Mass Spectrometry Analysis of Small Molecules

Metabolomics- A realm of small molecules (<1000 Da)

Coined in the 1990s:
Oliver, S. G., Winson, M. K., Kell, D. B. & Baganz, F. (1998).

Jeevan Prasain, PhD

What is metabolomics?

- **Identification and quantification of the complete set of metabolites in a biological system**
- **Quantitative measurement of the dynamic metabolite response of living systems to pathophysiological stimuli or genetic modification**
- **provides important insights into physiological and disease states and facilitate in depth understanding of underlying biochemical pathways. Metabolomics has the potential to provide valuable information on various patho-physiological conditions such as cancer, Alzheimer's and cardiovascular diseases.**

Key applications

- **Toxicity assessment/toxicology**
- **Functional genomics**
- **Nutrigenomics**
- **Herbal formulation, quality control and clinical trial.**

Metabonomics-NMR as analytical plateform

- **Prior to LC-MS based metabolomics, the small molecule -ome was analyzed by ^{1}H- and a lesser extent ^{13}C-NMR (Metabonomics)**
- **NMR is carried out on urine and plasma**
  - **Does not require chromatography, but sample has to carefully dried and redissolved in $^{2}H_2O$**
  - **Compounds analyzed by their chemical shifts**
  - **Assisted by very high magnetic fields to obtain resolution**
  - **Not very sensitive, but microliter probes now in use**

Metabolomics in the context of other omics

Genome → Transcriptome → Proteome → Metabolome → Phenotype

Genome → **What might be happening in a cell** (Proteome)

Metabolome → **Snapshot of the entire physiology**

Possible metabolites

Amino acids
Fatty acids
Phenolics
Prostaglandins
Steroids
Organic acids
Organic amines
Nucleosides
Nucleosides
Polyamines
Organic acids
Lipids

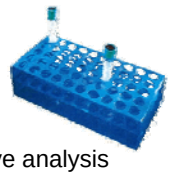
Steps involved in metabolomic analysis

- Profiling involves finding of all metabolites detectable to a selected analytical technique with statistically significant variations in abundance within a set of experimental and control groups.
- Identification of chemical structures of metabolites of interest after profiling
- Quantification and validation
- Interpretation of data making connections between the metabolites discovered and the biological conditions

Metabolomics is complementary to the other -omics and the combination of these three may provide important information about the status of a cell

Workflow for metabolome analysis

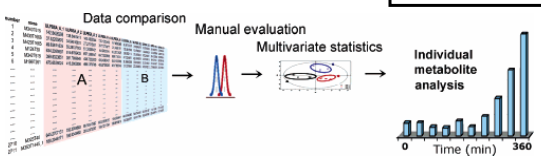
Sample collection → Treatment or Diseased group / Control group — Urine, plasma, tissue etc.



Sample preparation → Internal standard spiking for quantitative analysis
Extraction (liquid-liquid, ppt with or without hydrolysis)

Sample analysis → LC-MS and LC-MS/MS analysis
- Q1 scan using HPLC or UPLC/-TOFMS
- +ve/-ve ion mode- accurate mass measurement
- MS/MS experiments using a hybrid instrument Q-Trap

Data export → **Data analysis** Multivariate analysis, e.g. PCA



Marker identification

Points to be considered in LC-MS analysis

- **Choice of ionization mode - ESI vs. APCI +ve/-ve modes**
- **Choice of eluting solvent - methanol vs. acetonitrile**
- **Evaluation of spectral quality - what to look for in a good quality spectra**
- **Molecular ion recognition**

Sample preparation

Sample collection

↓

Quenching by liquid Nitrogen or cold methanol
(stops metabolism)

↓

Extraction of intracellular metabolites
(methanol, methanol-water for polar)
(chloroform or hexane for less polar)
(protein precipitation, supercritical fluid extraction)

↓

Concentration
(evaporation under vacuum, lyophilization, SPE)

MS acquisition strategy

Full scan (Q1 scanning) for total profiling of metabolites (+ve and –ve ion mode) ESI/APCI

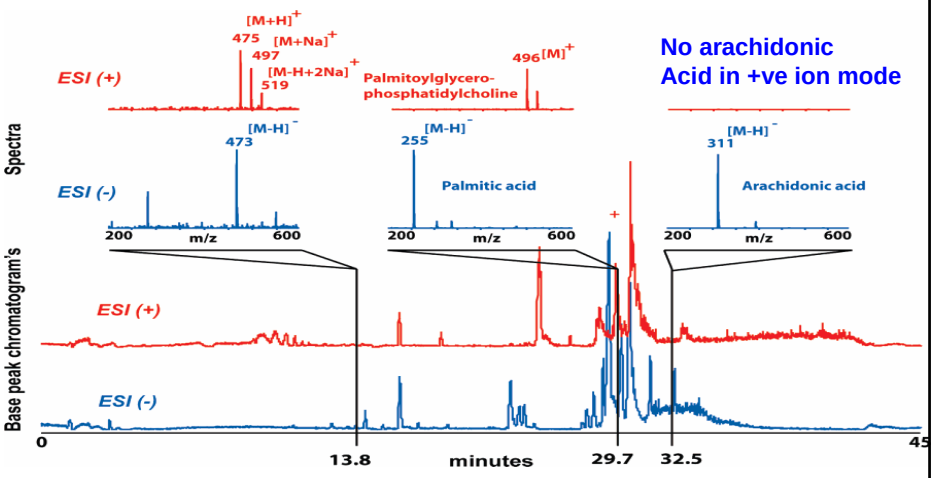
ESI- Effluent is charged and nebulized, for more polar compounds, e.g., conjugated metabolites.

APCI- Effluent is heated, but not charged- a corona discharge is needed. Good for less polar compound.

ESI is the most common ionization method

Advantage: non-selective and most ionizable ions are detected
Disadvantage: low sensitivity and detection of minor metabolites is compromised.

Increasing metabolite coverage using +ve and –ve ion mode

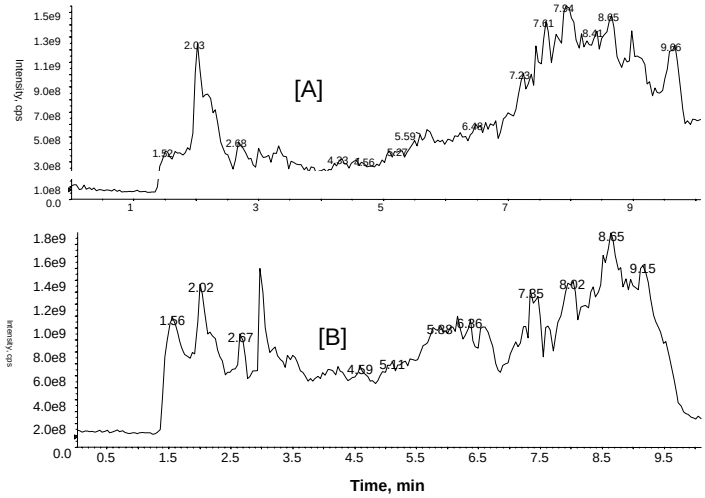


Representative Q1 scans of a methanolic extract of human blood serum

Source: Nordstrom et al. Analytical Chemistry, 2007

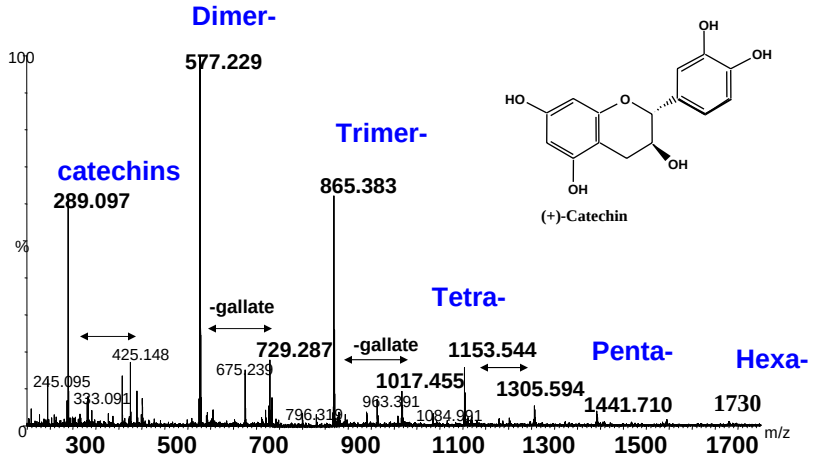
Does ionization mode affect the metabolite detection?

TIC obtained from grape seed extract treated urine operated in -ve Q1 [A] and +ve Q1 [B] modes

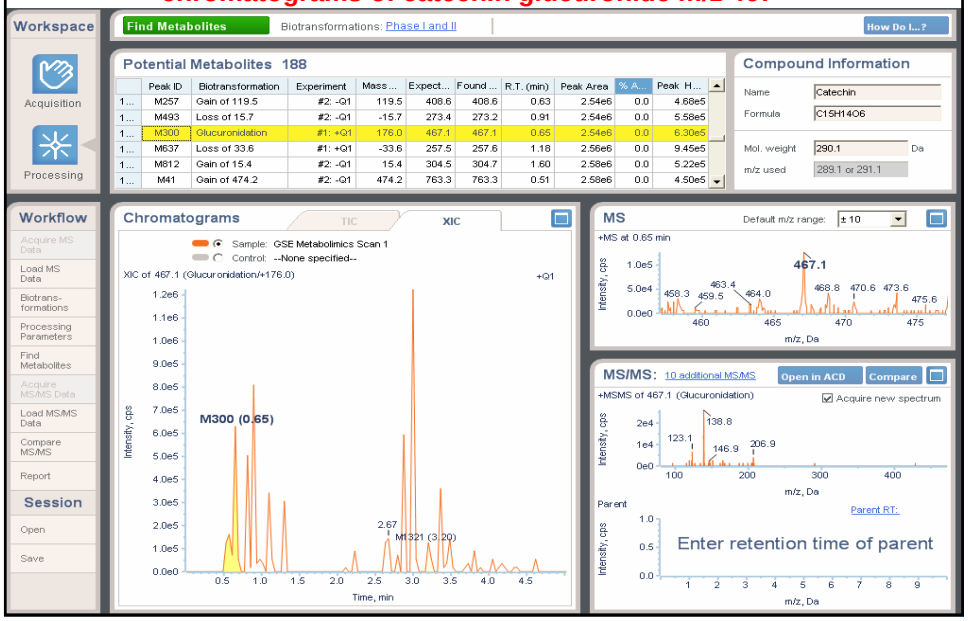


Visual inspection of the two TIC plots show that the two modes of ionization will generate different metabolomic information based on their ionization difference

Profiling of grape seed extract metabolites in ESI-MS Q-TOF –ve ion mode

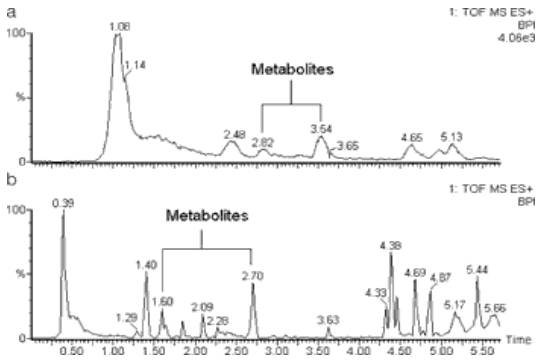


Metabolite ID software LightSight™ showing peak list and LC-MS/MS chromatograms of catechin glucuronide m/z 467



HPLC Vs. UPLC?

Full-scan ToF mass spectra for (a) the HPLC approach and (b) the UPLC approach for metabolites of dextromethorphan.



Demonstrates a 5-fold increase in the performance of the UPLC system over conventional HPLC

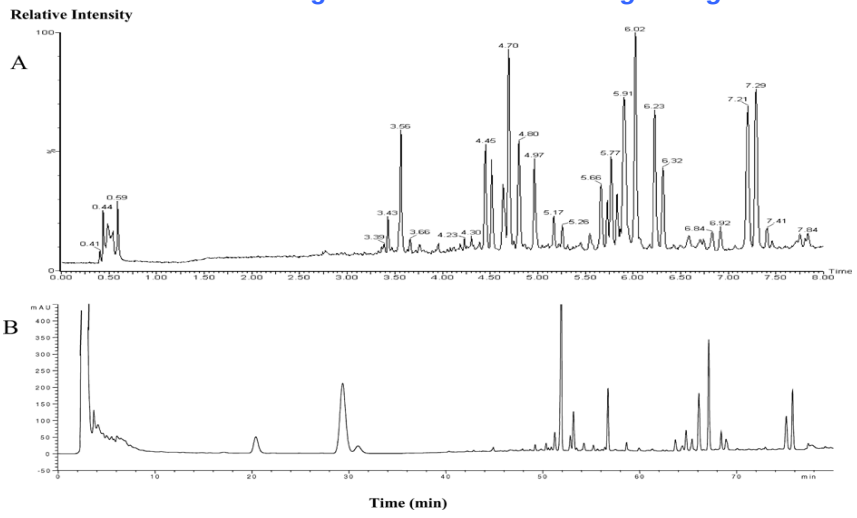
Source: Jose Castro-Perez, Rapid Communications in Mass Spectrometry
Volume 19, Issue 6, **Pages 843-848**

Metabolomics for herbal quality control

Metabolite profiling of ginseng in different herbal formulations based on metabolomic approach

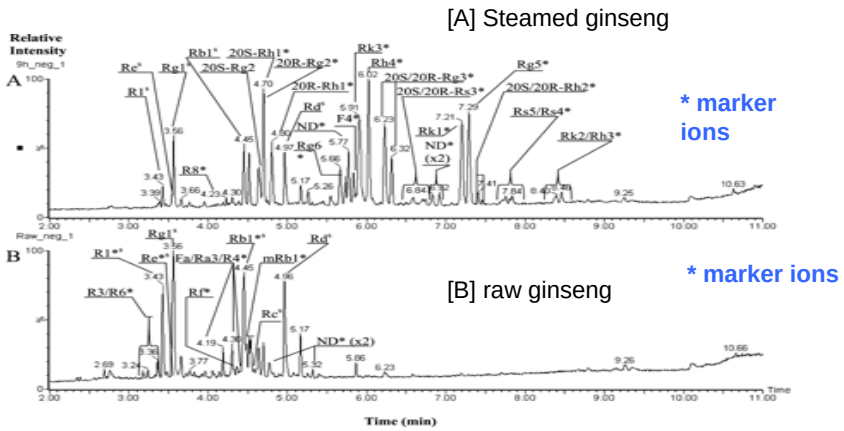
Metabolomics of raw and steamed *Panax notoginseng*

[A] UPLC/TOF-MS total ion current chromatogram (TIC) and HPLC-UV Chromatogram of steamed *P. notoginseng*



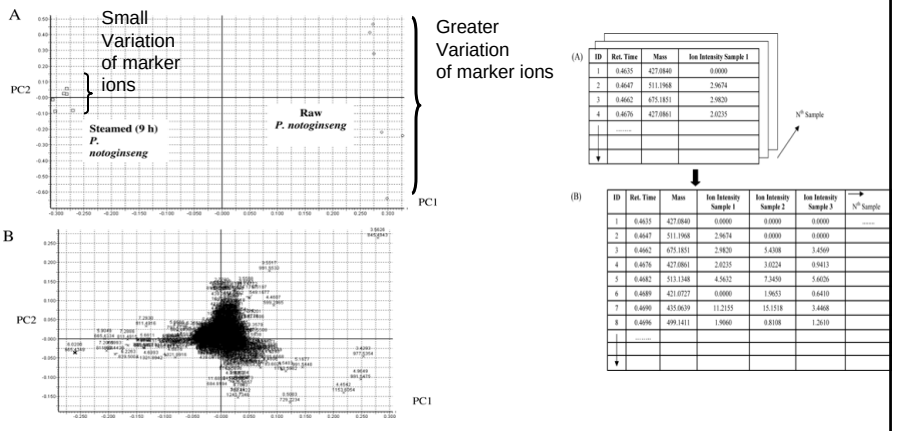
Source: Chan et al. Rapid Commun. Mass Spectrom. 2007, 21, 519-528

TIC of UPLC/TOF-MS analysis of [A] steamed ginseng and [B] raw ginseng



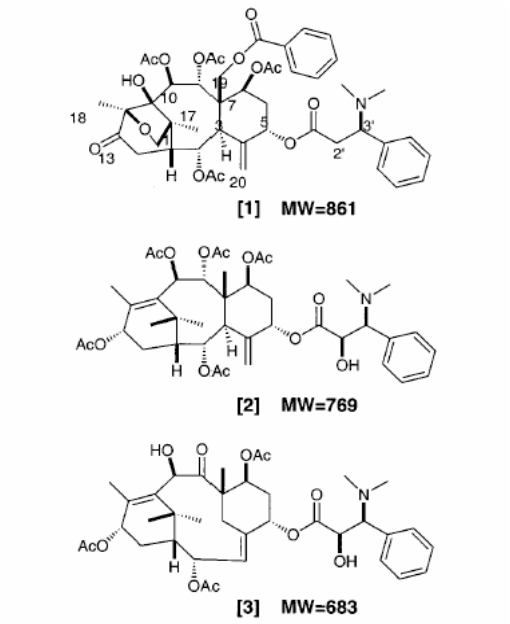
More than 200 metabolites were detected in both raw and steamed ginsengs. The concentration of Rg1, Re, Rb1, Rc and Rd in steamed ginseng was less than that of raw ginseng

[A] Score plot of raw and steamed groups and [B] loadings Plot obtained using pareto scaling with mean centering



Small variation of marker ions in steamed samples indicates that the steaming process might result in consistency of the levels of ginsenosides

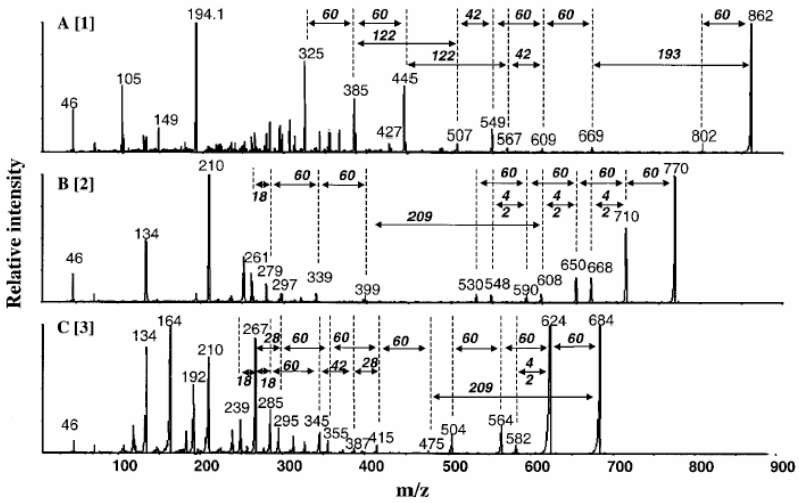
Conclusion- MS based metabolomic study is able to discriminate differentially processed herbs such as raw and streamed *P. notoginseng*



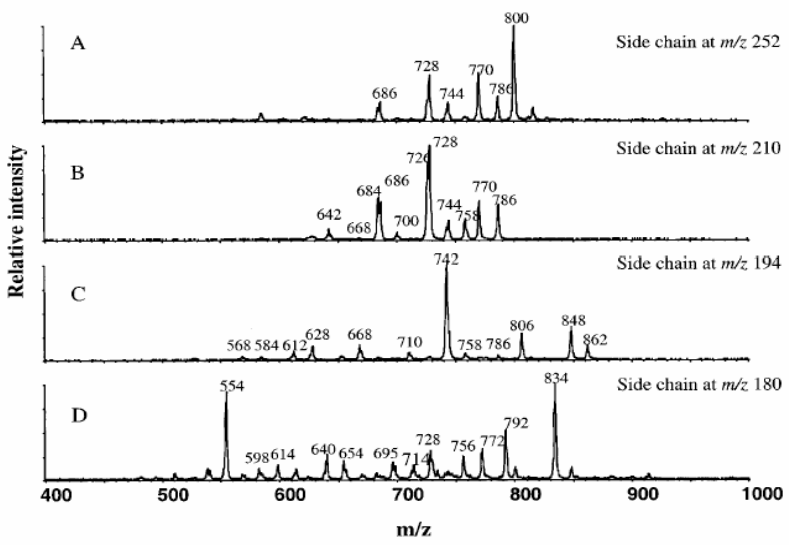
Profiling taxoids metabolites in the Yew plant *T. Wallichiana* extract based on tandem mass spectrometry

Prasain et al. Anal Chem, 2001

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

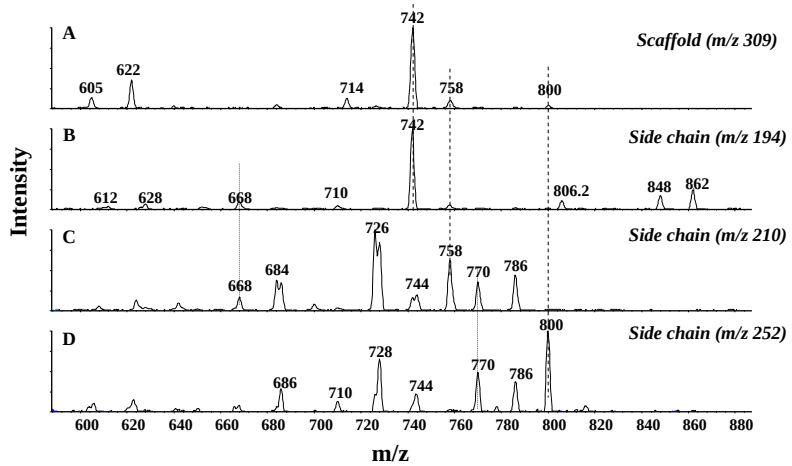


MS/MS precursor-scan spectra of typical alkaloid side chains to identify the basic taxoids compounds in an ethyl acetate extract of *T. wallichiana*.



Prasain et al. Anal Chem, 2001

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

Precursor-scan analysis of selected product ions allowed for the detection of 57 basic taxoids from the ethyl acetate extract of *T. wallichiana*, 45 of which have not been reported

Table 2. Basic Taxoids Identified in the Ethyl Acetate Extract of *T. wallichaina* and Their Constituent Alkaloid Side Chains[a]

alkaloid side-chain ions	basic taxoid precursor ions													
164	684	726												
180	554	598	614	640	654	695	714	756	772	792	834			
194	568	584	612	628	668	710	742	758	786	806	848	862		
196	570	584	630	654	670	672	712	714	730	744	756	772	808	850
210	642	668	684	686	700	726	728	742	744	758	770	786		
252	686	728	744	770	786	800								

[a] Boldface characters represent unreported basic taxoids and alkaloid side chains. A total of 44 out of the 57 identified basic taxoids are unreported and 2 out of the 6 alkaloid side chains were unreported.

Prasain et al. Anal Chem, 2001

Next experiments

- Collect different species of Yew plants and profile all taxoids
- Identify the marker ions
- Perform PCA for pattern recognition

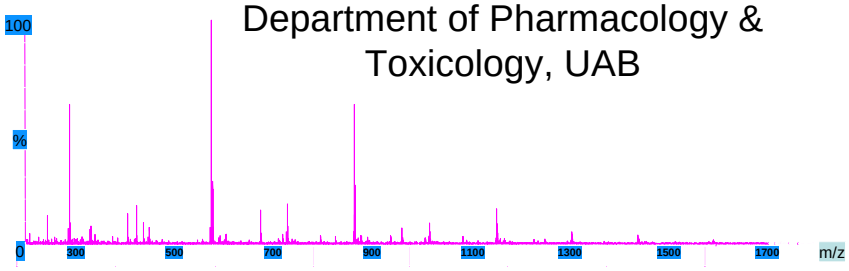
Conclusions

- LC-MS has been a powerful tool to provide useful means of generating metabolite profiles.
- LC-MS-based metabolomic approach is promising for the quality control of dietary supplements and discovery of novel markers in biomedical research.
- Identification of metabolites, data standardization, export and finding a biomarker is a real challenge

Mass Spectrometry in quantitative analysis of small molecules

Jeevan Prasain, Ph.D.

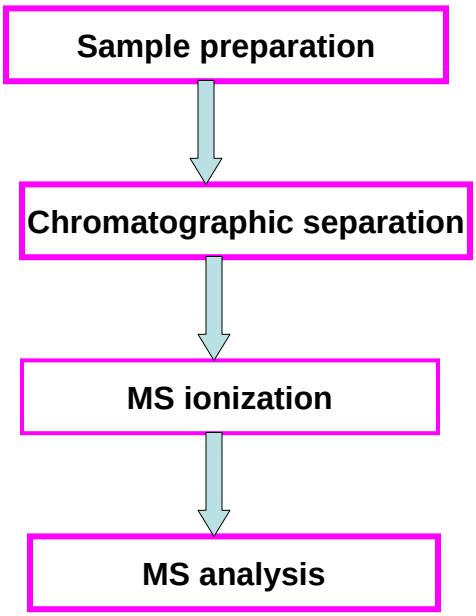
Department of Pharmacology & Toxicology, UAB



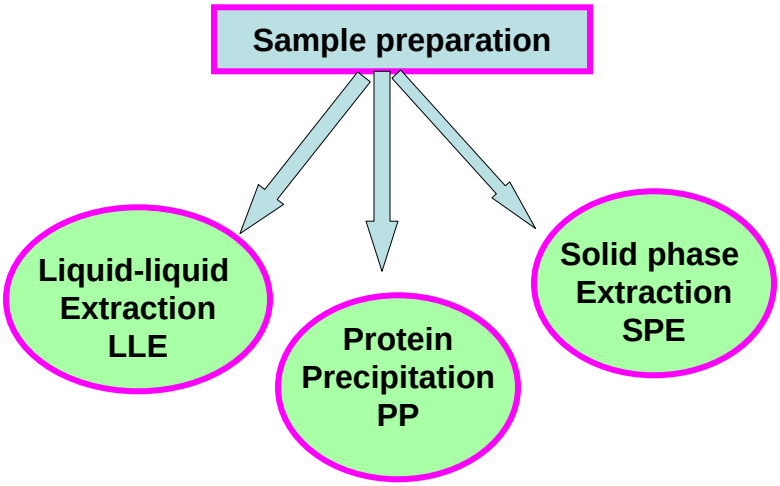
Class Overview

- **Introduction to bioanalysis**
- **Quantitative analysis of puerarin, EGCG and isoflavones in biological samples by LC-MS/MS**

Bioanalysis Flow Chart



Sample preparation is a crucial step in removing the interfering compounds from biological matrix

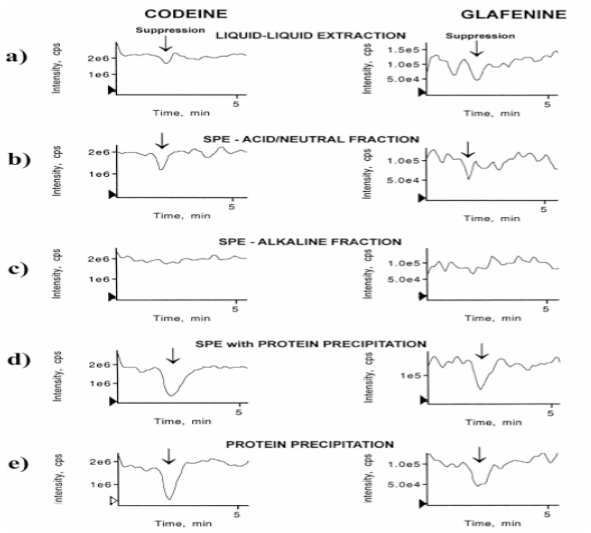


The method of choice will be determined by the sample matrix and the concentration of compounds In samples

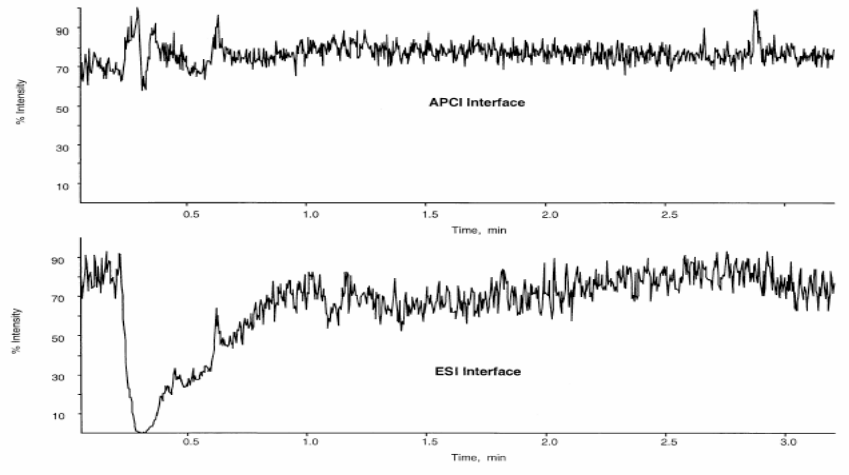
Properties of Good Internal Standards

- **Should not be found in the original sample**
- **The structure of the internal standard needs to be similar to the analyte.**
- **Provides data about your extraction process:**
  - **Hydrolysis of Methylumbelliferyl sulfate**
  - **Hydrolysis of Phenolphthalein glucuronide**
  - **Extraction Efficiency (Apigenin)**

Severe ion suppression effect for codeine and glafenin was observed with PPT and SPE-PPT



APCI is less prone to than ESI to the effects of ion suppression



King et al. J. Am Soc Mass Spectrom 2000

Analytical method validation

Should demonstrate specificity, linearity, accuracy, precision

- **EEstablish lower limit of quantification**
- **SStability (freeze/thaw)**
- **eEstablish robustness**

LC/MS/MS Method for Puerarin

Column:	**Waters X-Terra C18 with guard, 2.1 x 100 mm, 3.5 micron**
Mobile Phase A:	**10% MeCN + 10 mM NH4OAc**
Mobile Phase B:	**70% MeCN + 10mM NH4OAc**
Gradient:	**0 minutes = 100% A**
	6 minutes = 100% B
	7 minutes = 100% A
	10 minutes = Stop
Injection Volume:	**20 ul**
Flow Rate:	**0.2 ml/min split flow**
Mass Spectrometer:	**Negative Electrospray**
Mass Transitions:	**415/267 (Puerarin)**
	415/295 (Puerarin)
	269/149 (apigenin, IS)

What is calibration/standard curve?

A calibration curve is the relationship between instrument response and known concentration of the analyte.

Lower limit of quantification (LLOQ) - the lowest standard on calibration curve that should be at least 5 times the response compared to blank with precision of 20% and accuracy of 80-120%.

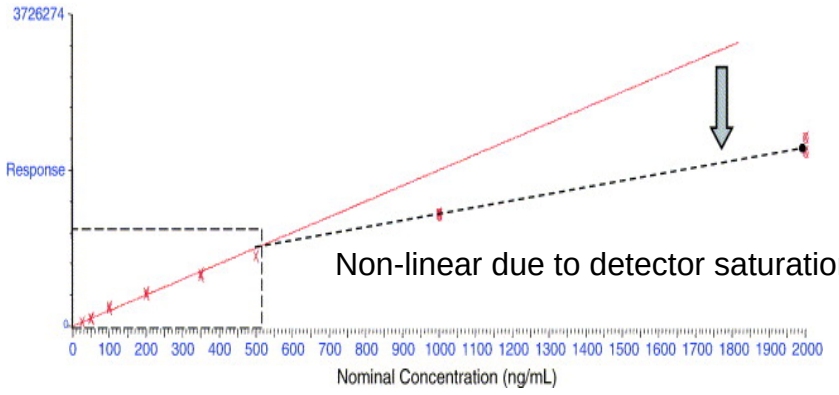
Table 1.
Summary of calibration curves (n =5)

Concentration (ng/ml)	Mean ± S.D.	CV (%)	Accuracy (%)
2.0	2.21 ± 0.16	7.00	110.7
5.0	5.22 ± 0.28	5.30	104.48
50	45.32 ± 2.53	5.60	90.64
500	473.60 ± 26.57	5.60	94.72
1000	1021.20 ± 71.53	7.00	102.12
5000	5340 ± 420.18	7.90	106.80

Mean r = 0.996

Prasain et al. Biomedical chromatography, 2007

Standard curve non-linearity is possible due to detector saturation, dimer/multimer formation, and or ESI droplet saturation at higher concentration



Source: Bakhtiar & Majumdar.
Journal of Pharmacological and Toxicological Methods, 2007

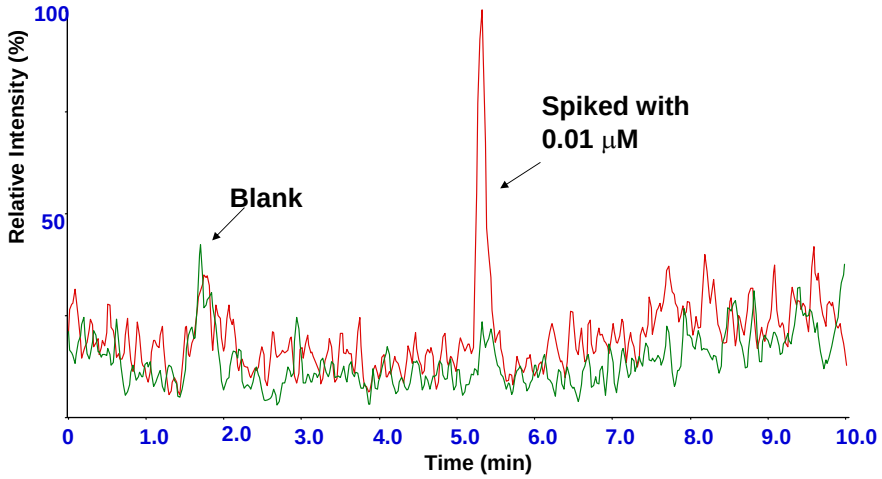
What are accuracy and precision?

Accuracy describes the closeness of mean test results obtained by the method to the true value (concentration of the analyte)
Precision - the closeness of individual measure of an analyte when the procedure is applied to multiple aliquotes, expressed in %CV = Std deviation/mean x 100

Table 2.
Assay validation characteristics of the method for the determination of puerarin in rat serum (n =5)

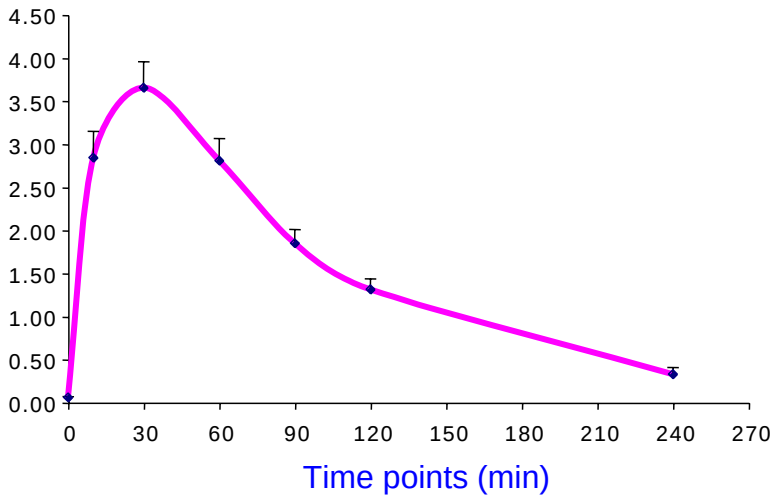
Concentration (ng/ml)	Mean ± S.D.	CV (%)	Accuracy (%)
2.0	2.21 ± 0.16	7.00	110.7
4.0	3.96 ± 0.30	7.90	99.20
8.32	7.32 ± 1.00	14.40	113.30
20	19.20 ± 1.20	6.30	96.00
200	203.20 ± 19.41	9.60	101.60
832	821.18 ± 55.86	6.80	101.31
2000	2240 ± 96.70	4.30	112.00

Specificity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample

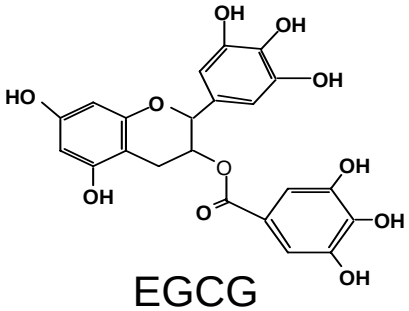


Ion chromatograms of a rat serum spiked sample (0.01 μM of puerarin) vs. blank serum

Average serum concentration of puerarin versus time after oral administration of 50 mg/kg puerarin



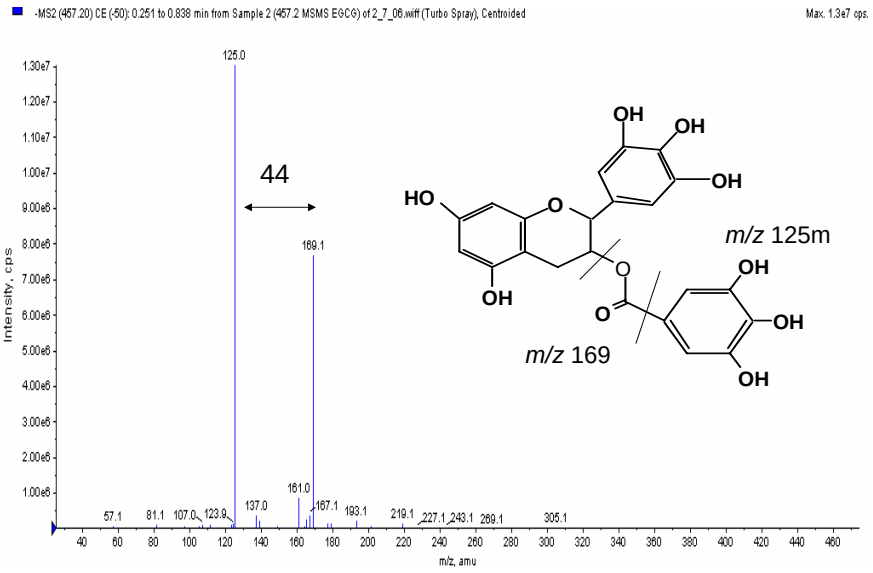
Analysis of tea catechin in biological samples



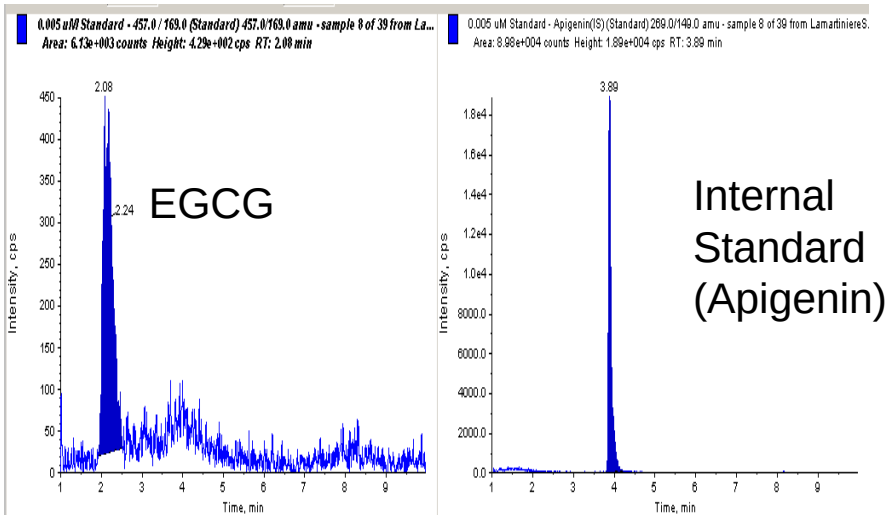
EGCG

$[M-H]^-$ *m/z* 457

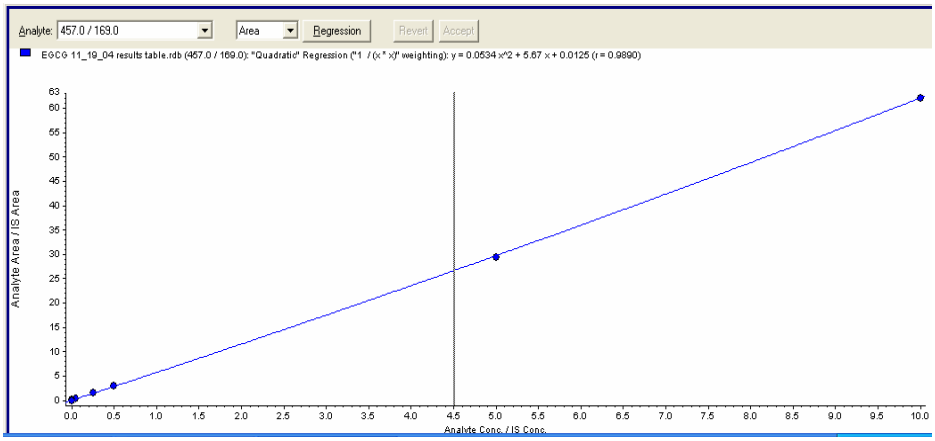
Product ion spectrum of the ion *m/z* 457



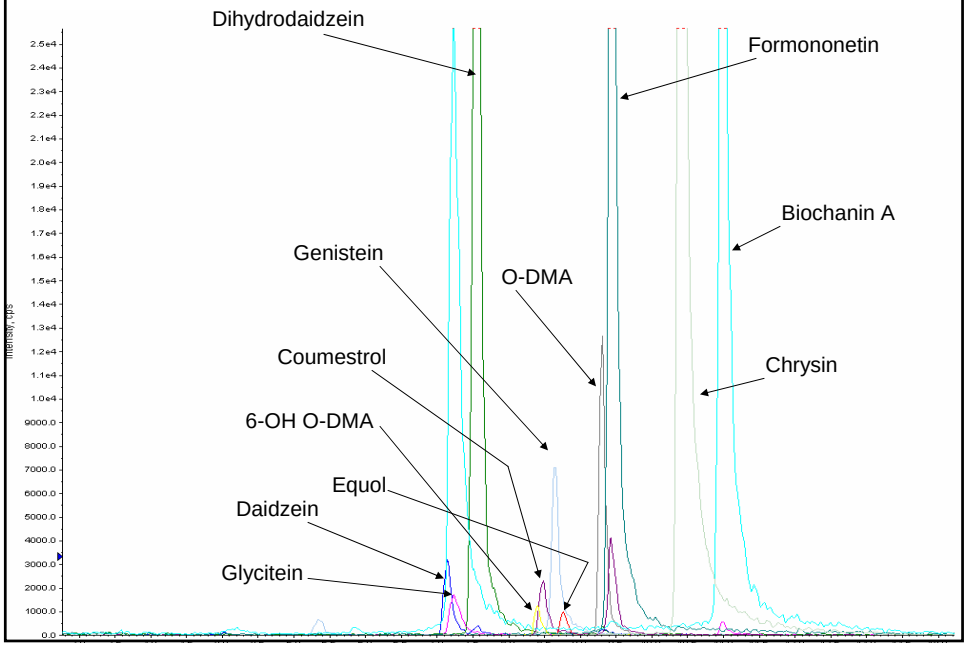
Typical chromatogram of a serum sample spiked with 5 nM after protein precipitation

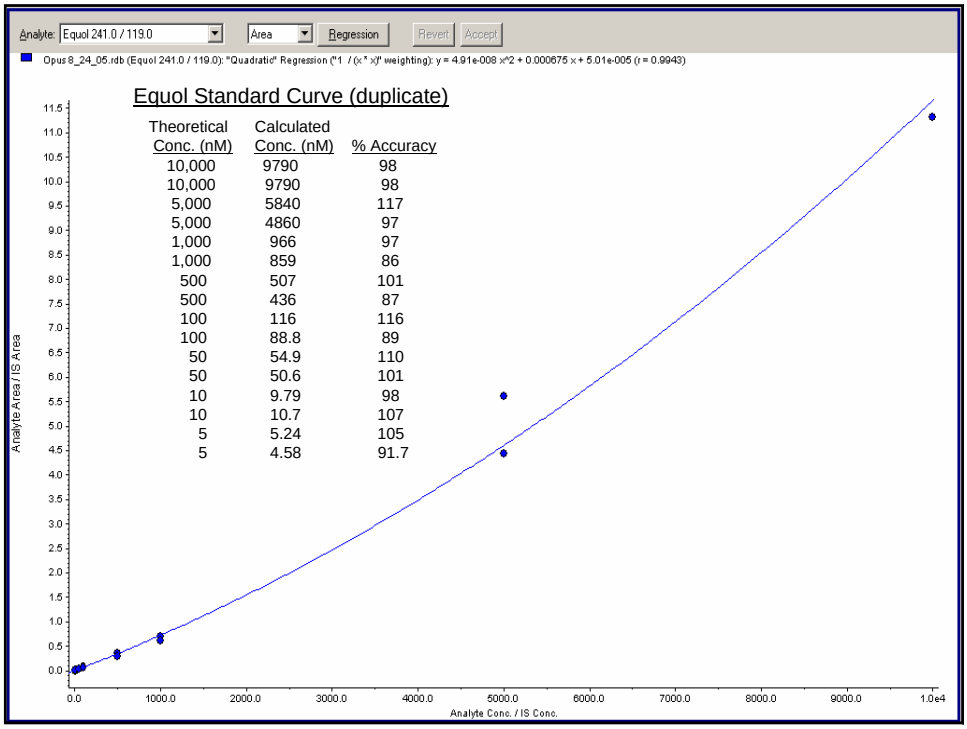


Calibration curve for EGCG (1-10,000 nM) after extracting from rat serum



Simultaneous Quantification of 10 Isoflavones in one run





Equol Standard Curve (duplicate)

Theoretical Conc. (nM)	Calculated Conc. (nM)	% Accuracy
10,000	9790	98
10,000	9790	98
5,000	5840	117
5,000	4860	97
1,000	966	97
1,000	859	86
500	507	101
500	436	87
100	116	116
100	88.8	89
50	54.9	110
50	50.6	101
10	9.79	98
10	10.7	107
5	5.24	105
5	4.58	91.7