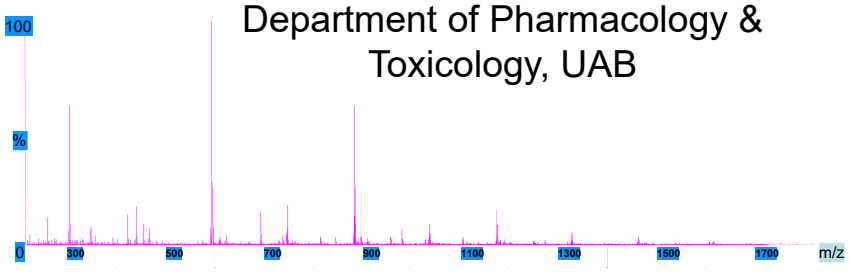
Quantitative analysis of small molecules in biological samples

Jeevan Prasain, Ph.D.

Department of Pharmacology & Toxicology, UAB



Class Overview

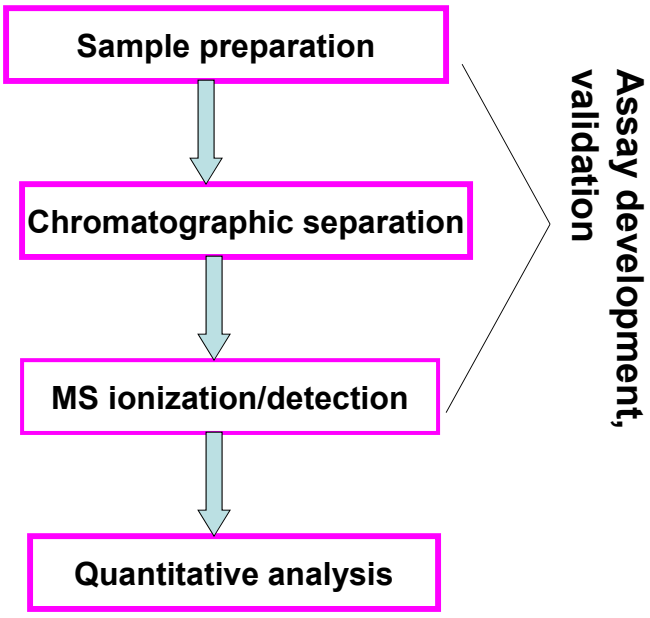
- **Introduction to method validation and LC-MS/MS analysis**
- **Quantitative analysis of puerarin, and phytoestrogens in biological samples by LC-MS/MS**

Untargeted metabolomics and method validation

- No guidelines for validating analytical part in untargeted metabolomics.
- Unbiased differential, comprehensive analysis of metabolites in a biological sample.
- Reproducibility in chromatographic as well as MS performance
- Comparison should be valid and the change in signals should be related to the concentration- i.e. precisely measured.
- Quality control samples, spiking with unnatural internal standard to monitor reproducibility
- Statistical analysis- similarity/differences between and within samples.

Naz et al., J Chrom A., 2014.

Bio-analytical works



Challenges in quantitative analysis of analytes

- Low concentrations of metabolites in a complex matrix
- Number of samples (eg.10-1000)/study
- Wide dynamic concentration range (pico to microgram/mL)

Problems encountered in LC-MS analysis
Matrix effect on Ion suppression?

- **The presence of endogenous substances from matrix, i.e., organic or inorganic molecules present in the sample and that are retained in the final extract**
- **Exogenous substances, i.e., molecules not present in the sample but coming from various external sources during the sample preparation**

Choice of Good Internal Standards

- A stable isotopically labeled IS is preferable.
- Is not found in the original sample
- In the absence of stable isotopically labeled internal std, the structure of the internal standard needs to be similar to the analyte and co-elute with the analyte.
- Should not react chemically with the analyte.

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

Choice of solvent

- Common organic solvents- Methanol and acetonitrile, water alone is poor solvent for ESI
- Acetonitrile vs methanol- acetonitrile (expensive), water/methanol creates more pressure than water/acetonitrile
- Elution strength- usually acetonitrile> methanol
- Methanol provide a more stable spray and better sensitivity than acetonitrile in negative ion mode.

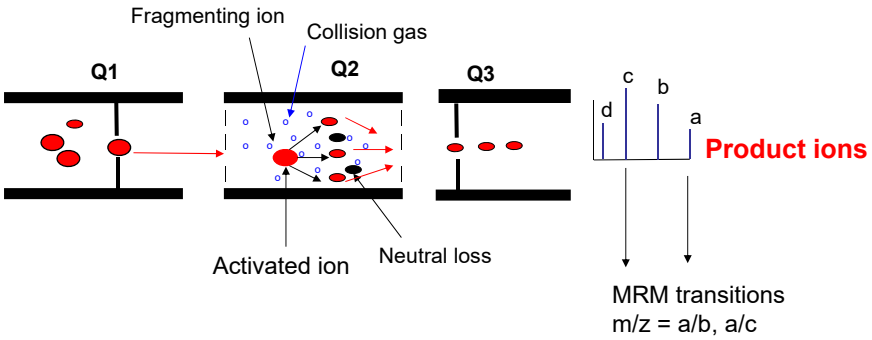
LC-MS analysis

HPLC

- Isocratic
- Gradient
- Reversed-nonpolar stationary, polar mobile
- Normal- polar stationary, nonpolar mobile
- HILIC- hydrophilic interaction

Common column- 100-200 mm long and 3-4.6 mm diameter
Smaller diameter offers better separation and sensitivity

Schematic of MRM/SRM experiments



Strength/weakness of MRM

- Targeted analysis
- Analyze multiple metabolites in a single run
- High sensitivity, specificity and reproducibility
- Gold standard for quantitative analysis
- Isobaric/isomeric overlap can be a problem- have same fragment ions therefore, same MRM mass transitions

Analytical method validation

- Should demonstrate specificity, linearity, recovery, accuracy, precision
- Lower limit of quantification, detection
- Stability (freeze/thaw)
- Robustness & ruggedness
- Matrix effects

Method validation..

- Specificity is established by the lack of interference peaks at the retention time for the internal standard and the analyte.
- Accuracy is determined by comparing the calculated concentration using calibration curves to known concentration. The LLQ is defined as the smallest amount of the analyte that could be measured in a sample with sufficient precision (%CV) and accuracy (within 20% for both parameters) and is chosen as the lowest concentration on the calibration curve.

Linearity

- It indicates the relationship between changed concentrations and proportional response
- R2> 0.95, with at least 5 concentration levels

Precision..

- The closeness of agreement between a series of measurements obtained from multiple samples of the homogenous sample.- Repeatability
- %CV

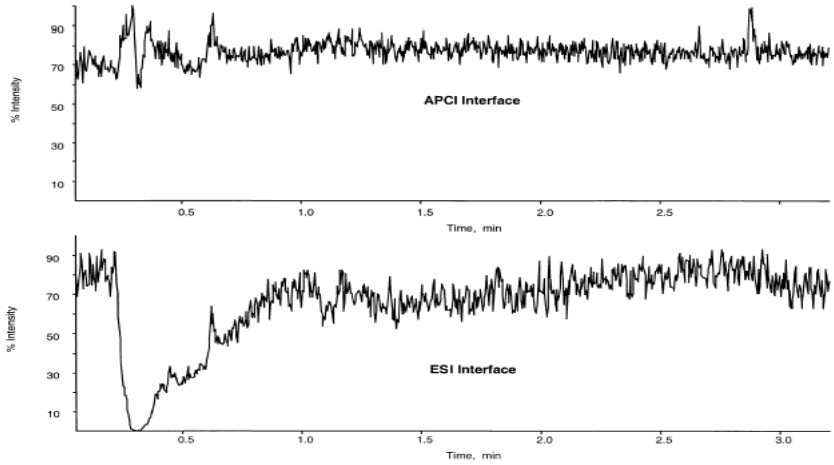
Robustness

- Ability to remain unaffected by small but deliberate variations in the LC-MS/MS method parameters- such as pH in a mobile phase, composition of solvents, different lots of column, flow rates etc.

Ruggedness

- Indicates degree of reproducibility of test results under a variety of conditions such as different labs, instruments and reagents etc.

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



King et al. J. Am Soc Mass Spectrom 2000

Eliminating matrix effects

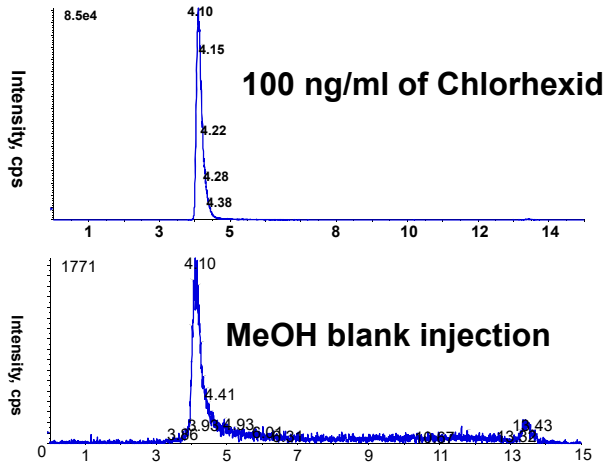
1. Preparing more cleaner samples.
2. Concentrating analyte of interest
3. Improve analytical system performance

% matrix effects

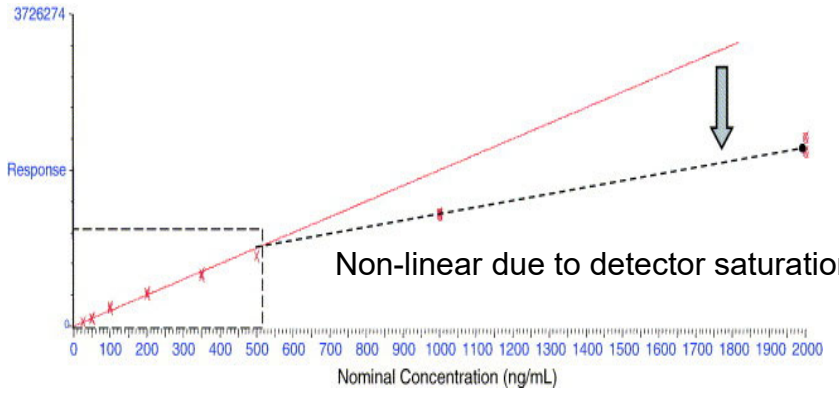
= [Response post-extracted spiked sample -1] x100
response non-extracted neat samples

Carry over - a big problem

Previously injected sample which appears upon subsequent analyses due to physico-chemical property of the sample, analysis system or both.



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

Recovery

- Recovery is a ratio of the detector response of an analyte from an extracted sample to the detector response of the analyte in post extracted sample (spiked sample)
- %RE = response extracted sample x100
  response post extracted spiked sample

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)

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

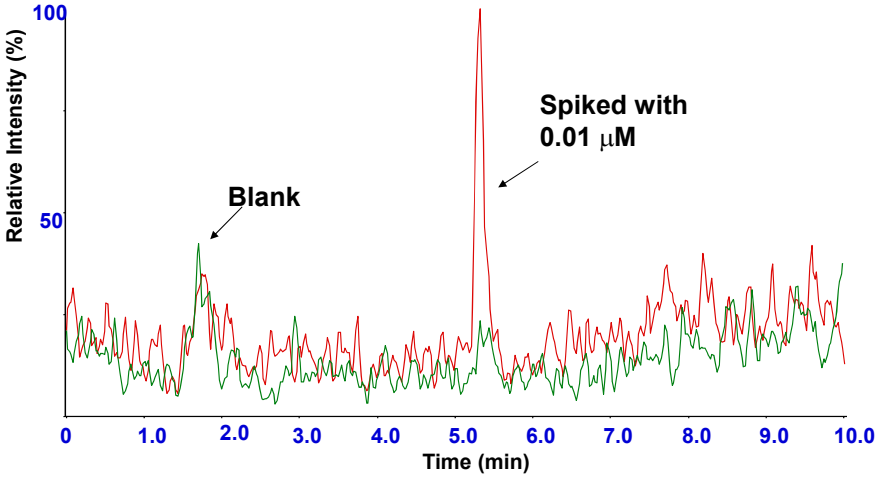
Prasain et al., Biomed. Chromatogra. 2007

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

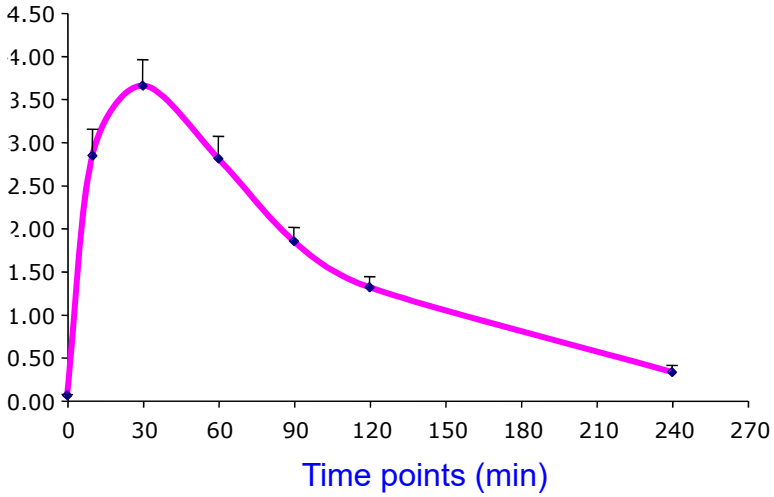
Concentration (ng/ml)	Mean ± S.D.	CV (%)	Accuracy (%)
2.0	2.21 ± 0.16	7.00	110.7
4.0	3.96 ± 0.30	7.90	99.20
8.32	7.32 ± 1.00	14.40	113.30
20	19.20 ± 1.20	6.30	96.00
200	203.20 ± 19.41	9.60	101.60
832	821.18 ± 55.86	6.80	101.31
2000	2240 ± 96.70	4.30	112.00

Prasain et al., Biomed. Chromatogra. 2007

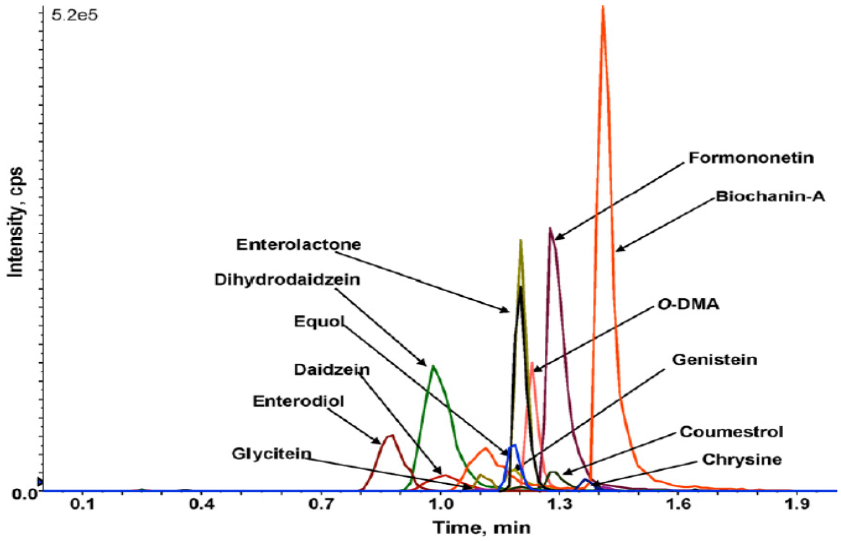
Ion chromatograms of a rat serum spiked sample (0.01 μM of puerarin) vs. blank serum



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

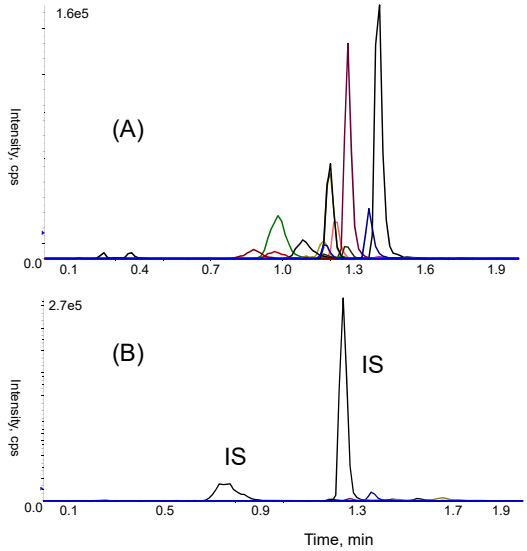


MRM chromatogram showing separation of 11 phytoestrogens using a 2 min run time



Prasain et al., J. Chrom B 2010

Specificity of the assay - no peaks from matrix



Prasain et al., J. Chrom B 2010

Calibration range and lower limit of Quantification (LLOQ) of analytes

Analyte	Calibration range (ng/ml)	LLOQ (ng/ml)
Equol	1 - 5,000	1
Daidzein	2 - 5,000	2
DHD	2 - 5,000	2
O-DMA	1 - 5,000	1
genistein	2 - 5,000	2
Glycitein	5 - 5,000	5
Formononetin	1 - 5,000	1
Coumetsrol	1 - 5,000	1
Bichanin-A	1 - 5,000	1
6-OH-ODMA	20 - 5,000	20
Enterodiol	2 - 5,000	2
Enterolactone	1 - 5,000	1

Prasain et al., J. Chrom B 2010

Precision and accuracy of quality control samples

Analyte	Nominal concentration (ng/mL)	Accuracy (%)			Precision (%CV)			Inter-day
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	
Equol	50	100.42	90.13	96.60	2.01	4.33	5.11	3.74
	500	103.30	99.85	114.66	2.31	5.61	1.93	2.97
	2000	97.60	89.90	103.96	6.11	10.61	10.13	8.34
Daidzein	50	99.98	102.73	94.04	4.35	6.44	8.23	6.62
	500	101.48	98.31	97.73	3.14	5.44	7.42	5.38
	2000	92.50	87.41	86.03	2.88	3.61	3.96	3.58
Dihydrodaidzein	50	103.00	100.15	101.66	3.94	1.43	4.99	3.63
	500	103.79	95.20	106.00	3.96	6.44	3.35	4.34
	2000	91.70	90.40	96.33	1.68	5.80	6.60	2.82
O-DMA	50	104.00	93.72	96.51	5.16	4.71	5.80	5.32
	500	105.67	93.78	102.33	3.22	9.42	5.54	5.84
	2000	101.20	93.57	100.93	5.53	5.37	6.53	3.63
Genistein	50	107.66	106.83	99.08	3.97	3.37	6.65	4.86
	500	97.50	88.90	91.36	5.40	3.61	5.60	4.96
	2000	95.13	92.28	93.38	2.63	3.97	4.17	3.59

Comparison of precision intra-day and inter-day

Prasain et al., J. Chrom B 2010

Mean recovery (%) of phytoestrogens following extraction

Conc. (ng/mL)	Equol	Dz	DHD	O-DMA	GN	Gly	Form	Cm	Bio	6-OH-ODMA	Ent	End
5	91.04	87.57	98.95	72.79		94.49	87.36		84.10		78.62	73.60
50	76.58	80.09	80.88	71.00		74.96	82.08	76.63	74.26		75.17	73.82
500	85.70	86.49	89.39	71.70		91.18	80.15	86.97	54.84		92.50	92.78
5000	87.32	79.57	95.02	81.97		92.45	93.22	81.52	67.67		92.30	77.70

Dz = daidzein, DHD = dihydrodaidzein, GN = genistein, Gly = glycitein, Form = formononetin, Bio = biochanin A, Ent = enterolactone
End = enterodiol

Prasain et al., J. Chrom B 2010

Conclusions

- The sensitive & accurate analysis of biological samples remains a significant challenge.
- Column temperature, LC column particles, gradient and run time can influence chromatographic separation.
- Method of validation is always performed with spiked matrix same as the biological sample following the validation criteria.