Quantitative analysis of small molecules in biological samples

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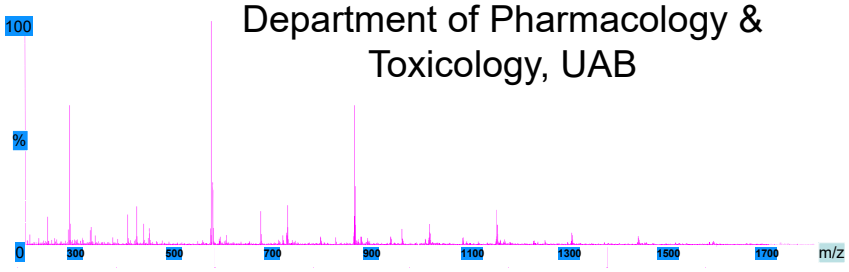


Class Overview

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

Validation

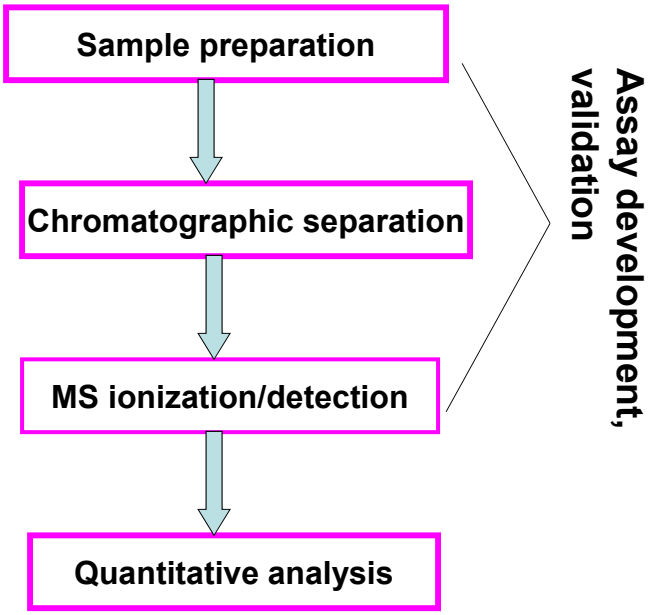
"All of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, Such as blood, plasma, serum, or urine, is reliable and reproducible for the intended use"

http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf

Untargeted metabolomics and method validation

- **No guidelines for validating analytical part in untargeted metabolomics.**
- **Unbiased differential, comprehensive analysis of metabolites in a biological sample.**
- **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.**

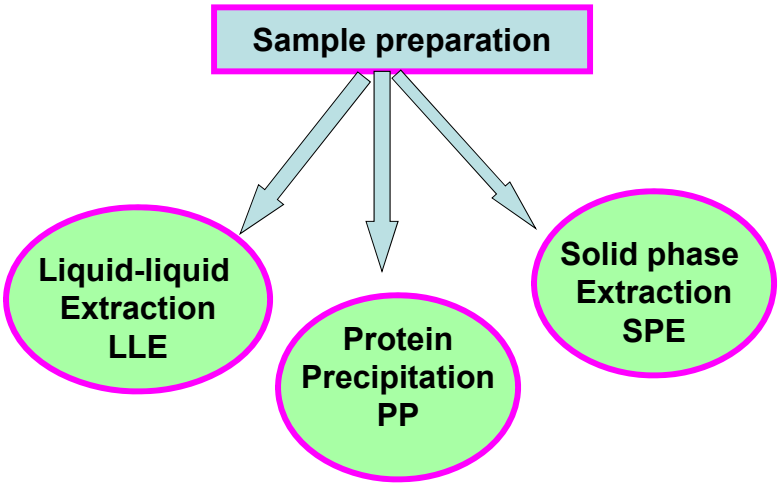
Bio-analytical workflow



Challenges in bioanalytical works

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

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.

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**

LC-MS analysis

HPLC

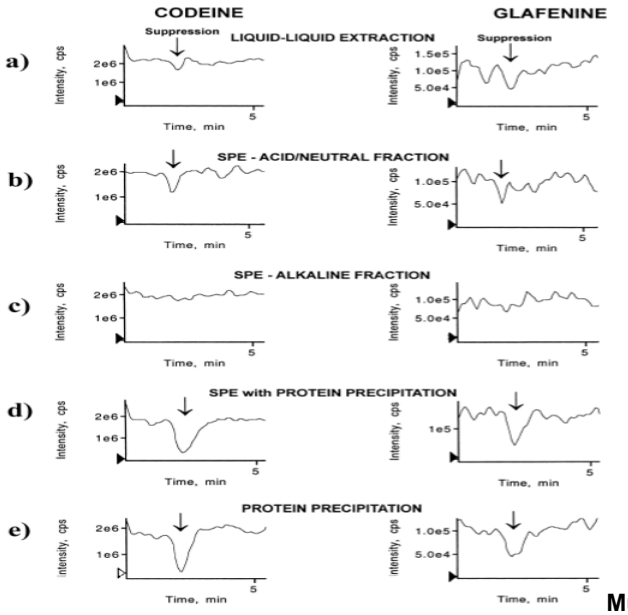
- Isocratic
- Gradient
- Reversed-nonpolar stationary, polar mobile
- Normal- polar stationary, nonpolar mobile
- HILIC- hydrophilic interaction

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

Choice of solvent

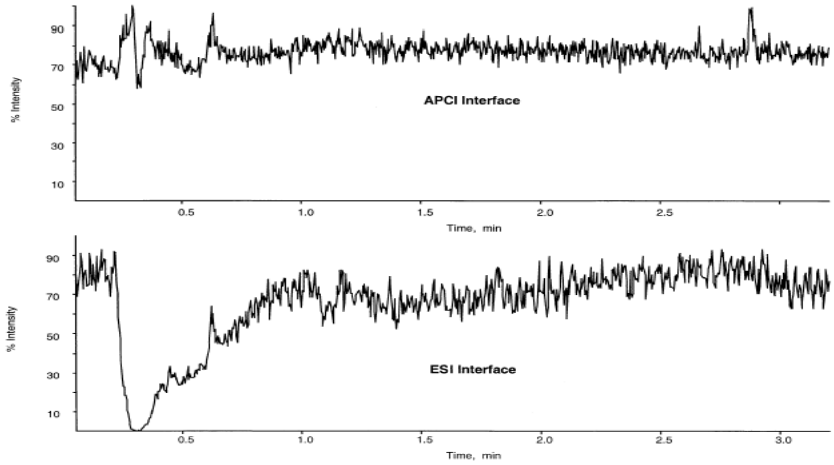
- Common organic solvents- Methanol and acetonitrile, water alone is poor solvent for ESI
- Acetonitrile vs methanol- acetonitrile (expensive), water/methanol creates more pressure than water/acetonitrile
- Elution strength- usually acetonitrile> methanol
- Methanol provide a more stable spray and better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

Severe ion suppression effect for codeine 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

Eliminating matrix effects

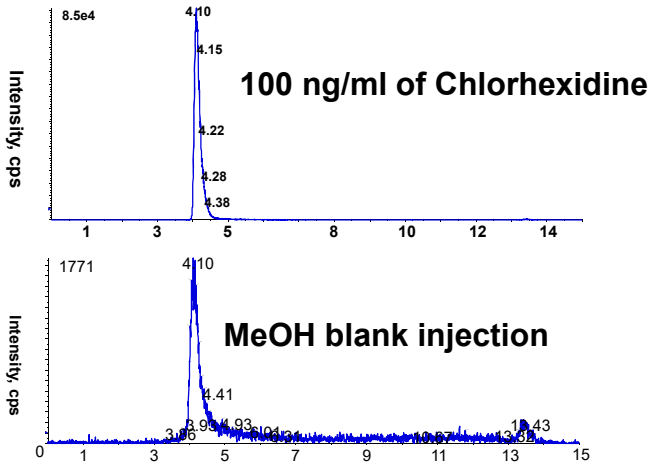
1. Preparing more cleaner samples.
2. Concentrating analyte of interest
3. Improve analytical system performance

% 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.



Analytical method validation

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

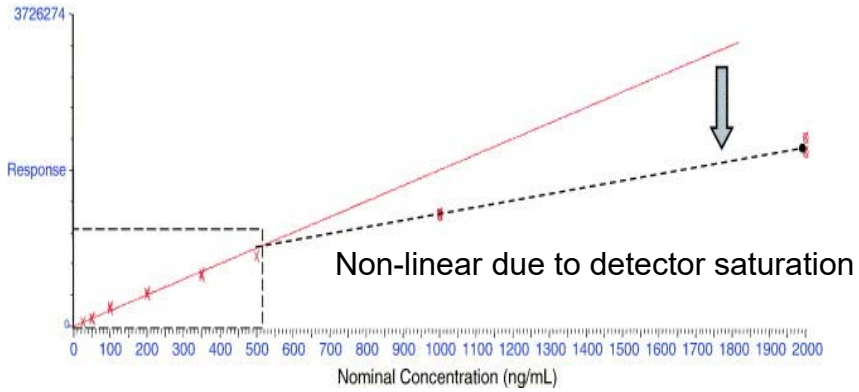
Method validation..

- **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

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

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.

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)

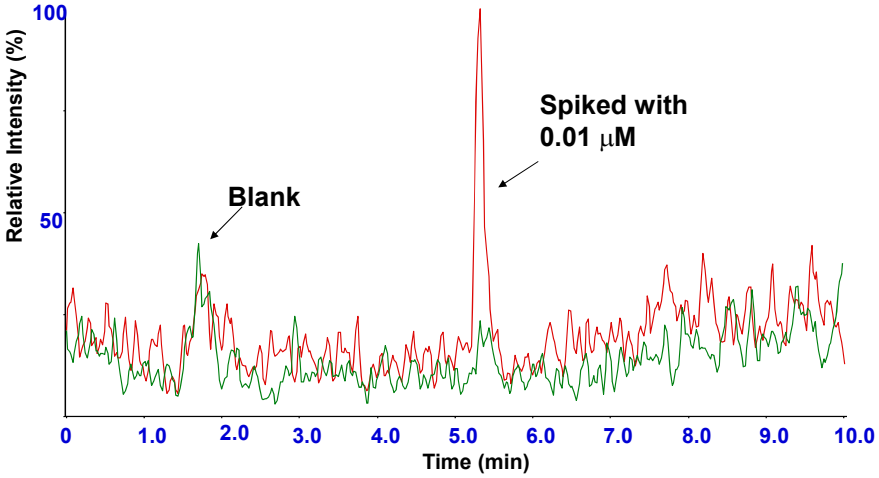
Concentration (ng/ml)	Mean ± S.D.	CV (%)	Accuracy (%)
2.0	2.21 ± 0.16	7.00	110.7
5.0	5.22 ± 0.28	5.30	104.48
50	45.32 ± 2.53	5.60	90.64
500	473.60 ± 26.57	5.60	94.72
1000	1021.20 ± 71.53	7.00	102.12
5000	5340 ± 420.18	7.90	106.80

Mean r = 0.996

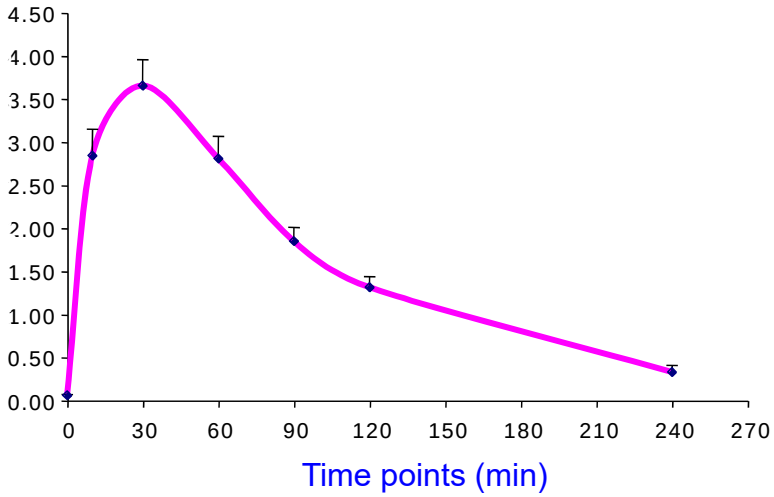
Table 2.
Assay validation characteristics of the method for the determination of puerarin in rat serum (n =5)

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 a 2 min run time

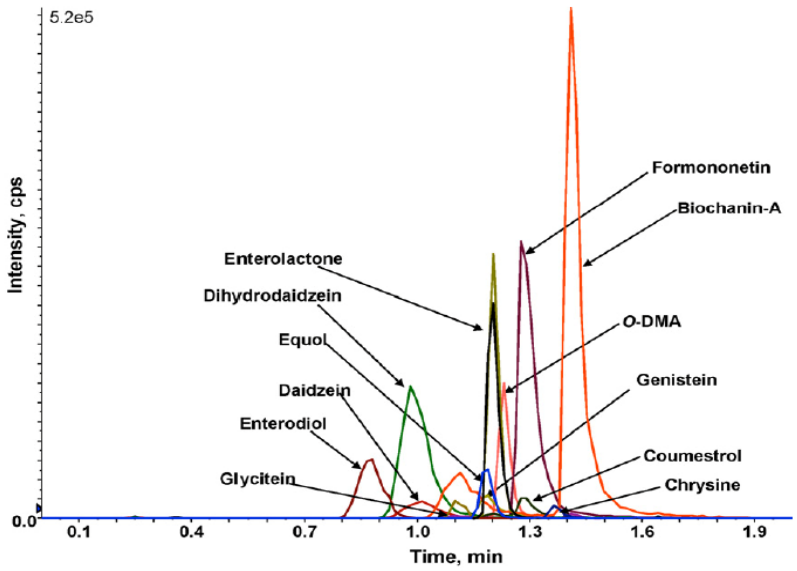


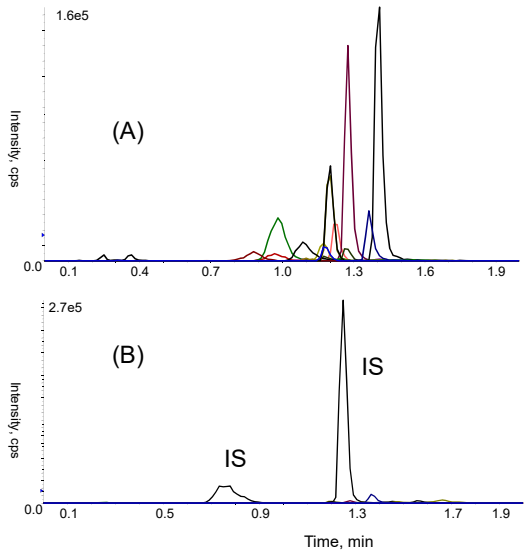
Table 1. MS/MS parameters optimized for phytoestrogens and internal standards

Analyte	Q1/Q3	Dwell (msec)	DP (V)	CE (eV)	CXP (V)
Equol	314/119	50	-65	-30	-5
Daidzein	253/132	50	-65	-55	-10
Dihydrodaizein	255/149	50	-50	-30	-9
O-DMA	257/108	50	-70	-40	-5
Genistein	269/133	50	-75	-40	-5
Glycitein	283/184	50	-65	-45	-5
Formononetin	267/251	50	-75	-35	-5
Coumestrol	267/91	50	-50	-50	-2
Biochanin A	283/268	50	-70	-30	-5
Enterolactone	297/253	50	-80	-30	-10
Enterodiol	301/253	50	-70	-30	-9
Phenophthalein	317/93	50	-50	-20	-5
4-MU	175/119	50	-50	-38	-4
Chrysin	253/143	50	-50	-50	-5

DP = Declustering potential
CE = Collision energy
CXP = Cell exit potential

Prasain et al., 2010

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 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

Table 5. Stability of quality control samples

Compound	Nominal Concentration (ng/mL)	Mean measured concentration (ng/mL) autosampler at 4 ^{0}C, 72h	long storage -20 ^{0}C, 2 months
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
Enterodiol	50	44.96 ± 3.45	46.84 ± 2.47
	500	488.16 ± 13.04	489.83 ± 20.79
	2000	1906.66 ± 68.89	1963.33 ± 119.27

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

Conclusions

- The sensitive & accurate analysis of biological samples remains a significant challenge.
- Although SPE and PPT can be HTS, LLE where extensive clean up is required, is less prone to matrix effects.
- 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.