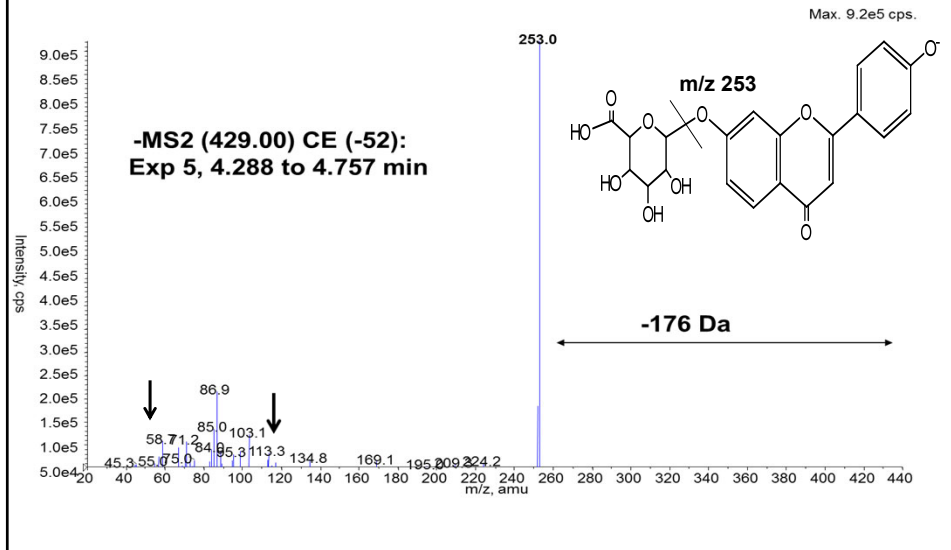


## Putative identification- a glucuronide conjugate of tetrahydroxybenzene



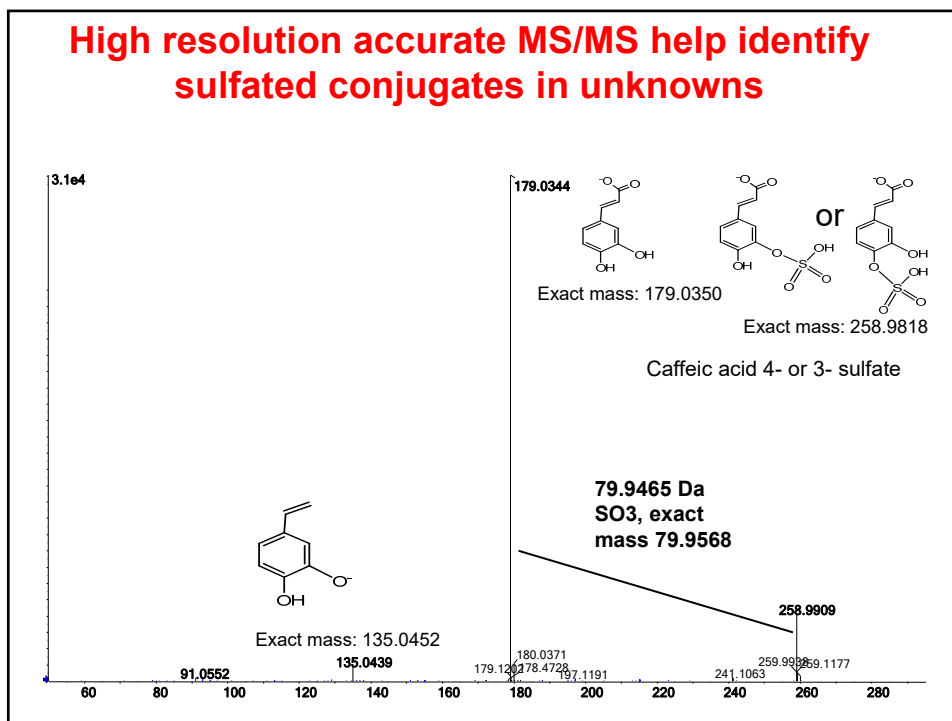
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## MSMS of m/z 429 indicate that it may be daidzein glucuronide



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## High resolution accurate MS/MS help identify sulfated conjugates in unknowns



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## Change in mass is associated with possible metabolic reaction

Metabolic rxn	Change in mass
Methylation	14
Demethylation	-14
Hydroxylation	16
Acetylation	42
Epoxidation	16
Desulfuration	-32
Decarboxylation	-44
Hydration	18
Dehydration	-18

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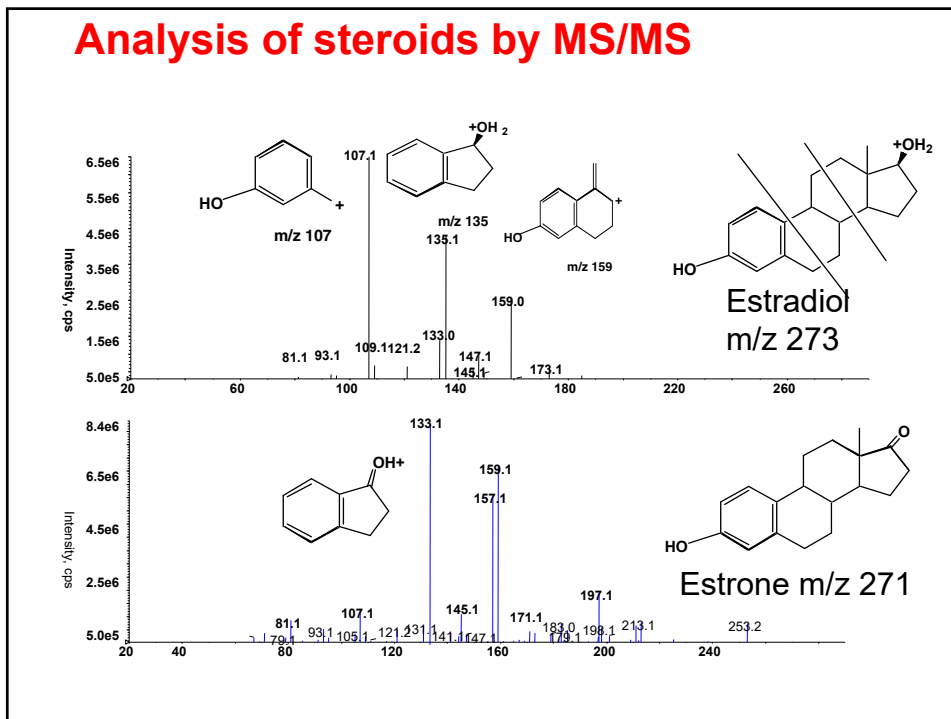
## Characteristic neutral loss and precursor ion scans for conjugated metabolites

Conjugate	Ionization mode	Scan
Glucuronides	pos/neg	NL 176 amu
Hexose sugar	pos/neg	NL 162 amu
Pentose sugar	pos/neg	NL 132 amu
Phenolic sulphate	pos	NL 80 amu
Phosphate	neg	Precursor of m/z 79
Aryl-GSH	pos	NL 275 amu
Aliphatic-GSH	pos	NL 129
taurines	Pos	Precursor of m/z 126
N-acetylcysteins	neg	NL 129 amu

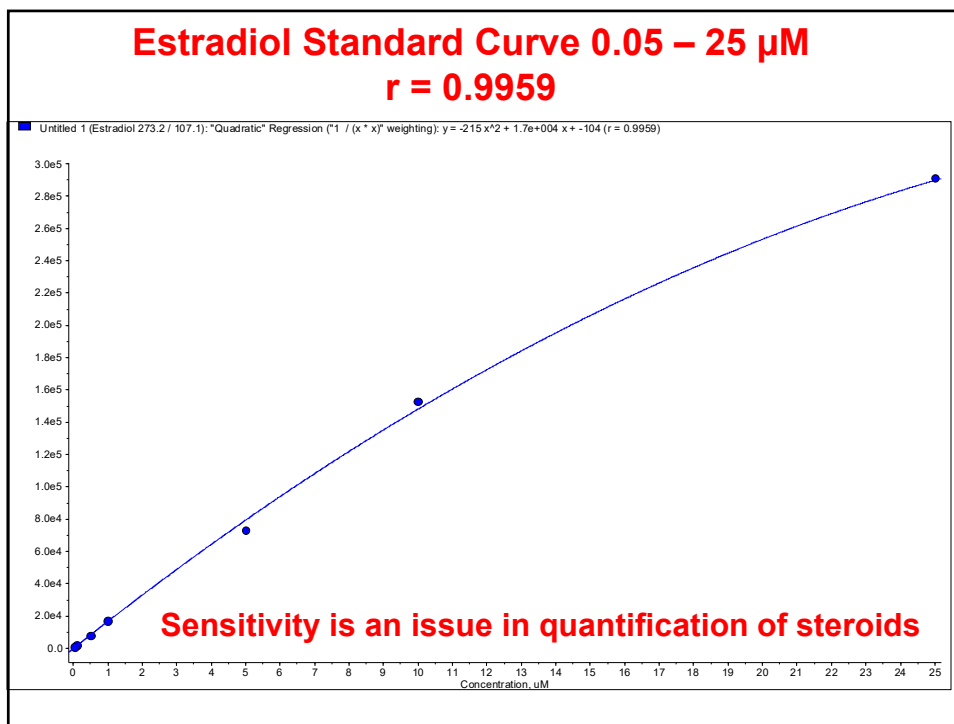
**NL = neutral loss.** **Kostiainen et al., 2003**

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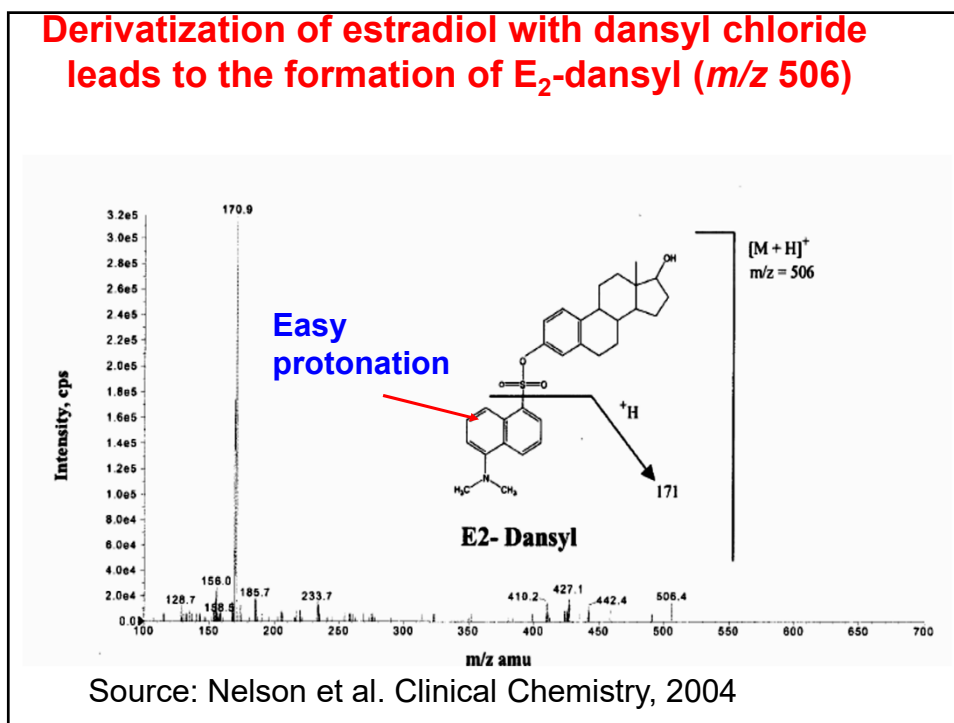
## Analysis of steroids by MS/MS



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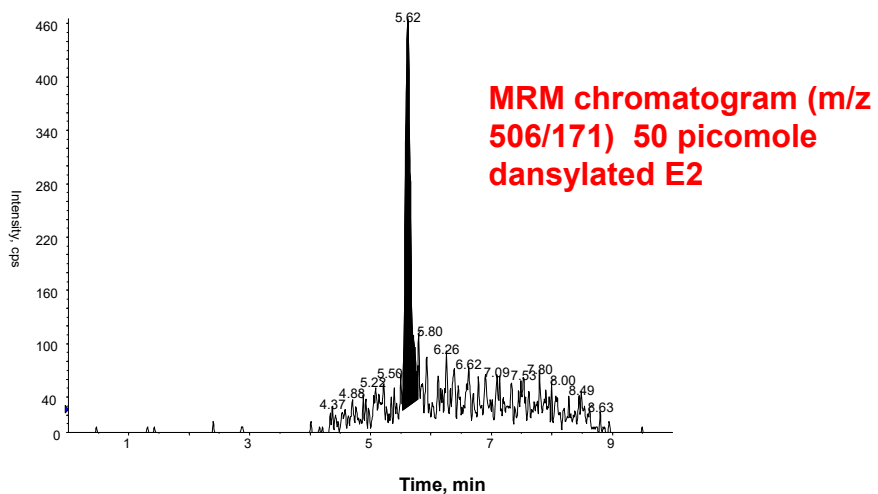


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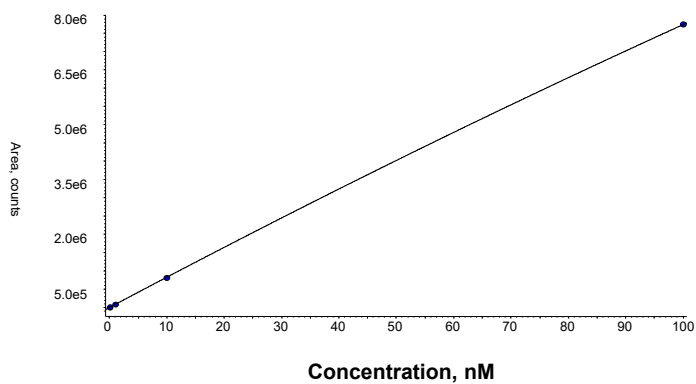
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## Derivatization tremendously helps increase sensitivity of E2



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## Calibration curve for dansylated E2 showing linearity from 0.005-100 nM concentration range ( $r = 0.999$ )

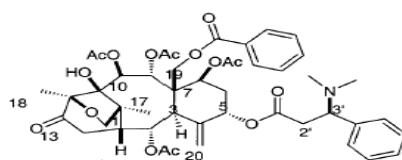


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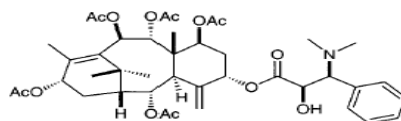
**Substructure analysis in ESI-MS/MS  
(dereplication and partial identification  
of natural products)**

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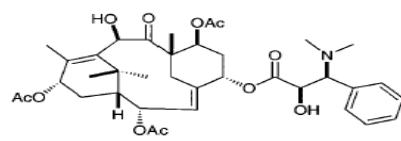
**Fragmentation of basic taxoids from *T. Wallichiana* extract**



[1] MW=861



[2] MW=769

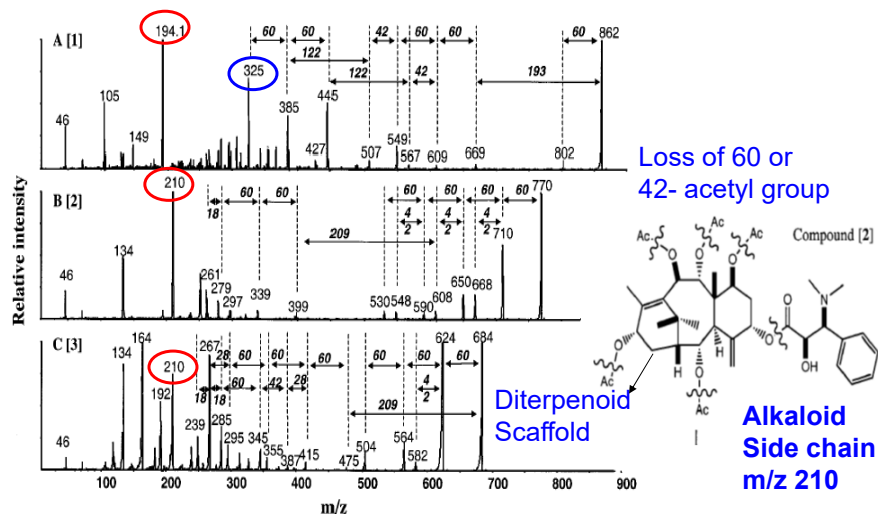


[3] MW=683

*Stefanowicz et al. Anal Chem, 2001*

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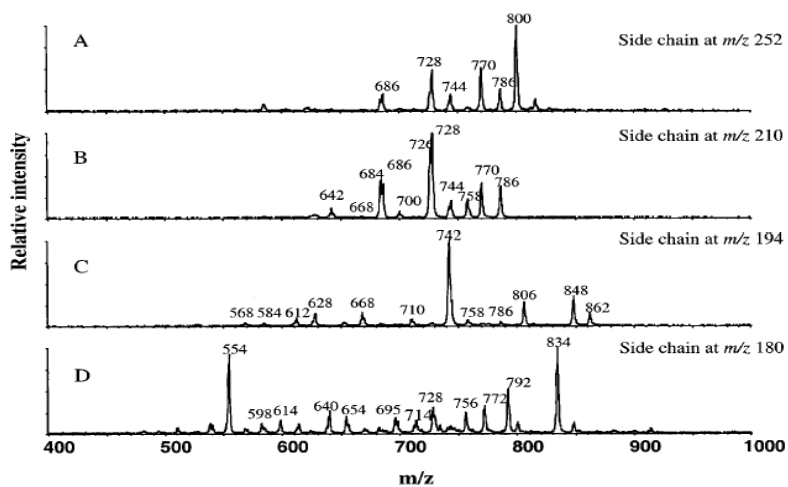
**ESI-MS/MS spectra of taxoids (1-3). Peaks  $m/z$  194 and 210 represent the intact alkaloid side chain.**



Stefanowicz et al. Anal Chem, 2001

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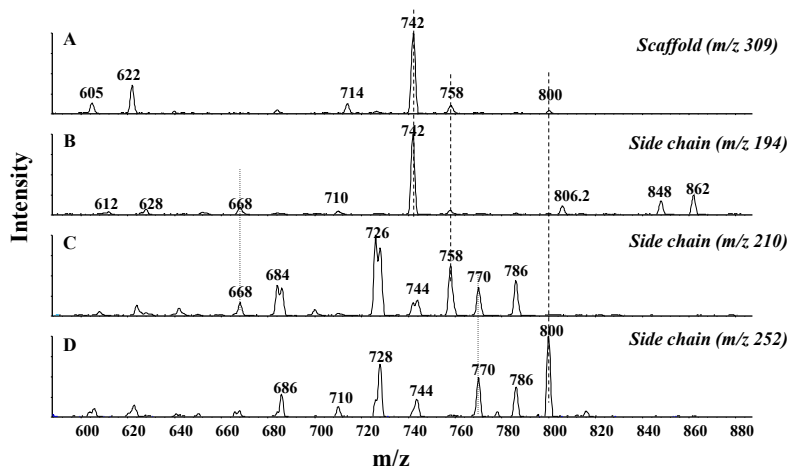
**MS/MS precursor-scan spectra of typical alkaloid side chains to identify the basic taxoids compounds in an ethyl acetate extract of *T. wallichiana*.**



Stefanowicz et al. Anal Chem, 2001

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## Comparison of precursor scan spectra obtained from the scaffold $m/z$ 309 and side chain $m/z$ 194, 210 and 252



Taxoids with scaffold  $m/z$  309 and alkaloid side chains are shown by dashed lines

Stefanowicz et al. *Anal Chem*, 2001

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## References

1. Electro spray Ionization Mass Spectrometry by Richard B. Cole.
2. Stefanowicz P, Prasain JK, Yeboah KF, Konishi Y. Detection and partial structure elucidation of basic taxoids from *Taxus wallichiana* by electrospray ionization tandem mass spectrometry. *Anal Chem*. 2001;73:3583-9.
3. [Prasain J.K., Wang C.-C., Barnes S. Mass spectrometric analysis of flavonoids in biological samples. \*Free Radical Biology & Medicine\*, 37: 1324-1350, 2004.](#)
4. William Griffiths. Tandem mass spectrometry in the study of fatty acids, bile acids and steroids. *Mass Spectrometry Reviews*, 2003;22:81-152.
5. Yi et al., *Anal Bioanal Chem*. 2006.

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