Qualitative and quantitative analysis/method validation in metabolomics

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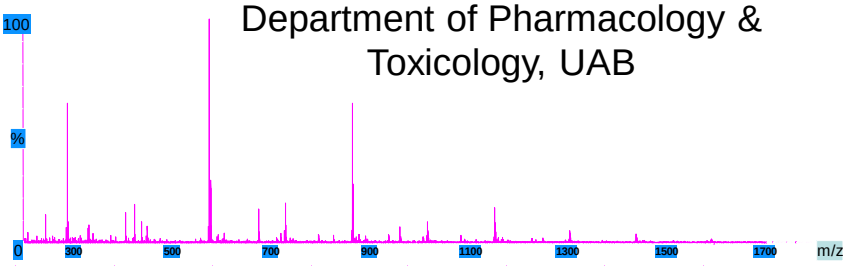
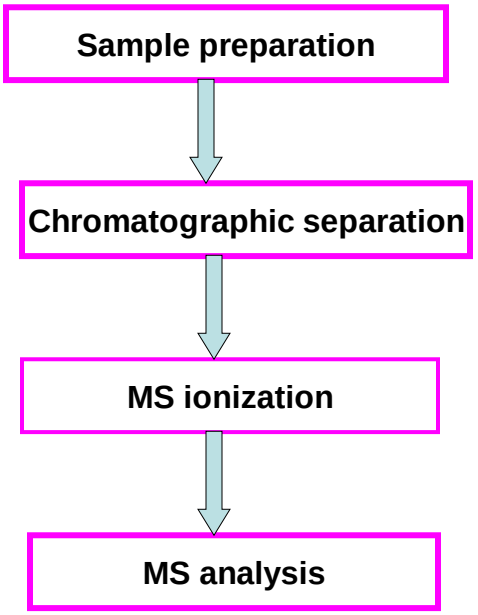


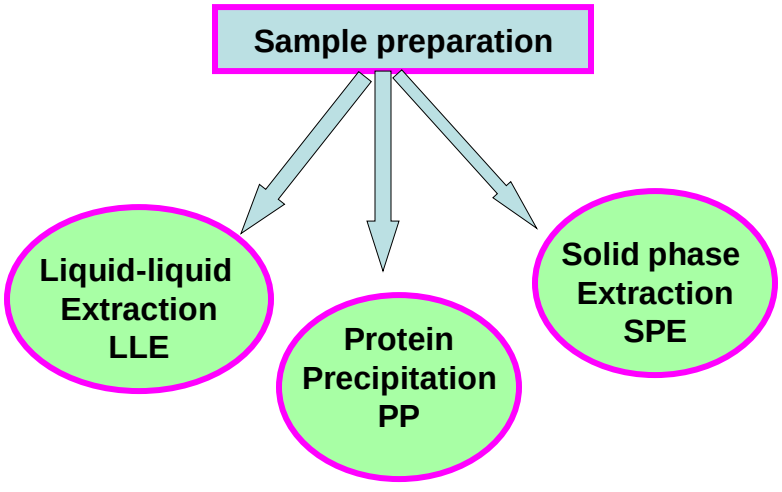
Class Overview

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

LC-MS/MS 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

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

Factors affecting ionization of analytes

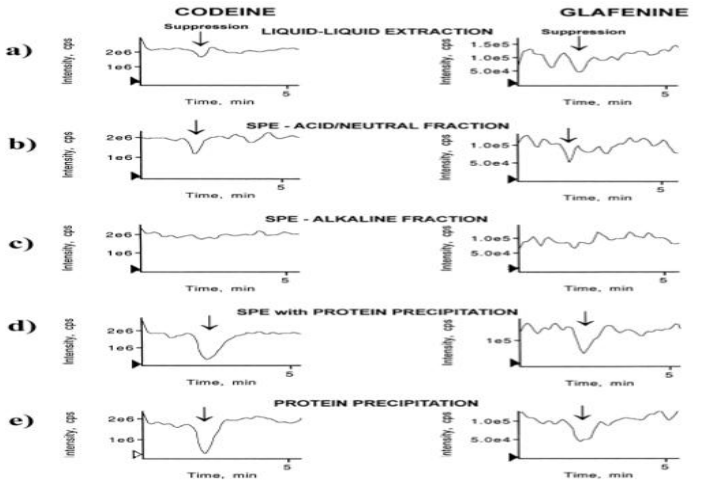
- Polarity and ionization potential of analytes (ESI vs APCI)
- Mobile phase composition (appropriate use of buffer such as acids and base), methanol vs acetonitrile, water composition in elution etc.
- Matrix components/salts
- Ion source of hardware

Problems encountered in LC-MS analysis
Matrix effect

Ion suppression?

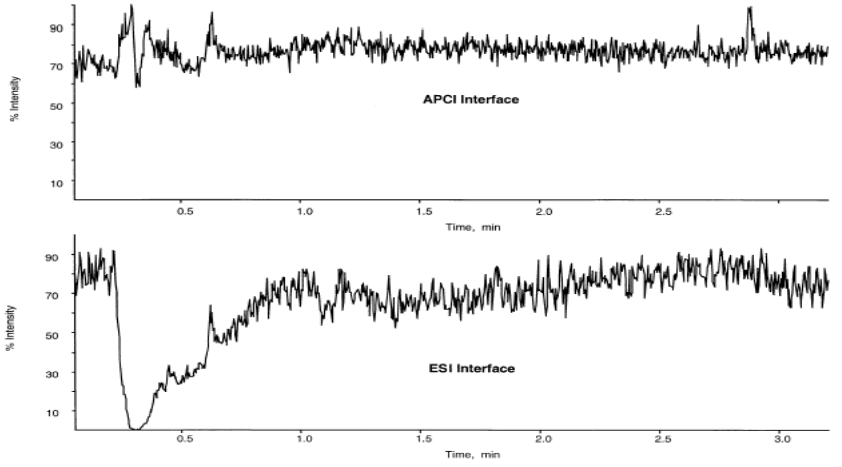
- the presence of endogenous substances from matrix, i.e. organic or inorganic molecules present in the sample and that are retrieved in the final extract
- exogenous substances, i.e. molecules not present in the sample but coming from various external sources during the sample preparation

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



Muller *et al. J. Chrom B (2002)*

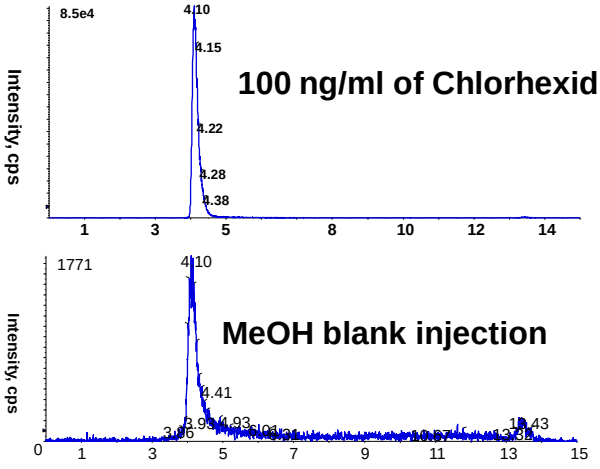
APCI is less prone to than ESI to the effects of ion suppression



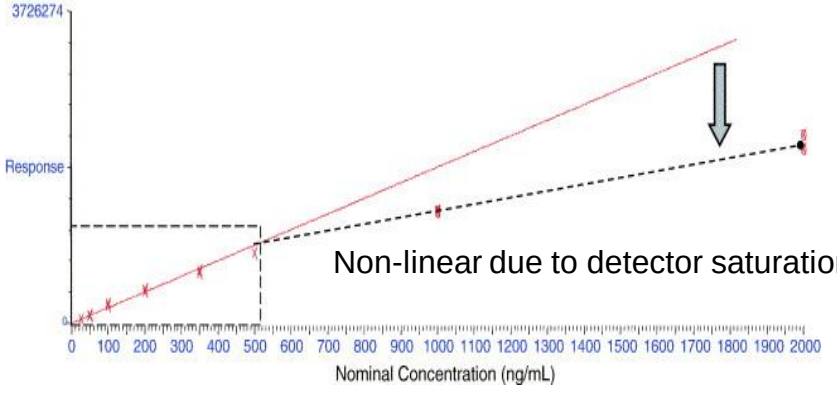
King et al. J. Am Soc Mass Spectrom 2000

Carry over a big problem?

Previously injected sample when 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

Why quantification of drug/drug metabolites in plasma/tissues PK studies is so important?

- An accurate and fast analytical method for measuring the concentrations of a compound in plasma or tissue is the first step in order to yield the PK of a compound
- Established assay for human sample analyses (plasma, serum or urine matrix) needs to be more rugged, robust and be able to withstand the test of time during this the longest phase of clinical development. The requirements and adherence to specificity, selectivity and stability will become very important

Analytical method validation

- Should demonstrate specificity, linearity, recovery, accuracy, precision
- Lower limit of quantification
- Stability (freeze/thaw)
- Robustness
- 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.**

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)

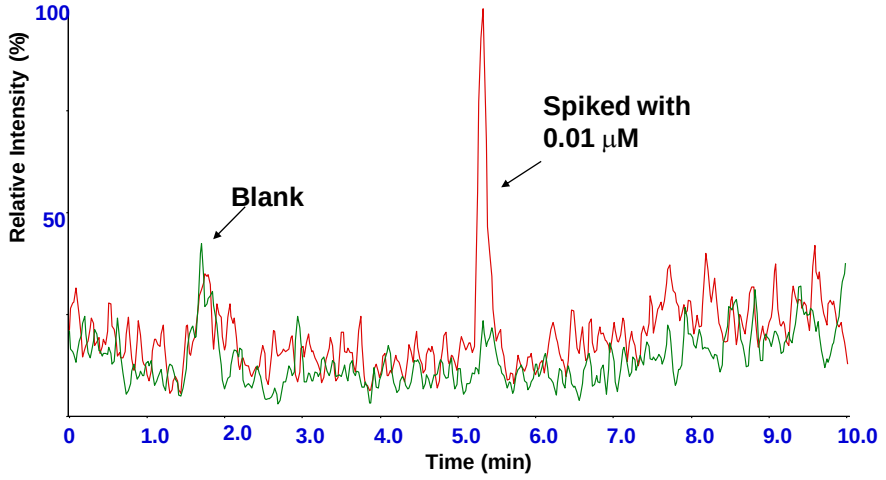
Concentration (ng/ml)	Mean ± S.D.	CV (%)	Accuracy (%)
2.0	2.21 ± 0.16	7.00	110.7
5.0	5.22 ± 0.28	5.30	104.48
50	45.32 ± 2.53	5.60	90.64
500	473.60 ± 26.57	5.60	94.72
1000	1021.20 ± 71.53	7.00	102.12
5000	5340 ± 420.18	7.90	106.80

Mean r = 0.996

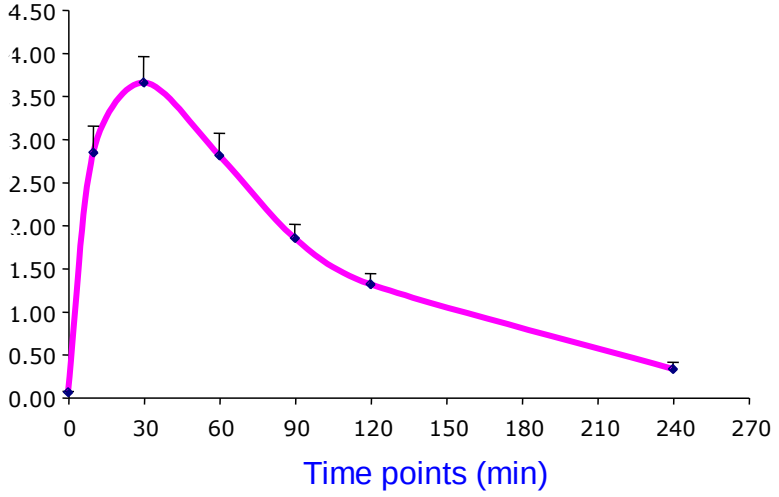
Table 2.
Assay validation characteristics of the method for the determination of puerarin in rat serum (n =5)

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

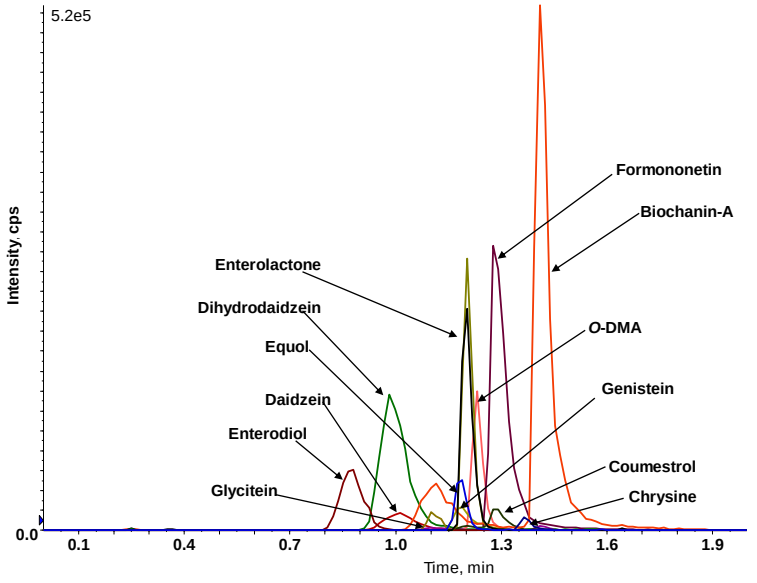
Ion chromatograms of a rat serum spiked sample (0.01 μM of puerarin) vs. blank serum



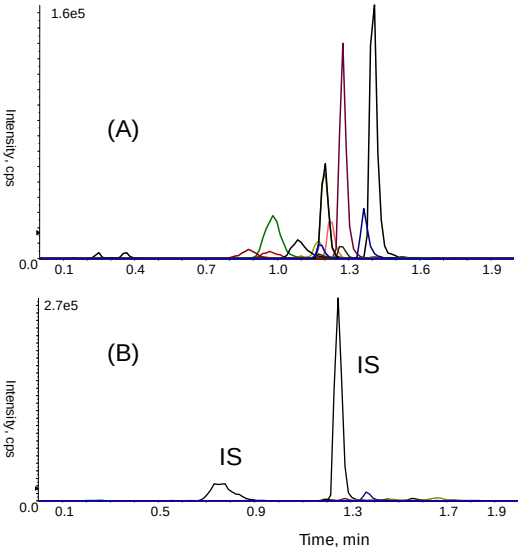
Average serum concentration of puerarin versus time after Oral administration of 50 mg/kg puerarin



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



Specificity of the assay- no peaks from matrix



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

Precision and accuracy of standards

Analyte	Concentration (ng/mL)	CV (%)	Mean accuracy (%)
Equol	1	9.26	95.82
	2	4.94	99.73
	20	1.86	100.00
	100	4.85	100.05
	200	3.33	106.32
	1000	4.79	100.72
	5000	2.38	98.47
Daidzein	1	6.84	101.56
	2	5.10	99.00
	20	6.83	100.08
	100	1.00	104.50
	200	5.92	101.48
	1000	5.86	99.48
	5000	3.67	96.90
Dihydrodaidzein	1	ND	ND
	2	13.49	97.62
	20	2.06	101.30
	100	5.13	104.05
	200	3.15	107.66
	1000	4.85	102.92
	5000	2.02	99.03
O-DMA	1	6.28	95.53
	2	6.15	96.53
	20	5.00	102.98
	100	2.47	101.55
	200	3.39	103.73
	1000	7.63	103.98
	5000	6.59	96.70
Genistein	1	10.47	104.16
	2	10.73	94.47
	20	6.23	99.18
	100	2.60	104.16
	200	2.72	107.66
	1000	6.72	106.34
	5000	4.62	102.38
Glycitein	1	3.08	114.83
	2	5.92	107.21
	20	7.94	93.91
	100	6.78	100.35
	200	4.10	105.93
	1000	2.90	95.82
	5000	6.21	102.05
Formononetin	1	4.39	100.72
	2	7.40	102.77
	20	8.52	101.18
	100	5.48	97.03

Precision and accuracy of quality control samples

Analyte	Nominal concentration (ng/mL)	Accuracy (%) Day 1	Day 2	Day 3	Precision (%CV) Day 1	Day 2	Day 3	Inter-day
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
	2,000	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
	2,000	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
	2,000	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
	2,000	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
	2,000	95.13	92.28	93.38	2.63	3.97	4.17	3.59

Equol	50	43.35 ± 2.50	45.68 ± 3.98
	500	487.80 ± 9.20	475.66 ± 30.16
	2000	1793.33 ± 67.42	1921.66 ± 94.74
Daidzein	50	47.03 ± 2.50	50.83 ± 1.87
	500	534.20 ± 21.05	491.66 ± 7.17
	2000	1848.33 ± 72.77	1861.66 ± 71.67
Dihydrodaidzein	50	45.55 ± 1.97	47.52 ± 5.23
	500	485.83 ± 26.35	219.20 ± 15.90
	2000	1738.33 ± 85.18	828.50 ± 27.01
O-DMA	50	48.31 ± 3.75	54.80 ± 5.67
	500	469.16 ± 24.01	534.66 ± 28.57
	2000	1861.66 ± 114.61	2151.66 ± 110.89
Genistein	50	50.90 ± 3.19	51.16 ± 3.34
	500	487.33 ± 33.15	497.33 ± 37.59
	2000	1875.00 ± 116.40	2190.00 ± 11.83
Glycitein	50	44.31 ± 2.44	40.15 ± 1.98
	500	481.00 ± 39.11	489.50 ± 28.26
	2000	1886.66 ± 87.10	2045.00 ± 191.91
Formononetin	50	47.36 ± 4.16	47.58 ± 3.22
	500	512.33 ± 26.41	507.66 ± 27.82
	2000	2018.33 ± 106.09	1925.00 ± 167.06
Coumestrol	50	46.26 ± 6.68	56.80 ± 2.37
	500	549.33 ± 36.74	498.00 ± 26.1
	2000	2120.00 ± 104.30	1905.00 ± 128.17
Biochanin A	50	52.47 ± 2.27	56.10 ± 1.49
	500	444.00 ± 29.81	523.00 ± 23.34
	2000	1893.33 ± 202.06	2130.00 ± 88.31